Implications of Threonine Hydrogen Bonding in the Glycophorin A Transmembrane Helix Dimer

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ABSTRACT The transmembrane helix of glycophorin A contains a seven-residue motif, LIxxGVxxGVxxT, that mediates protein dimerization. Threonine is the only polar amino acid in this motif with the potential to stabilize the dimer through hydrogen-bonding interactions. Polarized Fourier transform infrared spectroscopy is used to establish a robust protocol for incorporating glycophorin A transmembrane peptides into membrane bilayers. Analysis of the dichroic ratio of the 1655-cm^{-1} amide I vibration indicates that peptides reconstituted by detergent dialysis have a transmembrane orientation with a helix crossing angle of $<35°$. Solid-state nuclear magnetic resonance spectroscopy is used to establish high resolution structural restraints on the conformation and packing of Thr-87 in the dimer interface. Rotational resonance measurement of a 2.9-Å distance between the γ-methyl and backbone carbonyl carbons of Thr-87 is consistent with a *gauche*− conformation for the $\chi 1$ torsion angle. Rotational-echo double-resonance measurements demonstrate close packing (4.0 ± 0.2 Å) of the Thr-87 γ-methyl group with the backbone nitrogen of Ile-88 across the dimer interface. The short interhelical distance places the β-hydroxyl of Thr-87 within hydrogen-bonding range of the backbone carbonyl of Val-84 on the opposing helix. These results refine the structure of the glycophorin A dimer in membrane bilayers and highlight the complementary role of small and polar residues in the tight association of transmembrane helices in membrane proteins.

INTRODUCTION

The transmembrane domain of glycophorin A is largely composed of hydrophobic amino acids and has a seven-residue motif that mediates dimerization in membrane bilayers (Fig. 1). There has been considerable interest in establishing the detailed structure of the helix-to-helix contacts in the dimer interface to address the general mechanism for how hydrophobic helices associate in a sequence-specific manner in membrane environments (Lemmon and Engelman, 1994). Helix association is important for the folding of polytopic membrane proteins and for the oligomerization of membrane proteins having only a single transmembrane helix.

A striking feature of helix dimerization in glycophorin A is that it is mediated largely by hydrophobic residues (Lemmon et al., 1992a,b). Magic angle spinning (MAS) nuclear magnetic resonance (NMR) spectroscopy provided the first direct measurements of the helix-to-helix contacts in the glycophorin A transmembrane domain by demonstrating close packing of the side chain methyls of Val-80 and Val-84 against Gly-79 and Gly-83, respectively (Smith and Bormann, 1995). More recently, the structure of the helical dimer in detergent micelles has been determined by solution NMR and the dimer interface has been modeled using several unique interhelical restraints from nuclear Overhauser enhancements (NOEs) (MacKenzie et al., 1997). Four NOE restraints used in the modeling involve the only polar residue in the seven-residue dimerization motif, Thr-87 (Table 1). Interestingly, the refined structure in detergent micelles based on these restraints shows that Thr-87 does not form an interhelical hydrogen bond, but rather the β-hydroxyl group hydrogen bonds back to the carbonyl oxygen of Gly-83 on the same helix (MacKenzie et al., 1997).

The mutational studies that originally established the seven-residue dimerization motif in glycophorin A only indirectly addressed whether Thr-87 forms interhelical hydrogen bonds. The most revealing mutation, replacement of Thr-87 with valine, results in partial disruption of the helix dimer (Lemmon et al., 1992a,b). This mutation, which replaces the Thr-87 β-hydroxyl with a methyl group of roughly the same molecular volume, suggests that hydrogen bonding contributes to dimer stability. In their discussion of the mutational data, Lemmon et al. (1992b) indicated that the T87V mutation was consistent with both interhelical hydrogen bonding and close steric interactions.

The potential of threonine residues to form interhelical hydrogen bonds has general implications for the association and stability of membrane proteins. Threonines (and serines) are the most common polar residues found in the transmembrane domains of membrane proteins. This is because they are able to readily hydrogen-bond back to backbone carbonyls on the same helix (Gray and Matthews, 1984). However, these residues do not drive helix associa-

Submitted September 28, 2001 and accepted for publication November 29, 2001.

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0006-3495/02/05/2476/11 $2.00

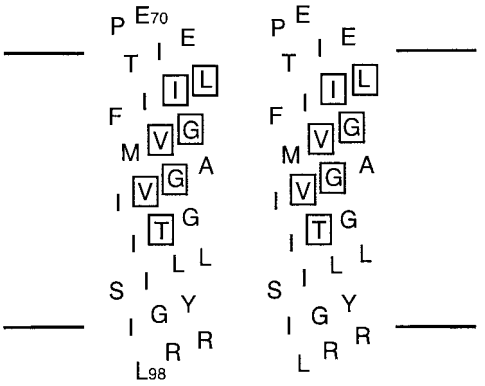


FIGURE 1 Transmembrane sequence of human glycophorin A from Glu-70 to Leu-98. The seven residues responsible for dimerization are boxed.

tion through interhelical hydrogen bonding as effectively as polar residues with longer side chains (e.g., Gln and Glu) or with more polar functional groups (e.g., Asn and Asp) (Choma et al., 2000; Zhou et al., 2001). In known crystal structures of membrane proteins, however, threonine and serine are abundant in helix interfaces, exhibit high packing values, and frequently form interhelical hydrogen bonds (Javadpour et al., 1999; Eilers et al., 2000; Senes et al., 2001). We have proposed that, when residues with small side chains (e.g., glycine and alanine) lie on the same face of an α helix as threonine and serine, the helices can pack more closely together, thereby facilitating the formation of stabilizing interhelical hydrogen bonds (Javadpour et al., 1999; Eilers et al., 2000). In the glycophorin A dimer, we have recently shown, using solid-state NMR, that close Gly-79–Gly-79 and Gly-83–Gly-83 packing occurs across the dimer interface (Smith et al., 2001). Such close glycine packing may allow Thr-87 to form interhelical hydrogen bonds.

TABLE 1 Interhelical distances and restraints involving Thr-87 from solution NMR measurements of the glycophorin A transmembrane dimer

Helix 1	Helix 2	Restraint (Å)	Modeled interhelical distance* (Å)
Val-84 (Hγ2)	Thr-87 (Hγ1)	2.1−2.7	2.0 (2.4)
Thr-87 (Hγ2)	Ile-88 (Hn)	2.2−4.5	3.8 (2.9)
Ile-88 (Hγ2)	Thr-87 (Hγ2)	2.2−3.2	3.0 (2.2)
Val-84 (Hα)	Thr-87 (Hγ1)	2.1−3.0†	3.8 (4.2)

Distances and restraints derived from observed solution NMR NOEs (MacKenzie, 1996).

*Distances are derived from the glycophorin A dimer coordinates in the Protein Data Bank (1AFO) by rotating the Val-84 or Thr-87 methyl groups to obtain a minimum ^{1}H$\cdots^1$H distance. Distances in parentheses are the minimum ^{1}H$\cdots^1$H distances obtained from the model of the glycophorin A dimer based on MAS NMR distance measurements (Smith et al., 2001).

†Restraint obtained from the 1AFO restraint file deposited in the Protein Data Bank.

In this paper, we use polarized infrared (IR) and solid-state NMR spectroscopy to establish the conformation and packing of Thr-87 in the glycophorin A transmembrane dimer. In polarized IR spectroscopy, the dichroic ratio of the 1655-cm^{-1} amide I vibration reports on the orientation of the glycophorin A helix. The helix orientation not only yields an important constraint on the crossing angle of the helices in the dimer, it provides a way to assay different methods for reconstituting the hydrophobic glycophorin A peptide into membrane bilayers. One of the motivations for the polarized IR studies is to re-visit the polarized IR measurements previously made on glycophorin A peptides, which yielded dichroic ratios of 1.66 (Bechinger et al., 1999) and 2.4 (Smith et al., 1994) using thin films. Because the observed dichroic ratio varies with film thickness in the thin film limit (Bechinger et al., 1999), it was not possible to calculate a value for the helix orientation in these studies. In the first section of this paper, we carry out a series of polarized IR measurements using thick multilayer films obtained with different reconstitution protocols. Bacteriorhodopsin (bR) is used as a control to determine the effective transition moment angle α needed for converting observed dichroic ratios into average helix orientations (see Appendix). Comparison of the dichroic ratios obtained using different reconstitution protocols identifies the best method for reconstituting the glycophorin A peptide into membrane multilayers in a homogeneous, transmembrane orientation.

In the second section of the paper, solid-state MAS NMR measurements are used to establish constraints on the packing of Thr-87 in the dimer interface of glycophorin A. One advantage of solid-state NMR is that high-resolution distances ($\pm$0.2–0.3 Å) can be measured in membrane bilayers out to $\sim$6 Å for ^{13}C$\cdots^{13}$C and ^{13}C$\cdots^{15}$N spin pairs. Two complementary MAS NMR methods are used to measure dipolar couplings between specifically ^{13}C- and ^{15}N-labeled sites in the glycophorin A transmembrane peptides. The dipolar couplings are directly related to internuclear distance and form the basis for determining the conformation and packing of Thr-87. Rotational resonance techniques for measuring ^{13}C$\cdots^{13}$C distances (Raleigh et al., 1988; Peersen et al., 1995) are used to define the conformation of the Thr-87 side chain. The Thr-87 conformation observed in the detergent micelle structure corresponds to the dominant rotamer (*gauche*−) for threonine in α-helices (Lovell et al., 2000), which allows the β-hydroxyl group to hydrogen-bond back to the i-4 carbonyl. Rotational-echo double-resonance (REDOR) NMR methods for measuring heteronuclear dipolar couplings (Gullion and Schaefer, 1989) are used to determine the interhelical distance between the γ-methyl group of Thr-87 and the backbone amide nitrogen of Ile-88. These positions are predicted to be in close proximity if the Thr-87 β-hydroxyl forms an interhelical hydrogen bond. Together the REDOR and rotational resonance NMR measurements address the structure of glyco-

TABLE 2 Polarized IR analysis of glycophorin A reconstitutions

	Method*			
	1	2	3	4
Dichroic ratio	1.9–2.2	2.4–2.6	2.6–3.2	2.8–3.4

*Method 1, cosolubilization of lipid and peptide in TFE; Method 2, same as method 1 followed by hydration, sonication and incubation above the lipid phase transition temperature; Method 3, SDS precipitation; Method 4, detergent dialysis followed by sucrose gradient purification.

phorin A dimer in the region of Thr-87, and provide insights into how hydrophobic transmembrane helices associate in membrane environments.

MATERIALS AND METHODS

Lipids and bR were obtained as lyophilized powders from Avanti Polar Lipids (Alabaster, AL) and Sigma Chemical (St Louis, MO), respectively. Isotopic ^{13}C- and ^{15}N-labeled amino acids were obtained from Cambridge Isotope Laboratories (Andover, MA) or Mass Trace (Woburn, MA).

Synthesis and purification of glycophorin A transmembrane peptides

Glycophorin A transmembrane peptides with the sequence shown in Fig. 1 were synthesized using solid-phase methods. The peptides were purified by reverse-phase high performance liquid chromatography as follows. Crude peptide (~8 mg) was dissolved in 1 mL trifluoroacetic acid, injected onto a reverse phase C4 high performance liquid chromatography column, and purified by gradient elution using millipore-filtered water (solvent A), 95% acetonitrile (solvent B) and 95% isopropanol (solvent C). The solvents each contained 0.1% trifluoroacetic acid. The initial aqueous solvent conditions (70% A, 12% B, 18% C) were gradually changed to a more hydrophobic composition (40% B, 60% C) in which the peptides elute. The elution was monitored by the optical absorbance at 280 nm. The solutions corresponding to the peaks were collected into several fractions. The fractions were then lyophilized and checked by mass spectrometry for purity.

Reconstitution of glycophorin A transmembrane peptides into membrane bilayers

To establish a reconstitution protocol for the glycophorin A transmembrane domain, several different methods were evaluated using polarized Fourier transform infrared (FTIR) spectroscopy. The first method involves cosolubilization of purified lyophilized peptide and lipid in organic solvent, trifluoroethanol (TFE) or chloroform. In this case, the glycophorin A transmembrane domain is codissolved in organic solvent with dimyristoylphosphocholine (DMPC). If the lipid–peptide solution is directly layered onto the IR plate and the solvent evaporated, the peptide forms α-helical secondary structure as indicated by the amide I frequency of 1655 cm^{-1}, but typically low dichroic ratios (1.9–2.2) are observed, indicating that the helix axis is randomly oriented relative to the surface of the attenuated total reflection (ATR) plate (Table 2).

Two variations of this method involved (1) rehydration of the lipid–peptide film, followed by brief sonication before layering on the IR plate, and (2) rehydration, sonication and incubation above the lipid phase transition. The dichroic ratio increases (2.4–2.6) if the sample is hydrated and incubated above the lipid-phase transition temperature for 12–48 h. The results without incubation are more variable.

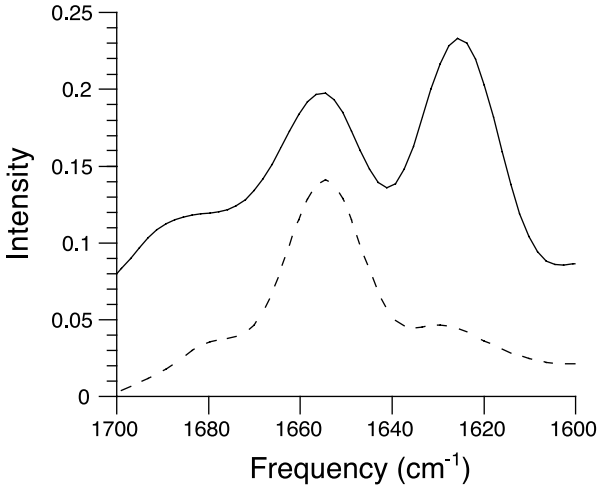


FIGURE 2 Transmission FTIR spectra of the glycophorin A transmembrane peptide. The spectrum obtained after detergent dialysis exhibits two components at 1655 and 1625 cm^{-1} (*solid curve*), which correspond to α-helical and extended β-structure, respectively. Only the helical band at 1655 cm^{-1} (*dashed curve*) is observed after sucrose gradient purification.

A second-general approach for reconstitution involves cosolubilization of the peptide, lipid, and detergent in organic solvent (usually TFE) to disperse the lipid and peptide. The TFE is removed by vacuum and the sample is rehydrated. The detergent can then be slowly removed either by dialysis or, if one uses sodium dodecylsulfate (SDS) as the detergent, by precipitation with KCl (Braiman et al., 1987). We generally found that the reconstitutions by SDS precipitation yielded high dichroic ratios (>2.8), but it was difficult to thoroughly remove the SDS, and we tended to lose peptide in the KCl precipitates. In the case of detergent dialysis, lipid, peptide (lyophilized), and detergent (octyl β-glucoside) were dissolved in TFE. Octyl β-glucoside is most often used because of its high critical micelle concentration, which makes it easier to remove by dialysis. The TFE was removed by passing a slow stream of argon gas over the solution and then placing under vacuum. The dry lipid/peptide/detergent mixture was rehydrated with phosphate buffer (10 mM phosphate and 50 mM NaCl, pH 7), such that the final concentration of octyl β-glucoside was 5% (w/v), and then stirred slowly at 4°C for at least 6 h. The octyl β-glucoside was then dialyzed for 24 h against phosphate buffer with repeated buffer changes using Spectra-Por dialysis tubing (3500-MW cutoff). The reconstituted membranes were sonicated briefly (4 × 15 s) in an ultrasonic bath and layered on a germanium crystal for IR measurements. Figure 2 presents the polarized IR spectrum of the glycophorin A transmembrane domain following detergent dialysis. The dominant peaks in the spectrum are at 1655 cm^{-1}, corresponding to α-helix and at ~1625 cm^{-1}, corresponding to extended β-structure.

It is possible to separate the two components observed in the IR spectrum by sucrose gradient ultracentrifugation on a 10–40% (w/v) gradient at 150,000 × g for 8–12 h at 5°C (palmitoleoylphosphocholine) or 15°C (DMPC). The reconstituted membranes form two discrete bands in the sucrose gradient, with the upper band having a more homogeneous appearance. The lipid-to-peptide ratio in the upper band is generally in the range of 40:1–60:1 as assessed by the intensities of the lipid carbonyl (1735 cm^{-1}) and protein amide I (1655 cm^{-1}) bands (Tamm and Tatulian, 1993). The lower band has an aggregated appearance and a lower lipid-to-peptide ratio (10:1–30:1). The sucrose in each band was removed by dialysis against phosphate buffer for 24 h. Importantly, after dialysis of the sucrose, the more homogeneous upper band exhibits only the α-helical

component (Fig. 2, *dashed curve*). Polarized IR measurements show that the peptide in the upper band has a transmembrane orientation.

For NMR measurements, the sucrose-free reconstituted membranes containing ~4 mg peptide were pelleted in an ultracentrifuge and then loaded into an NMR rotor as a very wet paste. The sample is then typically spun in an MAS rotor at 3–4 kHz for 30 min to further pellet the membranes and remove excess water. This step helps balance the rotor for high-speed MAS experiments. The level of hydration can be measured based on the intensity of the water ^{1}H resonances relative to those of the lipid and peptide (Zhou et al., 1999). The hydration levels after this procedure are typically in the range of 80–100% (w/w) water. At this level of hydration, lipid phase transition temperatures are not changed (Small, 1986).

Polarized infrared spectroscopy

Polarized ATR FTIR spectra were obtained on a Nicolet Magna 550, Protege 440, or Bruker IFS 66V/S spectrometer. Multilamellar vesicle dispersions at a concentration of 10 mg lipid/mL were spread on a germanium internal reflection element and dried using a slow flow of N_2 gas directed at an oblique angle to the ATR plate to form an oriented multilamellar lipid–peptide film. Each sample spectrum represents the average of 1000 scans acquired at a resolution of 1 cm^{-1} (bR) or 4 cm^{-1} (glycophorin A, KK-L24-KK). An appropriate background spectrum was subtracted in each case.

The dichroic ratio (R^{ATR}) is defined as the ratio between absorption of parallel ($A_\parallel$) and perpendicular ($A_\perp$) polarized light. The observed dichroic ratio is used to calculate the order parameter S_{meas} using the equation,

$$S_{meas} = \frac{E_x^2 - R^{ATR}E_y^2 + E_z^2}{E_x^2 - R^{ATR}E_y^2 - 2E_z^2}.$$

Order parameters of 1.0 and −0.5 correspond to helical orientations parallel and perpendicular to the membrane normal, respectively. The order parameter depends on the electric field amplitudes,

$$E_x = \frac{2(\sin^2\phi - n_{21}^2)^{1/2}\cos\phi}{[(1 - n_{21}^2)^{1/2}[(1 + n_{21}^2)\sin^2\phi - n_{21}^2]^{1/2}]} = 1.399,$$

$$E_y = \frac{2\cos\phi}{(1 - n_{21}^2)^{1/2}} = 1.514,$$

$$E_z = \frac{2\sin\phi\cos\phi}{[(1 - n_{21}^2)^{1/2}[(1 + n_{21}^2)\sin^2\phi - n_{21}^2]^{1/2}]} = 1.621,$$

where ϕ is the angle of incidence between the IR beam and the internal reflection element (45°), and n_{21} is the ratio between the refractive indices of the sample (n_2 = 1.43) and the internal reflection element (n_1 = 4.0) (Fringeli et al., 1989; Tamm and Tatulian, 1993; Wolfe and Zissis, 1978). These equations are based on the assumption that the thickness of the deposited film is much larger than the penetration depth (~1 μm) of the evanescent wave (Harrick, 1967). The films in our experiments are greater than ~10 μm in depth, calculated on the basis of the amount of lipid used per experiment (~2 mg), the area of the ATR plate covered (~500 mm^2), the area per lipid molecule (50–80 $Å^2$), and the thickness of a single bilayer (>50 Å). As a result, the thick film limit is applicable. This is important because the dichroic ratios observed for the amide I and II vibrations are very sensitive to the film thickness in the thin film limit (Bechinger et al., 1999).

The measured order parameter S_{meas} is related to three nested order parameters describing the average distribution of the helix angle relative to the membrane normal (S_{hel}), the orientation of the transition dipole moment for the amide I bands relative to the helix axis (S_{dip}), and disorder in the orientation of the membrane (S_{mem}),

$$S_{hel}S_{dip}S_{mem} = \left[\frac{3}{2\langle\cos^2\theta\rangle} - \frac{1}{2}\right]\left[\frac{3}{2\cos^2\alpha} - \frac{1}{2}\right]S_{mem} = S_{meas},$$

where θ is the angle between the helix director and the normal of the internal-reflection element, and α is the angle between the helix director and the transition-dipole moment of the amide I vibrational mode. For our calculations of helix orientation below, we use a value of α = 41.8° derived from parallel experiments on bR (see Appendix). The use of this value of α implies a value of S_{mem} between 0.8 and 0.9, and provides the only correction we apply for possible disorder in the sample (see Appendix).

Magic angle spinning NMR

MAS NMR measurements were made on a Chemagnetics or Bruker Avance 360 MHz spectrometer using a double-resonance probe from Doty Scientific (Columbia, SC) or Chemagnetics (Fort Collins, CO). ^{13}C spectra were acquired with ramped amplitude proton cross-polarization (Metz et al., 1994) using a contact time of 3 ms. Two-pulse phase modulation proton decoupling was used during acquisition (Bennett et al., 1995) with a field strength of 83 kHz. The recycle delay was typically 2.5–3.0 s. The NMR measurements were all carried out at a sample temperature of −10°C.

The pulse sequence and parameters for the rotational resonance experiment have been described previously (Peersen et al., 1995; Smith et al., 2001). The spinning frequency was maintained at 14,400 Hz ± 5 Hz, the n = 1 rotational resonance condition for the γ-$^{13}CH_3\cdots{}^{13}C{=}O$ spin pair. A 500-μs low-power pulse was used to selectively invert the carbonyl resonance before the variable mixing period during which magnetization exchange occurs between the two ^{13}C sites. Simulations were carried out with the program cc2Z (Levitt et al., 1990) using the anisotropy and asymmetry parameters for the carbonyl (10,630 Hz, 0.85) and methyl (1672 Hz, 0.32) carbons taken from the literature (Peersen et al., 1995).

The pulse sequence for the REDOR experiment used two ^{15}N dephasing pulses per rotor cycle and a single ^{13}C 180° refocusing pulse on the observe channel. XY8 phase cycling was used to minimize resonance offset effects (Gullion and Schaefer, 1991). REDOR experiments were carried out with 32, 48, and 64 rotor cycles. Acquisition of the S(full) and S(reduced) spectra were interleaved to help compensate for long-term spectrometer drift. The normalized echo differences, $\Delta S/S$(full), for these two experiments were analyzed using a Mathematica macro written and kindly provided to us by Terry Gullion (West Virginia University). The dipolar coupling (D) is related to the internuclear distance by the equation $D = \gamma_1\gamma_2 h/2\pi r^3$, where γ_1 and γ_2 are the gyromagnetic ratios of the coupled ^{13}C and ^{15}N spins, h is Planck's constant, and r is the internuclear distance. The calculated $^{13}C\cdots{}^{15}N$ dipolar couplings assume that reconstitution of the ^{13}C- and ^{15}N-labeled peptides in a 1:4 ratio results in 71% of the ^{13}C-labeled peptides forming ^{13}C:^{15}N heterodimers (see Results and Discussion).

RESULTS AND DISCUSSION

Orientation of the glycophorin A transmembrane helix in membrane bilayers

Table 2 summarizes the results of several different reconstitution protocols described in Materials and Methods. The method of detergent dialysis and sucrose gradient purification was found to consistently yield the highest dichroic ratios. To translate the experimentally determined dichroic ratios into helix orientation, we independently estimated the

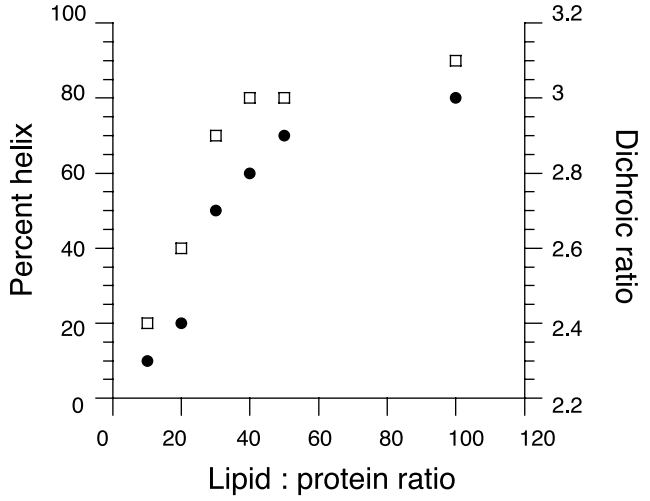


FIGURE 3 Helix content and orientation as a function of the lipid-to-peptide ratio. The percentage of the reconstituted peptide in helical secondary structure increases with an increase in the lipid:peptide ratio from 10:1 to 100:1 (*squares*). The percentage was determined by taking the ratio of the integrated intensity of the 1655-cm^{-1} band to the combined intensities of the 1625- and 1655-cm^{-1} bands. The samples were run after detergent dialysis and before sucrose gradient purification. For the helical component observed at 1655 cm^{-1} in Fig. 2, the measured dichroic ratio increases as a function of the lipid:peptide ratio (*circles*).

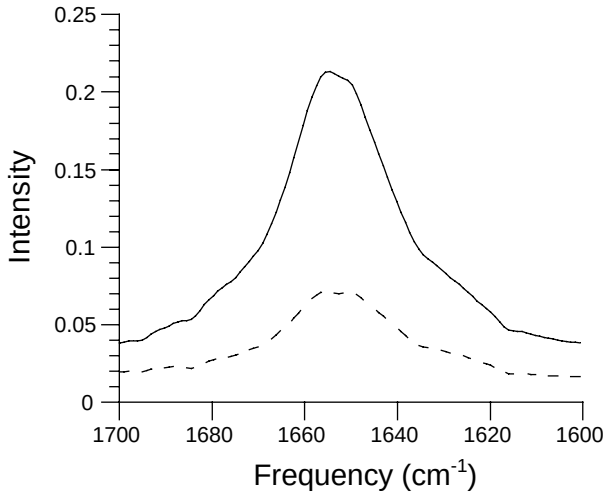


FIGURE 4 Polarized FTIR spectra of the glycophorin A transmembrane peptide using parallel (*solid line*) and perpendicular (*dashed line*) polarized light. The peptide was reconstituted by detergent dialysis and purified on a 10–40% sucrose gradient. The reconstituted membranes were layered on the ATR crystal from a D_2O solution. The dichroic ratio of the 1655 cm^{-1} band is 3.4.

angle α of the transition dipole moment from parallel measurements on bR (see Appendix). Nevertheless, several additional factors must be considered that may influence the measured dichroic ratio and, consequently, the calculated helix orientation. These include the lipid-to-protein ratio used in the reconstitution, the length and degree of unsaturation of the lipid acyl chains, and the amount of residual intensity in the amide I region due to water absorption.

Low lipid-to-protein ratios increase the NMR sensitivity by increasing the total protein in the sample. However, low ratios also decrease the amount of lipid solvating each peptide and lead to peptide aggregation. Figure 3 presents an analysis of the helical secondary structure (*squares*) and dichroic ratio (*circles*) as a function of the lipid-to-peptide molar ratio measured after peptide reconstitution. The lipid-to-peptide molar ratio was varied from 10:1 to 100:1, and the glycophorin A transmembrane peptide was reconstituted using detergent dialysis without sucrose gradient purification. As the lipid-to-peptide ratio increased, the intensity of the 1625-cm^{-1} band (extended β-strand) decreased relative to the 1655-cm^{-1} band (α-helix), indicating that increased lipid results in a higher percentage of α-helical peptide. The dichroic ratio of the helical component at 1655 cm^{-1} also increased with increasing lipid-to-protein ratio (*circles*). Sucrose gradient purification leads to higher lipid-to-protein ratios in the final samples because the gradient separates peptide that has aggregated or induced nonbilayer phase lipids. An analysis of the relative intensities of the lipid carbonyl vibration at 1735 cm^{-1} and the protein amide I vibration at 1655 cm^{-1} shows that the final lipid-to-peptide ratios are generally in the range of 40:1 to 60:1. Interestingly, very similar lipid-to-protein ratios were observed after sucrose gradient purification of rhodopsin (a 40-kDa membrane protein) reconstituted into vesicles of different unsaturated lipids by detergent dialysis of octyl β-glucoside (Jackson and Litman, 1982). Based on our analysis, we start with lipid-to-peptide molar ratios between 30:1 and 40:1, and rely on sucrose gradient purification to remove aggregated peptide.

To assess whether the lipid acyl chain length or degree of chain unsaturation has a pronounced effect on the reconstitution and transmembrane orientation of the glycophorin A peptides, three phosphatidylcholine lipids with saturated acyl chains were compared: dilaurylphosphotidylcholine, DMPC, and dipalmitoylphosphatidylcholine (DPPC). 1-palmitoyl, 2-oleoyl phosphatidylcholine provided a comparison as a phosphatidylcholine lipid having one unsaturated acyl chain. The glycophorin A transmembrane peptide was reconstituted into the pure lipid system using detergent dialysis and sucrose gradient purification starting with a lipid-to-peptide molar ratio of 40:1. There were no significant differences in the observed dichroic ratios (which fell into the range of 2.8–3.4) between the four different reconstitutions.

Finally, polarized IR spectra were obtained of the glycophorin A transmembrane peptide in DMPC after exchange with D_2O. One of the inherent problems with IR measurements of protein secondary structure is that water exhibits a broad vibration at 1640 cm^{-1} in the middle of the amide I region. However, the water contribution can be removed by exchange with D_2O. Figure 4 presents polarized IR spectra of the glycophorin A transmembrane peptide that has been

suspended in D_2O before layering on the ATR crystal. Based on the integrated intensity of the amide I band (or its Fourier deconvolution), the dichroic ratio is 3.4. This was the maximum dichroic ratio observed in four independent measurements and corresponds to a helix tilt angle θ of $\sim$17°.

The measured dichroic ratios of the glycophorin A dimer obtained with detergent dialysis and sucrose gradient purification are significantly higher than the value of 2.4 previously reported by our group (Smith et al., 1994) and the value of 1.6 reported by Bechinger et al. (1999). To directly test whether this reconstitution method provides a general, robust approach for incorporating hydrophobic peptides into membrane bilayers, we synthesized, purified, and reconstituted a model hydrophobic peptide, KK-L24-KK, into DPPC bilayers. The KK-L24-KK peptide has previously been used to address the assumptions underlying the thin film approximation in ATR-FTIR experiments (Axelsen et al., 1995). The observed dichroic ratio of the KK-L24-KK peptide was 3.1 $\pm$ 0.2, indicating that the method of detergent dialysis and sucrose gradient purification works well for generic hydrophobic peptides. For comparison, in polarized IR studies using supported monolayers, Axelsen et al. (1995) obtained a dichroic ratio of 2.09 for the KK-L24-KK peptide in DPPC using a 10:1 lipid-to-peptide molar ratio.

Conformation of the Thr-87 side chain

The χ1 torsion angle defines the conformation of the threonine side chain and the orientation of the β-hydroxyl group in the dimer interface. The intraresidue distance between the γ-methyl and backbone carbonyl in uniformly ^{13}C-labeled threonine is sensitive to the χ1 torsion angle and can be used to establish the side-chain conformation. This distance varies from $\sim$2.8 Å for the *gauche*$-$ conformation ($\chi1 \approx -60°$) to $\sim$4.0 Å for the *gauche*$+$ conformation ($\chi1 \approx +60°$). The dominant rotamer observed for threonine in α-helices has a χ1 angle of $-61°$, corresponding to the *gauche*$-$ conformation (Lovell et al., 2000).

Rotational resonance NMR was used to measure the γ-$^{13}CH_3\cdots{}^{13}C{=}O$ distance in the glycophorin A transmembrane peptide containing uniformly ^{13}C-labeled Thr-87. The MAS frequency was set to exactly match the frequency separation between the two ^{13}C resonances, i.e., the $n = 1$ rotational resonance condition. The $^{13}C{=}O$ resonance was inverted with a selective low power pulse, and magnetization exchange spectra were obtained for a series of mixing times between 100 μs and 10 ms. Several spectra were collected and averaged using a 100-μs mixing time to obtain a good initial point. The intensities of both resonances were measured as a function of the mixing time as described previously for free, uniformly ^{13}C-labeled threonine (Smith et al., 1996).

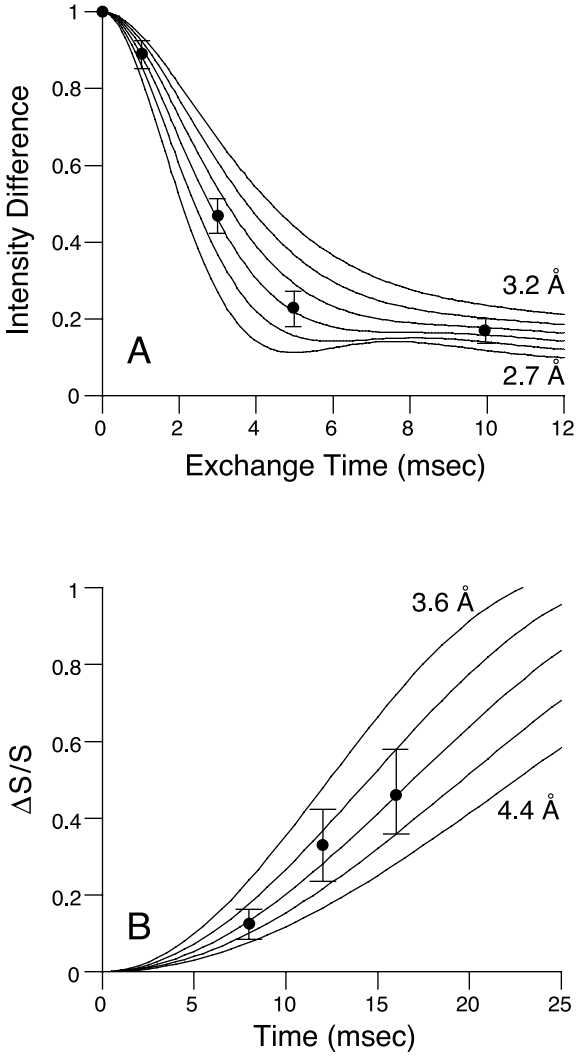


FIGURE 5 (*A*) Rotational resonance data and simulated exchange curves for Thr-87 γ-$^{13}CH_3\cdots{}^{13}C{=}O$ distance measurements. The experimental data were obtained at the $n = 1$ condition for the γ-$^{13}CH_3$ and $^{13}C{=}O$ resonances using mixing times of 100 μs, and 1, 3, 5, and 10 ms. The simulated curves were calculated using a zero quantum T_2 relaxation time of 1.6 ms and internuclear distances between 2.7 Å and 3.2 Å. (*B*) REDOR data and simulated dephasing curves for interhelical ^{15}NIle-88$\cdots$U–^{13}C–Thr-87 distance measurements. REDOR data were obtained with 32, (8 ms), 48 (12 ms), and 64 rotor cycles (16 ms). All data were corrected for reconstitution of the ^{13}C- and ^{15}N- labeled peptides in a 1:4 ratio, which results in 71% of the ^{13}C-labeled peptides forming ^{13}C:^{15}N heterodimers.

Figure 5 *A* presents the observed and simulated exchange curves for the γ-$^{13}CH_3$ and $^{13}C{=}O$ spin pair in glycophorin A reconstituted into DMPC bilayers at -10°C. The experimental data were fit using a zero quantum T_2 relaxation time of 1.6 ms, based on experiments with glycophorin A peptides containing single labels at 1–^{13}C–Phe-78 and 3–^{13}C–Ala-82 (Smith et al., 2001). The simulated curve obtained with a dipolar coupling of 255 Hz provides the best fit to the experimental data and corresponds to a γ-$^{13}CH_3\cdots{}^{13}C{=}O$ distance of 2.9 $\pm$ 0.2 Å, consistent with

a *gauche*− conformation of the $\chi 1$ torsion angle. Because the possible intraresidue distances are between 2.8 and 4.0 Å, the 2.9 ± 0.2 Å distance, at the short end of the distance range, indicates that there is limited conformational heterogeneity.

Interhelical hydrogen bonding of Thr-87

Interhelical packing of Thr-87 was addressed by measuring the distance between the Thr-87 side chain and the backbone amide of the opposing helix. In the structures of the glycophorin A dimer based on solid state (Smith et al., 2001) and solution (MacKenzie et al., 1997) NMR measurements, the closest side chain–backbone distance involving Thr-87 is between the γ-methyl group of Thr-87 and the amide nitrogen of Ile-88. These sites are 5.0 Å apart in the structure of the dimer determined in detergent micelles, but range from 3.5 to 4.0 Å apart in molecular dynamics simulations of the dimer structure obtained using solid-state NMR restraints involving Gly-79 and Gly-83 (Smith et al., 2001). The small difference between the membrane and detergent structures is critical because the interhelical spacing in the region of Thr-87 is too large in the detergent structure to allow direct interhelical hydrogen bonding. The closer packing of Thr-87 in the membrane structure results from a smaller helix crossing angle and rotation of the glycophorin A helix to place Gly-79 and Gly-83 more directly in the dimer interface.

Uniformly ^{13}C-labeled Thr-87 and ^{15}N-labeled Ile-88 were incorporated separately into glycophorin A peptides by chemical synthesis, and the peptides were combined in membrane reconstitutions in a 1:4 molar ratio. Assuming the peptides dimerize quantitatively in a head-to-head fashion, the 1:4 dilution results in 71% of the U–^{13}C–Thr-87 peptide forming heterodimers with the ^{15}N–Ile-88 peptide. This assumption is supported by previous solid-state NMR measurements of short interhelical distances (4–5 Å) between glycophorin A peptides reconstituted into membrane bilayers (Smith and Bormann, 1995; Smith et al., 2001), as well as by resonance energy transfer (Fisher et al., 1999) and analytical ultracentrifugation (Fleming et al., 1997) measurements in detergent micelles, which yield dimer dissociation constants of 80 and 240 nM, respectively.

REDOR spectra were obtained with 32, 48, and 64 rotor cycles using a MAS frequency of 4000 ± 5 Hz. Figure 6 presents the full and reduced echo spectra of U-^{13}C Thr-87 glycophorin A complexed with 1-^{15}N Ile-88. The reduced echo spectrum was obtained with 48 rotor cycles of dephasing pulses on the ^{15}N channel. The full and reduced spectra are overlaid to highlight the small but diagnostic differences caused by the dephasing pulses. The dominant resonances in the spectrum result from natural abundance ^{13}C from the lipid and protein. The γ-methyl resonance from Thr-87 is observed at 19.6 ppm (marked with an asterisk), whereas the Cα and Cβ resonances are observed as a broad band at

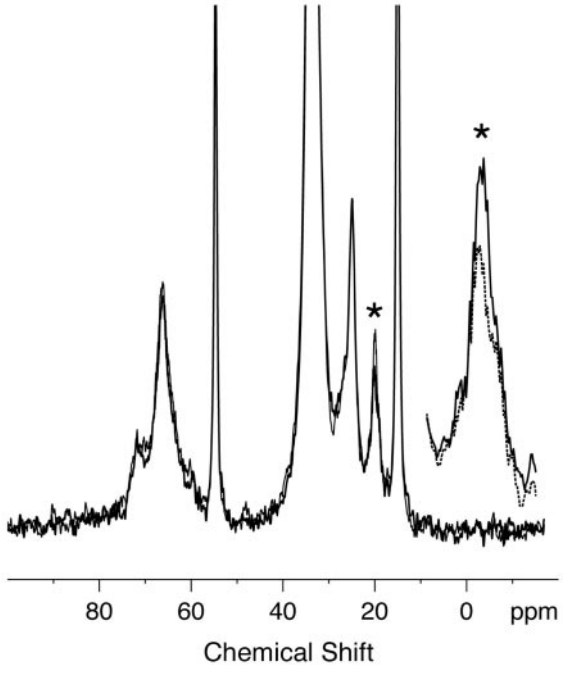


FIGURE 6 ^{13}C REDOR spectra of U–^{13}C Thr-87-labeled glycophorin A complexed with ^{15}N–Ile-88-labeled glycophorin A. The full and reduced spectra are overlaid. The inset shows the γ-CH_3 resonance at 19.6 ppm. The reduced spectrum was obtained with 48 rotor cycles of ^{15}N dephasing pulses. The MAS frequency was maintained at 4000 Hz ± 5 Hz. The proton decoupling field strength was 83 kHz.

~66 ppm. The most significant difference is observed in the γ-CH_3 resonance at 19.6 ppm, and is shown in the inset. The normalized echo difference, $\Delta S/S$(full), was observed to be 0.23 using 48 rotor cycles.

The normalized echo difference, $\Delta S/S$(full), can be related to the dipolar coupling by simulating the REDOR dephasing curve (Fig. 5 *B*). The measured $\Delta S/S$(full) values after correcting for the reconstitution ratio were 0.13, 0.33, and 0.46 for 32, 48, and 64 rotor cycles, respectively. The data corresponds to an effective dipolar coupling of 45 Hz and an internuclear distance of 4.0 ± 0.2 Å. Importantly, if there is a distribution of monomers and dimers in the reconstituted membranes, the actual internuclear distance would be shorter than 4.0 Å because both the monomers and dimers contribute to the S(full) intensity, but only the dimers are responsible for ΔS intensity. The 4.0-Å distance is distinctly smaller than the 5.0-Å distance predicted by the NMR structure in detergent micelles, and is consistent with tight packing of the γ-methyl group with Ile-88 and interhelical hydrogen bonding of the β-hydroxyl group of Thr-87 across the dimer interface.

Structure of the glycophorin A dimer

Figure 7 presents a molecular model of the glycophorin A dimer in the region of Thr-87. The model was previously

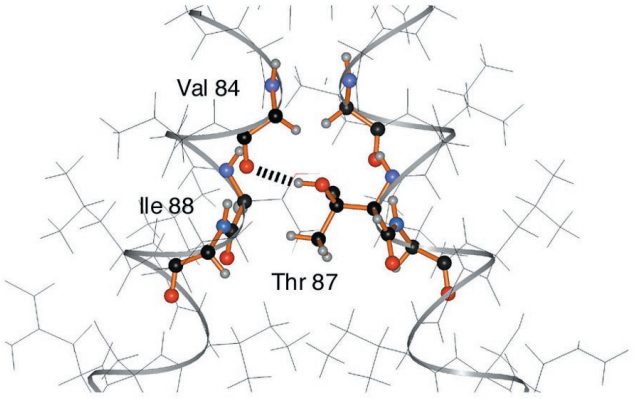


FIGURE 7 Molecular model of the glycophorin A structure showing the proposed structure of Thr-87 in the dimer interface of glycophorin A. The dimer model was generated using the computational search algorithm developed by Brünger and Adams (Adams et al. 1996) and the following NMR restraints (Smith et al.; 2001): Gly-79 ($^{13}CH_2$)···Gly-79 ($^{13}C{=}O$), 4.1 Å; Gly-83 ($^{13}CH_2$)···Gly-83 ($^{13}C{=}O$), 4.3 Å; Gly-79 ($^{13}CH_2$)···Ile-76 ($^{13}C{=}O$), 4.8 Å; Gly-83 ($^{13}CH_2$)···Val-80 ($^{13}C{=}O$), 4.3 Å; Gly-79 ($^{13}C{=}O$)···Val-80 ($^{13}CH_3$), 4.0 Å; Gly-83 ($^{13}C{=}O$)···Val-84 ($^{13}CH_3$), 4.0 Å.

developed using molecular dynamics simulations and distance restraints involving Gly-79 and Gly-83 (Smith et al., 2001), but not Thr-87. In the simulations, the Gly restraints result in structures exhibiting hydrogen bonding between the Thr-87 side chain hydroxyl group and the backbone carbonyl of Val-84 across the dimer interface. The distance between the γ-methyl group of Thr-87 and the amide nitrogen of Ile-88 ranges from 3.5 to 4.0 Å consistent with the interhelical REDOR measurement of 4.0 Å. The simulated structures are also consistent with the NMR results on the conformation of the Thr-87 side chain. Interestingly, the $\chi 1$ torsion angle is *gauche*− in both the membrane and detergent structures, indicating that this conformation can accommodate both intrahelical and interhelical hydrogen bonding.

The polarized IR measurements of the dichroic ratio provide an important constraint on the dimer structure, namely the crossing angle between the helices. The helix tilt angle θ measured for the glycophorin A helix is ~17° (relative to the bilayer normal), which corresponds to a crossing angle between the helices in the dimer of 34°. The helices in both the detergent and membrane structures have right-handed crossing angles. However, the crossing angle is predicted to be slightly less (~35°) in the membrane structure than in the detergent structure (~40°).

The structure in Fig. 7 is also consistent with the mutational results placing the seven residues in the dimer interface, and with the NOE correlations involving Thr-87, which provided distance restraints for modeling the dimer in detergent micelles (Table 1). Two of the restraints involve contacts between Ile-88 and Thr-87 across the dimer interface.

The differences in the transmembrane dimer structures based on solid-state MAS and solution-state NMR measurements may originate from at least two sources. First, detergents may not perfectly mimic membrane bilayers. The interior of detergent micelles are more aqueous than the interior of membrane bilayers. As a result, in micelles Thr-87 may be solvated, and interhelical hydrogen bonding, if present, may be mediated by water. Mackenzie and Engleman (1998) point out that polar residues may be destablilizing in detergents because the helix can unravel and hydrogen bonds can be made with water. Second, detergent micelles and membrane bilayers differ in their geometry. Spherical micelles may favor dimer structures with larger crossing angles than in planar bilayers.

Role of threonine in helix association

The structural studies on glycophorin A raise the general question of the role of small (Gly and Ala) and polar residues (Ser and Thr) in the association of transmembrane helices. The importance of polar residues in driving transmembrane helix association has been highlighted in a series of recent papers investigating model hydrophobic peptides (Choma et al., 2000; Gratkowski et al., 2001; Zhou et al., 2001). The general finding has been that Asn, Asp, Gln, and Glu are the most effective residues in mediating helix association. Ser and Thr are largely ineffective, ranking close to Leu. However, the polyleucine or "leucine zipper" peptides used in these studies have large bulky side chains making it difficult for Thr and Ser, which have small side chains, to contact one another. Moreover, the more polar Asn, Asp, Gln, and Glu residues are found to occur only rarely in the hydrophobic regions of known membrane protein sequences, indicating that these residues do not typically mediate helix association. In contrast, we have recently shown that Ser and Thr are common and tightly packed in the helix interfaces of membrane proteins of known structure (Eilers et al., 2000).

Perhaps most revealing of the role of threonine hydrogen bonding in stabilizing the glycophorin A dimer is the study by Russ and Engelman (2000) to establish which residues can substitute for the seven key interfacial amino acids in the dimer. In a screen of transmembrane domains that form high-affinity homo-oligomers based on the right-handed dimerization motif of glycophorin A, a randomized sequence library (LeuLib) yielded 47 of 49 high-affinity isolates with glycine at positions 79 and 83. Of these 47 isolates, there were 27 with threonine at position 87. None of the high-affinity isolates had valine at position 87. Valine is isosteric with threonine, but lacks the β-hydroxyl group, and would be expected to substitute for threonine if only van der Waals interactions were important in stabilizing the helix dimer. All of the other isolates had serine, glycine, or

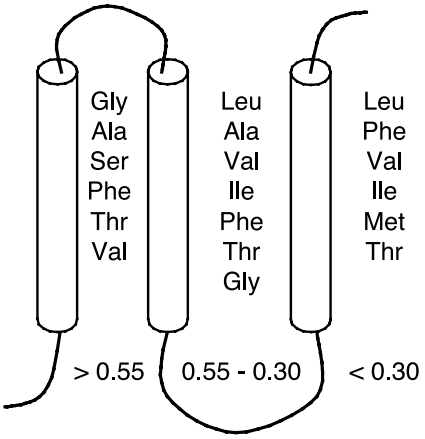


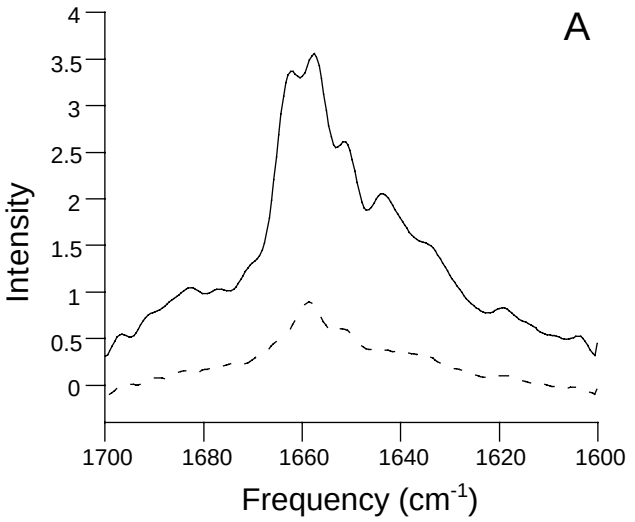
FIGURE 8 Occurrence of amino acids in helical membrane proteins based on packing. The figure lists amino acids with high (>0.55), intermediate (0.55–0.30) and low (<0.30) packing values in order of abundance (Eilers et al., 2000). Four of the five most abundant amino acids in membrane proteins with packing values greater than 0.55 have small or polar side chains. These four amino acids have the highest propensity for occurring in helix-to-helix interfaces in helical membrane proteins (Eilers et al., 2002). Only amino acids with occurrences greater than 5% are listed.

alanine at position 87, emphasizing the role of small and polar residues.

Figure 8 highlights the high occurrence of small and polar residues observed in the helix interfaces of membrane proteins by listing the most abundant residues with high (>0.55), intermediate (0.55–0.30) and low (<0.30) packing values (Eilers et al., 2000, 2002). The high occurrence and high propensity of small and polar residues in helical membrane proteins compared to soluble α-bundle proteins has led us to propose that small residues allow close packing of transmembrane helices and facilitate the formation of interhelical side chain–side chain or side chain–backbone hydrogen bonds (Javadpour et al., 1999; Eilers et al., 2000). The structure of the transmembrane interface of glycophorin A with Thr-87 in a position to form interhelical hydrogen bonds provides strong support for this proposal. The importance of this study is that the glycophorin A system is simple because only seven residues are involved in stabilizing the dimer structure, and the measurements can be made in membrane bilayers. The most closely packed residues in the glycophorin A interface, Gly and Thr, are abundant in the interfaces of polytopic membrane proteins, suggesting that similar interactions occur in these systems.

APPENDIX: POLARIZED IR OF BACTERIORHODOPSIN

The polarized FTIR studies have led us to re-evaluate the value of the angle (α) between the helix director and the transition dipole moment of the amide I vibrational mode. Values for α in the literature range from 29 to 40° (Bradbury et al., 1962; Miyazawa and Blout, 1961; Tsuboi, 1962). In calculating helix orientations, higher estimates of α result in higher order parameters and lower helix tilt angles (Bechinger et al., 1999; Citra and



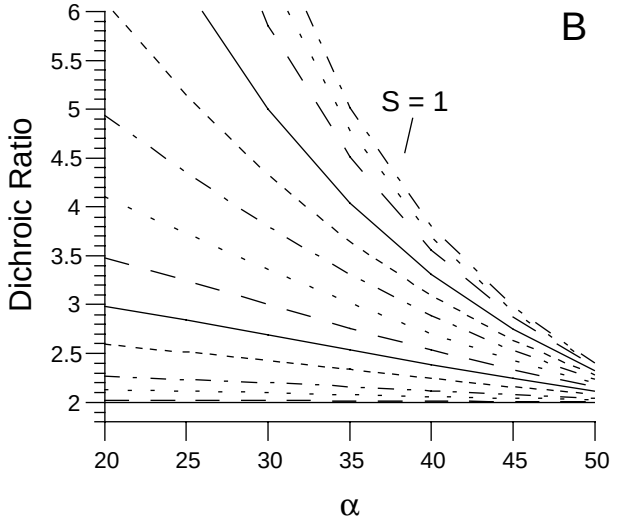


FIGURE 9 (*A*) Polarized ATR-FTIR spectra of bR. Fourier self-deconvolution of the amide I region from 1600–1700 cm^{-1} obtained with parallel and perpendicular polarized light. (*B*) Dependence of the dichroic ratio (R^{ATR}) on the transition moment orientation α. The calculated curves are for order parameters varying from $S = 1$ to $S = 0$.

Axelsen, 1996). The difficulty in determining α has generally been associated with two factors: there is no robust model membrane protein having known secondary structure and orientation, and the measured dichroic ratio depends on the thickness of the sample. For our studies, we have used bR as a model system for analyzing polarized IR data. The most compelling reasons for using bR as a model system are that: the structure is known (Grigorieff et al., 1996; Luecke et al., 1999), the protein is readily available from a commercial source, and purple membrane is a two-dimensional lattice of bR that orients readily (Rothschild and Clark, 1979).

Figure 9 *A* presents the ATR-FTIR spectra of bR between 1700 and 1600 cm^{-1} obtained with 0° and 90° polarization. The amide I vibration is centered at 1662 cm^{-1} and can be decomposed into two components at 1663 and 1658 cm^{-1} using Fourier self-deconvolution. Curve fitting of the bR 90° and 0° IR spectra allows us to determine the dichroic ratio of the intense 1658-cm^{-1} component alone. The assumption is that this band is

due to the transmembrane α_I helices in bR. The observed dichroic ratio of the 1658-cm^{-1} component is 3.5, similar to that determined previously using ATR methods (Cabiaux et al., 1997). From the refined structure of bR, the average tilt angle of the transmembrane helices is 14° (Grigorieff et al., 1996). This leads to a value of 41.8° for the transition moment angle α using the equations in Materials and Methods. This represents an upper limit for α because the observed dichroic ratio may be reduced due to disorder in membrane orientation.

Figure 9 *B* presents a plot of the dichroic ratio as a function of the transition moment angle α for different effective order parameters. As the membrane order parameter goes from $S = 1$ to $S = 0$, the value of α appears to decrease for a fixed dichroic ratio. Disorder in membrane and helix orientation in layered purple membrane has previously been estimated from measurements of the mosiac spread (Rothschild and Clark, 1979), which yield an effective order parameter of S_{mem} between 0.8 and 0.9, which corresponds to values for α between 38.5° and 40.4°, which brackets the value of 39° previously determined by Tsuboi (1962). This analysis supports the use of the "thick film" approximation and values used for the refractive indices of the phospholipid bilayer. More recently, Marsh et al. (2000) have used a method based on analyzing the total integrated intensities of the parallel and perpendicular polarized components of the amide I band of an α-helical copolymer, poly(γ-methyl-L-glutamate)$_x$-co-(γ-*n*-octadecyl-L-glutamate)$_y$. They estimate an angle of 38° for the amide I transition moment relative to the helix axis. Importantly, this method is not strictly dependent on the orientation of the polymer relative to the ATR element, only that the compound is strictly α-helical.

Finally, for our studies, we assume that membrane disorder in the glycophorin A reconstitution is at least equal to that of bR (i.e., the purple membrane sheets that contain bR are likely to orient much better than the membrane vesicles reconstituted with glycophorin A). The most common method for establishing the order of the membranes is by measuring lipid order parameters. Lipid order parameters are obtained from the lipid methylene symmetric and asymmetric stretching modes. However, we generally do not correct for membrane disorder based on the observed lipid order parameters because we often observe situations in which the lipid order parameters are very good, but the helix orientation is poor. Instead, we make measurements of several independent reconstitutions, base our calculations on the maximum observed dichroic ratio, and use the value of $\alpha = 41.8°$ taken from the measurements on bR that are run in parallel.

This work was supported by a grant to S.O.S. from the National Institutes of Health (GM-46732). We gratefully acknowledge the W.M. Keck Foundation for support of the NMR facilities in the Center for Structural Biology at Stony Brook.

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