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Determination of helix–helix interactions in membranes by rotational resonance NMR

(glycophorin A/magic angle spinning)

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ABSTRACT **Dimerization of human glycophorin A in erythrocyte membranes is mediated by specific interactions within the helical transmembrane domain of the protein. Rotational resonance NMR provides a unique approach for obtaining high-resolution structural data in membrane systems and has been used to establish intermolecular contacts in the glycophorin A dimer by using hydrophobic peptides that correspond to the transmembrane sequence. Magnetization exchange rates were measured between [^{13}C]methyl labels in the hydrophobic sequence -G^{79}-V^{80}-M^{81}-A^{82}-G^{83}-V^{84}- located in the middle of the transmembrane domain and specific [^{13}C]carbonyl labels along the peptide backbone across the dimer interface. Significant magnetization exchange was observed only between V^{80} ($^{13}CH_3$) and G^{79} ($^{13}C{=}O$) and between V^{84} ($^{13}CH_3$) and G^{83} ($^{13}C{=}O$), indicating that these residues are packed in the dimer interface in a "ridges-in-grooves" arrangement.**

Membrane proteins frequently span the cell membrane in α-helical stretches of hydrophobic amino acids. Helix–helix interactions are important in stabilizing the folded form of a protein with multiple membrane-spanning domains (1) and have been shown to drive the dimerization of proteins having only a single transmembrane domain (2–4). High-resolution structures that reveal helix packing arrangements and specific contacts have been reported for only a few membrane proteins because of problems associated with obtaining well-ordered crystals for diffraction studies (5–7). Magic angle spinning (MAS) NMR methods provide an alternative approach for structural measurements on membrane systems. MAS yields high-resolution NMR spectra of membrane proteins in bilayer environments (8), and in the past 5 years several different strategies have been developed for measuring weak dipolar couplings in MAS experiments (9–15). Internuclear distances are derived from measurements of dipolar couplings providing constraints for generating and evaluating structural models. Rotational resonance (RR) is a MAS NMR approach that selectively restores homonuclear dipolar couplings by spinning the sample so that an integral multiple of the MAS frequency is equal to the chemical shift difference ($\Delta\omega$) between two NMR resonances (9, 12). In this paper, RR NMR distance measurements are used to establish helix–helix contacts between peptides corresponding to the transmembrane (TM) domain of human glycophorin A in bilayers.

Glycophorin A is an integral membrane protein that spans erythrocyte cell membranes in a single stretch of 23 predominantly hydrophobic amino acids (16). The TM sequence forms a well-defined folded domain that is bounded by charged glutamate or arginine residues and is responsible for dimerization of the protein (4, 17). The TM domain itself contains no charged groups, indicating that dimerization is mediated by hydrogen bonding and/or van der Waals interactions. Silverberg *et al.* (18) found that methyl carboxylation of methionine-81 in the TM domain prevented dimerization, while recent mutagenesis studies have shown that dimerization can be disrupted by conservative changes of several specific hydrophobic amino acids in the TM sequence, although not M^{81} (19, 20). Direct experimental evidence establishing which residues are packed in the dimer interface and their arrangement can be obtained by RR NMR.

MATERIALS AND METHODS

Peptides (29 residues long) were synthesized incorporating ^{13}C-labeled amino acids and purified by reverse-phase HPLC as described (21). The sequence NH_3-PEITLIIFG^{79}V^{80}M^{81}-A^{82}G^{83}V^{84}IGTILLISYGIRRLI-COOH incorporates the TM domain of the protein and the numbering scheme corresponds to that of human glycophorin A. Peptides bearing single ^{13}C labels in the side-chain methyl groups of V^{80}, M^{81}, A^{82}, and V^{84} and the backbone carbonyl groups of G^{79} and G^{83} were synthesized along with a peptide containing two ^{13}C labels at the backbone carbonyl groups of M^{81} and A^{82}. The peptides were reconstituted into ^{2}H-labeled dimyristoyl phosphatidylcholine ([^{2}H]DMPC) (Avanti Polar Lipids) by detergent dialysis. Pure peptide and DMPC were separately dissolved in 2% octyl β-glucoside and then combined in a 20:1 lipid/peptide molar ratio, typically with 15 mg of peptide, and stirred slowly overnight at 4°C. The octyl β-glucoside was dialyzed with Spectra-Por 3 dialysis tubing (M_r 3000 cutoff) for 1–2 days against phosphate buffer (10 mM phosphate/150 mM NaCl, pH 7). The samples were then pelleted, made up to a vol of ≈3 ml, and sonicated in 3–5 freeze–thaw cycles. The resulting vesicles were purified by centrifugation overnight at 80,000 × *g* on 20–70% sucrose gradients. Homogeneous bands containing peptide were collected and washed twice with buffer. The membranes were pelleted and transferred into a 5-mm NMR rotor. The NMR data were collected on these hydrated multilamellar dispersions at either −50°C or 5°C. Both temperatures are below the phase transition temperature of pure DMPC. Lowering the temperature reduces rotational diffusion of the peptides, ensuring that the full dipolar couplings are measured in the RR experiment.

Peptides with a single [^{13}C]methyl label were reconstituted into [^{2}H]DMPC bilayers in a 1:5 molar ratio with peptides ^{13}C-labeled at either one or two backbone carbonyl sites. This ensures that >90% of the [^{13}C]methyl-labeled peptides that form dimers are paired with a [^{13}C]carbonyl-labeled peptide. The abundant $^{13}C{=}O$ carbonyl resonance is selectively inverted with a low-power 500-μs pulse, and the intensity of only the $^{13}CH_3$ resonance is monitored during the magnetization exchange period since it is difficult to accurately determine the intensity of the nonexchanging $^{13}C{=}O$ population.

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Abbreviations: DMPC, dimyristoyl phosphatidylcholine; MAS, magic angle spinning; RR, rotational resonance; TM, transmembrane.

MAS NMR experiments were performed on a Chemagnetics CMX 360-MHz spectrometer with a 5-mm high-speed double resonance probe from Doty Scientific (Columbia, SC). The sample spinning speed was kept constant to within 5 Hz. The pulse sequence used for the RR NMR experiments has been described and uses variable amplitude cross polarization during the contact time (21). The decoupling power was set to a field strength of 83 kHz during the variable delay and acquisition periods.

RESULTS AND DISCUSSION

The strategy for establishing which residues are packed in the dimer interface of glycophorin A by RR NMR has been to target one turn in the helical transmembrane sequence from G^{79} to V^{84}. We have previously shown by circular dichroism and by Fourier transform infrared and RR NMR spectroscopy that the glycophorin A TM peptides are helical and oriented largely perpendicular to the bilayer plane (21). The distance between two ^{13}C labels is estimated by the intensity changes observed in a magnetization exchange experiment with RR to enhance the exchange rate. In the intermolecular experiments described below, magnetization exchange rates were measured between the [^{13}C]methyl groups of V^{80}, M^{81}, A^{82}, and V^{84} and the backbone [^{13}C]carbonyls of G^{79}, M^{81}, A^{82}, and G^{83}.

Fig. 1 presents RR NMR spectra of glycophorin A peptides $^{13}CH_3$-labeled at V^{80} reconstituted in [^2H]DMPC with peptides $^{13}C{=}O$ labeled at G^{79}. Only the upfield region of the spectrum containing the V^{80} $^{13}CH_3$ resonance is shown. The spectra were obtained at the $n = 2$ resonance condition (ω_r = 6.842 kHz) at mixing times of 0.5, 5, 10, 20, and 30 ms. Deuteration of the lipids reduces the natural abundance ^{13}C signals from the acyl chain carbons in the ^1H-^{13}C cross polarization experiment by eliminating the directly bonded ^1H spins. Nevertheless, natural abundance signals from the peptides are observed in the region between 0 and 40 ppm along with residual signals from the lipid methylene and methyl carbons resulting from cross polarization with protons on the transmembrane peptide. The assignment of the V^{80} resonance is readily made by comparison with the spectrum obtained from unlabeled peptide (21). The V^{80} resonance shows a clear loss of intensity as a function of time, while those resonances not at RR do not change in intensity.

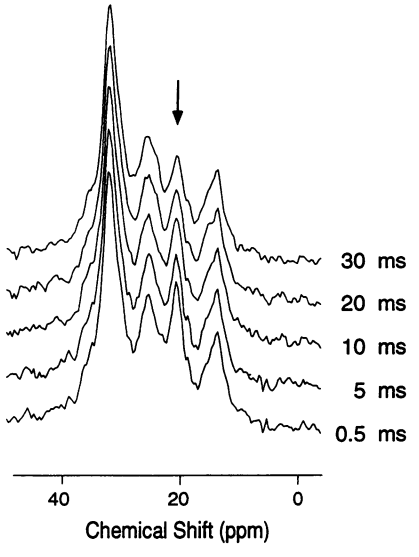

FIG. 1. RR NMR spectra of the glycophorin A TM domain in membrane bilayers illustrating intermolecular magnetization transfer from the backbone $^{13}C{=}O$ of G^{79} to the side-chain $^{13}CH_3$ of V^{80}. Only the upfield region of the spectrum containing the V^{80} $^{13}CH_3$ resonance (arrow) is shown. The spectra at mixing times of 0.5, 5, 10, 20, and 30 ms were obtained at the $n = 2$ resonance condition spinning at 6.842 kHz. Each spectrum represents the average of 2048 scans and is processed with 25-Hz exponential line broadening. Temperature was maintained at −50°C to restrict rotational motion of the dimer. Similar results were obtained at 5°C.

There are several combinations of ^{13}C-labeled peptide experiments that are possible. We present data from the four combinations that would yield close ^{13}C contacts in symmetric dimers. The integrated intensity changes of the methyl resonances for V^{80}, M^{81}, A^{82}, and V^{84} are plotted in Fig. 2 for each of these four reconstitutions both on (solid symbols) and off (open symbols) RR. The off RR curves serve as controls for estimating the intensity changes that are not due to the RR process. The largest RR-driven changes are observed for the V^{80} ($^{13}CH_3$)/G^{79} ($^{13}C{=}O$) reconstitution, while the M^{81} and A^{82} curves show no significant transfer over the off RR measurements. One other combination of peptides [A^{82} ($^{13}CH_3$)/G^{83} ($^{13}C{=}O$)] was run but did not exhibit significant transfer.

The observation of magnetization exchange between G^{79} and V^{80} and between G^{83} and V^{84} on different peptides provides direct evidence that these residues are in the dimer interface and also confirms that the glycophorin A TM domains associate in membrane bilayers. Previous measurements of glycophorin dimerization have largely been carried out in sodium dodecyl sulfate micelles (4, 19, 20). Only rough distance estimates, however, can be derived from these experiments since the extent of dimerization is not known. An upper limit on the distance estimate is ≈6 Å for these $n = 2$

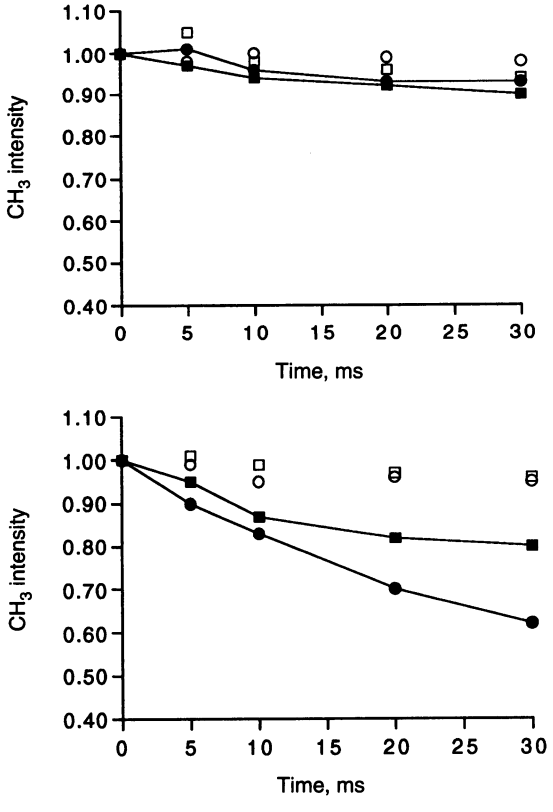

FIG. 2. RR NMR magnetization transfer curves for the region between G^{79} and V^{84} in the glycophorin A TM domain. Intensity changes observed in the $^{13}CH_3$ resonances of V^{80}, M^{81}, A^{82}, and V^{84} are plotted as a function of the magnetization exchange time. Experiments both on (solid symbols) and off (open symbols) RR are shown. (*Upper*) A^{82} (■) and M^{81} (●) methyl-labeled peptides reconstituted with peptides bearing double $^{13}C{=}O$ labels at M^{81} and A^{82}. (*Lower*) V^{80} (●) and V^{84} (■) methyl-labeled peptides reconstituted with peptides bearing single $^{13}C{=}O$ labels at G^{79} and G^{83}, respectively.

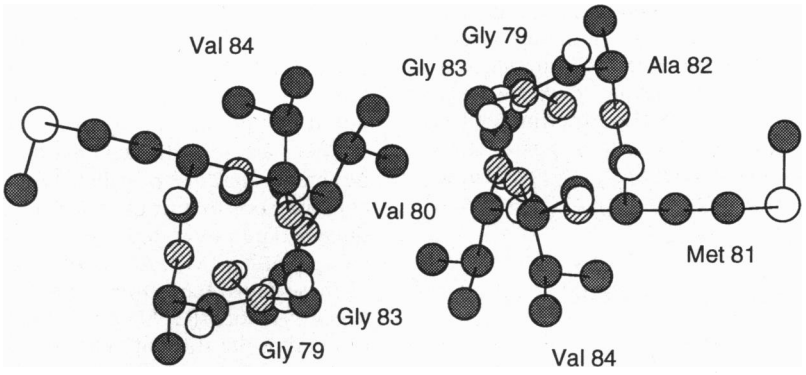

FIG. 3. Molecular model of the region between G^{79} and V^{84} of the glycophorin dimer suggesting a ridges-in-grooves packing arrangement.

experiments; RR-driven intensity changes would not be observed for longer internuclear distances. A lower limit on the distance estimate of 2–3 Å corresponds to the $^{13}CH_3$ and $^{13}C{=}O$ groups being in van der Waals contact. The intensity changes observed in the V^{80} methyl resonance would correspond to a V^{80}–G^{79} distance of 5.0 ± 1 Å based on a simple comparison with RR curves of $^{13}CH_3$–$^{13}C{=}O$ groups at known distances in crystalline model peptides (22, 23) if one assumes that the monomer–dimer equilibrium strongly favors dimer formation. Fluorescence resonance energy transfer measurements between labeled glycophorin A TM peptides support this assumption and argue that the peptides dimerize in a parallel fashion (24). The uncertainty in the distance estimate given above arises from several factors. First, the magnetization exchange rates depend on the relative orientations of the $^{13}CH_3$ and $^{13}C{=}O$ groups at the $n = 2$ RR condition (9, 22). These orientations are not independently known, although the carbonyl is constrained to lie roughly along the helix axis. Second, only in the case of the alanine $^{13}CH_3$ group is the side-chain position restricted relative to the peptide backbone. For valine and methionine, the distance measurements reflect an average over all side-chain conformations, although valine is exceptional in having predominantly just one rotamer population (25). The most significant drawback of the valine measurements is that only one of the two valine methyl groups bears a ^{13}C label. Consequently, the distance estimated above reflects the average distance between the $^{13}C{=}O$ group and the two valine methyl groups.

Fig. 3 presents a model of the helix–helix interactions in the region of the transmembrane dimer from G^{79} to V^{84} that is consistent with the NMR measurements. The V^{80} and V^{84} side chains pack against G^{79} and G^{83}, respectively, in a "ridges-in-grooves" geometry, a packing arrangement that has been described by Chothia *et al.* (26). In their analysis of known protein structures, a preference is found for coiled-coil geometries having helix crossing angles in the range of −27° and −74°. The NMR data are consistent with mutagenesis results which show that dimerization is sensitive to conservative changes at G^{79}, V^{80}, G^{83}, and V^{84}. For example, substitution of these residues with alanine either completely or partially disrupts dimerization, while substitution of M^{81} with alanine has no effect (19, 20). The mutagenesis data (19, 20) and modeling studies using molecular dynamics simulations (27) both indicated that the glycophorin A TM domain can be modeled as a right-handed coiled-coil having ≈3.9 residues per helical turn and a crossing angle of −30°. This coiled-coil structure places the residues G^{79}, V^{80}, G^{83}, and V^{84} more closely together in the dimer interface than is possible with straight canonical α-helices packed against one another. The model shown in Fig. 3 incorporates the right-handed coiled-coil geometry but differs from that previously proposed by rotating the helices to bring the V^{80} and V^{84} side chains into the dimer interface. More recent simulations of the glycophorin dimer agree with this packing arrangement (P. Adams and A. Brünger, personal communication). The closest V^{80} ($^{13}CH_3$) ↔ G^{79} ($^{13}C{=}O$) and V^{84} ($^{13}CH_3$) ↔ G^{83} ($^{13}C{=}O$) distances are calculated to be 4.0 and 4.1 Å, respectively.

Lemmon *et al.* (28) have shown that seven residues contribute directly to dimerization of glycophorin A. Besides the four residues mentioned above, the critical residues include L^{76}, I^{77}, and T^{87}. RR distance measurements to further refine the structural model presented in Fig. 3 and to determine how these three additional residues pack in the dimer interface remain to be done. One key question is whether the T^{87} hydroxyl group hydrogen bonds across the dimer interface or to the same helix. Besides T^{87}, the only other polar residues in the TM sequence are S^{92} and Y^{93}. These would be predicted to face the lipids in the right-handed coiled-coil model. We have previously observed magnetization exchange between the ^{13}C OH label of Y^{93} and a $^{13}CH_2$-labeled site on dipalmitoyl phosphatidylcholine consistent with this model (29). Tyrosine and tryptophan residues appear to be favored at the membrane interface of integral membrane proteins (30), and it is interesting to note that tyrosine substitutions disrupt dimerization even at those positions—for instance, A^{82}—that are not in the dimer interface (19, 20).

In summary, the observations of magnetization exchange between G^{79} and V^{80} and between G^{83} and V^{84} across the dimer interface constrain the structure of the glycophorin A dimer. They rule out the possibility that M^{81} is in the interface as suggested by methyl carboxylation of this residue (18). The estimated distances are consistent with more recent simulations of a right-handed coiled-coil structure for the dimer that position the valine side chains in the dimer interface and not splayed out from the interface as previously suggested (26). These intermolecular RR measurements in a membrane environment provide the foundation for studying helix–helix interactions in a range of other membrane proteins, such as hydrogen-bonding interactions in the neu receptor TM domain (31, 32) or electrostatic interactions in T-cell receptor complexes (3). These measurements are currently most effective for studying specific interactions at sites that can be specifically labeled. Two-dimensional NMR methods or isotope-directed editing will be needed to extend these types of distance measurements to multiply labeled proteins.

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