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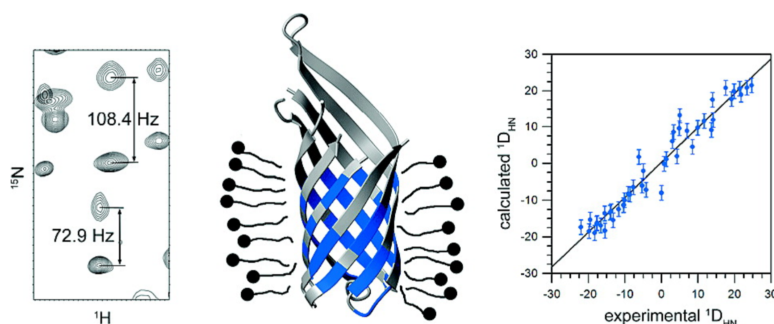

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Charged Gels as Orienting Media for Measurement of Residual Dipolar Couplings in Soluble and Integral Membrane Proteins

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Abstract: Measurement of residual dipolar couplings for membrane proteins will dramatically improve the quality of the structures obtainable by solution NMR spectroscopy. While there has been some success in achieving alignment of membrane-bound peptides, there has been very limited success in achieving alignment for functional membrane proteins. Herein, we demonstrate that charged polyacrylamide-based copolymers are suitable for obtaining weak alignment of membrane proteins reconstituted in detergent micelles. Varying the copolymer compositions, we prepared positively, zwitterionic, and negatively charged gels that are very stable at low concentration and can be used for obtaining weak alignment by compression in an NMR tube. Application of this method is demonstrated for the integral membrane protein OmpA in DPC micelles.

Introduction

While a wealth of structural information on soluble proteins has been generated in the recent past, the determination of structures of integral membrane proteins has lagged far behind. This is a reflection of the significant challenges associated with structural studies of this class of proteins. One of the most exciting recent developments in the application of solution NMR has been the successful application of these methods to the determination of the structures of a number of integral membrane proteins in detergent micelles. Application of TROSY-based experiments for uniformly deuterated proteins [1] has extended the size limit for studies of membrane protein–detergent complexes beyond 100 kDa[2] and made possible structure determinations of moderate-sized integral membrane proteins. The number of successful applications of high-resolution NMR for integral membrane proteins is growing, and numerous new systems are being characterized.[2,3] Current NMR methodology can now routinely define backbone structures of moderate-sized β-barrel proteins.[4–6] Proteins containing α-helical membrane spanning motifs have thus far proven more challenging targets for structural analysis. To date, only a handful of small helical polypeptides limited to the presence of two membrane spanning fragments have been structurally characterized by NMR.[7–9] The recent resonance assignment of the α-helical 40 kDa trimeric protein DAGK bodes well for future success in this area.[2]

Solution NMR structural studies of integral membrane proteins reconstituted in detergent micelles requires high-level deuteration, resulting in a paucity of NOE information. This limits the quality of the structure determination that can currently be achieved for this class of proteins. Methyl protonation in a background of high-level deuteration has been employed to collect additional NOE information for large soluble proteins.[10] This approach has been applied to the structure determination of the integral membrane protein OmpX, resulting in a significant improvement in the quality of the structure determination.[11] An alternative approach for improving the accuracy of these structures would be by way of collection of residual dipolar couplings (RDC) which have proven their utility in improving structural accuracy[12–14] and have been applied particularly in the setting of highly deuterated proteins with limited NOE information.[15] RDCs contain information about the orientation of internuclear vectors relative to the magnetic field and can be

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readily employed to improve structural accuracy.[12−14] For fully deuterated proteins, a large set of backbone RDCs can be easily collected using TROSY-based triple resonance approaches.[16] In addition, the recent measurement of side-chain $^{13}C-^{13}C$ dipolar couplings for a large protein suggests the potential to derive side-chain structural information from these highly deuterated proteins as well.[17]

To measure RDCs, it is necessary to introduce a weak alignment of the protein molecules.[12−14] Partial orientation of globular proteins has been achieved using several types of orienting media including bicelles[18] and filamentous phage.[19] None of these systems is suitable for alignment of membrane proteins reconstituted in detergent micelles. There is a clear need for alignment media which is stable and effective in the context of protein−detergent micelles. The recent development of polyacrylamide hydrogels[20,21] for alignment of soluble proteins provides just such a medium. The serious limitation of such gels is the high concentration of polyacrylamide necessary to ensure mechanical stability. Thus, even for relatively weak alignment, this will affect the rotational diffusion of the proteins leading to extensive broadening of the NMR signals as well as to difficulties in diffusing the protein samples into the gels. Substantial reductions in the gel concentration to ameliorate these effects result in very small degrees of alignment which are not useful for structure determination. As a consequence, very few examples of such measurements of RDCs using gels have been reported, and they are all for relatively small peptides. These include a short peptide from HIV-1 gp41[22] and small α-helical motifs from Vpu[23] and the Pf1 coat protein.[24] To date, there have been no reports of significant RDCs measured for a membrane protein larger than 10 kDa. Previous attempts to induce a sufficient level of alignment of integral membrane proteins in polyacrylamide gels were not successful.[2,6] It is clear that there is a significant need for reliable effective alignment media for integral membrane proteins.

Applications of polyacrylamide gels for alignment have been significantly expanded by modification of the gel composition, for example, by addition of a charged component to the gel.[25] Such charged copolymers undergo strong electroosmotic swelling in aqueous solutions and retain high mechanical stability, even at very low gel concentration. Gel properties can be further modified by altering the copolymer composition. Indeed, it was recently demonstrated that gels with opposite charge produced different alignments of the protein.[26]

Herein, we have examined a series of charged copolymer gels that are suitable for inducing structurally useful degrees of alignment of integral membrane proteins in detergent micelles. These gels have proven to be stable for extended periods at elevated temperature and to induce high degrees of alignment of a 20 kDa integral membrane protein. To test the broad range of gel compositions we developed, we first examined the alignment of the globular domain of a mutant of the N-terminal domain of doublecortin (N-DCXmut).[27] Subsequently, we have successfully utilized several copolymer gels to achieve high degrees of alignment of the integral membrane protein OmpA in detergent micelles.

Experimental Section

Protein Samples. The N-terminal domain of doublecortin containing residues 45−150 (N-DCX) and its double mutant K134D, K135D (N-DCXmut), were prepared as described previously.[27] Samples for NMR spectroscopy contained 0.2 mM ^{15}N-labeled protein in 50 mM sodium phosphate buffer, pH 6.0. If indicated, samples also contained 150 mM NaCl. Uniformly ^{2}H, ^{15}N and ^{2}H, ^{13}C, ^{15}N-labeled OmpA were prepared as described previously.[4] One millimolar protein solutions in 600 mM DPC, 10 mM potassium phosphate buffer, pH 6.3, and 50 mM NaCl were used for all NMR experiments.

Data Collection and Analysis. NMR spectra were collected on Varian Inova 500 and 600 MHz spectrometers. Measurements for N-DCX and OmpA have been done at 30 and 50 °C, respectively. For determination of $^{1}D_{HN}$ residual dipolar couplings, the standard IPAP-HSQC experiment was employed.[28] Due to the low polymer concentration, suppression of acrylamide signals was not necessary.[21] A TROSY-HNCO-based experiment was employed for measurement of C′−Cα dipolar couplings.[16] NMR spectra were processed in NMRPipe[29] and analyzed in Sparky (Goddard, T. D.; Kneller, J. M. University of California, San Francisco). Fitting of experimental RDCs to crystal structures was performed using Pales.[30] Evaluation of experimental error was done using a jack-knife procedure by random elimination of 10% of the data and repeating the calculations 100 times.[31] A statistical measure of the extent to which two sets of residual dipolar couplings are similar was obtained by calculating Pearson's correlation coefficient, *r*.

Gel Preparation. Preparation of copolymer polyacrylamide gels was performed in a manner similar to that described previously.[25] Stock solutions of 40% acrylamide and *N*,*N*′-methylenebisacrylamide in a 19:1 ratio (Bio-Rad) were mixed with 40% charged acrylate derivatives containing *N*,*N*′-methylenebisacrylamide in a 19:1 ratio. The mixtures were diluted with 10xTBE buffer (0.9 M TRIS, 0.9 M borate, 0.02 M EDTA, pH 8.2) to a final 7% concentration. Polymerization was initiated by the addition of 0.15% ammoniumperoxide sulfate and 1% tetramethylethylenediamine (TEMED). To introduce negative charge, we used acrylic acid (Sigma-Aldrich, Inc.) or 2-acrylamido-2-methyl-1-propanesulfonic acid (AMPS, Sigma-Aldrich, Inc). Positive charge was introduced by addition of (3-acrylamidopropyl)-trimethylammonium chloride (APTMAC, Sigma-Aldrich, Inc.) or *N*-(2-acryloamido-ethyl)triethylammonium iodide (NAETEAI, Sigma-Aldrich, Inc.). Subsequently, zwitterionic copolymers were obtained by the use of equivalent amounts of acrylic acid and APTMAC. The polymerization of 130 μL of the mixtures was carried out overnight in plastic tubes with 3.2 mm diameter. This volume was selected to keep the ratio between length and diameter of polymerized gel as 5 to 1. Polymerized

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gels were extensively washed in deionized water (three cycles over a period of 2 days). During the washing procedure, the gels experienced a significant increase in size due to strong electroosmotic swelling. The gels were dried over a 2 day period at 30 °C on a plastic support wrapped with polyvinylidene chloride (PVDC) foil. To assemble the NMR sample, the dried gel was transferred to a Shigemi tube and protein solution was added. For a nonrestricted gel in a 4.2 mm NMR tube, the final length reached 21 mm. Vertical compression of the gels was achieved by inserting the Shigemi plunger into the tube and limiting the final length between 12 and 19 mm. After overnight equilibration, the samples were ready for NMR experiments. Reduction of the gel compression and thus the degree of protein alignment could be easily achieved by simply adjusting the plunger position.

Gel Nomenclature. The following nomenclature was employed to describe the gel composition: (1) the first number indicates the w/w concentration of the charged component; (2) the second symbol denotes positive (+), negative (−), or zwitterionic (±) copolymer; and (3) the final symbols describe charged components, A, acrylic acid; S, AMPS; M, APTMAC; and E, NAETEAI. For example, 25+M identifies a positively charged copolymer containing 25% of APTMAC and 75% of acrylamide; 50±MA identifies a zwitterionic copolymer based on 50% content of 1:1 mixture of APTMAC/acrylic acid and 50% acrylamide.

Theoretical Background

Residual dipolar coupling in weakly aligned rigid systems can be described as

$$D_{IS} = \frac{-\mu_0 h \gamma_I \gamma_S}{8\pi^3 {r_{IS}}^3}\left[\frac{1}{2}A_{zz}(3\cos^2\theta - 1) + \frac{1}{2}(A_{xx} - A_{yy})\sin^2\theta\cos 2\phi\right] \quad (1)$$

where γ_I and γ_S are the gyromagnetic ratios of the interacting nuclei separated by distance r_{IS}, and μ_0 is the magnetic permeability in a vacuum.[13] The average orientation of the molecule in solution is defined by alignment tensor A with principal components A_{xx}, A_{yy}, and A_{zz}. The orientation of an IS internuclear vector relative to the principal axes of the molecular alignment tensor is expressed in the polar angles θ and ϕ. In the principal axes frame, the alignment tensor can be conveniently decomposed into an axial component $A_a = {}^1/_2 A_{zz}$ and rhombicity $R = {}^2/_3(A_{xx} - A_{yy})/A_{zz}$. If only the ${}^1D_{HN}$ couplings are considered, then eq 1 can be further simplified to

$$^1D_H N = D_a\left[(3\cos^2\theta - 1) + \frac{3}{2}R\sin^2\theta\cos 2\phi\right] \quad (2)$$

where D_a represents the magnitude of the alignment tensor normalized to interaction between H and N nuclei assuming a constant distance r_{HN}. If the structure is known, the alignment parameters D_a and R can be determined from fitting experimental values of RDCs using singular value decomposition.[32] This also yields the transformation necessary to convert molecular coordinates to principal axes frame represented in three Euler angles α, β, and γ. For evaluation of the agreement between predicted and observed RDCs, the quality factor is typically calculated:[33]

$$Q = \mathrm{rms}(D^{calc} - D^{obs})/\mathrm{rms}(D^{obs}) \quad (3)$$

The quality factor offers a convenient criterion to validate compatibility between structures and RDCs measured in solution.

Results and Discussion

Alignment in Compressed Gels. While the application of polyacrylamide gels for the alignment of soluble proteins has been well-established,[20,21,34,35] the application of this technique for integral membrane proteins is significantly more demanding. Large membrane protein−detergent complexes require the preparation of gels at low concentration while retaining high homogeneity. It would also be desirable to have the flexibility to easily tune the alignment by simple changes in the gel geometry in the assembled NMR sample. These criteria cannot be satisfied when using regular polyacrylamide hydrogels mainly because of the high polymer concentrations required for these gels. However, gels with charged copolymers[25,26] undergo electroosmotic swelling in aqueous solutions and retain high mechanical stability at low polymer concentrations.

To prepare gels suitable for achieving alignment of integral membrane proteins, we first evaluated a series of copolymers based on acrylamide and charged derivatives of acrylic acid. Various compositions of monomers have been mixed at 7% concentration and polymerized in plastic tubes with a diameter smaller than that of the NMR tube. During an extensive washing in deionized water, we observed strong swelling and an increase in the volume of all charged gels. The samples for NMR experiments were prepared by placing the dried gel in a Shigemi tube followed by addition of protein solution. For introduction of gel anisotropy, we applied vertical compression using the Shigemi plunger in a manner similar to that described previously.[20,21] Adjustment of the position of the plunger was used to accurately control gel compression and concentration. Due to strong swelling, the final gel concentration was always lower than the initial 7%. It is also worthwhile to note that this approach to sample preparation ensures very high homogeneity, making it possible to utilize automatic gradient shimming and resulting in very high-quality NMR spectra.

The resulting charged copolymer polyacrylamide gels were initially evaluated using a small soluble protein, the N-terminal domain of doublecortin,[27] with two point mutations introduced (N-DCXmut). Alignment of N-DCXmut in a broad range of gel compositions is summarized in Table 1. The calculation of alignment tensor parameters was based on fitting of the experimental residual dipolar couplings to the N-DCXmut crystal structure (Kim, M.; Derewenda, U.; Derwenda, Z., unpublished results). To quantitatively assess the fit quality, we calculated quality factors, Q.[33] For 12 of the 13 copolymer compositions examined, Q values between 15% and 20% were obtained, indicating a very high degree of consistency in the datasets. One copolymer composition (50−A) gave a slightly higher Q value of 24% mainly as a result of signal broadening due to strong interaction with the protein.

The degree of protein alignment was easily controlled by the extent of gel compression. To generate useful degrees of alignment for the DCX domain, the copolymer concentration in the NMR samples was kept in the range between 3.7% and

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Table 1. Analysis of RDCs and Calculation of Alignment Parameters for N-DCXmut

gel type[a]	D_a (Hz)[b]	R[b]	Q (%)[b]	α[c]	β[c]	γ[c]	Nr[d]	conc (%)[e]
75+M	15.4 ± 0.2	0.11 ± 0.01	15.6 ± 0.8	28.5 ± 0.4	−27.4 ± 0.3	27.0 ± 4.1	42	4.2
75+M NaCl	9.4 ± 0.1	0.15 ± 0.02	17.4 ± 0.8	26.1 ± 0.4	−25.2 ± 0.3	−14.0 ± 3.3	41	4.2
75+E	4.2 ± 0.1	0.21 ± 0.02	20.0 ± 0.8	34.9 ± 0.4	−23.4 ± 0.4	40.0 ± 1.6	46	5.3
50+M	14.1 ± 0.1	0.14 ± 0.01	15.2 ± 0.6	26.7 ± 0.3	−24.9 ± 0.3	4.4 ± 3.2	42	4.2
50+M	12.9 ± 0.1	0.15 ± 0.02	18.0 ± 0.7	28.1 ± 0.4	−25.5 ± 0.4	9.6 ± 2.6	46	4.0
50+M T = 5%	7.5 ± 0.1	0.14 ± 0.02	18.3 ± 0.9	28.7 ± 0.4	−25.7 ± 0.3	12.3 ± 2.9	43	3.8
25+M	10.5 ± 0.1	0.45 ± 0.02	18.5 ± 1.0	9.1 ± 0.9	22.4 ± 0.5	90.4 ± 0.9	39	4.5
25+M NaCl	7.2 ± 0.1	0.45 ± 0.02	18.4 ± 1.0	−9.5 ± 0.6	18.4 ± 0.3	93.9 ± 1.0	43	4.9
50±MA	9.6 ± 0.1	0.55 ± 0.01	17.0 ± 0.9	7.1 ± 0.7	22.1 ± 0.4	88.6 ± 0.6	42	4.5
25−A	13.8 ± 0.1	0.40 ± 0.01	15.5 ± 0.9	9.8 ± 0.5	22.3 ± 0.3	88.2 ± 1.0	39	4.2
50−A	2.9 ± 0.1	0.30 ± 0.02	24.0 ± 1.1	10.0 ± 0.9	28.6 ± 0.5	93.1 ± 1.6	40	3.3
50−A NaCl	7.5 ± 0.1	0.64 ± 0.02	19.2 ± 1.0	−7.6 ± 0.9	26.4 ± 0.3	81.6 ± 0.5	39	4.0
50−S	11.8 ± 0.1	0.18 ± 0.01	16.6 ± 0.9	21.3 ± 0.5	19.2 ± 0.4	95.1 ± 2.1	39	3.7

[a] Type of gel medium used for alignment. Samples with addition of 150 mM salt are labeled NaCl; T = 5% indicates that the initial gel concentration was 5% instead of the typical 7%. [b] Alignment tensor parameters are calculated from the best fit of experimental values of $^1D_{HN}$ to the crystal structure of N-DCXmut. Errors were estimated using the jack-knife procedure by random elimination of 10% of the data. [c] Euler angles defining the orientation of alignment tensor relative to PDB coordinates; α, β, and γ correspond to rotation about the x, y, and z axes. [d] Number of RDCs used for the analysis. [e] Final gel concentration.

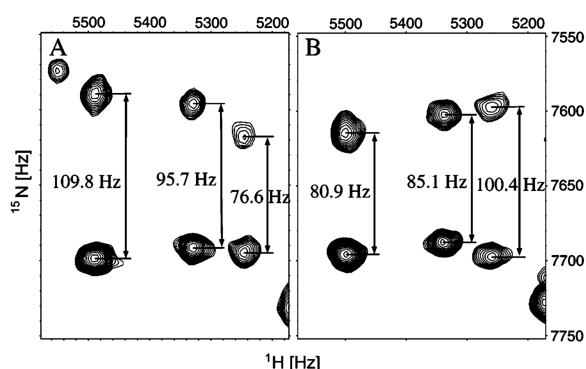

Figure 1. IPAP-HSQC spectra for N-DCXmut showing values of $^1D_{HN}$ coupling constants in two different gels: (A) zwitterionic (50±MA) and (B) positively charged (50+M).

5.0%. For example, the compression of 50+M gels to a final size of 15 mm produces a 4.2% gel. Under these conditions, the alignment tensor of N-DCXmut yields D_a^{HN} = 14.1 Hz. Subsequent release of the plunger to 16 mm reduced the gel concentration and the alignment to 4.0% and D_a^{HN} = 12.9 Hz, respectively. Furthermore, the degree of alignment significantly depends on the gel composition (Table 1). The magnitude of the alignment tensor for N-DCXmut is typically larger than 7.5 Hz and extends up to 15 Hz for the 75+M copolymer gel. This makes possible convenient and accurate measurement of RDCs with absolute values up to 30 Hz. Selected regions of the $^{15}N-^1H$ HSQC-IPAP spectra recorded for N-DCXmut in two different gels are presented in Figure 1. Two distinct alignments of the protein are demonstrated for 50±MA and 50+M copolymer gels.

Because the alignment of larger proteins, particularly large detergent−membrane protein complexes, will require lower gel concentrations, we tested the feasibility of decreasing the initial gel concentration from 7% to 5%. Stable gels were obtained; however, significantly stronger compression was necessary to achieve a significant degree of alignment of the N-DCXmut protein. Comparison of the alignments in 50+M gels polymerized from initial 5% and 7% concentration and compressed to ~4% gives rise to D_a^{HN} = 7.5 and 12.9 Hz, respectively, tensor magnitudes for N-DCXmut (Table 1). Using this approach, we were able to demonstrate the ability to obtain stable gels even at concentrations below 2.5% (data not shown).

Another critical component in the applicability of these gels for dipolar coupling measurements is their long-term stability, particularly at the elevated temperatures often employed for membrane protein samples. Replicates of the experiments carried out clearly show that samples are stable for extended periods of time without affecting the degree of alignment, making possible the measurement of heteronuclear coupling constants using various 3D experiments (see below).[16] Furthermore, the temperature stability was evaluated by recording replicates of spectra for OmpA at 50 °C which also showed no changes in the measured values over the course of 2 weeks. All of our data clearly indicate these copolymer gels have all that is necessary to study demanding systems such as membrane proteins.

Effects of Gel Charge. Protein orientation in the anisotropically compressed or stretched charged gels is determined by both steric effects and electrostatic interactions.[26,36] Indeed, it has been demonstrated that alignment in negatively and positively charged gels can be significantly different.[26] Application of two different alignments reduces degeneracy inherent in dipolar couplings [37] and significantly improves the accuracy of calculated structures.[38] To modify the composition of polyacrylamide gels and introduce differing charge, we have introduced a number of different copolymers. For negatively charged gels, we have copolymerized both acrylic acid and 2-acrylamido-2-methyl-1-propanesulfonic acid (AMPS). Positively charged copolymers have been obtained by copolymerization with 3-(acrylamidopropyl)-trimethylammonium chloride (APTMAC) or *N*-(2-acryloamidoethyl)triethylammonium iodide (NAETEAI). Methyl versus ethyl substitution on the positively charged nitrogen will result in a difference in the shielding of the positive charge resulting in an alteration of electrostatic interactions. We have also combined positive and negative copolymers to generate zwitterionic copolymer gels. All of the gels undergo electroosmotic swelling in water and could be successfully used to prepare NMR samples.

Analysis of protein alignments as a function of gel charge indicates that in general two unrelated alignments can readily

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be produced (Table 1). In the case of N-DCXmut, this can be achieved even without inversion of the gel charge. Comparison of N-DCXmut alignments in various conditions indicates that a change in protein orientation occurs between 25+M and 50+M gels. Thus, varying the content of APTMAC between 25% and 50% is sufficient to produce different alignments. The two sets of dipolar couplings measured for N-DCXmut in 50+M and 25+M are completely unrelated with a correlation coefficient r = −0.1. Similarly, Zweckstetter et al.[36] have shown substantial changes in orientation with varying salt concentration with phage alignment. They effectively modeled this showing that the magnitude of the quadrupole and dipole contributions to alignment varies with ionic strength. These contributions will also vary with charge density on our gels, providing a rationale for the observed behavior.

The degree of protein alignment in copolymer polyacrylamide gels is clearly affected by surface charge density. With the same degree of compression and gel concentration, stronger orientation is achieved in gels with a higher content of the charged component. Comparison of the 75+M, 50+M, and 25+M gels reveals a gradual decrease in alignment magnitude (Table 1). While for a 4.2% 75+M gel, we achieved $D_a^{HN} = 15.4$ Hz, a value of 10.5 Hz was obtained for a 4.5% 25+M gel. This observation can be used to minimize the gel concentration necessary to produce the desired level of orientation. Effects of surface charge density were further verified by comparing gels based on two positively charged acrylamide derivatives, APTMAC and NAETEAI. The alignment of DCXmut in a 75+E gel is almost 4 times weaker relative to a 75+M gel, even if the former is at a relatively high gel compression (Table 1). The positive charge of the quaternary amine group in the trimethylammonium derivative (APTMAC) is less shielded relative to the triethylammonium group (NAETEAI). As a consequence, APTMAC-based gels have stronger surface charge and yield higher degrees of alignment.

One drawback of charged orienting media is the risk of the unfavorable electrostatic interactions with the protein resulting in degradation of the quality of the NMR spectra. Our experiments revealed that strong interactions between various proteins and the gels are common (data not shown). Indeed, our recent study on syntenin PDZ domains indicated that strong interactions could be avoided only in gels with substantial positive charge (unpublished results). Thus, the availability of a broad range of conditions is essential to find media that are effective for a particular NMR sample. For example, strong interaction between N-DCXmut and the 50−A gel resulted in significant line-broadening in the NMR spectra. Such unfavorable electrostatic interactions may be quenched by addition of salt (see below). Because compression of the gel under these conditions results in severe degradation of the quality of the NMR data, we were limited to small degrees of compression resulting in weak alignment with $D_a^{HN} = 2.9$ Hz. Interestingly, good-quality NMR spectra and a reasonable alignment could be obtained for a 50−S gel ($D_a^{HN} = 11.8$ Hz). Comparison of RDCs measured in the two negative gels, 50−A and 50−S, yields a correlation coefficient $r = 0.75$ and substantially changed rhombicities (Table 1). Interestingly, a similar effect was observed for OmpA (see below).

Effects of Ionic Strength. Ionic strength is one of the crucial parameters affecting electrostatic-based alignment.[36] Indeed, 50−200 mM salt is often necessary to maintain stability of proteins in solution. High ionic strength will have a significant effect on the magnitude of the alignment in charged gels, particularly those with low polymer concentrations.[25] We observe this influence of ionic strength; however, in 4% copolymer gels this effect is not particularly strong. Addition of 150 mM NaCl decreases the magnitude of the alignment tensor of N-DCXmut from 15.4 to 9.4 Hz for a 75+M gel at the same level of compression. The decreased alignment due to diminished electrostatic interactions can be compensated by higher degrees of gel compression.

The addition of salt may be favorable in cases where quenching the electrostatic interactions is necessary to facilitate measurement in charged media. As reported previously, 250 mM NaCl was necessary to measure RDCs for ubiquitin aligned in phage media.[39] As mentioned above, strong interaction of N-DCXmut with the 50−A gel limited the degree of compression we could utilize, and only weak alignment could be obtained ($D_a^{HN} = 2.9$ Hz). Addition of 150 mM NaCl combined with stronger compression overcame this problem and resulted in an increased alignment with $D_a^{HN} = 7.5$ Hz (Table 1).

As observed by Zweckstter et al. [36] for phage-based alignment, we see cases where only the alignment magnitude is affected by ionic strength and others where the alignment tensor is affected. The correlation between two data sets recorded for N-DCXmut in 75+M gel in the absence and the presence of 150 mM NaCl is very high with $r = 0.98$. In contrast, distinct differences were found for the 50−A gel where the correlation coefficient between sets of RDCs collected with and without salt is $r = 0.88$. This change was accompanied by an increase in rhombicity from 0.30 to 0.64.

Multiple Alignments as a Result of Protein Modification. Changing the composition of the alignment medium is the most convenient way to affect the alignment. However, strong interaction with a protein may limit the application of a broad range of gel conditions. For example, only two gels with strong positive charge, 50+M and 75+M, could be used for alignment of the syntenin PDZ domains (unpublished results). As a consequence, a second orientation was not obtained. Thus, if the gel composition cannot be varied, an alternative option may be protein modification to alter the electrostatic interactions.

To test this possibility, we compared N-DCXmut with wild-type N-DCX. Two substitutions in N-DCX, K134D, and K135D lead to a substantial decrease in the calculated pI from 8.0 to 4.9 and significantly affect the surface charge distribution. The structure of the N-DCX domain is not perturbed to any significant degree by these mutations with only slight changes observed in the loop regions. Indeed, the experimental values of $^1D_{HN}$ for N-DCX fit very well to the crystal structure of N-DCXmut, and the quality factors are only slightly worse (Table 2). To assess whether these point mutations affected the alignment, we prepared three gels: 75+M, 50±MA, and 50−A. The correlation coefficients between data sets recorded for N-DCX and N-DCXmut aligned under the same conditions are 0.83, 0.69, and 0.37, respectively. Despite very similar degrees of alignment for N-DCX and N-DCXmut in the 75+M gel, the orientation of the modified protein was affected (Tables 1 and 2). The differences are more pronounced for the 50±MA and 50−A gels. Interestingly, unlike N-DCXmut, the wild-type

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Table 2. Analysis of RDCs and Calculation of Alignment Parameters for Wild-Type N-DCX[a]

gel type	D_a (Hz)	R	Q (%)	α	β	γ	Nr	conc (%)
75+M	14.1 ± 0.2	0.12 ± 0.01	18.9 ± 0.8	9.0 ± 0.5	−43.5 ± 0.4	7.7 ± 3.8	48	4.0
5±MA	11.4 ± 0.1	0.53 ± 0.02	16.9 ± 0.7	−28.7 ± 0.7	8.0 ± 0.4	85.6 ± 1.0	49	5.3
50−A	7.6 ± 0.1	0.38 ± 0.02	17.7 ± 1.0	−34.9 ± 0.6	3.4 ± 0.4	71.4 ± 1.4	48	4.2

[a] Table description is identical to that in Table 1.

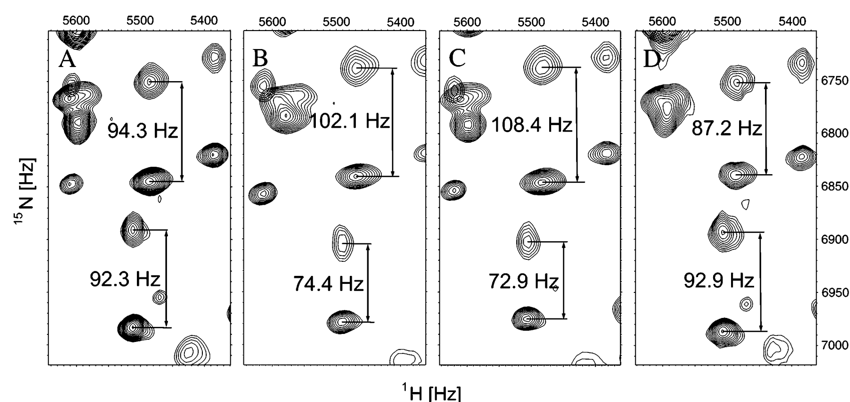

Figure 2. Sections of IPAP-HSQC spectra recorded for ^{2}H,^{15}N-OmpA. Comparison of $^1D_{HN}$ couplings for isotropic sample (A) and three different gels: 50−A (B), 50−S (C), and 50+M (D).

Table 3. Alignment of OmpA in Three Different Copolymer Polyacrylamide Gels

gel type	D_a (Hz)[a]	R[a]	Q (%)[b]	Q (%)[c]	α[d]	β[d]	γ[d]	Nr[e]	conc (%)[f]
50−A	9.3 ± 0.2	0.05 ± 0.02	23.3 ± 1.2	30.1 ± 1.3	96.6 ± 0.6	32.6 ± 0.6	−19.6 ± 13.2	43	3.5
50−S	10.8 ± 0.2	0.57 ± 0.03	22.6 ± 1.3	28.0 ± 2.0	81.7 ± 1.0	7.7 ± 0.8	−85.4 ± 1.0	45	4.0
50+M	12.9 ± 0.2	0.06 ± 0.01	23.9 ± 1.8	27.1 ± 2.0	−65.1 ± 1.2	−75.1 ± 0.3	168.3 ± 7.3	43	3.3

[a] Alignment tensor parameters are calculated from the best fit of experimental values of $^1D_{HN}$ to the high-resolution OmpA crystal structure (PDB code 1qjp). Errors were estimated using the jack-knife procedure by random elimination of 10% of the data. [b] Quality factors calculated for 1.65 Å resolution OmpA structure (PDB code 1qjp). [c] Quality factors calculated for 2.5 Å resolution OmpA structure (PDB code 1bxw). [d] Euler angles defining the orientation of alignment tensor relative to PDB coordinates; α, β, and γ correspond to rotation about the *x*, *y*, and *z* axes. [e] Number of RDCs used for the analysis. [f] Final gel concentration.

N-DCX domain does not show strong interaction with negative media, and reasonable alignment in a 50−A gel was obtained (D_a^{HN} = 7.6 Hz).

In general, protein modification, albeit more laborious, can be considered as an alternative approach for the generation of alternative alignment. For systems such as integral membrane proteins or multidomain proteins, a second alignment may be of critical importance for solving structural ambiguities. Because the site of such mutations needs to be carefully selected, this approach may require a preliminary structure of the protein or a reliable homology model. Appropriate targets for mutagenesis include solvent-exposed charged residues, preferably in loop regions, and the N- and C-termini. For example, the alignment tensor may be affected by addition (or removal) of poly-histidine tags to the protein termini.[40]

Alignment of Integral Membrane Protein OmpA in DPC Micelles. To test the feasibility of inducing weak alignment of OmpA in DPC micelles, we prepared several samples in different gel conditions. Preparation of aligned samples was carried out in the same manner as we did for the N-DCX domain. Interestingly, the swelling of the gels in the presence of protein−detergent complex was fast, and highly homogeneous samples were obtained. A lower gel concentration and compression were sufficient to induce alignment of OmpA relative to the N-DCX domain. Typically, satisfactory levels of alignment were obtained in gels with 3−4% copolymer concentrations. The high quality of the IPAP-HSQC spectra recorded for OmpA in three different gels is shown in Figure 2. Because the upfield component of the doublet exhibits a moderate degree of broadening, we were able to extract couplings from standard IPAP-HSQC spectra. Due to the high cost of triply labeled OmpA, we have initially recorded only 2D spectra using ^{2}H,^{15}N-labeled OmpA. Because of severe overlap in the 2D spectra, a reliable analysis was limited to approximately one-third of all amides. The use of triple-labeling and 3D NMR spectra will significantly increase the number of resonances that can be characterized. The alignment achieved for OmpA permits the measurement of $^1D_{HN}$ couplings up to 25 Hz.

Three data sets have been analyzed for ^{2}H, ^{15}N-labeled OmpA aligned in 50−A, 50−S, and 50+M copolymers. Alignment tensor parameters calculated from fitting of the measured RDCs to the high-resolution crystal structure of OmpA (PDB code 1qjp) are summarized in Table 3. Each data set shows a different alignment and very good agreement with the crystal structure with quality factors below 24%. The alignment of OmpA in the 50−S gel is characterized by D_a^{HN} = 10.8 Hz and a large rhombicity, R = 0.57. Measured values of $^1D_{HN}$ span the range between −22 and +25 Hz. Comparison of the experimental and calculated RDCs for the 50−S gel is presented in Figure 3A. The amides with isolated chemical shifts used for the analysis are located in the β-barrel portion of the protein and are shown in black (Figure 3B).

Because the structural analysis of membrane proteins may rely heavily on achieving multiple different alignments, we

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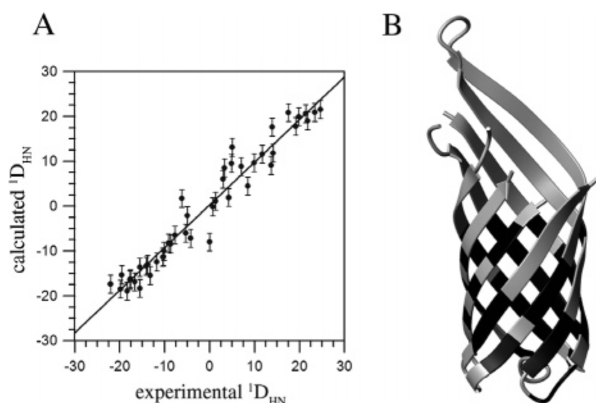

Figure 3. Analysis of $^1D_{HN}$ residual dipolar couplings for OmpA. (A) Compatibility between experimental values of $^1D_{HN}$ and those predicted from the crystal structure (PDB code 1qjp); (B) high-resolution crystal structure of OmpA (1qjp) with selected residues used for the analysis of RDCs (shown in black).

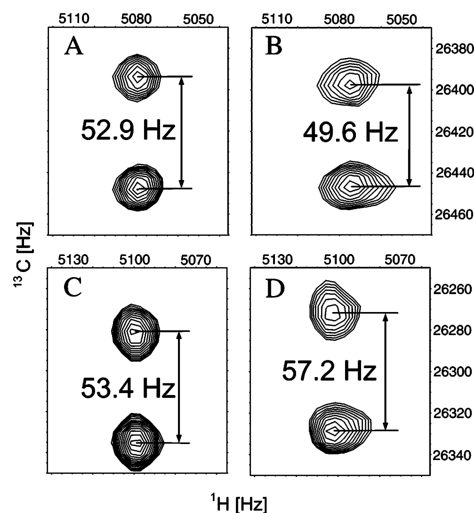

Figure 4. Selected sections from the 3D TROSY-HNCO experiment for measurement of $^1J_{C'C\alpha}$ coupling constants recorded for ^{2}H,^{13}C,^{15}N-labeled OmpA. The $^1J_{C'C\alpha}$ coupling constants measured in isotropic samples (A and C) and the corresponding values recorded in 50−S gel (B and D, respectively).

tested various gel conditions with OmpA to establish the ability to achieve multiple alignments with a membrane protein. Distinctly different alignments of OmpA were obtained with two types of negatively charged gels, 50−S and 50−A (Table 3). The two data sets show moderate correlation with $r = 0.72$. Lower compression of the 50−A gel may account for the slightly weaker degree of alignment ($D_a^{HN} = 9.3$ Hz). Because the alignment is weakly rhombic ($R = 0.05$), the values of $^1D_{HN}$ range between −10 and +20 Hz. We also tested the alignment of OmpA in positively charged gels. A significantly different orientation of the protein−micelle complex was obtained in a 50+M gel (Table 3). The alignment obtained at relatively low, 3.3%, gel concentration corresponds to $D_a^{HN} = 12.9$ Hz and experimental values of $^1D_{HN}$ between −10 and +25 Hz. Compatibility between the RDCs and the crystal structure is again very high with $Q = 23.9\%$.

Detailed studies of membrane protein structures by NMR and X-ray crystallography may suffer from the non-native environments in which the structural studies are carried out. Thus, measurement of RDCs for OmpA makes it possible to directly compare the structures in micelles in solution and in the crystalline state. The close agreement observed between the RDCs and the crystal structure of OmpA indicates that the structures in solution and in the crystal closely resemble one another. Indeed, we carried out the RDC analysis for two OmpA crystal structures determined at 1.65 Å (PDB code 1qjp) and 2.5 Å (PDB code 1bxw) resolution. Despite similar backbone structures (RMSD between Cα is 0.42 Å), better agreement between experimental and predicted RDCs is observed for the high-resolution structure (Table 3). The quality factors calculated for the 1.65 Å structure are from 3% to 7% lower than for the 2.5 Å structure (Table 3). This observation is in agreement with previous reports for globular proteins [13] and is a strong indication of the accuracy of the RDC data.

Although the $^1D_{HN}$ data are sufficient to demonstrate the alignment of membrane proteins in charged copolymer polyacrylamide gels, structure refinement will significantly rely on additional heteronuclear residual dipolar couplings. To assess the feasibility of measuring additional heteronuclear RDCs from 3D experiments, we prepared a sample of ^{2}H,^{13}C,^{15}N OmpA in a compressed 50−S gel. Selected regions of the TROSY-based HNCO experiment for determination of $^1D_{C'C\alpha}$ couplings are shown in Figure 4.[16] These data clearly indicate that high-quality triple resonance data can be collected on integral membrane proteins using these gels. Furthermore, this experiment also demonstrates the necessary long-term stability of the gels at elevated temperatures, an essential prerequisite for the collection of the ensemble of dipolar couplings needed for structure calculations.

Conclusions

In this report, we focused on the development of a series of charged copolymer polyacrylamide gels that would be suitable for inducing weak alignment of macromolecules for measurement of RDCs, particularly for integral membrane proteins. We have developed a wide range of different charged copolymer gels for application as alignment media. These gels show excellent homogeneity and long-term stability even at elevated temperatures. Studies using a small soluble protein clearly established the ability to achieve multiple unique alignments by manipulation of the copolymer composition. Subsequently, we have utilized these gels to achieve meaningful degrees of alignment for the integral membrane protein OmpA, the first such demonstration for a membrane protein of this size. We have also shown that we can achieve multiple unique alignments of OmpA by manipulating copolymer composition, which will greatly assist in the structure determination of this class of proteins.

As expected, the alignment of OmpA in the compressed gels is primarily orthogonal relative to the magnetic field. Thus, the values of the RDCs are smaller than for axially aligned samples at the same level of compression.[35] However, the advantage of soaking and compression of the dried gel directly in the NMR tube is the ability to obtain stable and highly homogeneous samples even at low polymer concentrations. The mechanical stability of uncharged 2−3% gels will not be sufficient to use other methods relying on gel manipulation.[35] We show that the degree of OmpA alignment in such compressed gels is more than adequate for structure calculations, with measured values of $^1D_{HN}$ up to 25 Hz. Because of the low gel concentration, the diffusion of the protein−detergent complex within the gel is

not significantly affected, which makes it possible to record high-quality 3D spectra and carry out measurement of multiple heteronuclear RDCs. In general, the charged copolymer polyacrylamide gels described herein should significantly extend application of residual dipolar couplings for large and complex systems, and particularly for integral membrane proteins.

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