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α-Helical, but not β-sheet, propensity of proline is determined by peptide environment

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ABSTRACT **Proline is established as a potent breaker of both α-helical and β-sheet structures in soluble (globular) proteins. Thus, the frequent occurrence of the Pro residue in the putative transmembrane helices of integral membrane proteins, particularly transport proteins, presents a structural dilemma. We propose that this phenomenon results from the fact that the structural propensity of a given amino acid may be altered to conform to changes imposed by molecular environment. To test this hypothesis on proline, we synthesized model peptides of generic sequence H_2N-(Ser-Lys)$_2$-Ala-Leu-Z-Ala-Leu-Z-Trp-Ala-Leu-Z-(Lys-Ser)$_3$-OH (Z = Ala and/or Pro). Peptide conformations were analyzed by circular dichroism spectroscopy in aqueous buffer, SDS, lysophosphatidylglycerol micelles, and organic solvents (methanol, trifluoroethanol, and 2-propanol). The helical propensity of Pro was found to be greatly enhanced in the membrane-mimetic environments of both lipid micelles and organic solvents. Proline was found to stabilize the α-helical conformation relative to Ala at elevated temperatures in 2-propanol, an observation that argues against the doctrine that Pro is the most potent α-helix breaker as established in aqueous media. Parallel studies in deoxycholate micelles of the temperature-induced conformational transitions of the single-spanning membrane bacteriophage IKe major coat protein, in which the Pro-containing wild type was compared with Pro^{30} → Ala mutant, Pro was found to protect the helix, but disrupt the β-sheet structure as effectively as it does to model peptides in water. The intrinsic capacity of Pro to disrupt β-sheets was further reflected in a survey of porins where Pro was found to be selectively excluded from the core of membrane-spanning β-sheet barrels. The overall data provide a rationale for predicting and understanding the structural consequences when Pro occurs in the context of a membrane.**

Proline is unique among the 20 naturally occurring amino acids in mammalian systems in that its side chain cyclizes back to the backbone amide, leaving one of its dihedral angles (ϕ) fixed at *ca.* −65° (1, 2). The structural consequence of this special arrangement is that the Pro residue is rarely found at the center of periodic secondary structures such as the α-helix and β-sheets in globular proteins (3, 4). When it does occur in an α-helix, however, Pro is usually located in the first turn, acting presumably as an N-capping residue (5). Proline is detrimental to the α-helical conformation for the several reasons. First, it lacks an amide proton on an X-Pro (X = any amino acid) bond to participate in helix stabilization through intramolecular H-bonding (6). Second, the bulkiness of its pyrrolidine ring places steric constraints on the conformation of the preceding residue in the helix (7). Third, as a secondary amide, Pro is a relatively polar residue that displays an enhanced tendency to form strong H-bonds in nonperiodic structural motifs such as Pro-induced β-turns (8) and γ-turns (9). Similarly, proline disfavors the β-sheet structure, because it lacks one potential H-bond donor and its ϕ angle is not compatible with standard β-sheets.

Notwithstanding these observations in water-soluble globular proteins, the Pro residue is found to be widely distributed in the putative transmembrane (TM) domains of many integral membrane proteins (10–12), regions believed to be α-helical in conformation (13–15). Helicity has been confirmed in at least two cases, namely bacteriorhodopsin (16) and the photosynthetic reaction center of *Rhodopseudomonas viridis* (17). In both cases, however, multiple prolines are found in several of the TM domain helices. Williams and Deber (6) proposed that prolines occurring in the TM domain may have one or more structural and/or dynamic roles depending on the local environment imposed by the solvating matrix and the parent membrane proteins. The fact that intramembranous prolines are often conserved among homologous proteins has led to the suggestion that these prolines may provide ligand binding sites for cations (12, 18). Prolines in TM domains of active protein transporters and channels, which are usually concentrated on the hydrophilic (interior) faces of the pore-forming helices, are also implicated in gating of the channels through helix kinking (11, 19). Brandl and Deber (10) further hypothesized that the cis-trans isomerization of an X-Pro peptide bond buried within the membrane can provide the reversible conformational change requisite for the regulation (opening/closing) of a transport channel. The relatively comparable energies for the cis and trans forms render the cis conformer much more likely than in other peptide bonds (1, 20).

Whereas extensive efforts have been devoted to the elucidation of proline functions in individual membrane proteins (reviewed in ref. 6), studies aimed specifically at exploring the structural propensity of proline in the molecular environment imposed by the membrane proteins and lipids are limited. A fundamental question is: how can the Pro residue—a well-known helix-breaker in globular proteins—be accommodated into TM α-helices without major structural readjustments of the rest of the protein (21)? In an early study concerning the helical propensity of amino acids in the membrane environment (22), we were able to show that apart from the primary sequence, the molecular environment is the most important factor in determining peptide conformation (22, 23). The frequent occurrence of proline in putative TM helices suggests that the structural propensity of proline may be altered in the membrane environment as compared with that in water. In the present work, this assumption is tested using Pro-containing peptides and model membrane proteins designed/engineered specifically to investigate changes of proline conformation in

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Abbreviations: TM, transmembrane; LPG, lysophosphatidylglycerol; TFE, trifluoroethanol.

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response to modifications of the environment. A series of 20-residue peptides of prototypical sequence H_2N-(Ser-Lys)$_2$-Ala-Leu-Z-Ala-Leu-Z-Trp-Ala-Leu-Z-(Lys-Ser)$_3$-OH (Z = Ala or Pro) were thus synthesized, and their conformations analyzed by circular dichroism (CD) spectroscopy in both aqueous and nonpolar media as represented by lipid micelles and organic solvents. In addition, the single Pro residue in the putative TM segment of bacteriophage IKe major coat protein was mutated to Ala. The conformations of the IKe wild-type and mutant coat proteins were compared at various temperatures in deoxycholate micelles. Results from these combined studies reveal that proline is not an intrinsic α-helix breaker in the membrane, but it is a potent β-sheet breaker in both aqueous and membranous media. A survey conducted on a set of the membrane protein porins, which form membrane-spanning β-barrels, similarly indicated that Pro is a residue selectively excluded from the center of TM β-sheets.

MATERIALS AND METHODS

Peptide Synthesis. Peptides were synthesized by solid phase techniques on a Biolynx 4170 automatic peptide synthesizer (LKB) using Fmoc (9-fluorenylmethoxycarbonyl) chemistry (24, 25). Amino acids were coupled as their pentafluorophenyl esters with an equivalent amount of HOBt (1-hydroxybenzotriazole) added, except for the case of serine, in which Fmoc-Ser(O-t-butyl)-ODhbt (ODhbt, 3,4-dihydro-3-hydroxy-4-oxo-1,2,3-benzotriazole) was used. Cleavage of peptides from the resin was accomplished by treating peptide resins for 3–5 hr in trifluoroacetic acid/2-mercaptoethanol/anisol (95:2.5:2.5) at ambient temperature. Crude peptides were dissolved to saturation in 0.5 M HCl, and the resulting cloudy mixture was extracted with one-third volume of ethyl ether three times to remove low molecular weight organic contaminants. Purification of peptides was carried out on a Delta-Pak C4 RCM column (25 mm × 100 mm; Waters), using a linear elution gradient of 10–25% of acetonitrile (containing 0.1% trifluoroacetic acid) in the first 10 min, followed by 25–35% acetonitrile in the second 10 min, and 35–50% acetonitrile in the final 10 min. Main peak fractions were collected and further chromatographed on a Vydac C4 analytical HPLC column (4.6 mm × 250 mm), from which a single peak was obtained for each peptide. Characterization of peptides was performed by amino acid analysis, and analytical purity was assessed by liquid chromatography and mass spectrometry.

CD Measurements. CD spectra were recorded on a Jasco-720A spectropolarimeter equipped with a Neslab variable temperature control unit, using cuvettes of 1-mm path length. Peptide concentration was typically 30 μM, as determined by quantitative amino acid analysis. Peptides were assumed to be monomeric under the experimental conditions, as suggested by the the corresponding NMR spectra and by the observation that CD spectra were independent of peptide concentration in the range of 20–300 μM (data not shown). The aqueous buffer used for CD experiments contained either 10 mM sodium chloride and 10 mM sodium phosphate at pH 7.0 or 20 mM sodium acetate, with pH adjusted to 11.3 with 1 M NaOH. SDS (Sigma) and lysophosphatidylglycerol (LPG; Sigma) micellar solutions were prepared by dissolving desired amounts of lipids into either of the above buffers and were directly used for sample preparations. Micellar solutions were routinely checked for background absorbance, from which it was noted that light scattering was insignificant at concentrations up to 50 mM SDS and LPG. Noise that did arise was averaged out by running several CD scans (usually six scans) for each peptide.

Site-Directed Mutagenesis. Strategies used to mutagenize the TM domain residues of bacteriophage IKe major coat protein were as reported (26). IKe single-stranded DNA was isolated from phage and employed directly as the template to allow for direct expression of mutant coat proteins from the IKe genome. An oligo designed to subject Pro^{30} to saturation mutagenesis (3′-AGAGTCTGAACCNNNCAACATTGCT-GAC-5′), was prepared with 25% of each base supplied at the three bases coding for the Pro residue. Transformation was by the $CaCl_2$ method, with cells plated on 2 × YT plates, held at 25°C overnight and then transferred to 37°C for 8 hr to generate large, distinct plaques. Sequencing of individual plaques was performed using the T7 kit (Pharmacia) based on the dideoxy method (27). Approximately 150 plaques were sequenced for each of the oligos; mutagenesis efficiency was ≈30%. The mutant protein Pro^{30}-Ala generated from the above saturation mutagenesis was purified as described (28).

RESULTS

Design of Pro-Containing Model Peptides. We designed and synthesized peptides mimicking the general features of a single-spanning membrane protein (22, 23, 29). Specifically, the 20-mer parent peptide, designated 0P for no proline, was fashioned with a 10-residue stretch of hydrophobic amino acids (underlined in Table 1) consisting largely of Ala and Leu. These residues are expected to promote interactions with the membrane-mimetic media used in this study. The hydrophobic segment was flanked by hydrophilic residues Ser and Lys at both peptide termini (Table 1). These latter residues rendered the peptide(s) water-soluble so that peptide synthesis and purification were greatly facilitated. Substitution of Ala residues by Pro at positions 7, 10, and 14 of the hydrophobic segment, either individually or in combination, generated peptides 1P, 2P, and 3P, containing one, two, and three prolines, respectively. Variations of Pro content in the peptides made it possible to examine the influence of Pro residues on peptide conformation both individually and collectively.

Comparison of Peptide Conformations in Aqueous and Micellar Solutions. The secondary structure-forming potential of the peptides in various solvents was readily examined by CD spectroscopy. Spectra shown in Fig. 1 were obtained in three distinct solvent systems: aqueous buffer, pH 11.3 (Fig. 1*a*); 10 mM SDS micelles, pH 11.3 (Fig. 1*b*); and 10 mM LPG micelles, pH 7.0 (Fig. 1*c*). The latter two systems were employed to provide a membrane-like environment for the peptides. Similar approaches have been used in related studies (22, 23, 29).

Peptide 0P, which contains no prolines in its hydrophobic core, was the only peptide in the group displaying partial α-helical structure in aqueous buffer. This was reflected by the negative absorbencies of its CD spectrum at 208 and 222 nm (Fig. 1*a*), characteristic of an α-helix (30). In comparison, a single substitution of Ala^{10} by Pro in peptide 0P resulted in a complete loss of α-helicity for peptide 1P (Fig. 1*a*). Similarly, peptides 2P and 3P exhibited essentially random conformations under the same conditions. These results support the notion that proline is an effective α-helix breaker, as noted in globular proteins (2–4).

Table 1. Amino acid sequences of Pro-containing model peptides

Peptide	Amino acid sequence
0P	H_2N-(Ser-Lys)$_2$-Ala-Leu-**Ala**-Ala-Leu-**Ala**-Trp-Ala-Leu-**Ala**-(Lys-Ser)$_3$-OH
1P	H_2N-(Ser-Lys)$_2$-Ala-Leu-**Ala**-Ala-Leu-**Pro**-Trp-Ala-Leu-**Ala**-(Lys-Ser)$_3$-OH
2P	H_2N-(Ser-Lys)$_2$-Ala-Leu-**Pro**-Ala-Leu-**Pro**-Trp-Ala-Leu-**Ala**-(Lys-Ser)$_3$-OH
3P	H_2N-(Ser-Lys)$_2$-Ala-Leu-**Pro**-Ala-Leu-**Pro**-Trp-Ala-Leu-**Pro**-(Lys-Ser)$_3$-OH

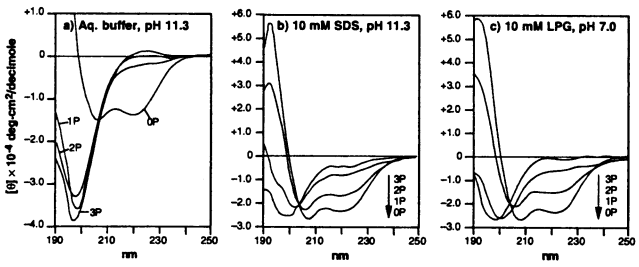

FIG. 1. CD spectra of peptides 0P, 1P, 2P, and 3P at 25°C in aqueous buffer, pH 11.3 (*a*); 10 mM SDS, pH 11.3 (*b*); and 10 mM LPG, pH 7.0 (*c*). Peptide concentration was 30 μM. Curves are as indicated in the diagram.

In contrast, peptides in micellar solutions assumed generally greater contents of α-helix than in water. In particular, peptide 0P was found to be almost fully α-helical in both SDS and LPG micelles (Fig. 1 *b* and *c*), whereas peptide 1P exhibited appreciable α-helicity under the same conditions (Fig. 1*a*). To assess the impact of the positive charges of Lys residues on peptide–lipid interactions and on peptide conformation, the pH of the micellar media was raised from 7.0 to 11.3. This did not appear to affect the secondary structure of the peptides significantly (Fig. 1 *b* and *c*), suggesting that the α-helical character observed for peptide 1P originates primarily from its mid-hydrophobic segment, where the single Pro is located. These results indicate that Pro does not disrupt the α-helical structure in the membrane environment as effectively as in water. Nonetheless, peptides 2P and 3P displayed minimal α-helicity under the same conditions (Fig. 1 *b* and *c*), an observation that may stem from the decreased hydrophobic interaction of the peptides with the interior of the micelles as a result of Ala to Pro substitutions. Indeed, Trp fluorescence indicated that peptides 2P and 3P associated only superficially with the SDS micelles (data not shown). Alternatively, the incapability of peptides 2P and 3P to form an α-helix in SDS and LPG micelles may suggest that the collective effect of more than one Pro in a short peptide segment is helix-destabilizing.

Helix Formation by Pro-Containing Peptides in Organic Solvents. Unlike lipid micelles, organic solvents such as alcohol provide a homogeneous, low dielectric environment mimicking the hydrophobicity of the core of a lipid bilayer (24). In general, peptides in alcohol form an α-helix more readily than in water, because intramolecular hydrogen bonds tend to be strengthened in the low dielectric environment of alcohol (31, 32). Another advantage of using organic solvents is that the polarity of the medium can be easily controlled to examine how the helical propensity of proline changes with solvent polarity (24). For this purpose, three organic solvents—methanol, trifluoroethanol (TFE), and 2-propanol—were employed in the present study. In 90% methanol, a relatively polar solvent, each of the four peptides assumed secondary structures (Fig. 2*a*) similar to those observed in SDS and LPG micelles (Fig. 1 *b* and *c*), suggesting that peptides in the latter media are likely situated at the water–micellar interface due to the short length of the hydrophobic core. However, helicities of peptides 1P and 2P were greatly increased as the dielectric of the organic solvent was decreased. Specifically, in 94% TFE, peptides 1P and 2P adopted largely α-helical structures comparable in magnitude with that of peptide 0P (Fig. 2*b*). The difference in percent helicity between peptides 0P and 1P, as calculated using ellipticity values at 222 nm (24), is only 15%, whereas the difference is <25% between peptides 0P and 2P. Similar results were obtained for the peptides in 90% 2-propanol (Fig. 2*c*). These data substantiated the notion that the Pro residue can participate in helical structure formation under optimal conditions (such as in the low dielectric environment of organic solvents)—*e.g.*, up to 20% of Pro residues can be

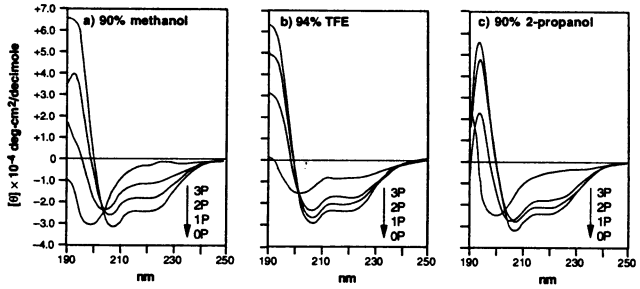

FIG. 2. CD spectra of peptides 0P, 1P, 2P, and 3P at 25°C in 90% methanol (*a*); 94% TFE (*b*); and 90% 2-propanol (*c*). Peptide concentration was 30 μM. The pH values of the solutions were ≈4.5 and were left unadjusted.

accommodated into a 10-residue hydrophobic core without significantly compromising the overall helicity of the recipient peptide (2P). Interestingly, peptide 3P remained largely random in all solvent systems tested, suggesting that there exists a limit to the number of prolines that can be incorporated into a peptide segment without disrupting its helical character. Apparently, none of the Pro-containing peptides formed the polyproline II helix (33).

Thermal Stability of Pro-Containing α-Helices. To determine the thermal stability of helices formed by peptides 1P and 2P, we conducted temperature experiments with peptide solutions in 2-propanol; this solvent was preferred over either methanol or TFE because it is less volatile. Peptide samples were heated gradually, and CD spectra were recorded at regular temperature intervals to assess the corresponding helix–coil equilibrium. Input of thermal energy should be expected to unfold the α-helix formed by a short peptide. This effect was observed for peptide 0P, which underwent a linear transition from α-helix to coil as the temperature of the medium was raised (Fig. 3). In contrast, peptides 1P and 2P exhibited nonlinear transition patterns (Fig. 3). Peptide 1P experienced a rapid loss of α-helicity with increasing temperature in the range of 0–30°. This pattern was replaced by a slower process of structural transition as the temperature was

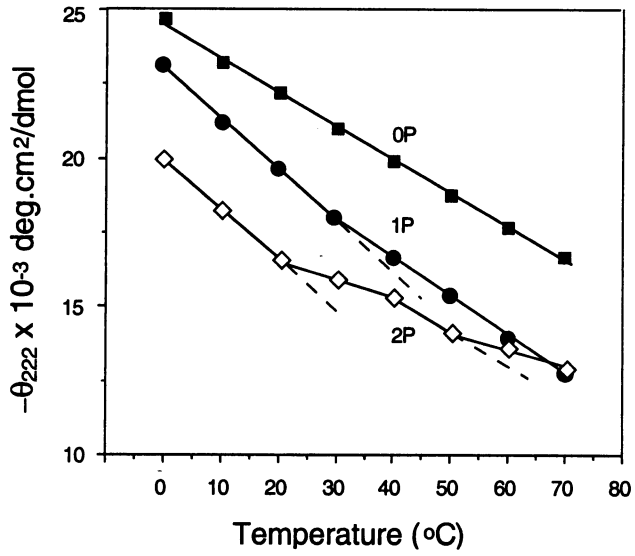

FIG. 3. Temperature dependence of peptide helicity in 2-propanol. Peptide helicity is represented in the diagram by CD absorbencies at 222 nm, taken from the respective CD spectra recorded for the peptides at various temperatures. Peptide concentration was 30 μM. Points corresponding to peptide 0P is represented by filled squares, 1P by filled circles, and 2P by open diamonds. Duplicate experiments were performed for each peptide, with a deviation in each data point of less than ±1% in ellipticity (222 nm).

further increased above 30°. It is possible that the single Pro in peptide 1P is responsible for retaining the α-helical character of the peptide at elevated temperatures. If so, one would expect that the two Pro residues in peptide 2P to do the same. This was indeed the case. As shown in Fig. 3, the helicity of peptide 2P was observed to decrease rapidly between 0° and 20°. Helicity changed very little between 20° and 40° before decreasing again between 40° and 50°. Above 50°, there was again a gradual decrease in helicity for peptide 2P.‖ It is unlikely that the deviations of peptides 1P and 2P from a linear helix-to-coil transition were due to cis-trans isomerization of the Leu-Pro peptide bond in the peptide. NMR studies by Raleigh *et al.* (34) on a synthetic peptide showed that the X-Pro bond was predominantly in the trans configuration, and that the ratio of cis to trans isomer populations was independent of temperature between 5° and 70°C (34). These results suggest that Pro is capable of stabilizing the helical conformation at elevated temperatures relative to Ala, as similar temperature effects were not observed for peptide 0P, which contains Ala instead of Pro residues at positions 7 and 10 (Fig. 3). This effect is best shown in Fig. 4, where peptide 1P is more helical than 2P at 0°, but assumes essentially equal helicity to 2P when temperature is raised to 70°. It is likely that each point in Fig. 3 represents a state of thermodynamic equilibrium for the peptide, because the observed transition pattern was completely reversible (data not shown). We further noted that peptide 3P did not display similar features in parallel experiments since it was largely random at temperatures as low as 0°. Taken together, these results suggest that Pro can protect against the loss of peptide helicity at elevated temperatures.

Proline Disrupts β-Sheets in Both Aqueous and Membrane Environments. To examine the β-sheet-forming propensity of proline in the aqueous medium, peptide samples were heated in an alkaline buffer containing 20 mM sodium acetate, pH 11.3. Similar conditions were employed to induce β-sheet formation in poly(Lys) (35, 36). As the present series of peptides contains several Lys residues, it might therefore be expected that β-sheet structure can, in principle, be initiated at the peptide termini and propagate through the sequence. Indeed, peptide 0P was observed to undergo a clear transition from α-helix to β-sheet when the temperature was increased to ≈40° (Fig. 5*a*). Further increases in temperature to 80° did not alter the CD pattern observed at 40°, suggesting that stable β-sheet structure was already formed at 40°. In contrast, none of the Pro-containing peptides was capable of forming β-sheets under similar conditions (Fig. 5 *b* and *c*). This phenomenon may be a genuine reflection of the strong β-sheet-destabilizing effect of proline in water, as observed in globular proteins (3).

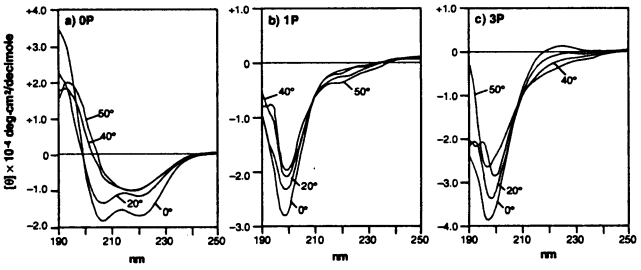

FIG. 5. Temperature dependence of CD spectra for peptides (*a*) 0P, (*b*) 1P, and (*c*) 3P in aqueous buffer, pH 11.3. Peptide concentration was 30 μM. Peptide 2P displayed similar behavior to 3P (data not shown).

To test whether the same structural property of proline is retained in the membrane environment, we examined the conformations of the IKe coat protein and one of its TM mutants, $Pro^{30} \rightarrow Ala$, in the membrane-mimetic environment of 30 mM deoxycholate micelles, an approach used previously to address the stability of TM helices (26, 28, 37). At room temperature, both the wild-type and mutant IKe protein formed essentially complete α-helical structure in deoxycholate micelles (Fig. 6 *a* and *b*). When the temperature was raised to 95°, a clear structural transition from α-helix to typical β-sheet was observed for the mutant (Fig. 6*b*), whereas the wild-type IKe remained α-helical (Fig. 6*a*). SDS/PAGE analysis indicated that the mutant protein formed high-molecular weight β-aggregates at 95°, whereas the wild-type IKe coat protein remained monomeric under the same conditions (data not shown). Therefore, a single mutation from Pro to Ala in the TM segment of the IKe major coat protein was sufficient to allow induction of β-sheet at elevated temperatures. Thus, the Pro residue was capable of retaining the IKe protein in an α-helical conformation in micelles and protecting against its transition to the β-sheet conformation.

Proline Is Selectively Excluded from Porin β-Barrels. The above studies on model peptides and the IKe coat proteins suggest that proline's destablizing effect on β-sheets may be manifested in nature as an exclusion of proline from β-structures. To test this hypothesis, an initial survey was conducted on porin proteins, which form large, weakly selective pores in membranes through β-barrel formation (Fig. 7). High β-sheet

‖To test if the "breakpoints" in the helix–coil transition profile observed for peptide 2P (Fig. 3) relate to the specific location of the Pro residues in the peptide sequence, we synthesized two additional peptides—designated $2P_{APP}$ and $2P_{PAP}$—where the Pro residues are now substituted into Z-positions 7, 10, and 14 as indicated. Preliminary parallel experiments conducted on peptides $2P_{APP}$ and $2P_{PAP}$ resulted in similar patterns of helix–coil transition in 2-propanol, with CD spectra of each peptide exhibiting helix–coil transition curves with two breakpoints observed at similar temperatures as for peptide 2P.

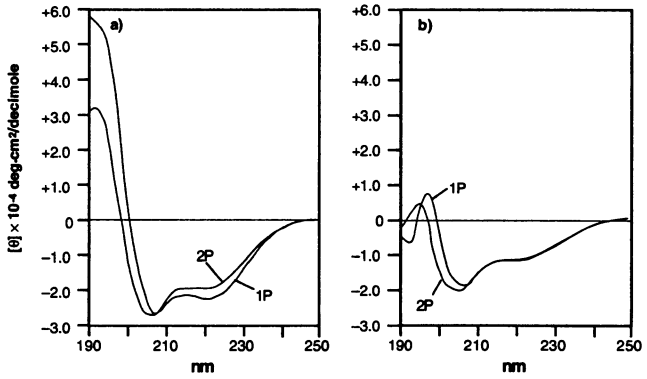

FIG. 4. CD spectra of peptides 1P and 2P in 2-propanol at (*a*) 0°C and (*b*) 70°C. Peptide concentration was 30 μM. Curves are as labeled on the diagram.

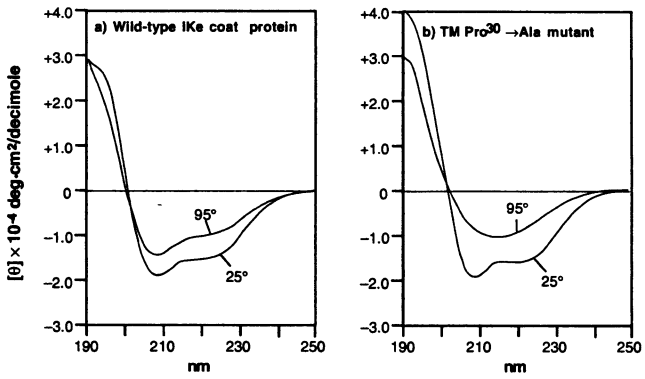

FIG. 6. CD spectra of (*a*) wild-type IKe major coat protein and (*b*) mutant $Pro^{30} \rightarrow Ala$ IKe in 40 mM deoxycholate micelles (pH 9.0) at 25°C and 95°C. Protein concentration was 1 mg/ml in a cell of path length 0.1 cm.

content combined with low sequence homology between species make porins an ideal family to chart the occurrence of proline within and around β-strands. The crystallographic structures of five such proteins have been determined and form the basis of this survey (38–41); three additional porins have also been included where structural information has been provided through biochemical, immunological, and predictive means (42–44). As seen in Fig. 7, Pro is selectively enriched in membrane-flanking regions, where it may facilitate the reversal of the peptide backbone by promoting turn formation, but it is excluded from the core of the β-barrels—an observation consistent with its role as an effective β-sheet breaker (see also Fig. 7 legend).

DISCUSSION

TM proline residues have been the focus of extensive research since this residue was observed to be enriched in the TM segments of many protein channels and transporters (6, 10). In globular proteins, proline has been extensively studied for its relevance to protein folding (1, 6, 45–47). More recently, proline-rich regions have been found in a vast number of proteins engaged in biological processes, such as signal transduction and transcription initiation, where Pro occurs either in an extended conformation or in polyproline II helices (48). Appreciation of the roles proline may play in such a diverse array of proteins prompts a detailed characterization of its structural properties in various molecular environments.

Using model peptides and small membrane proteins in conjunction with statistical analyses, we were able to demonstrate that proline effectively disrupts the α-helical structure in water, but it is not, in essence, an α-helix-breaker in membrane environments. In addition, Pro is seen to disrupt β-sheets irrespective of the solvent media. Our results are consistent with those of Strehlow *et al.* (49), in which a single Pro was introduced into various positions of the C-peptide helix of RNase A. This group found that incorporation of Pro effectively truncated the peptide helix in water, except when in the first turn of the helix (49). Contrary to this observation, Vogel reported that a Pro residue placed in the center of a hydrophobic peptide Dns-(Ala-Aib-Ala-Aib-Ala)$_4$Trp-OMe (Dns- = dansyl-) did not significantly reduce the helical content of the peptide in either methanolic solutions or in membranes composed of lipid vesicles (50). These two studies similarly demonstrate that the structural propensity of proline is a function of the molecular environment.

It is conceivable that proline can be a helix-former under certain circumstances. The ϕ-torsion angle of proline, although restricted at around $\approx -65°$, falls within the range for α-helices, whereas its other torsional angle, ψ, while more flexible, can, in principle, adopt values in the α-helical range (51). Intramolecular hydrogen bonds are generally strengthened in the low dielectric environments imposed by lipids or organic solvents, which may compensate, in part, for the inability of proline to form H-bonds with the carbonyl group from the preceding turn of the α-helix. However, the most striking finding of the present study is that not only can proline be supportive to an α-helix, it can also stabilize the helical conformation at elevated temperatures. This unique structural feature of proline can be explained using a thermodynamic description. For individual amino acids, the free energy of a helix–coil transition, ΔG, can be divided into two functions: the corresponding changes in enthalpy (ΔH) and in entropy (ΔS). These functions are related to each other through the equation, $\Delta G = \Delta H - T\Delta S$. The contribution of entropy to free energy is thus intimately related to temperature (T). Therefore, at low temperature, the term $T\Delta S$ plays a relatively less significant role than ΔH to free energy. However, as the significance of $T\Delta S$ increases proportionally with temperature, it may replace ΔH as the determining factor to ΔG at a certain point characteristic of the system. As proline has the least

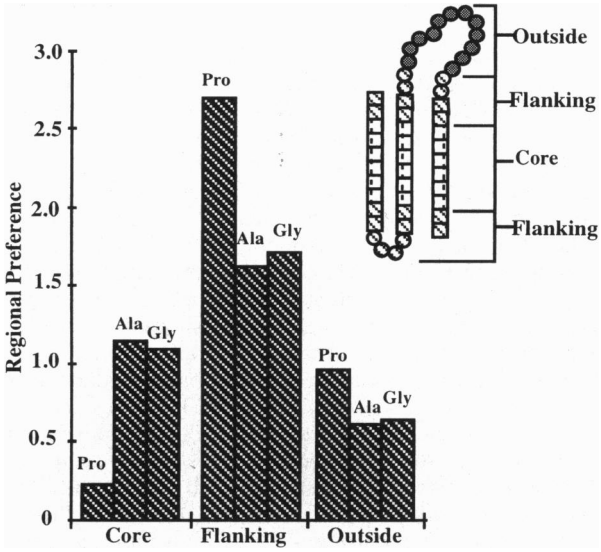

FIG. 7. Survey of proline occurrence in porin β-barrel proteins. Proteins included in the study were: *Escherichia coli* OmpF P02931 (38), *E. coli* PhoE P02932 (38), *E. coli* LamB P02943 (41), *R. blastica* Porin P39767 (40), *R. capsulatus* Porin P31243 (39), *Pseudomonas acidovorans* Omp 32 P24305 (42), human VDAC P21796 (44), and yeast VDAC P04840 (43). (*Inset*) Residue classification is illustrated by representing three β-sheets within a TM β-barrel. Squares denote residues designated to be in a β-conformation, and circles show amino acids outside the sheets in turns or loops. Flanking residues are hatched and encompass four residues on either side of the dotted "core" β-sheet region. The remaining dark circles are designated as "outside" residues. This residue classification was used in the survey of 2578 porin amino acid residues where the numbers of Pro, Ala, or Gly residues in the core region were calculated as a percent of the sum of all amino acids in that region. Similar calculations were performed for flanking and outside regions to give frequencies of Pro, Ala, or Gly in each region. Parallel calculations were also performed to determine the frequency of Pro, Ala, or Gly in all regions together. These "total" frequencies were then used to normalize the frequencies of each amino acid, and the resultant "regional preference" is represented in the y axis of the bar graph. The regional segregation of the raw data was analyzed using a student's unpaired t test at 95% confidence, which established that Pro was selectively excluded from the core of the β-barrels ($P < 0.005$), while Ala and Gly showed no statistically significant difference between their distribution in the core and throughout all regions ($P > 0.05$). The test also confirmed the apparent enrichment of all three amino acides in the flanking regions ($P < 0.05$). Depletion of Ala and Gly in the outside regions was also found to be statistically significant versus their frequencies in the full protein.

entropy loss in a helix-to-coil conformational transition because the motion of its side chain is constrained, this rationale may explain the observation that proline can slow down the process of helix-to-coil transition in peptides 1P and 2P at elevated temperatures, whereas alanine (in peptide OP), one of the most noted helix-formers, cannot do so.

The concept of proline as a temperature-dependent helix-stabilizer is depicted in the model presented in Fig. 8. For the sake of simplicity, we assume (*i*) that a peptide containing a single proline at the centre position can adopt only two conformational states, the α-helix and coil, and (*ii*) that the α-helical state predominates at low temperatures. The temperature-induced helix-to-coil transition generally initiates from the peptide terminus. Thus, as temperature rises, regions in the peptide relatively distant from the Pro locus would be expected to undergo a rapid transition to the coil state. It is during this period (the low temperature range; Fig. 3) that the greatest loss of α-helicity for peptides 1P and 2P in 2-propanol is observed. Until the temperature reaches a certain point, the integrity of helical conformation at the proline locus is main-

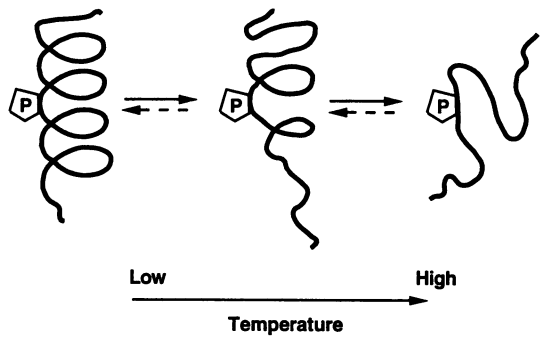

FIG. 8. Model for temperature-induced helix–coil transition in a Pro-containing peptide. A proline residue in the core of a TM helix is designated by a pentagon fused to the peptide backbone. As temperature increases, regions most distant from the Pro locus fray in helicity at a more rapid rate than Pro-adjacent regions. At higher temperatures, the helicity of the full segment is eventually melted.

tained. When that point is passed, however, the proline site begins to lose its own helical character, but at a significantly slower rate than the flanking regions. Presumably, the minimal gain in entropy for proline in a helix-to-coil transition does not particularly favor the coil state for the Pro locus, compared with the flanking regions that contain no proline. In addition, the bulk of the proline side chain restricts the thermal motion of the preceding residues in the helix, which renders the unfolding of the Pro-locus even more energy-consuming. In globular proteins, it has similarly been shown that introduction of proline into appropriate site(s) can increase thermal stability, due probably to a decrease in the unfolding entropy of the protein (43, 52). Thus, it should be possible to engineer the Pro residue into the α-helical regions of a protein to increase its thermal stability, assuming caution is taken to avoid interference with the native fold or the function of the protein.

In contrast to the temperature-dependent stabilization of helical structure, the Pro residue is found to disrupt β-structures in both aqueous solutions and membrane environments. Consistent with this observation, Pro was seen to be selectively excluded from the core (membrane-interactive) positions in the β-strands of porins (Fig. 7). The effect of proline as a strong β-sheet breaker was similarly apparent in a study with the Alzheimer disease-associated amyloid peptide β/A4, where the introduction of Pro into a fragment of β/A4 abolished the ability of the peptide to form β-sheet aggregates (53). The environment-independent detrimental effect of proline on β-sheets likely stems from the fact that the fixed Pro ϕ-torsion angle is not compatible with regular β-sheets. These properties of Pro differ from those of Gly, another potent breaker of the β-sheet structure in globular proteins (3, 4). We have shown in previous studies that a peptide bearing three Gly residues at the Z positions (refs. 7, 10, and 14; Table 1) is capable of forming both α- and β-structure depending on the molecular environment (54).

The β-sheet breaking characteristics of Pro may impart specific features to protein structure and folding. For instance, proline may help to protect against immature protein aggregation (*i.e*, in the form of β-sheets) during the folding of nascent polypeptide chains in the cell. Furthermore, Pro can be engineered into suitable sites in a protein by site-directed mutagenesis to counter the aggregation (as β-sheets) inherent of hydrophobic/membrane proteins. Similar approaches can also be adopted to improve the level of protein expression where Pro could act to disrupt the formation of "amorphous" protein aggregates *in vivo* (53).

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