Communication

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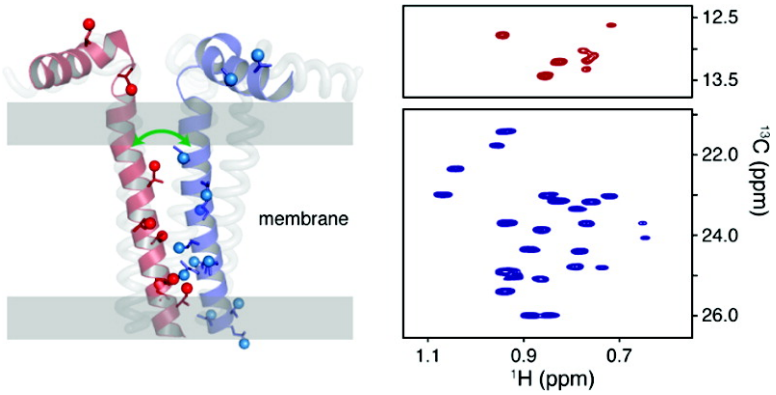

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Asymmetric Methyl Group Labeling as a Probe of Membrane Protein Homo-oligomers by NMR Spectroscopy

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Homo- and hetero-oligomeric membrane protein interactions are central to many cellular events, including signal transduction, post-translational modification, and regulation of receptors, channels, and several other integral membrane proteins.[1] Since the majority of membrane proteins are helical, these interactions often involve helix−helix packing, with interdigitation of hydrophobic side chains (Leu, Val, Ile, Ala) to form strong van der Waals interactions, that is, the "knobs-into-holes" model proposed by Crick.[2] Yet probing these hydrophobic binding surfaces remains a formidable challenge for structural techniques.

NMR is a powerful tool for investigating dynamic and functional interfaces of soluble proteins.[3,4] Chemical shift mapping, saturation transfer, paramagnetic relaxation enhancement, and cross-relaxation (nuclear Overhauser effect, NOE) are among the NMR methods currently used. By probing the through-space cross-relaxation of dipolarly coupled nuclei, NOE is considered a *direct* probe of molecular interfaces. Intermolecular NOEs are detected using isotope-filtered experiments[5] in differentially labeled proteins.[6,7] While applied with success to several medium size complexes thanks to the implementation of transverse relaxation optimized spectroscopy (TROSY) and partial protein deuteration, these methods are very insensitive. Jasanoff also introduced an asymmetric labeling method, where the detection of intermolecular NOEs in homo-oligomers relies on the comparison of two ^{13}C-edited NOESY spectra.[8] As with the isotope-filtered experiments, this approach is severely limited by spectral overlapping of the aliphatic groups, the need to acquire two NOESY spectra, and the ability to detect only short-range distances (∼6 Å).

Recently Kay and co-workers introduced the selective methyl labeling techniques in conjunction with methyl-NOESY pulse sequences, pushing both sensitivity and resolution of NMR up to molecular weights of ∼1 MDa.[9−11] The isotopic labeling of [U-^{2}H, ^{13}C, ^{15}N] with $^{13}CH_3$ methyl labels at Ile, Val, and Leu allowed for the identification of NOEs between methyl groups to give tertiary restraints in soluble proteins with hydrophobic cores at high-resolution. However, for hetero-oligomeric protein interactions, it is difficult to resolve intermolecular NOEs on the basis of this labeling scheme because of severe overlap of the resonances,[12] and in the case of symmetric homo-oligomers this problem is exacerbated by the magnetic equivalence of the methyl groups.

In this Communication, we present an asymmetric isotopic labeling strategy for probing unambiguously membrane protein binding interfaces in homo-oligomers. We show that by using methyl-edited NOESY spectroscopy and extensive protein perdeuteration it is possible to detect interprotein distances up to 10−12 Å. This technique is highly sensitive, rapid, and requires only one NOESY spectrum. As a proof of principle, we apply this technique to the homo-pentameric membrane protein phospholamban (PLN), defining the leucine−isoleucine zipper interface between its monomers with atomistic resolution.

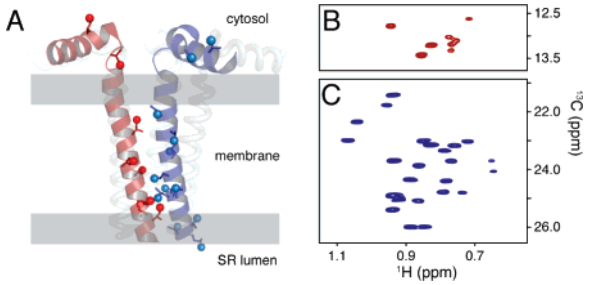

Figure 1. (A) Isotopic labeling scheme as shown on the pinwheel model of PLN.[17] The red monomer of PLN is labeled [U-^{2}H, ^{12}C, ^{14}N, $^{13}CH_3$-Ile$^{\delta1}$], while the blue monomer is labeled [U-^{2}H, ^{12}C, ^{14}N, $^{13}CH_3$-Val$^{\gamma1,2}$, $^{13}CH_3$-Leu$^{\delta1,2}$]. Owing to the precursor chosen, only one of the methyl groups in the Val and Leu sample is $^{13}CH_3$. Constant-time HSQC experiment on the 1/1 mixed sample showing Ile$^{\delta1}$ (red, B) and Val$^{\gamma1,2}$/Leu$^{\delta1,2}$ (blue, C) methyl resonances.

PLN is an integral membrane protein embedded within the sarcoplasmic reticulum (SR) membrane. As a monomer (active state),[13,14] PLN inhibits the SR Ca-ATPase (SERCA), but also exists in a pentameric storage state.[13] Domain II of PLN (residues 31−52) is helical and embedded in the hydrophobic core of the membrane. A leucine−isoleucine zipper is responsible for holding the transmembrane domains of PLN pentamer together.[1a,15] While sparse, intermolecular NOEs have been detected for PLN subunits involving connectivities with highly dispersed ^{1}H chemical shifts (i.e., $d_{H\alpha H\beta}$, $d_{H\alpha H\gamma}$),[16] but no distance restraints have been reported between the methyl groups of the zipper motif.

To refine the oligomerization surface of PLN, we expressed and purified two wild-type PLN samples: (1) [U-^{2}H, ^{12}C, ^{14}N] and $^{13}CH_3$ at the Ile$^{\delta1}$ and (2) [U-^{2}H, ^{12}C, ^{14}N] and $^{13}CH_3$ at the Leu$^{\delta1/2}$/Val$^{\gamma1/2}$. This labeling scheme provides well-resolved resonances for the two samples, where Leu$^{\delta1/2}$/Val$^{\gamma1/2}$ have chemical shifts significantly different from Ile$^{\delta1}$ (BMRB data bank, http://www.bmrb.wisc.edu). After purification, the two PLN samples were mixed to a 1/1 molar ratio and heated to 60 °C, allowing for stochastic mixing (details are given in the Supporting Information). A constant-time HSQC and a modified version of the methyl-NOESY experiment (^{13}C-ω_1, ^{13}C-ω_2, ^{1}H-ω_3)[11] were performed at 40 °C at a ^{1}H frequency of 599.54 MHz.

A schematic of the isotopic labeling is shown in Figure 1a, where the red monomer of PLN corresponds to sample 1 ($^{13}CH_3$-Ile$^{\delta1}$) and the blue to sample 2 ($^{13}CH_3$-Val$^{\gamma1,2}$/Leu$^{\delta1,2}$). Note that due to the isotopic labeling of the α-ketoisovalerate precursor, only one of the two methyl labels of Leu or Val is $^{13}CH_3$, while the other is $^{12}CD_3$. Figure 1b shows the HSQC spectrum of the mixed PLN sample, displaying the peaks corresponding to the Ile$^{\delta1}$ (red) and Val$^{\gamma1,2}$/Leu$^{\delta1,2}$ (blue) monomers.

The methyl-NOESY experiment[11] was used to detect intermolecular NOEs between the monomeric subunits within the pentamer assembly. Mixing periods of 200, 300, and 400 ms were used to monitor the build-up of the NOE peaks, eliminating the possibility of spin diffusion. In this experiment, the NOEs (indicated in green)

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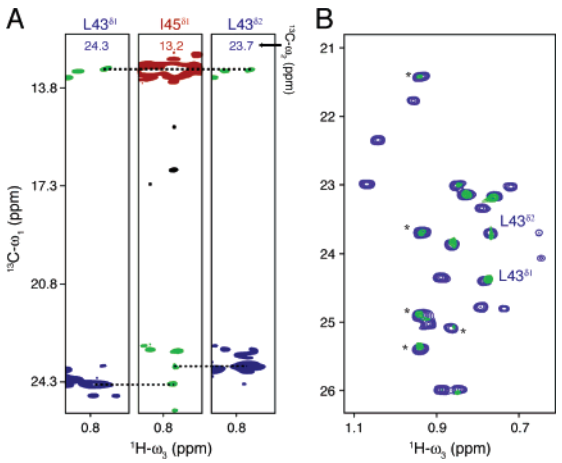

Figure 2. Intermolecular NOEs detected for pentameric PLN. (A) ^{13}C-ω_1/^1H-ω_3 strip plots showing ^{13}C-ω_2 planes corresponding to L43$^{\delta1}$, Ile45$^{\delta1}$, and L43$^{\delta2}$ (left to right). The green peaks are the intermolecular NOEs while the red and blue peaks are diagonal peaks. Dashed lines indicate connectivities between NOEs and diagonal peaks. (B) Overlap of ^{13}C-ω_2 planes for the Ile methyl region (12−14 ppm). The blue resonances are the same as in the Val/Leu HSQC shown in Figure 1a. Green peaks indicate all intermolecular NOEs from this experiment with a NOESY mixing time of 400 ms. Owing to the representation of ω_2 overlap, several of the green peaks encompass more than one intermolecular NOE. The asterisks indicate NOEs with the DPC detergent.

are given at resonance positions of [$^{13}C_i$-ω_1, $^{13}C_j$-ω_2, ^1H_j-ω_3] with the diagonal peaks appearing at [$^{13}C_i$-ω_1, $^{13}C_i$-ω_2, ^1H_i-ω_3] frequencies. The subscripts *i* and *j* indicate labels located on different monomeric subunits. Figure 2a shows strip plots indicating intermolecular NOEs between Ile-45$^{\delta1}$ and both Leu-43$^{\delta1}$ and Leu-43$^{\delta2}$. The dashed lines between the strip plots denote the connectivity between the diagonal (either blue or red) and NOE (green) peaks.

Since the [^{1}H, ^{1}H] NOESY mixing period is sandwiched between two ^{13}C editing blocks, any peaks detected within the Leu/Val methyl region of the spectrum ($\omega_1 \approx 21-26$ ppm) in an Ile methyl plane ($\omega_2 \approx 12-14$ ppm) indicate an intermolecular NOE. Figure 2b depicts this, showing the ω_1/ω_3 2D Leu/Val methyl region with the ω_2 dimension summed over the Ile methyl region (12−14 ppm). All of the green peaks shown within Figure 2b are intermolecular NOEs. The spectrum shown in black is that from the Leu/Val HSQC shown in Figure 1b. As is seen within Figure 2b, it is straightforward to distinguish the peaks involved in the quaternary assembly of the PLN pentamer from those that are not involved in the binding interface. Note that several of the green resonances within Figure 2b have more than one intermolecular NOE to an Ile methyl group. From the resolution offered by these spectra, we can easily discern not only the protein−protein interface, but also with atomistic detail which of the Val and Leu methyl group diastereomers are involved in binding (Figure 2a). Finally, since the resonances of the DPC are well resolved, it is possible to assign the NOEs between the hydrocarbon core of the micelles and the methyl groups of the protein side chains, confuting the extended structure of PLN previously reported.[16]

In addition to the unambiguous nature of the spectra, another advantage of this perdeuterated labeling strategy is the ability to probe long-range interactions between protein interfaces. From the pinwheel model of pentameric PLN,[17,18] the intermolecular NOEs observed within Figure 2 correspond to a range in distances from 4 to 12 Å. Such long distances have previously been observed by Koharudin et al.[19] and Sounier et al.[20]

In conclusion, we report a new labeling strategy for the determination of intermolecular membrane protein distances. The beauty of this method is its high sensitivity coupled with unambiguous and straightforward interpretation of the NMR spectra. While we report the application to Leu/Val and Ile, this strategy can be easily extended to various combinations of selective methyl labeling schemes that can include Met, Leu, Val, Ile, and Ala, and even backbone ^{15}N labeling in combination with selected methyl labeling.

Recent analysis of the genomes suggests that the majority of the proteins assemble into symmetric homo-oligomers.[21] While the structural information of the monomers within the oligomeric, quaternary structures will be easily derived from the current NMR technology, intermonomer distances will be possible thanks to asymmetric labeling strategies (such as the one presented here) in combination with segmental labeling.[22−24] Of course, homo-oligomers represent the worst-case scenario for unambiguous assignments of intermolecular NOEs. Given the recent successes of methyl labeling technology,[9−11] this asymmetric labeling method will allow for accurate distance measurements as well as for rapid assessment of topological supramolecular assemblies of soluble and membrane-bound proteins.

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Supporting Information Available: Sample preparation, details of NMR experiments, additional spectra, and observed NOEs. This material is available free of charge via the Internet at http://pubs.acs.org.

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