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Proline Residues in Transmembrane Helices: Structural or Dynamic Role?†

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Proline is the least likely of the 20 amino acids to be present in an α-helix on the basis of analysis of soluble proteins (Chou & Fasman, 1978; O'Neil & DeGrado, 1990), and since it is generally located in hydrophilic environments (Richardson & Richardson, 1989), it would not be expected to occur within a membrane. Nonetheless, Pro has been found to occur with relatively high frequency in the putatively α-helical transmembrane (TM) segments of many integral membrane proteins that function as receptor subunits or transporters (Brandl & Deber, 1986). While the distribution of Pro in membranes appears to be random with respect to position within TM segments (Brandl & Deber, 1986), TM Pro residues tend to be conserved among homologous proteins [e.g., Vilsen et al., (1989)].

We examine the potential roles of Pro residues in TM helices and assemble this material into a framework upon which the function(s) of individual membrane-buried Pro residues can be assessed. In the ensuing discussion, we categorize the effect(s) of Pro in a TM helix as *structural* and/or *dynamic*. Structural roles include purely static effects such as that arising from a kinked helix, as well as electronic effects that stem from increased local polarity. Dynamic roles, which describe the participation of Pro in conformational change, are in two categories: cis–trans isomerization of Xaa-Pro peptide bonds and Pro-mediated interconversions among all-trans conformational states.

Structural Effects of Proline Residues in Transmembrane Helices. Proline is the only mammalian imino acid. As such, its side chain is bonded to the tertiary nitrogen in a cyclic pyrrolidine ring. This effectively fixes the backbone dihedral phi (ϕ) angle at the preferred α-helical value, $-60°$, and leaves the psi (ψ) angle flexible [with two preferred (so-called cis'/trans') regions] but hindered. As well, the bulky ring constrains the conformation of adjacent residues (Carver & Blout, 1967). Another helix-destabilizing feature is that the Pro nitrogen lacks a proton so that the Xaa-Pro peptide bond cannot participate in H-bonding to a neighboring carbonyl group; in helical segments, this situation accordingly frees up the carbonyl group four residues away for participation in alternative H-bonding schemes. Further, the oxygen atom of the C=O of the residue preceding Pro (i.e., the Xaa C=O) is more electronegative than those preceding other amino acids (Veis & Nawrot, 1970), with the result that this carbonyl group has an enhanced tendency to accept and form strong H-bonds. This effect is evidenced widely by its participation in Pro-containing β-turns (Smith & Pease, 1980) and γ-turns (Deber et al., 1990a).

X-ray crystallographic data from soluble proteins have provided direct information concerning the structural consequences of Pro occurrence in an α-helix. The angle by which helices in globular proteins bend due to the presence of Pro has been estimated to be 20–30° (Barlow & Thornton, 1988; Sankararamakrishnan & Vishveshwara, 1990; Richardson & Richardson, 1989), although Pro causes only a minor disruption in H-bonding in the helices (Barlow & Thornton, 1988). A proline-induced kink in a surface helix of globular proteins may function in helical packing by bending the helix around the protein core (Woolfson & Williams, 1990). Given that such structural attributes of Pro residues would be expected to be inherently independent of protein type, these observations from soluble proteins should extend to membrane-buried Pro residues. Thus, the presence of Pro-induced kinks appears consistent with the results on a membrane protein that has been studied in detail by X-ray crystallography, the photosynthetic reaction center (Deisenhofer et al., 1985), in which a proline-induced kink in helix C of the reaction center L subunit was observed. Henderson et al. (1990) used electron cryomicroscopy to obtain a high-resolution structure of bacteriorhodopsin (bR)—the light-driven proton pump of the *Halobacterium halobium* purple membrane [for a minireview, see Khorana (1988)]—and similarly observed that Pro residues, which occur in helices B (Pro_{50}), C (Pro_{91}),

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and F (Pro_{186}) of bR's seven TM helices, produced kinks in these three helices. In an analysis of Pro-induced kinks in TM helices, von Heijne (1991) noted that such helices tend to be oriented with the Pro-containing convex side packed against neighboring TM helices and suggested that Pro serves to generate bent helices that may suit specific packing motifs.

Dynamic Effects of Proline Residues in Transmembrane Helices. Pro is the only mammalian residue for which the cis peptide bond is energetically accessible, due largely to destabilization of the trans Xaa-Pro conformation relative to Xaa-Yaa trans peptide bonds (Zimmerman & Scheraga, 1976). Cis Xaa-Pro peptide bonds have been documented in a number of globular proteins (Stewart et al., 1990), where they tend to occur in preferred patterns of local sequence and can alter chain direction through "type VI" turns (Frommel & Preissner, 1990; Richardson & Richardson, 1989). The importance of Pro dynamic roles in soluble proteins is demonstrated by the involvement of Pro cis–trans isomerization in the slow phase of protein folding (Brandts et al., 1975), in giving rise to conformational heterogeneity (Evans et al., 1987; Kikuchi et al., 1988), and in the catalytic activity of peptidyl–prolyl cis–trans isomerases (PPIases) (Fischer et al., 1989; Takahashi et al., 1989). PPIases have received considerable attention since the discovery that cyclophilin, the protein target of the immunosuppressant drug cyclosporin A, is a PPIase that catalyzes the isomerization of Xaa-Pro peptide bonds (Fischer et al., 1989; Takahashi et al., 1989).

Pro dynamic roles may similarly be important in the context of membrane proteins. Brandl and Deber (1986) proposed that ligand-mediated cis–trans isomerization of intramembranous Xaa-Pro bonds may regulate transport channel opening and closing. This notion is supported by the finding that both cis and trans states are energetically accessible on the basis of computer modeling of the Pro-containing peptide segment $(Ala)_8$-Leu-Pro-Phe-$(Ala)_8$ in a low-polarity ("TM") environment (Deber et al., 1991); the potentially drastic change in protein chain direction caused by isolated cis–trans isomerization of the Xaa-Pro peptide bond in a TM helix is alleviated by adjustments to adjacent Leu ψ and Phe ϕ angles. The rate of Xaa-Pro isomerization has been observed to vary widely from several seconds to fractions of a second as a function of peptide chain length and/or the nature of adjacent residues (Brandts et al., 1975; Galardy et al., 1982; Grathwohl & Wuthrich, 1981; Lin & Brandts, 1984; Kordel et al., 1990). Interconversions in the millisecond time scale would be required for the regulation of transport channel opening and closing [e.g., Montal et al. (1990)].

In instances where intramembranous cis–trans isomerization would produce disruptive chain movement within the membrane and/or is kinetically inefficient for channel regulatory events (Deber et al., 1990a), the flexibility about the backbone angles of adjacent residues as well as interconversions between favored states at the hindered Pro ψ angle should be considered. In a ligand-activated process, Pro may convert a preexisting α-helix into a number of all-trans structures that differ essentially in the H-bonding patterns of the Pro N-adjacent carbonyl (Deber et al., 1991). This type of chain fluctuation would be particularly opportune in an environment that favors intramolecularly H-bonded structures, as exists in the low dielectric within a bilayer. The rate of interconversion would be expected to be sufficiently rapid for ligand-activated processes since it would simply require rotation about one or more single bonds. The possibility of conformational interconversion may be least partially explain the relative ease with which Pro is replaced in site-directed mutagenesis experiments [see, e.g., Alber et al., (1987, 1988)].

Determination of the Function of Transmembrane Proline Residues. (A) Structural Roles. Experimental work to examine the functional role(s) of Pro in TM helices is summarized in Table I. Khorana and his associates have undertaken an extensive study of site-directed bR mutants, in which the three TM Pro residues of bR were variously mutated (Ahl et al., 1988; Mogi et al., 1989). These prolines were not essential for bR proton pumping activity since function was not eliminated when the residues were mutated. These studies, together with electron cryomicroscopy work that demonstrates the occurrence of kinks due to Pro (Henderson et al., 1990), suggest that Pro may be involved in structural interactions between the helices. Computer modeling has been used to predict that the function of Pro in the TM helices of bR is to create rigidity so as to optimize the position of functionally important residues (Sankara-Ramakrishnan & Vishveshwara, 1989). In their review, Oesterhelt and Tittor (1989) similarly concluded that Pro serves a structural function in maintaining the rigidity of TM helices in halorhodopsin and bR. Pro was conserved in a potential dolichol binding site of yeast glycosyltransferase, and the similarity of this pocket to the retinal binding pocket in bR was noted (Albright et al., 1989).

Studies of membrane-buried prolines in other proteins also support Pro structural roles. Site-directed Pro mutants of the Ca^{2+}-ATPase of the sarcoplasmic reticulum impacted Ca^{2+} uptake (Vilsen et al., 1989). One suggestion is that Pro may participate (i.e., via the Xaa-Pro C=O) in the Ca^{2+} binding site and that Pro_{308} and Pro_{803} may insert kinks in their respective helices to position the Ca^{2+} carboxylic binding sites (Vilsen et al., 1989). Pro mutants of *Escherichia coli lac* permease implicated the functional importance of a chemical property of Pro, such as hydropathy or residue bulk (Lolkema & Puttner, 1988; Consler et al., 1991).

The structural role of TM Pro may, in some cases, result from its polar character. The absence of a proton on the Pro nitrogen has led to the description of this atom as a "proton hole" (Dunker, 1982) that may function in binding positively charged species during their translocation (Brandl & Deber, 1986). Pro mutants of the *E. coli* melibiose carrier led to the suggestion that Pro may contribute to proton/Li^+ recognition and translocation (Kawakami et al., 1988). Pro_{28} of *lac* permease (putatively in helix I) was determined to be important for substrate binding and recognition (Consler et al., 1991). Since a binding role has direct implications with respect to function, several authors studying cytochromes have favored this hypothesis; for example, Pro may function in proton abstraction (Howell, 1989) and/or interact locally with protons or ions during their transport across the membrane (Pakrasi et al., 1988).

(B) Dynamic Roles of Proline. There has as yet been no specific experimental observation of cis–trans isomerization of an Xaa-Pro peptide bond within a TM helix. Indirect evidence that isomerization does not occur has been afforded by the ease with which Pro could be mutated in several systems (Ahl et al., 1988; Lolkema et al., 1988; Mogi et al., 1989; Vilsen et al., 1989; Consler et al., 1991), although we note that the techniques employed in these studies generally assay activity but do not directly detect conformational change.

Whether or not cis–trans isomerization of TM Pro is involved directly in membrane protein dynamics, the involvement of Pro isomerization in other functions such as protein folding should be considered. Thus, the Pro_{323}-to-Ser mutation of the β-adrenergic receptor led to two smaller immunoreactive polypeptides rather than the single product usually observed,

Table I: Structural and Dynamic Effects of Membrane-Buried Proline Residues

protein	technique	proline (→ mutant)	phenotype	proposed effect(s) of TM Pro	reference
bacteriorhodopsin (*H. halobium*)	SDM[a]	P_{50} → G, A	normal chromophore and proton pumping with alterations in chromophore regeneration	may allow for structural interaction between helices	Mogi et al. (1989)
	SDM	P_{91} → G, A			Mogi et al. (1989)
	SDM	P_{186} → G, A, V, L	reduced proton pumping and altered chromophore regeneration		Mogi et al. (1989); Ahl et al. (1988)
	SDM + FTIR	P_{186} → G, A, V, L		Pro(s) undergo conformational change during the photocycle on the basis of frequency shifts	Rothschild et al. (1990)
	^{15}N label + FTIR				
	^{15}N label + FTIR				Rothschild et al. (1990)
	^{13}C NMR				Gerwert et al. (1990)
	electron cryo-microscopy			all X-Pro peptide bonds are trans	Deber et al. (1990b)
	computer modeling			inserts kink in helix	Henderson et al. (1990)
Ca^{2+}-ATPase (sarcoplasmic reticulum)	SDM	P_{308} → A, G; P_{803} → A	reduced affinity for Ca^{2+}	provides rigidity to fix position of neighboring residues	Sankara-Ramakrishnan and Vishveswara (1989)
	SDM	P_{312} → A, G	no Ca^{2+} transport	P_{308} and P_{803} may promote Ca^{2+} binding sites by participating in binding Ca^{2+} or by kinking the helices	Vilsen et al. (1989)
	SDM	P_{312} → L	7% of wild-type Ca^{2+} transport	P_{312} may cause a kink that facilitates the phosphoenzyme intermediate conformational transition	Vilsen et al. (1989); Vilsen et al. (1989)
F_1F_o-ATPase (*E. coli*)					
subunit c	SDM	P_{43} → S, A	reduced growth on glucose and less sensitivity to DCCD inhibition	P_{43} involved in functional coupling and binding of F_1 to F_o	Miller et al. (1989)
subunit a	SDM	P_{190} → Q, R		P_{190} affects F_1F_o-ATPase assembly and H^+ translocation	Vik et al. (1988)
	SDM	P_{190} → N	similar to wild type		Vik et al. (1988)
lac permease (*E. coli*)	SDM	$P_{31,123,280,327}$ → A, G	retains significant transport activity	contributes requisite hydropathy/bulk	Lolkema et al. (1988); Consler et al. (1991)
	SDM	$P_{31,123,280,327}$ → L	no lactose transport		
	SDM	$P_{61,89,97,220}$ → A, G, L	retains significant transport activity		
	SDM	P_{28} → A, G, L, S	no lactose transport	participates in substrate binding/recognition	
melibiose carrier (*E. coli*)	SDM	P_{142} → S	Li^+ resistant and no H^+ coupling	participates in recognition or transport of H^+ or Li^+	Kawakami et al. (1988)
	SDM	P_{128} → S	loss of transport activity		Kawakami et al. (1988)
	SDM	P_{122} → S	Li^+ resistant and no H^+ coupling		Yazyu et al. (1984)
β-adrenergic receptor	SDM	P_{323} → S	44- and 50-kDa immunoreactive proteins obtained rather than usual 67-kDa protein	involved in processing event such as folding	Strader et al. (1987)

[a] SDM, site-directed mutagenesis.

suggesting that Pro_{323} was involved in a receptor processing event such as folding (Strader et al., 1987). Another intriguing possibility is suggested by the *ninaA* visual transduction mutant of *Drosophila melanogaster*, which is associated with a 10-fold reduction in the level of rhodopsin (Shieh et al., 1989). The mutant protein was found to be a PPIase, and it was proposed that the protein may affect the processing of rhodopsin or that it may function as a Pro isomerase involved in the conformational changes during visual transduction (Shieh et al., 1989). Pro cis–trans isomerization is also important in the context of the protein conformational multiplicity that arises when conformers have either the cis or trans conformation at a particular residue, such as in staphylococcal nuclease (Evans et al., 1978) and in the Val_{110}-to-Pro mutant in human lyso-

zyme, which generated four components with different specific activities (Kikuchi et al., 1988). Intramembranous Pro residues could similarly establish protein conformational heterogeneity.

An expanding body of experimental evidence, obtained principally from studies that employ vibrational spectroscopy, now indicates that Pro in TM helices may, in some cases, participate in discrete conformational transitions. Rothschild et al. (1989, 1990) studied a number of site-directed bR Pro mutants (see Table I) with Fourier transform infrared (FT-IR) spectroscopy to assess structural changes occurring during the bR photocycle and concluded that one (or more) TM Pro residues undergoes a structural change; the largest changes were observed in the vibrational spectra of Pro_{186} bR mutants. These authors suggested that strains in the retinal binding pocket due to retinal isomerization may be relieved by Xaa-Pro movements (Rothschild et al., 1990). Gerwert et al. (1990) employed FT-IR to examine ^{15}N-labeled bR and observed structural changes at the peptide bonds of two Pro residues and proposed that they may function as "hinges". FT-IR has also been used to study rhodopsin, and conformational changes at one or more Pro's were similarly observed (Ganter et al., 1989). Site-directed mutation of Pro_{312} in helix M4 of the Ca^{2+}-ATPase resulted in a defective phosphoenzyme intermediate (E_1P to E_2P) conformational transition; Vilsen et al. (1989) suggested that movement of helix M4 may be central to this transition, with Pro_{312} functioning by introducing a defect in helix packing.

Conclusion. To resolve the function(s) of particular Pro residues in membrane proteins, detailed resolution of Pro in TM helices must be obtained—likely by techniques that have not traditionally been applied to membrane proteins, such as X-ray crystallography (Deisenhofer et al., 1985), and by 1-D and 2-D NMR spectroscopy (Bax, 1989). As well, conformational changes associated with ligand binding and/or photoactivation should be further characterized by techniques such as FT-IR (Braiman & Rothschild, 1988), phase-lifetime spectroscopy (Sinton & Dewey, 1988), low-temperature reversed-phase HPLC (Henderson & Mello, 1990), and Trp fluorescence quenching (Eftink et al., 1989; Jang & El-Sayed, 1989). Meanwhile, it is apparent from data in Table I and the discussion above that membrane-buried proline residues may be expected to have one or more structural and/or dynamic roles, with the function of a particular Pro mediated by its local environment (i.e., within the nonpolar bilayer) and by the requirements of its parent membrane protein. These roles stem from the capacity of Pro to exert characteristic structural, electronic, and conformational effects.

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