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Weak alignment of membrane proteins in stressed polyacrylamide gels

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Abstract

Residual dipolar couplings are important as angular constraints for the structure determination of membrane proteins in micelles. Strained polyacrylamide gels are one of the few available mechanisms available for inducing the requisite weak alignment for these samples. However, their use is frequently limited by the ability to incorporate proteins and buffer solutions into the gel matrix. The implementation of several methods of incorporating membrane proteins into gels are described. Conditions for copolymerizing the protein in the absence of a change in pH are detailed. Electrophoresis is also shown to be a useful method to incorporate proteins. Weak alignment of the protein–micelle complex in the gel matrix is subsequently achieved using either vertical or radial compression. The magnitude of alignment can be controlled by altering the gel concentration, the acrylamide/bisacrylamide ratio, and the compression ratio. The alignment tensor can be altered relative to uncharged polyacrylamide gels by copolymerizing samples with acrylamide/acrylic acid to incorporate negative charges in the strained polyacrylamide gel to provide an alternate orientation
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1. Introduction

Residual dipolar couplings (RDCs) and other orientational constraints measured from solution NMR spectra of weakly aligned samples are widely used in the structure determination of globular proteins and their complexes. They have dramatically improved essentially all aspects of protein structure determination by solution NMR spectroscopy. The potential impact of these constraints on structure determination of membrane proteins in micelles is even greater because of the difficulty in measuring and assigning ''long-range'' NOEs in these systems. However, there have been only a few applications to membrane proteins, largely because of difficulties encountered in the preparation of suitable samples. In this article, we describe several ways to prepare weakly aligned samples of membrane proteins in stressed polyacrylamide gels that overcome many of the problems encountered in previous studies by ourselves and others.

Following the initial demonstration that weak alignment of globular proteins in solution can be induced by the presence of magnetically aligned bicelles [1], a wide variety of media have been developed for this purpose, including solutions of the filamentous bacteriophages fd [2] and Pf1 [3]; mixtures of cetylpyridinium halide and hexanol [4,5]; mixtures of *n*-hexanol and alkylethylene glycol [6]; and purple membrane fragments [7,8]. Unfortunately, these media are incompatible with membrane proteins because their accompanying lipids are destructive to the bacteriophage media, interfere with some of the mixtures, or merge with the lipids in the bicelles. We

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have previously used lanthanide ions to induce weak alignment of membrane proteins in micelles [9,10], but this approach results in only modest degrees of alignment and is fraught with complications due to the presence of paramagnetic ions in the solutions.

Polyacrylamide gels provide an alternative approach to the preparation of weakly aligned samples of membrane proteins. Strain-induced alignment in gel (SAG) [11–13] employs either vertical or radial compression of the gel in order to alter the pore shape and induce preferential alignment of the protein. Polyacrylamide gels are chemically inert, therefore, the samples are stable over a wide range of temperature, ionic strength, and pH [12]. Further, the extent and direction of alignment can be "tuned" by physically altering the mechanical compression or the gel composition [11,12]. Typically, the protein is allowed to diffuse into the gel [12–14], and this has enabled the preparation of samples of several membrane proteins for NMR experiments [13,15–17]; however, it requires a relatively large amount of material, and in our experience is not routinely applicable to membrane proteins. Moreover, it can be extremely time consuming to prepare the many samples that may be needed to find suitable conditions because the temperature [18], ionic strength [19], lipid composition and concentration, and size of the polypeptide all affect the ability of the protein to diffuse into the gel. And even after optimization of all of these parameters it still may not be feasible to generate a sample compatible with the requirements of NMR experiments because of poor incorporation, heterogeneity, or excessive line broadening.

We have implemented several alternative methods to incorporate membrane proteins into a polyacrylamide gel and induce weak alignment for the measurement of ^{15}N–^{1}H backbone RDCs and other orientationally dependent frequencies. Because they do not depend on simple diffusion, these methods are applicable to a broader range of proteins, lipids, buffers, and other conditions. The methods of sample preparation are illustrated with the 50-residue fd coat protein because it has features of a typical membrane protein including a long hydrophobic transmembrane helix and a short amphipathic helix that lies on the surface of the bilayer that are connected by a short loop [20,21].

2. Experimental section

2.1. Sample preparation

The expression, isolation, and purification of the membrane-bound form of uniformly ^{15}N-labeled fd coat protein have been previously described [22]. Isotropic samples for solution NMR spectroscopy consist of 1 mM fd coat protein, 500 mM sodium dodecyl-d_{25} sulfate (SDS, Cambridge Isotope Laboratories, Andover, MA), 40 mM NaCl, 10% D_2O, at pH 4.0.

Co-polymerized gel samples were prepared from a stock solution of 30% w/v acrylamide and 0.8% w/v *N*,*N*′-methylenebisacrylamide (National Diagnostic, Atlanta, GA). Immediately prior to use, all stock solutions (H_2O, D_2O, and acrylamide) were degassed under high vacuum (50 mBar) for 5 min while sonicating at 40 °C. Samples were prepared by mixing the appropriate amount of acrylamide, water, SDS, NaCl, D_2O, fd (from a 50 mg/ml stock solution), and adjusting the pH to generate a 500 μl sample of 1 mM fd coat protein, 500 mM SDS, 40 mM NaCl, 10% D_2O, at pH 4.0 with the desired acrylamide concentration. Chemical polymerization was initiated by the addition of 0.08% w/v ammonium persulfate (APS) and 0.002% *N*,*N*,*N*′,*N*′-tetramethylethylene diamine (TEMED) and the pH verified. Samples were subsequently incubated at least 4 h at 40 °C to ensure complete polymerization. As an alternative, the APS was replaced by 0.06% riboflavin and the sample incubated overnight at room temperature under strong fluorescent light to allow photo-polymerization. This has the advantage over APS of not being adversely affected by pH, but does require more care since the samples take longer to polymerize [23]. Degassing is crucial because oxygen is needed to convert riboflavin to the active form but also inhibits free radical formation [24]. Samples for radial compression were polymerized directly in a funnel device similar to that developed by Bax and coworkers [11], squeezed into an open bottom NMR tube, and sealed with the supplied plug (New Era). The presence of detergent provided adequate lubrication to ensure that the gel is not adversely affected during the process of stretching. Vertical compression was achieved by first co-polymerizing the sample in a medium wall NMR tube (524-PP, Wilmad), breaking off the bottom of the tube, and carefully transferring the gel into a screw cap NMR tube (535-TR-7, Wilmad). Approximately 50 μl buffer is added, and the gel compressed using the device shown in Fig. 2 until the sample exhibited strong optical birefringence; alignment is verified by observation of a splitting in the deuterium lock signal. Samples slowly lost birefringence over a period of several weeks, but this generally does not adversely affect the measurements since the data are typically acquired within 12 h of sample preparation. Older samples could be easily resurrected by applying additional force with the nylon screw.

The samples copolymerized in the presence of acrylic acid were prepared using the same protocol except that a solution consisting of 27% w/v acrylamide (EM Science), 3% acrylate (99% solution, Aldrich), and 0.4% w/v *N*,*N*′-methylenebisacrylamide (EM Science) was prepared and used in place of the acrylamide stock solution. After degassing all solutions, samples consisting of 1 mM fd coat protein, 500 mM SDS, 40 mM NaCl, 5%

polyacrylamide gels (PAG) were copolymerized at 42 °C overnight using 0.1% w/v APS and 0.002% TEMED in a 5 mm "Bax" device. The sample was subsequently squeezed into an open bottom tube and sealed with the supplied plug.

Samples for electrophoresis were prepared using the following protocol. A 60 mM KOH/acetic acid buffer (pH 4.0), 500 mM SDS, 7% polyacrylamide gel were poured at the bottom of a medium wall NMR tube (Wilmad). Polymerization was initiated by adding 0.002% TEMED and 0.06% riboflavin and incubating overnight at room temperature under strong fluorescent light. A small hole was prepared at the bottom of the tube by rubbing the tube vigorously across fine sandpaper. The tube was then inserted into the apparatus shown in Fig. 6 with the anode and cathode reservoirs filled with standard native Tris/glycine running buffer. A 500 μl NMR sample of fd coat protein (1 mM protein, 500 mM SDS, and 40 mM NaCl, pH 4.0) was prepared with 10 μl of loading dye (50% glycerol and 0.02% w/v xylene cyanol) and the sample loaded on the surface of the gel. The sample was electrophoresed into the gel at 165 V until the dye migrated to the bottom of the NMR tube, which typically required about 2 h. The NMR tube was subsequently removed from the apparatus and the running buffer aspirated from the surface of the gel. The bottom of the tube was sealed using either a small drop of epoxy or a plug. In order to obtain a deuterium lock signal 50 μl of D_2O was added and the sample incubated overnight to allow uniform diffusion of the D_2O into the gel. Isotropic data were subsequently acquired before transferring the gel to a screw cap NMR tube and compressing as described above.

2.2. NMR spectroscopy

Experiments were performed at 40 °C on a Bruker DMX spectrometer with a ^1H resonance frequency of 600.1 MHz. IPAP-HSQC spectra [25] were acquired with suppression of the acrylamide NH_2 signals [14]. All spectra were processed using nmrPipe [26] and peak positions were determined using the picking software in Sparky.

2.3. Calculations.

The identification of helical regions using Dipolar Waves was performed as described previously [16]. Unconstrained nonlinear optimization was used to fit the amplitude and phase of a sinusoid with a periodicity of 3.6 and residues were designated as part of a continuous helix if they fit within the average error of the measurements (0.5 Hz). Kinks were readily identified as an abrupt change in the average value, amplitude and/or phase of the sinusoid fitted to the experimental data as plots of RDCs as a function of residue number.

3. Results

3.1. Co-polymerization

Some lipids self-assemble in the presence of water to form micelles. With optimization, protein-containing micelles or small "isotropic" bicelles can be used for solution NMR studies. It can be difficult to diffuse protein-containing micelles or bicelles into polyacrylamide gels, probably because of the large hydrodynamic radius of the protein–lipid complexes. In our experience, the propensity of these samples to soak into a gel depends on many factors including the type of lipid, concentration of lipid, ionic strength, and size of protein. Frequently the volume of the rehydrated gel remains considerably smaller than its original size; the resulting increase in gel density not only adversely affects rotational diffusion, but also creates spatial inhomogeneities in the sample. This phenomenon has also been observed with soluble globular proteins [13]. In some cases it can be overcome by adjusting the gel composition to increase the pore size, however this is limited by the difficulties associated with handling low percentage gels. At acrylamide concentrations less than 4% the gels are not mechanically stable.

Co-polymerization of the protein, lipid, and acrylamide components provides a way to overcome problems associated with diffusing protein–lipid complexes into polyacrylamide gels [11,27]. This approach has the advantages that it is very rapid since it does not rely on sample diffusion, and it is not limited to conditions that are amenable to gel swelling. Copolymerization is associated with a dramatic increase in pH that has previously been compensated for by using a high concentration of buffer (300 mM) that is subsequently removed, or with the direct addition of HCl [13], however, large local changes in pH cause many membrane proteins to precipitate and form intractable aggregates. The change in pH results from the 0.5% quaternary amine TEMED that serves to stabilize the free radicals required for polymerization. Since dissolved oxygen represents the most common quenching agent, samples were prepared that had been thoroughly degassed under high vacuum, eliminating the requirement for TEMED. Initial trial samples polymerized very well but yielded spectra with multiple peaks indicating that the gel was not uniform or there were other sources of heterogeneities in the protein molecules. Subsequently, it was determined that degassing the buffers in conjunction with the addition of a very small amount of TEMED (0.002%) generated uniform samples without the problematic changes in pH. The two-dimensional ^1H–^{15}N HSQC spectra of fd coat protein in SDS micelles in aqueous solution and co-polymerized with 7% polyacrylamide compared in Fig. 1 are very similar. This indicates that the process of co-polymerization and the presence of any unreacted reagents

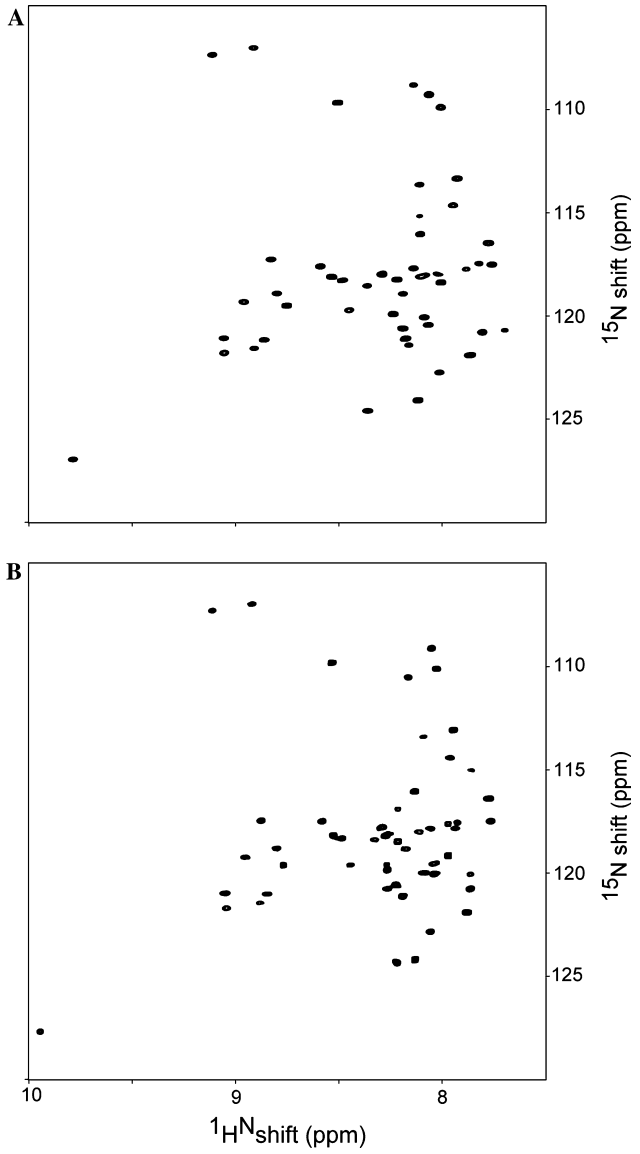

Fig. 1. Amide region of the ^{1}H/^{15}N HSCQ spectrum of 1 mM uniformly ^{15}N-labeled fd coat protein in 40 mM NaCl, 500 mM SDS at pH 4.0 and 40 °C. (A) Sample in the absence of acrylamide. (B) Sample copolymerized in a 7% polyacrylamide gel.

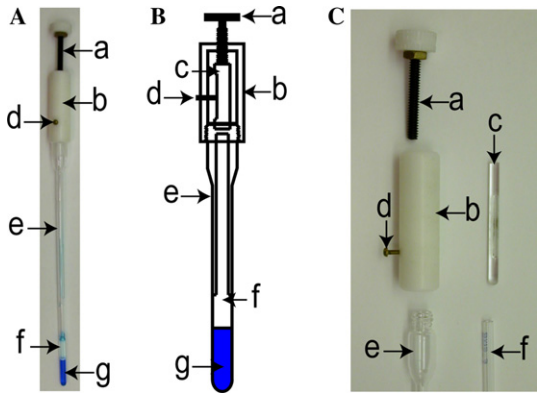

Fig. 2. Apparatus for compressing hydrated polyacrylamide gels. Photograph (A) and schematic drawing (B) of the device. (C) Photograph of disassembled apparatus. The various components are: (a) large nylon screw; (b) delrin cylinder threaded at the top for the nylon screw and at the bottom for the NMR tube; (c) glass rod notched at one side; (d) small brass screw; (e) screw cap NMR tube; (f) Shigemi plunger; and (g) compressed polyacrylamide gel.

or by-products does not result in any significant changes in the protein. Similar to previous observations, the resonance intensities were minimally reduced (approximately 10%) compared to nonpolymerized samples [11].

The application of mechanical force to stretch or compress the polyacrylamide gel generates weak alignment in the samples. Radial compression to stretch the gel is easily achieved by copolymerizing the sample directly in the funnel-like "Bax" device [13]; the inner diameter of the device is slightly larger than that of an NMR tube, and the gel stretches in the Z-direction as it is squeezed into an open-ended NMR tube, which is subsequently sealed with a plug. Not only does this approach provide a rapid and reliable method of incorporating the protein into the gel, but also since the gel is never physically manipulated it is feasible to copolymerize gels containing a very low percentage of acrylamide. It is expected that the larger pore sizes found in the lower percent gels will be vital for applications to larger polypeptides. If the magnitude of alignment is too great, the ^{1}H–^{1}H dipolar coupling increases the linewidth and prevents the facile measurement of RDCs [28].

Similarly, the application of vertical compression by applying pressure with a Shigemi plunger to a polyacrylamide gel (PAG) has also been shown to induce weak alignment [11,12]. In the case of membrane proteins this method has only proven successful when pressure was applied during the swelling process [17]. Briefly, the gel is prepared with a slightly smaller diameter than the NMR tube and dried by placing the plug in a 37 °C oven overnight; the protein solution is subsequently soaked into a dry gel using a Shigemi plunger to restrict its length. Initial attempts to compress a hydrated gel failed to produce satisfactory results because the gel would gradually expand forcing the plunger out of the tube as it returned to its original length. The process of compression would frequently destroy the gel or the Shigemi tube. The device developed to facilitate the homogeneous compression of fully hydrated gels in the Z-direction is shown in Fig. 2. It consists of six pieces: a screw cap NMR tube, a Shigemi plunger, a delrin cylinder threaded at the top to match the nylon screw and at the bottom to the NMR tube, a glass rod (notched on one side), a brass screw, and a large nylon screw. The susceptibility matched Shigemi plunger is inserted above the gel in the screw cap NMR tube and the delrin case screwed onto the end. Delrin proved to be the most effective material for this application since it is nonmagnetic, relatively light, and could withstand the mechani-

cal force. Rotation of the nylon screw forces the glass rod and plunger down and compresses the gel. The small brass screw isolates rotation from the sample by applying pressure against the notch in the glass rod. This is necessary to prevent twisting of the gel. Rotation of the plunger generally resulted in significant damage to the gel and interfered with compression. The Shigemi plunger was kept sufficiently long so that it could be easily removed with forceps. The advantage of this approach is that it enables the *J*-couplings and chemical shifts to be measured in the same gel environment before and after the application of mechanical stress to induce weak alignment. This eliminates the need to make multiple samples, and it is particularly important to be able to accurately compare the chemical shifts in order to identify the small frequency shifts due to weak alignment [17]. Initial trials were conducted directly in the screw cap tube; the samples were copolymerized and subsequently compressed until they exhibited optical birefringence. The maximum splitting of the deuterium resonance observed was ~1 Hz, and the maximum ^{1}H–^{15}N RDCs observed were relatively small (~2 Hz). The application of additional compression failed to increase the magnitude of the RDCs and only expelled water from the gel. The absence of a cleft between the gel and the NMR tube seemed to limit the anisotropic compression. In order to overcome this limitation the sample was first copolymerized in a medium wall NMR tube (3.4 mm ID), the isotropic values measured, and the gel subsequently transferred to the screw cap tube (4.2 mm ID) for compression. The presence of detergent enabled the gel to be readily removed from the NMR tube, but for standard samples it may prove beneficial to siliconize (Sigma) the glass walls prior to polymerization to facilitate easy extraction of the gel. Samples were slowly compressed until the gel uniformly filled the NMR tube and exhibited strong optical birefringence and a deuterium splitting of several hertz.

Fig. 3 shows a portion of the amide region of a ^{1}H–^{15}N IPAP HSQC spectrum of uniformly labeled fd coat protein in copolymerized PAG for isotropic, compressed, and stretched samples. This spectral region was selected for display because it contains resonances from residues in the transmembrane and amphipathic helices as well as the loop region. The upfield and downfield components of the ^{15}N doublets are overlaid for each sample. Comparison of spectra obtained before and after copolymerization demonstrated a modest 3.8 Hz average broadening of the ^{1}H linewidth, which indicates that the samples are reasonably homogeneous. This increase in linewidth is somewhat larger than the <2 Hz previously reported for soluble protein samples prepared by allowing the protein to diffuse into the acrylamide gel [11]. Higher viscosity of protein-containing micelle solutions in the pores of the gel, the large size of the protein micelle complex, or the presence of un-reacted reagents may account for this effect. The application of mechanical stress to induce weak alignment resulted in a further 2.5 Hz average increase in the linewidth and is associated with ^{1}H–^{1}H dipolar couplings previously observed in weakly aligned gel samples [11,14]. This did not affect the ability to measure RDC's since the spectral quality was sufficient to unambiguously resolve all resonances in the spectrum.

Since residual dipolar couplings translate into an alteration in the magnitude of the *J*-coupling, they are readily easily measured by determining the difference in the splitting obtained under isotropic and anisotropic conditions. The center and left panels in Fig. 3 show that it is feasible to easily measure large heteronuclear

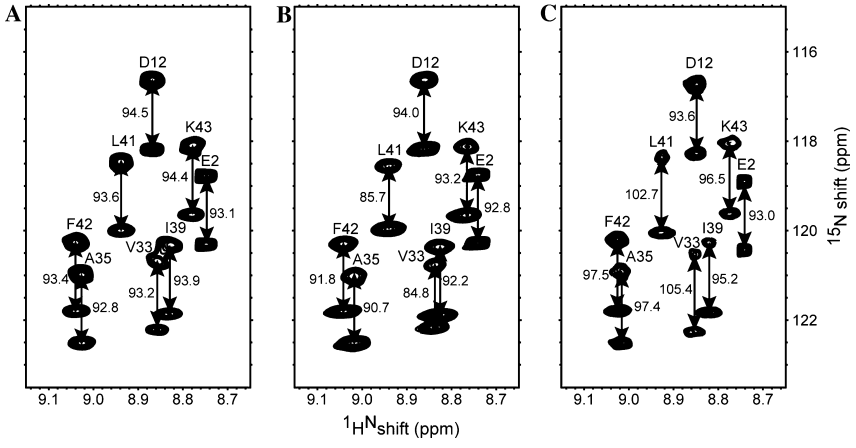

Fig. 3. Expanded region of the $^{1}H/^{15}N$ IPAP-HSQC spectrum of 1 mM fd coat protein solubilized in 500 m SDS and copolymerized in polyacrylamide gel. This region was selected since it contains peaks from all domains of the fd structure, which consists of an amphipathic helix extending from residues A7 to T19 and a transmembrane domain from A25 to K48 that is kinked after residue G38. The In-phase and Anti-phase spectra are overlapped and the corresponding splittings are shown. (A) Isotropic sample in 7% polyacrylamide gel in a 3.4 mm ID NMR tube. (B) Same sample transferred to a 4.2 mm ID screwcap NMR tube and compressed using the device shown in Fig. 2. (C) 5% polyacrylamide sample copolymerized in a 5 mm Bax device and squeezed into a 4.2 mm ID NMR tube.

RDCs. Samples with RDCs of a similar magnitude have been used to measure the smaller ^{13}C–^{15}N and ^{13}C–^{13}C interactions [29]. Notably, the RDCs have the opposite sign between the compressed and stretched samples. This is more obvious in plots of experimental ^{1}H–^{15}N RDCs as a function of residue number with the corresponding Dipolar Wave fits superimposed (Fig. 4). Since Dipolar Waves map the anisotropic RDCs to the periodicity of α-helices it is relatively simple to identify helical regions by plotting a sinusoid with the 3.6 residues per turn period found in ideal helices [16,30]. Using these oscillations as a strict criterion both samples identified the same helical regions: the amphipathic helix starting at A7 and ending at T19 and the transmembrane helix extending from A25 to K48. The transmembrane domain exhibits a kink after G38, manifested as a sharp change in the amplitude and phase of the sinusoid and has been found previously in both SDS micelles and lipid bilayers [16]; interestingly, this feature exists when this protein is in the intact bacteriophage where this region is in intimate contact with the DNA [31]. The irregular patterns of the dipolar couplings at the termini and the loop connecting the amphipathic and transmembrane helices make it easy to precisely identify helical regions. The magnitudes of the couplings at the termini and the loop are relatively small and reflect scaling of the RDCs since there is evidence that these residues exhibit local backbone dynamics when the protein is in micelles [20].

The compressed and stretched samples are expected to produce different anisotropies within the gel because of the oblate and prolate shapes of the cavities that are formed. Fig. 4 shows that the RDCs generated using these two different methods are mirror images about zero, and result in a 180° phase change in the Dipolar Waves, which is consistent with the orientation tensor becoming inverted between these two samples. This is more clearly shown in Table 1 that shows the average value, amplitude, and phase of the Dipolar Waves from each of the helical regions. The results demonstrate the ability to easily identify the kink at G38 because the average value and amplitude of the Dipolar Wave change between regions 25–38 and 39–48. Comparisons between the compressed and stretched samples indicate that the average value (Aa) for each of helices has the opposite sign and the phase (ϕ) differs by approximately π. The same degree of compression should scale the alignment by −2 in oblate cavities generated by squeezing the gel and orienting the alignment frame along the B_0 versus prolate pores generated by compression. It is very difficult to prepare stretched and compressed samples using with the identical extent of alignment, nonetheless, the results presented here are consistent with the expected result. The average value of the Dipolar Waves change sign between the two samples and despite decreasing the gel percent in the stretched sample to reduce the alignment and avoid excessive line broadening, the average value and amplitude are considerably larger.

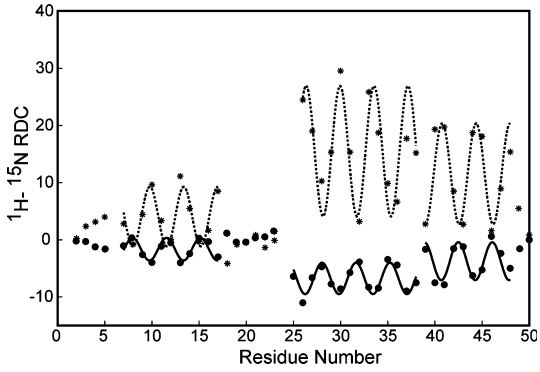

Fig. 4. RDCs as a function of residue number for 1 mM fd coat protein in SDS micelles and the corresponding Dipolar Wave (line). The ● and solid line are the results obtained from a 7% acrylamide, 0.8% bisacrylamide gel copolymerized in a 3.4 mm ID NMR tube, transferred to a 4.2 mm ID screwcap tube, and compressed. The * and dashed line correspond to 6% acrylamide gel, 0.4% bisacrylamide gel samples copolymerized in a 5 mm device and squeezed into a 4.2 mm ID NMR tube.

Studies using soluble proteins have demonstrated a correlation between the magnitude of alignment and both the extent of gel stretching and gel density. A similar trend exists for fd coat protein in micelles (Table 2). A 6% PAG copolymerized in a 5 mm funnel device and squeezed into a 3.9 mm ID NMR tube generates a sample aligned where only the peaks from the mobile termini and loop are present in the spectra since the other resonances are too broad to be observed. Decreasing either the total gel percent or the crosslink ratio decreases the magnitude of alignment as measured by the splitting of the deuterium resonance, with the former eliciting the greater effect. Alternatively this can be accomplished by squeezing the gel into a larger diameter NMR tube and decreasing the ratio of compression. As a result the magnitude of orientation can be easily manipulated by altering the percent gel, the percent crosslink, and the squeezing ratio.

3.2. Acrylic acid

A dipolar coupling constrains the orientation of the internuclear vector to two inverted cones about the unique axis of the alignment frame [32], and this range of orientations consistent with each experimental datum makes it impossible to uniquely determine the relative orientation from a single set of RDCs [33,34]. In the case of a helix the problem simplifies to fourfold degeneracy [35]. A unique solution can be obtained with additional information such as a limited number of long-range NOEs [33,34,36] or paramagnetic restraints [37] since the number of degenerate solutions scales as 4^{n-1}, where n is the number of helical domains. However, it

Table 1
Average value (Aa), Amplitude (Am), and phase (Phi) from dipolar wave fits[a]

Sample	Helix 1 residues 7–19			Helix 2 residues 25–38			Helix 3 residues 39–48		
	Aa (Hz)	Am (Hz)	Phi (rad)	Aa (Hz)	Am (Hz)	Phi (rad)	Aa (Hz)	Am (Hz)	Phi (rad)
Compressed[b]	−1.88	1.92	0.28	−7.265	2.96	2.78	−3.65	3.33	2.82
Stretched[c]	5.39	4.87	3.36	16.365	11.68	5.82	10.65	8.84	5.82
Acrylic acid[d]	1.08	1.19	3.37	5.29	1.82	0.46	3.59	3.07	5.71
Electrophoresis[e]	−2.24	2.33	0.078	−5.80	4.63	3.23	−1.26	5.66	2.90

[a] The values were calculated by using an unconstrained nonlinear optimization fit of the data to following equation: dipolar wave $= \mathrm{Aa} + \mathrm{Am} \times \sin\left(\frac{2\times pi}{3.6} \times \text{residue number} + \mathrm{Phi}\right)$.
[b] Compressed sample prepared by co-polymerizing 1 mM fd in a 7% acrylamide, 0.8% bisacrylamide gel in a 3.4 mm ID NMR tube, transferring to a 4.2 mm ID screwcap tube, and compressing.
[c] Stretched sample prepared by co-polymerizing 1 mM fd in a 6% acrylamide gel, 0.4% bisacrylamide gel in a 5 mm device and squeezing into a 4.2 mm ID NMR tube.
[d] Sample prepared by copolymerizing 1 mM fd in a 4.5% polyacrylamide, 0.5% acrylic acid, and 0.4% bisacrylamide gel in a 5 mm device and squeezing into a 3.9 mm ID NMR tube.
[e] Compressed sample prepared by electrophoresis a 1 mM fd in a 7% acrylamide, 0.8% bisacrylamide gel in a 3.4 mm ID NMR tube, transferring to a 4.2 mm ID screwcap tube, and compressing.

Table 2
Comparison of the magnitude of alignment for fd coat protein copolymerized in polyacrylamide gels

Total gel percent	Crosslink ratio[a]	Diameter ratio[b]	Max RDC (Hz)	D_2O splitting (Hz)
5	75:1	0.78[c]	35.09	3.5
5	150:1	0.78	21.47	1.8
4	75:1	0.78	10.15	1.0
6	75:1	0.78	nd[d]	nd
6	75:1	0.85[e]	25.86	2.5
7	37.5:1	1.22[f]	−11.01	1.3

[a] Ratio of acrylamide:bisacrylamide.
[b] Final diameter of NMR tube/initial diameter of gel.
[c] 5 mm ID funnel device squeezed into 3.9 mm ID NMR tube.
[d] Only peaks from the mobile regions of the protein were observed.
[e] 5 mm ID funnel device squeezed into 4.2 mm ID NMR tube.
[f] 3.4 mm ID NMR tube and compressed to 4.2 mm.
nd–not determined.

has also been shown that these degeneracies can be resolved by measuring RDCs in two or more alignment media that generate noncoincident order tensors [32,38,39]. Since the cones are tilted with respect to each other, the number of solutions is limited to the intersecting regions and this can be used to significantly increase the accuracy of structure determination [40]. With an adequate number of RDCs and two different orientations it is feasible to determine protein structures solely using RDC data [41,42].

A number of approaches have been developed to manipulate the orientation and/or rhombicity of the molecular alignment tensor including: using different alignment media [8], addition of a poly-His tag [32], altering the pH [32], doping bicelles with charged lipids [32,38], and preparing gels containing a mixture of acrylamide/acrylate [43]. In the later method the inclusion of a small amount of negatively charged acrylate was found to alter the alignment tensor compared to standard acrylamide gels. However, this is accompanied by a profound increase in the osmotic swelling of gel—up to 60 times their original volume—in a process that is highly dependent on the percent acrylate, sample pH, and ionic strength. Samples of 1 mM fd coat protein in 500 mM SDS, 40 mM NaCl failed to adequately soak into dried gels containing as little as 5% acrylate relative to acrylamide. This trend was also the case for 100 mM samples of negatively charged 1-myristoyl-2-hydroxy-*sn*-glycero-3-[phospho-RAC-(1-glycerol)] (LMPG), a detergent that has recently been noted for its ability to effectively solubilize membrane proteins for use in NMR studies [44]. The zwitterionic detergents dodecylphosphocholine (DPC) and 1,2-dicaproyl-1-*sn*-glycero-3-phosphocholine (DHPC) seemed to readily soak into acrylic acid containing gels, but the protein would precipitate unless the gels were exhaustively washed. Copolymerizing the protein with a 90:10 mixture of acrylamide/acrylic acid eliminated the need to rely on the protein diffusing into the gel and enabled the pH to be adjusted prior to polymerization. The plot of RDCs vs residue number and the corresponding Dipolar Wave fits for fd coat protein copolymerized in a 5% PAG in the presence and absence of 10% acrylic acid are shown in (Fig. 5) and demonstrate significant

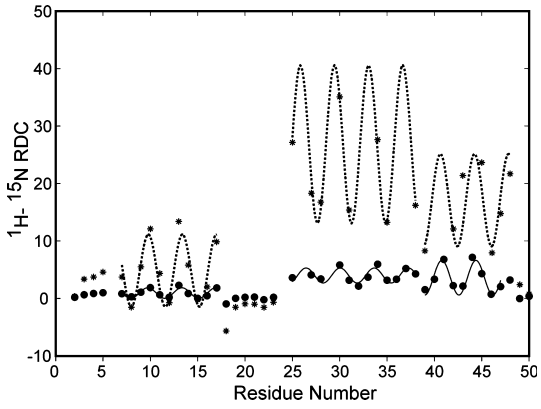

Fig. 5. Plot of RDCs versus residue number and the corresponding Dipolar Wave for 1 mM fd coat protein copolymerized in 5% acrylamide, 0.4% bisacrylamide gels. Weak alignment was achieved by preparing samples in a 5 mm ID device and squeezing into a 3.9 mm ID NMR tube. The * and dashed line correspond to the standard polyacrylamide matrix. The ● and solid line represents the results obtained by substituting 10% acrylamide with acrylate so the sample consists of 4.5% polyacrylamide, 0.5% acrylic acid, and 0.4% bisacrylamide.

changes in the Dipolar Waves. As in the previous data sets, the helical regions remain identical between all samples. The decrease in the maximum values probably represents a decrease in the magnitude of orientation, and is a manifestation of the decreased mechanical stability that has been previously noted for acrylic acid containing gels [43]. Comparisons of the average value, amplitude, and phase of the Dipolar Waves reveal significant differences in the samples containing acrylic acid (Table 1). This is the most obvious for helix 2 (residues 25–38) where there is a significant change in the amplitude and phase relative to the squeezed sample in the absence of acrylic acid. Since the average value, amplitude, and phase of the Dipolar Wave reflect helical orientation within the alignment frame these changes indicate that either the conformation has been altered or that the protein is at an alternative orientation. While the former possibility cannot be ruled out, comparison of the HSQC spectra does not reveal any significant changes and previous studies using significantly higher concentrations of acrylate did not show any evidence of conformational changes in the proteins.

3.3. Electrophoresis

Electrophoresis of proteins through polyacrylamide gels is standard laboratory practice that can serve as an active mechanism for loading protein-containing micelles into gels. The electrophoresis apparatus is shown in Fig. 6. The polyacrylamide gel is prepared in a medium wall NMR tube that has a small hole cut at the bottom to provide contact with the anode buffer. After filling the upper and lower reservoirs with running buffer, the protein is carefully layered onto the surface of

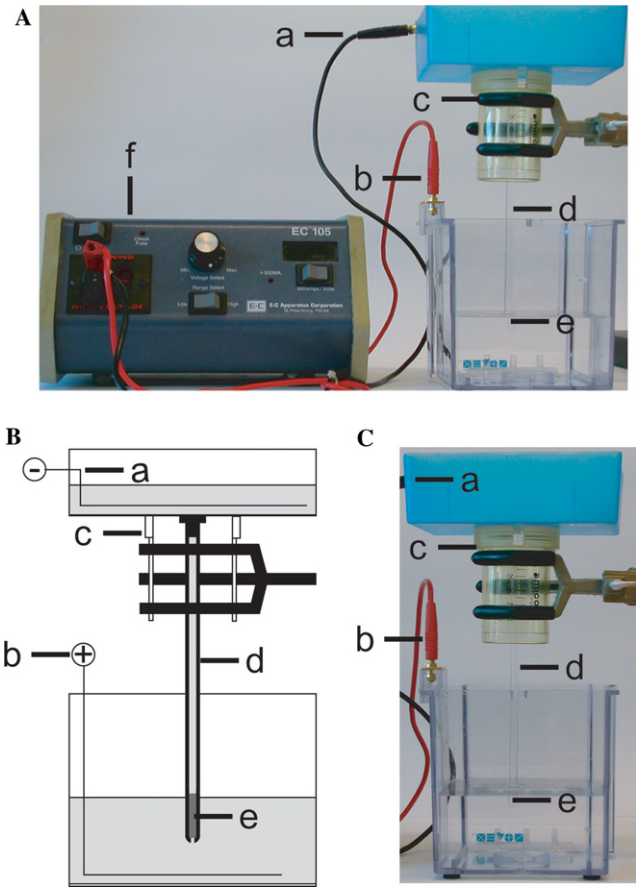

Fig. 6. Electrophoresis apparatus for incorporating a 1 mM sample of fd coat protein into a 7% polyacrylamide gel directly in an NMR tube that could subsequently be compressed to generate RDCs. (A) Photograph of the apparatus. (B) Schematic drawing and (C) close-up photograph. The apparatus consists of: (a) cathode and buffer reservoir; (b) anode and buffer reservoir; (c) large cylinder and clamp to support the upper buffer chamber; (d) 3.4 mm ID NMR tube with a small hole in the bottom; (e) polyacrylamide gel; and (f) power supply. The upper buffer chamber has a small hole cut in the center and a standard NMR tube cap is sealed to the bottom of the reservoir using epoxy after cutting a hole in the top of the cap. This readily facilitates the connection of the NMR tube to the apparatus in addition to sample loading and the flow of electrical current. The bottom of the NMR tube is simply suspended in the bottom chamber.

the gel. Current is applied, and electrophoresis proceeds until the indicator dye travels to the bottom of the gel. Since the dye migrates faster than the protein, the running time has to be calibrated empirically. The gel matrix is poured without a stacking gel; as a result, the protein runs as a diffuse band, approximately the same height as the loading solution and readily incorporates into the gel. Following electrophoresis, the running buffer is removed from the NMR tube, the bottom sealed, and a 50 μl drop of D_2O added. Fig. 7B shows the spectrum obtained for 1 mM fd coat protein, 500 mM SDS, and 40 mM NaCl, pH 4.0 after allowing the sample to incubate overnight at 42 °C. The ^1H–^{15}N HSQC spectrum is virtually identical to that obtained in solution

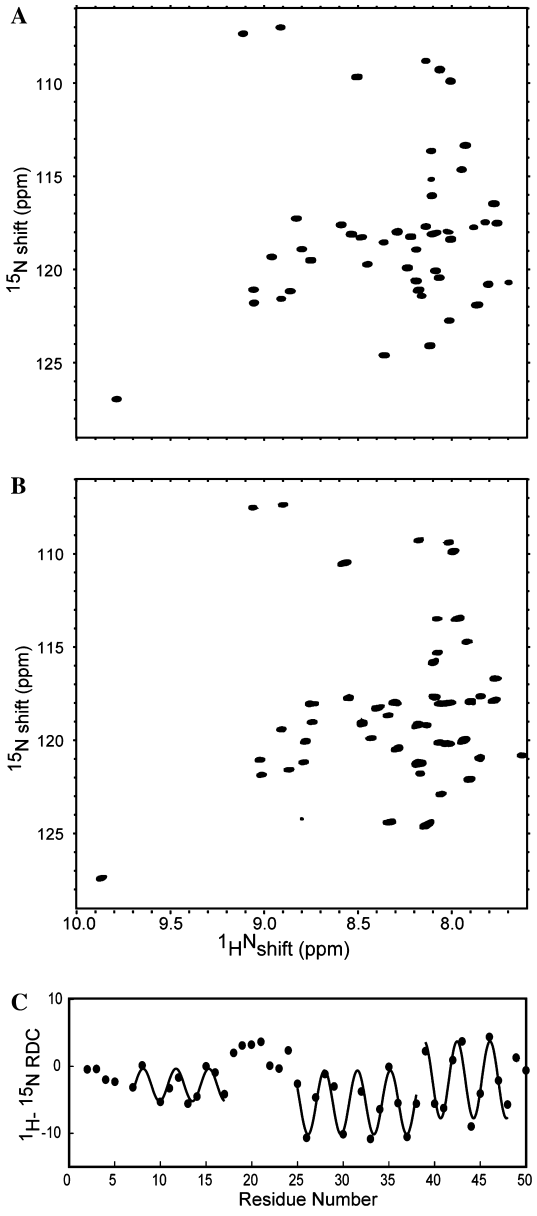

Fig. 7. Amide region of the $^{1}H/^{15}N$ HSCQ spectrum of 1 mM uniformly ^{15}N-labeled fd coat protein in 40 mM NaCl, 500 mM SDS at pH 4.0 and 40 °C obtained before (A) and after electrophoresis (B) into a 7% polyacrylamide gel. The later spectrum was acquired with NH_2 suppression to reduce the natural abundance signal from the gel. (C) Plot of RDCs versus residue number obtained from sample B after compression. The solid line corresponds to the Dipolar Wave fit.

(Fig. 7A) indicating that the protein was successfully incorporated into the gel matrix in its native conformation. Furthermore, the presence of 1% glycerol and 0.0004% xylene cyanol did not adversely affect the protein or the spectra.

The RDCs obtained by compressing the gel using the same protocol described above for copolymerized samples are shown in Fig. 7C along with the Dipolar Wave fits. The same helical regions are identified as in the previous samples. Interestingly, the phase of the Dipolar Waves are different relative to the copolymerized compressed samples indicating that this method may also provide access to an alternative orientation (Table 1). The similarity of HSQC with the one obtained in solution suggests that the changes do not reflect alterations in protein structure.

fd coat protein in SDS is very similar to the SDS–PAGE gels routinely used to separate proteins on the basis of mass. This may explain observations that membrane proteins frequently run anomalously compared to soluble proteins—not only have these very hydrophobic proteins been suggested to bind more SDS and consequently have an altered mass/charge ratio [45,46] but also helical membrane proteins appear to remain folded.

4. Discussion

The measurement of RDCs and other orientationally dependent frequencies are crucial for structure determination of membrane proteins in micelles by solution NMR spectroscopy because it is difficult to observe and assign long-range NOEs in these samples. Since stressed polyacrylamide gels can induce the required alignment and are suitable for studying proteins with high concentrations of lipids and at elevated temperatures [13,15–17] they provide an alignment medium suitable for membrane proteins in micelles. However, the incorporation of protein-containing micelles into a gel by diffusion has been problematic [12–14,16,17]. The swelling process is generally slow (overnight to several days) and frequently requires a significant amount of material. Further, not all samples are amenable to this approach since the ability to soak into the gel is highly dependent on the temperature [18], ionic strength [19], choice of lipid, and size of the protein. The preparation of a homogeneously folded, stable, membrane protein NMR sample generally requires considerable effort since a significant number of variables must be taken into consideration [47–50]. In addition to the usual sample variables of pH, ionic strength, and temperature there are several additional components including: the type of lipid, lipid concentration, and protein-to-lipid ratio. Moreover, it is necessary to optimize for the ability to incorporate the protein into the gel. The methods described in this article provide alternatives to the earlier techniques for incorporating protein-containing micelles into gels. They are not limited by diffusion and are not as strongly affected by the protein, the type of lipid, or ionic strength of the sample. In all cases the application of mechanical strain generates weak alignment suitable for the measurement of RDCs. The same helical regions were quickly and precisely identified using Dipolar Waves for all samples. Since Dipolar Waves are very sensitive to deviations from ideality, such as curvature and kinks [16], the ability to fit a pure sinusoid to the

RDCs indicates that the helices in fd coat protein are nearly ideal, except for the kink at residue 38. These findings indicate that the φ/ψ angles for each residue in the helix are nearly identical and are consistent with those found in high resolution structures [51,52].

Copolymerization is a generally applicable method for incorporating membrane proteins into polyacrylamide gels. By removing the free radical quenching oxygen it is feasible to prepare samples without the requirement for TEMED, which eliminates the large change in pH. While this method is not limited by processes of diffusion, it is important to note that samples containing free radical quenching agents will not be amenable to copolymerization [60]. Mechanically stressed polyacrylamide gels are capable of weakly aligning protein-containing micelles for measurements of RDCs. Similar to globular proteins [11,14], the magnitude of the alignment could be controlled by altering the anisotropic compression or the chemical composition of the polyacrylamide gel. Further, the alignment tensor principal axis orientations was found to be independent of the gel concentration, but was altered by changing the type of compression (stretched vs compressed). The mechanism that determines orientation in a strained gel is generally considered to be similar to the steric hindrance observed for the bicelle system [1] since the principal axes of the alignment tensors of these two media are frequently coincident [11,14]. Alignment in bicelles can be modeled as a system where the bicelles act as walls and alignment occurs during collisions [53]. However, the interactions among the protein molecules, solvent, and polyacrylamide that determine orientation are not well understood. A gel consists of a web of polymer chains that are randomly crosslinked, and the protein contacts the acrylamide chains through diffusion. These matrix interactions may explain the line broadening that occurs upon copolymerization. Regardless of the details of the mechanism, the steric model appears to be a reasonable approximation for the observed behavior of membrane proteins since one would expect that a helix should orient perpendicular to the field in a compressed gel and scale by -2 in a stretched gel. A similar trend was noted in the current system of helical membrane proteins.

The qualitative steric model provides an explanation of why a membrane protein in a micelle environment exhibits similar behavior as a globular protein. Electrostatic interactions are unlikely to affect the alignment since the gel matrix is uncharged and the shape of the molecule should be the primary determinant of orientation. The model of micelles as spherical assemblies is inconsistent with the ability to weakly align these proteins; unlike a cylinder, a sphere striking a wall should not exhibit a preferred orientation. However, the classical spherical micelle model is generally only considered an average and the true shape is significantly more irregular [48,54]. Since it has been demonstrated that proteins can dramatically affect the number of lipid molecules in the micelle and the critical micelle concentration [55–58] it has been suggested that the protein determines the micelle size and shape [48,59]. This is consistent with the results presented here where the hydrophobic requirements of fd coat protein distort the micelle to generate a prolate ellipsoid or discoidal architecture, making it susceptible to alignment through a steric mechanism.

Electrophoresis provides an alternative method to incorporate proteins into a gel matrix. In this approach the charge of the protein facilitates electrophoresis by allowing the electric current to force the molecule into the polyacrylamide. In the present example the charge was conferred by the negatively charged SDS molecules in the micelles. This approach should be applicable to any membrane protein in a charged lipid micelle or any soluble protein with a net charge. However, care must be exercised in the case of charged membrane proteins studied in zwitterionic or neutral lipids. The uncharged molecules will not experience the same force as the protein and while it is feasible that the strong hydrophobic attraction is sufficient to compel the lipid to accompany the protein into the gel this requires further experimentation.

^{1}H–^{15}N RDCs are attractive as input for structure determination because they economically provide very precise angular constraints. They only require uniform ^{15}N labeling, have a large magnitude, and are readily measured. This approach is very rapid since only backbone resonance assignments are required, and by using Dipolar Waves the helical regions can readily be identified. However, ^{1}H–^{15}N RDCs are generally only used for structural refinement because the degeneracy associated with these measurements is insufficient to generate a single structure [32–35]. This problem can be resolved in the case of molecular fragments, such as helices, using only N^{15}–H^{1} RDCs and two orientations [38]. It has been shown that preparing a gel consisting of a mixture of acrylamide/acrylate alters the alignment tensor compared to standard acrylamide gels [43]. Under these conditions the presence of the negative charge in the gel introduces an electrostatic component to the steric alignment and alters the alignment tensor. While this appears to represent a viable approach to overcoming the degeneracy problem we have found that negatively charged lipids are not amenable to swelling; the presence of acrylate can alter the pH and cause the protein to precipitate; and the large increase in osmotic swelling [43] can increase the magnitude of alignment to the extent that it is impossible to measure the RDCs. Copolymerizing the sample in a mixture of acrylamide/acrylate overcomes these problems and demonstrates that it is relatively easy to incorporate samples into the gel and obtain RDCs under conditions that are not amenable

to soaking. The change in the phase and amplitude of the Dipolar Waves in the presence of acrylate suggests that an alternative orientation has been obtained.

Copolymerization and electrophoresis are complementary methods for transferring protein-containing micelles into polyacrylamide gels; the subsequent application of mechanical stress results in weak alignment suitable for the measurement of RDCs. They represent a significant improvement over previous methods since they are considerably faster and, more importantly, are applicable to larger proteins and higher concentrations of lipids. By manipulating the composition of the gel, such as including charged molecules, it is possible to obtain alternate alignments that have the potential to enable structure determination based completely on the measurement of RDCs. Both of these methods have advantages for the incorporation of membrane proteins into acrylamide gels, and ultimately the system of choice is dependent on the requirements of the protein and consequently the sample conditions.

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