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Communication

Inter- and intramolecular contacts in a membrane protein/surfactant complex observed by heteronuclear dipole-to-dipole cross-relaxation

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ABSTRACT

Heteronuclear dipole-to-dipole cross-relaxation has been applied to exploring intermolecular interactions and intramolecular spatial proximities in a large supramolecular structure comprised of a β-barrel membrane protein, OmpX, in complex with a polymeric surfactant, amphipol A8-35. The experiments, performed in either the laboratory or the rotating frame, reveal the existence of intermolecular contacts between aromatic amino acids and specific groups of the polymer, in addition to intra-protein dipolar interactions, some of them involving carbonyl carbons. This study opens the perspective of collecting by NMR spectroscopy a new kind of through-space structural information involving aromatic and carbonyl ^{13}C atoms of large proteins.

1. Introduction

Nuclear cross-relaxation is of central importance in NMR structural biology and is particularly suitable to studying large macromolecules at high magnetic field strengths [1]. Whereas homonuclear cross-relaxation is commonly used, heteronuclear dipolar cross-relaxation spectroscopy has been hitherto exclusively applied to small organic and inorganic compounds (e.g. Ref. [2]), because of the dependence of the experimental sensitivity on the magnetogyric ratio of the detected nucleus. In addition, the distances between protons in a biomolecule are in general shorter than the distance between a ^{1}H and a non-bonded hetero-nucleus, making the usage of ^{1}H–^{1}H nuclear Overhauser effect (NOE) spectroscopy more pertinent. However, homonuclear cross-relaxation is not always applicable in biomolecular systems. For example, intermolecular contacts in binary macromolecular complexes cannot be observed using ^{1}H–^{1}H NOEs if one of the molecules is perdeuterated.

In the present study, the potential of exploiting heteronuclear cross-relaxation effects was tested, in either the laboratory or the rotating frame, using as a model system the integral outer membrane protein X (OmpX) from *Escherichia coli*, in complex with an amphiphilic polymer, amphipol (APol) A8-35 [3] (Fig. 1). APols are extremely mild surfactants designed to handle membrane proteins in aqueous solutions in the absence of detergent [4]. They were recently successfully employed to fold membrane proteins to their native state [5], including G-protein coupled receptors [6]. NMR studies of hydrophobic interactions between β-barrel proteins and various surfactants have already been reported, describing either direct contacts between protonated methyl groups and the hydrophobic tails of the surfactant [7], or indirect effects due to varying the amide proton ($^{1}H^{N}$) dipolar environment of surfactant-exposed residues [8,9]. Here, direct intermolecular hydrophobic contacts between ^{13}C atoms in the side chain of aromatic residues and ^{1}H nuclei of the surfactant are evidenced by ^{13}C-detected cross-relaxation experiments. Despite the use of a room-temperature probe, measurements are sensitive enough, due to the favorable relaxation properties of deuterated and carbonyl carbons. In addition, intra-protein inter-residue dipolar contacts involving carbonyl ^{13}C nuclei and aliphatic protons are identified.

2. Experimental

2.1. APol synthesis

Fully protonated and partially deuterated forms of APol A8-35 (HAPol and DAPol, respectively; Fig. 2) were obtained by grafting hydrogenated or deuterated octylamine and isopropylamine groups onto a hydrogenated poly(acrylic acid) precursor, respectively [3,10].

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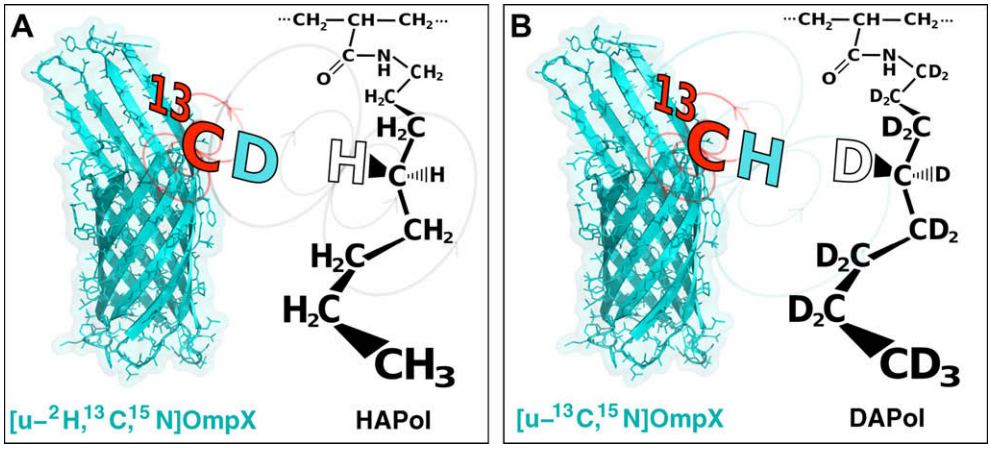

Fig. 1. Schematic view of the two NMR samples employed in this study. In blue, X-ray structure of OmpX (PDB ID: 1QJ8), and in black, an octylamide moiety part of APol A8-35. Sample A: perdeuterated and ^{13}C, ^{15}N-labeled OmpX in complex with fully protonated APol A8-35 (HAPol). Sample B: fully protonated ^{13}C, ^{15}N-labeled OmpX in complex with APol A8-35 whose alkyl side-chains are perdeuterated (DAPol) (the chemical structures of HAPol and DAPol are described in Fig. 2). Heteronuclear dipolar coupling is pictured by two interacting magnetic fields looping around ^{13}C and ^{1}H nuclei. OmpX cartoon was realized with the open-source software PyMOL [14].

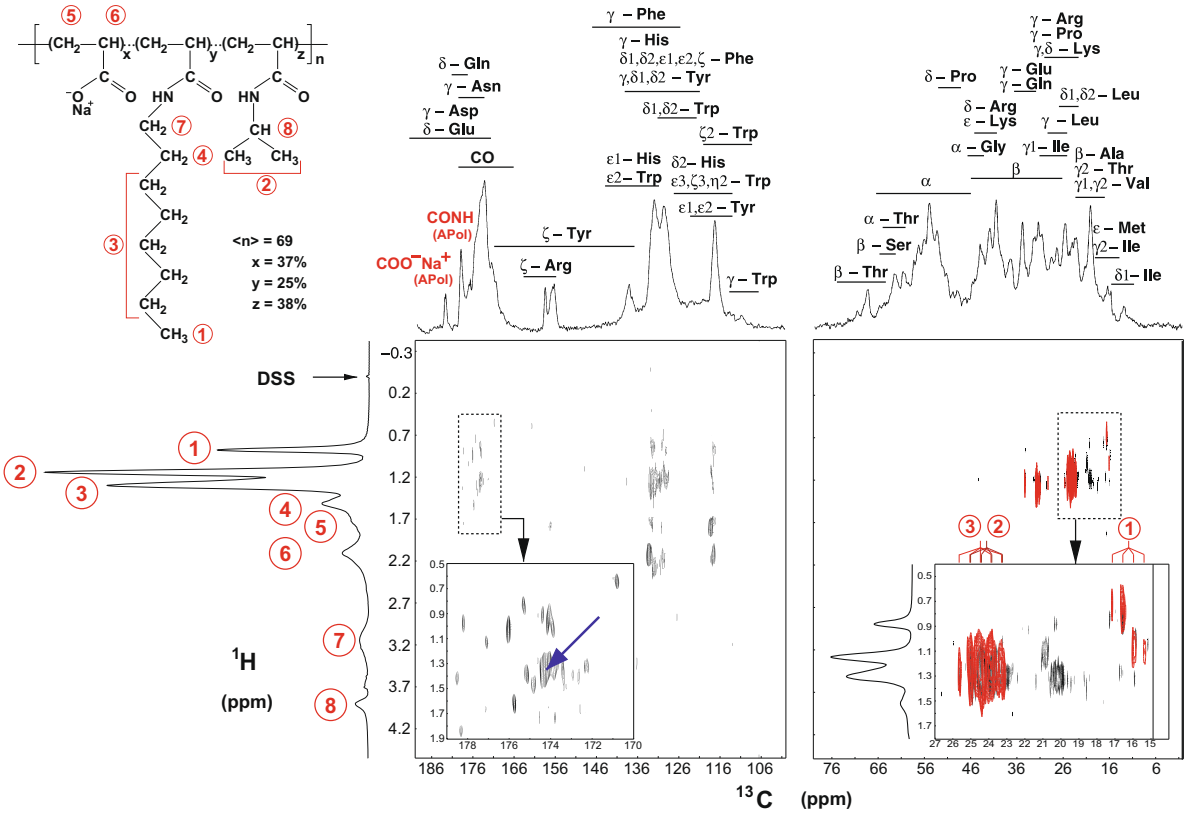

Fig. 2. Superimposition of two sets of 2D [^{1}H, ^{13}C]-HOESY data, obtained, respectively, on [u-^{2}H, ^{13}C, ^{15}N]OmpX/HAPol complexes (in black) and on HAPol alone (in red), both samples being in 100%-D_2O buffer. For a better representation, the direct ^{13}C dimension has been split into two parts: an aliphatic zone (from 0 to ~80 ppm) and a carbonyl and aromatic zone (from 100 to 190 ppm). On top and left, corresponding 1D ^{13}C and ^{1}H spectra of the complex are shown. ^{13}C protein nucleus labels and their respective statistical chemical shift SD [23] are indicated next to the 1D ^{13}C spectrum, as well as two identified ^{13}C APol resonance lines (in red). Circled red numbers refer to APol ^{1}H and ^{13}C chemical shift resonances reported on the chemical structure of A8-35 displayed in the upper left corner. The insets correspond to enlarged views of the regions delimited by the corresponding dotted frames. The blue arrow indicates the ^{1}H and ^{13}C carrier frequencies used in the 1D shROESY experiment described in Fig. 4.

2.2. Protein expression and purification

Uniformly ^{2}H-, ^{13}C-, ^{15}N-labeled OmpX(H100N) overexpression in *E. coli* and purification of inclusion bodies were similar to procedures already described [11,12], using D_2O (^{2}H > 99%)-based minimal growth media with 2 g/l [^{2}H, ^{13}C]-D-glucose (^{2}H > 97%, ^{13}C > 98%) and 1 g/l $^{15}NH_4Cl$ (^{15}N > 99%). All isotopically enriched compounds were purchased from Spectra Stable Isotopes (Columbia, MD). The percentage of deuteration of OmpX was estimated to be ~99% on the basis of mass spectroscopy measurements (see Supplementary Data, Fig. S1), assuming the protein to be labeled at 99% with ^{15}N and ^{13}C. A fully protonated [u-^{15}N, ^{13}C]OmpX was also produced. Inclusion bodies were solubilized in 6 M urea, 20 mM Tris–HCl, 5 mM EDTA, pH 8.5. OmpX was refolded by slow dilution [12]

in a solution of *n*-octylpolyoxyethylene (C_8POE, Bachem). The final protein concentration was 0.3 g/l in 1.5% (wt/vol) C_8POE.

2.3. Preparation of OmpX NMR samples

Starting from OmpX solubilized in C_8POE, the following NMR samples were prepared: [u-^{2}H, ^{13}C, ^{15}N]OmpX trapped by HAPol, and [u-^{13}C, ^{15}N]OmpX trapped by DAPol (Fig. 1). OmpX trapping with APol was performed as reported for tOmpA in Ref. [9] at a 1:4 protein/APol ratio (wt/wt). In order to remove all traces of C_8POE, we performed 10 cycles of dilution/concentration on an Amicon centrifugal filter unit (10 kDa cutoff, Millipore) with a 20 mM phosphate buffer, 100 mM NaCl, 10 mM EDTA–d_{16} (^{2}H > 98%, CDN isotopes), 0.05% NaN_3, pH ~8.0 in 100% D_2O. The characteristic signal of the polyethyleneglycol polar moiety of C_8POE was undetectable in ^{1}H NMR spectra. The following final OmpX/APol NMR samples were obtained: 1.3 mM [u-^{2}H, ^{13}C, ^{15}N]OmpX/HAPol and 0.6 mM [u-^{13}C, ^{15}N]OmpX/DAPol. The correct folding of APol-trapped OmpX was verified from a 2D [^{15}N, ^{1}H]-TROSY spectrum [13].

2.4. NMR spectroscopy

All NMR experiments with OmpX were carried out at 303 K on a Bruker Avance II 700 spectrometer equipped with a 5-mm triple resonance (TXI) gradient probe. The following parameters were used for the individual experiments: 2D [^{1}H, ^{13}C] heteronuclear NOE spectroscopy (HOESY) [data size 36 (t_1) × 16,384 (t_2) complex points, $t_{1max}(^1H)$ = 5 ms, $t_{2max}(^{13}C)$ = 213 ms, ^{13}C 90-degree pulse length = 20 μs, ^{13}C carrier frequency = 80 ppm, 4096 transients per increment, mixing time = 1 s, (proved to be the best compromise between cross-relaxation effect and experiment time, as tested by preliminary 1D ^{13}C{^{1}H-aliphatic} steady-state NOE experiments and during 2D HOESY set up), instrument time = 3.8 days]. Phase cycling for quadrature detection, ^{2}H decoupling (WALTZ-16 decoupling achieved with a 700 Hz field during the acquisition period) and pulsed-field gradients were added to the original sequence [15,16] (Fig. S2). Prior to Fourier transformation, the data were multiplied along the t_1-dimension with a cosine-bell window function and in the t_2-dimension with an empirically optimized exponential function (Line Broadening factor (LB) = 1 Hz in the aliphatic ^{13}C region and LB = 25 Hz in the carbonyl and aromatic ^{13}C region); 1D selective [^{1}H, ^{13}C] heteronuclear rotating frame NOE spectroscopy (shROESY) experiment [17] [2,048 complex points, t_{1max} = 213 ms]. The two continuous-wave matched spin-locking radio-frequency (RF) fields were simultaneously applied on ^{1}H and ^{13}C spins with identical amplitudes, i.e. $\gamma(^1H) \times B_{RF}(^1H)/2\pi = \gamma(^{13}C) \times B_{RF}(^{13}C)/2\pi$, where $\gamma(^1H)$, $\gamma(^{13}C)$, $B_{RF}(^1H)$ and $B_{RF}(^{13}C)$ are, respectively, the ^{1}H and ^{13}C magnetogyric ratios and the amplitudes of the RF fields applied on the ^{1}H and ^{13}C channels. Chemical shifts are referenced to an internal standard of 2,2-dimethyl-2 silapentane-5-sulfonate sodium salt (DSS). Data processing was performed with NMRPipe software [18] and spectra analyzed with NMRView [19].

2.5. Solvent-accessible surface calculation

The solvent-accessible surface per ^{13}C nucleus [20] was evaluated on the 20 OmpX NMR conformers (PDB ID: 1q9f [21]) using AREAIMOL (CCP4i software, version 6.0.2 [22]).

3. Results

A 2D [^{1}H, ^{13}C] heteronuclear NOE spectroscopy (HOESY) experiment [15,16] with [u-^{2}H, ^{13}C, ^{15}N]OmpX in complex with fully protonated APol (HAPol) (sample *A* in Fig. 1) was performed (Fig. 2). Some of the cross-peaks observed are also present in an identical experiment carried out on the surfactant alone at the same concentration (spectrum in *red*, Fig. 2), and hence correspond to intra-surfactant dipolar interactions. The high amount of methylene and methyl groups in HAPol indeed makes them detectable in the HOESY spectrum even with natural abundance ^{13}C level ([APol] = 100 g/l). A control experiment indicated that none of the observed peaks is due to aliasing. In addition, identical experiments performed with a ^{13}C carrier frequency shifted to either 35 or 150 ppm do not show additional peaks at the edges of the spectral region displayed in Fig. 2.

In the aromatic ^{13}C chemical shift range (116–135 ppm), numerous peaks are observed in the HOESY experiment (Fig. 2). In the same experiment recorded on a ^{13}C-labeled, fully protonated OmpX in complex with A8-35 whose octyl and isopropyl chains are perdeuterated (DAPol) (sample *B*, Fig. 1), the cross-peaks lying between 0.6 and 1.6 ppm in the ^{1}H dimension are not observed, thus establishing their intermolecular character (Fig. 3). The existence of contacts between ^{13}C nuclei of aromatic rings in the protein and alkyl chains of the surfactant is consistent with the structure of OmpX, which features aromatic rings exposed at the transmembrane surface, potentially accessible to APol alkyl chains, and displaying a large solvent-exposed surface per ^{13}C nucleus (25–30 Å^2). Interestingly, interactions of the protein with APol octyl chains seem to involve more extensively the last five methylene groups rather than the terminal methyl, nor the first two CH_2 groups (Figs. 2 and 3). Because of the large size of the complexes, ^{1}H–^{1}H spin-diffusion along the APol alkyl chains could conceivably give rise to some cross-peaks observed in the aromatic or aliphatic regions. However, HOESY experiments carried out with varying mixing time τ_m between 0.1 and 1 s indicate no variation in volume ratio between peaks facing the ^{1}H methyl and methylene resonances (line numbered 1 and 3 in Figs. 2 and 3). The optimized mixing time τ_m is rather long (1 s) compared to values usually employed in homonuclear ^{1}H–^{1}H cross-relaxation experiments

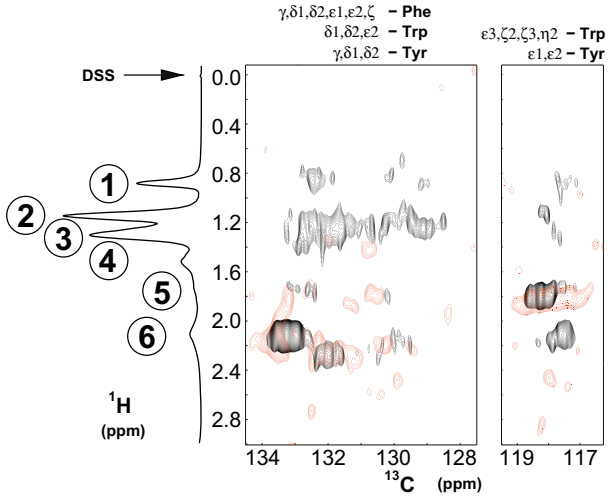

Fig. 3. Aromatic ^{13}C region of the 2D [^{1}H, ^{13}C]-HOESY experiment. The spectrum in black represents an enlarged view in the aromatic ^{13}C region of perdeuterated [u-^{13}C, ^{15}N]OmpX/HAPol 2D HOESY spectrum (from Fig. 2). The overlying spectrum in red is obtained upon performing an identical experiment with a fully protonated [u-^{13}C, ^{15}N]OmpX in complex with a partially deuterated APol (DAPol; see Fig. 2). On the left of the 2D spectra is displayed the 1D spectrum of the perdeuterated OmpX in complex with HAPol, the circled numbers along the 1D ^{1}H spectrum referring to the APol ^{1}H chemical shift resonances reported on the chemical structure of A8-35 in Fig. 2. Putative ^{13}C protein nuclei resonating in this region are specified above the 2D spectra. ^{13}C histidine nuclei are not mentioned since the mutated OmpX variant used here (H100N) does not contain any His residue [11].

because of the weaker heteronuclear ^{1}H–^{13}C dipolar interaction as compared to that between two interacting protons. A longer τ_m can partially compensate for a lower cross-relaxation rate constant.

In the carbonyl ^{13}C region from 170–180 ppm, very weak cross-peaks can be seen (Fig. 2). These are also observed with a protonated, ^{13}C-labeled OmpX in complex with DAPol (Fig. S3). This strongly suggests that, despite a good matching with ^{1}H HAPol resonances, they more likely arise from intramolecular dipolar interactions between carbonyl ^{13}C atoms and aliphatic ^{1}H in OmpX, which has a residual protonation level of ~1% as determined by MALDI–TOF (Fig. S1, they could not be due to intra-HAPol interactions as HAPol alone does not give rise to any correlation in the carbonyl ^{13}C region (Fig. 2). Another kind of cross-relaxation experiment, namely a selective [^{1}H, ^{13}C] heteronuclear rotating frame NOE spectroscopy (shROESY) experiment [17] was carried out (Fig. 4). Two matched low-amplitude spin-locking radio-frequency (RF) fields were set on the chemical shifts of the ^{1}H and ^{13}C nuclei indicated with a blue arrow in Fig. 2, i.e. at frequencies that do not correspond to either COOH or CONH ^{13}C APol resonances. RF-amplitudes of 10 Hz cause the emergence of an intense resonance peak (Fig. 4B), which confirms through-space dipolar proximity between a carbonyl atom and aliphatic ^{1}H nuclei. No such peak is observed in an identical experiment realized with a ^{1}H carrier frequency shifted from 1.24 ppm to a peak-free region (0.02 ppm), attesting that the resonance observed does not arise from a selective adiabatic excitation. These observations make the heteronuclear cross-relaxation phenomenon potentially attractive to detect spatial proximities between carbonyl carbons and methyl groups. Indeed, interatomic distances below 3 Å can be found on the 20 NMR OmpX conformers between carbonyl ^{13}C atoms and methyl groups ^{1}H. Most of these short distances involve side chain carbonyls.

Additional contacts are observed in two other regions of the HOESY spectrum of the perdeuterated, ^{13}C-labeled OmpX/HAPol complexes. Based on the present data, they could originate from inter- or intramolecular interactions. In the aromatic ^{13}C region, in a ^{1}H range comprised between 1.6 and 2.5 ppm (Fig. 3), the occurrence of intermolecular cross-peaks would indicate that protons of the APol backbone contact aromatic rings. Alternatively, these peaks could reflect inter-residue contacts between aromatic ^{13}C-nuclei and residual ^{1}H from non-aromatic residues, in which case they would contain information about the 3D structure of the protein. In the aliphatic region (see inset in Fig. 2), cross-peaks concern exclusively methyl ^{13}C nuclei, as judged from the ^{13}C chemical shift range concerned [12,23,24]. Despite an apparent good agreement with ^{1}H APol resonance lines and a very low amount of protons in the protein, residual ^{1}H involved in dipolar interactions with carbonyl ^{13}C atoms should give rise to cross-peaks with their covalently bound ^{13}C in this region.

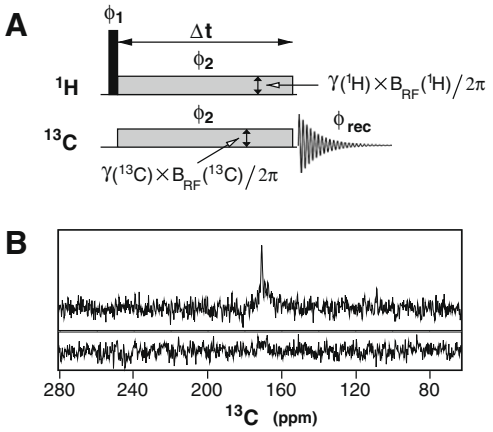

Fig. 4. Heteronuclear dipolar contact observed in the carbonyl ^{13}C region by a selective cross-relaxation experiment in the rotating frame. (A) 1D [^{1}H, ^{13}C]-shROESY pulse sequence scheme. Two continuous-wave matched spinlocking RF fields with amplitudes $\gamma(^{1}H) \times B_{RF}(^{1}H)/2\pi = \gamma(^{13}C) \times B_{RF}(^{13}C)/2\pi$ are applied simultaneously during Δt on ^{1}H and ^{13}C spins. Phase cycles are $\phi 1 = x, -x, -x, x, y, -y, -y, y$; $\phi 2 = -y, y, y, -y, x, -x, -x, x$; $\phi_{rec} = x, -x, -x, x, y, -y, -y, y$. (B) 1D ^{13}C shROESY spectra of [u-^{2}H, ^{13}C, ^{15}N]OmpX/HAPol. $\Delta t = 0.2$ s, $\gamma(^{1}H) \times B_{RF}(^{1}H)/2\pi = \gamma(^{13}C) \times B_{RF}(^{13}C)/2\pi = 10$ Hz at the carrier frequencies indicated with the blue arrow in Fig. 2, i.e. 171.95 ppm (^{13}C) and 1.24 ppm (^{1}H) (upper spectrum) and 171.95 ppm (^{13}C) and 0.02 ppm (^{1}H), in a peak-free zone (lower spectrum), 60k transients per experiment.

4. Discussion

Nuclear cross-relaxation is of central importance in NMR structural biology [1]. Dipolar cross-relaxation spectroscopy, in which magnetization is stored along the *z*-axis, is particularly suitable to studying large macromolecules at high magnetic field. 2D HOESY was first tested in 1983 [15,16]. Despite improvements in molecular biology and the advent of higher magnetic fields, this approach until now has been applied mostly to small-sized molecules, except for a recent study of protein/ligand complexes using ^{19}F NMR [25]. In general, in small molecules, i.e. in the fast-motion limit, the NOE is advantageously exploited through 1D or 2D steady-state saturation methods, where the largest effect can be expected; 2D or 3D NOE spectroscopy at high magnetic fields, on the other hand, is well-indicated to investigate macromolecular architectures [26].

Moreover, ^{13}C-detected experiments realized with a perdeuterated protein offer some benefits. In the case of ^{13}C–^{1}H spin-pairs, it is well-known that the substitution of protons by deuterons substantially increase carbon relaxation times (e.g. [27]). With large deuterated proteins whose methyl groups of Ile, Leu, and Val residues are optimally labeled [28], the heteronuclear dipolar cross-relaxation effect could be of interest, due to the favorable ^{13}C methyl relaxation properties. In that case, ^{13}C directly detected heteronuclear NOE experiments could be advantageously combined to specific COSY-based approaches [29] and/or methyl-TROSY experiments [30]. In addition, carbons without directly attached protons usually exhibit advantageous relaxation properties [31,32], and direct detection on carbonyl carbons could help to resolve resonance overlap. Furthermore, it has been shown, more than a decade ago, that homonuclear NOE between adjacent ^{13}C nuclei could advantageously replace scalar transfer in large proteins [33]. Recent technical improvements, such as the advent of cryogenic probes with an inner coil for detection of ^{13}C and/or cryogenically-enhanced preamplifiers, have made the ^{13}C nucleus more attractive [34–36]. Using optimized instrumentation, the time required to carry out the 2D HOESY experiment reported here could be cut down from 3.8 days to a day or less. Moreover, the simple experiments described here can be expanded to obtain or confirm ^{13}C assignments by adding a spin-lock and/or a second evolution period, or to suppress directly-attached ^{1}H–^{13}C correlations with a scalar coupling filter. With ^{13}C direct-detection experiments, the large one-bond ^{13}C homonuclear scalar coupling ($^{1}J_{CC} = 35–55$ Hz), as well as various smaller homonuclear scalar couplings such as $^{2}J_{CC}$, $^{3}J_{CC}$, and $^{4}J_{CC}$, affect the resolution in the acquisition dimension. Thus, to help the identification of the NOE observed, in particular in overlapped peaks regions like the ^{13}C CH_3 region (Fig. 2) or the ^{13}C aromatic one (Fig. 3), it is needed to simplify the NOE patterns observed. Maximum entropy reconstruction [37] or methods based on a spin-state selection [38] could be advantageously employed to suppress homonuclear ^{13}C one-bond scalar coupling constants in the direct dimension.

APols were designed to bind to the transmembrane surface of membrane proteins by multiple attachment points [3], which renders their non-covalent association with proteins virtually irreversible in the absence of competing surfactant: membrane protein/APol complexes, as a consequence, are remarkably stable [4,39,40]. In the presence of an excess of free APol and at the salt concentration used here, free and bound APols exchange on the minute scale [40]. Hence, the intermolecular NOE observed here are unlikely to be time dependent transferred NOE between bulk APol molecules and OmpX. On the other hand, it is likely that APol-bound octyl chains rapidly move about the transmembrane domain of OmpX, which, depending on the rate of this chemical exchange, could give rise to exchange-transferred NOE.

In summary, in this study, contacts between protons of methylene and methyl groups of the surfactant and carbons of aromatic rings of the protein are clearly revealed. This makes heteronuclear NOE spectroscopy particularly suitable to look at interactions implying such residues. In the case of membrane protein/amphipol complexes, this approach thus provides the first detailed molecular view of the way specific APol moieties interact with specific protein side chains. Inter-residue vicinities involving carbonyl ^{13}C and methyl ^{1}H groups, however, are also detectable. The present work therefore establishes that the study of heteronuclear cross-relaxation effects in macromolecules or macromolecular complexes makes it possible to collect through-space inter- and intramolecular structural information involving aromatic and carbonyl ^{13}C nuclei.

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Appendix A. Supplementary data

MALDI–TOF spectrum of [u-^{2}H, ^{13}C, ^{15}N]OmpX/HAPol complex (Fig. S1), the 2D [^{1}H, ^{13}C]-HOESY NMR pulse sequence scheme (Fig. S2), and a zoom in the ^{13}C carbonyl region of two sets of 2D [^{1}H, ^{13}C]-HOESY experiments carried out one on [u-^{2}H, ^{13}C, ^{15}N]OmpX/HAPol and the other on fully protonated [u-^{13}C, ^{15}N]OmpX/DAPol complexes.

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jmr.2008.11.017.

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