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Charged acrylamide copolymer gels as media for weak alignment

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Abstract

The use of mechanically strained acrylamide/acrylate copolymers is reported as a new alignment medium for biomacromolecules. Compared to uncharged, strained polyacrylamide gels, the negative charges of the acrylamide/acrylate copolymer strongly alter the alignment tensor and lead to pronounced electroosmotic swelling. The swelling itself can be used to achieve anisotropic, mechanical strain. The method is demonstrated for the alignment of TipAS, a 17 kDa antibiotic resistance protein, as well as for human ubiquitin, where alignment tensors with an $A_{ZZ,NH}$ of up to 60 Hz are achieved at a gel concentration of 2% (w/v). The alignment can be modulated by the variation of pH, ionic strength, and gel concentration. The high mechanical stability of the swollen gels makes it possible to obtain alignment at polymer concentrations of less than 1% (w/v).

Residual tensorial couplings have become standard parameters for probing structure and dynamics in biological macromolecules (Tolman et al., 1995; Tjandra and Bax, 1997). The prerequisite to obtain such orientation-dependent information in solution NMR is the weak alignment of biological macromolecules. Several liquid crystalline media have been introduced that achieve weak alignment of solute biomacromolecules by transient steric or electrostatic interactions. Recently, it has been shown that also mechanically strained polyacrylamide gels can be used as the carrier medium for such alignment experiments (Tycko et al., 2000; Sass et al., 2000; Chou et al., 2001). The highly inert gels allow for applications in hostile environments such as the conditions used for protein unfolding (Shortle and Ackerman, 2001). Alignment in the electrically neutral polyacrylamide is mainly caused by steric interactions and results in alignment tensors that are similar to uncharged lipid bicelles. Embedding of oriented, charged purple membranes (Sass et al., 2000) or filamentous phages (Trempe et al., 2002) into the acrylamide gels causes alignment by electrostatic interactions and leads to markedly different orientation tensors and therefore gain of additional information. In this communication we show that such electrostatic alignment can also be achieved in mechanically strained acrylamide/acrylate copolymers. The highly negatively charged gels exhibit strong electroosmotic swelling (Flory, 1953; Horkay et al., 2000) – an effect, which can be used advantageously to apply anisotropic mechanical strain to the gels. The swelling also results in increased mechanical stability. This makes it possible to achieve alignment by mechanical strain at gel concentrations of less than 1% (w/v), whereas uncharged acrylamide gels are difficult to handle at concentrations of less than 4% (w/v).

Preparation of acrylamide/acrylate copolymer gels was performed in analogy to polyacrylamide gels. A stock solution of acrylic acid/bisacrylamide was prepared as an aqueous solution of 29.2% w/v acrylic acid (MERCK) and 0.78% w/v N,N′-methylenebisacrylamide (BIS, SERVA) with the pH adjusted to the pK of acrylic acid (4.25) by the addition of one molar equivalent of NaOH. The commercially available premix of 29.2% w/v acrylamide and 0.78% w/v BIS (APPLICHEM) was used as a second stock solution for an acrylamide/bisacrylamide mixture. Aliquots of both stock solutions were mixed in specific ratios to obtain relative concentrations of acrylic acid in the range of 0–100% of the total acrylic

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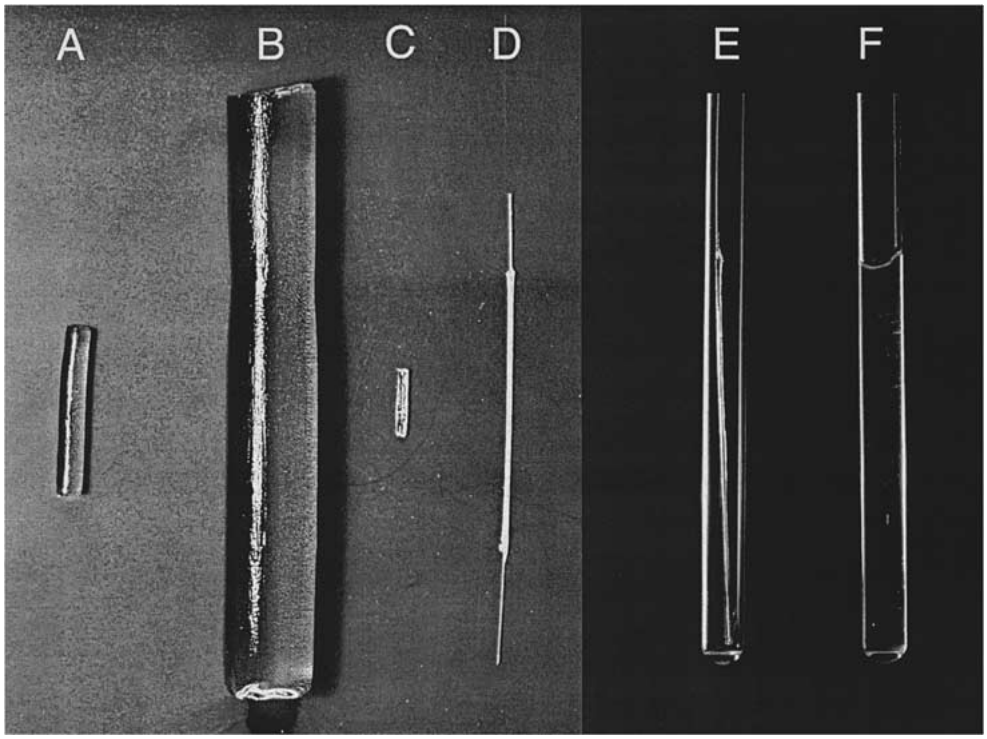

Figure 1. Swelling behavior of a 7% (w/v) 50% acrylate/50% acrylamide copolymer gel and preparation of an anisotropic NMR samples by stretch-drying. A: Gel after polymerization from a 300 μl solution in a 3.5-mm diameter plastic tube. B: Gel after washing in deionized MilliQ water. C: Gel dried on non-adhesive support. D: Gel dried on thin glass capillary stuck through center of the swollen gel. E: Dried gel on glass capillary in 5 mm NMR tube. F: Gel after reswelling with NMR sample solution and removal of the glass capillary.

acid/acrylamide monomers. Typically, these mixtures were diluted to a total monomer concentration of 7–10% w/v by a high ionic strength buffer (10×TBE, i.e., 0.9 M Tris, 0.9 M borate, 0.02 M EDTA, pH 8.2). Polymerization was then started at room temperature by the addition of 1% tetramethylethylenediamine (TEMED) and 0.15% ammonium peroxodisulfate. Polymerizations were either carried out on 300 μl reaction mixtures placed in a plastic tube of 3.5 mm inner diameter that was closed with parafilm on one end, or as 50 μl volumes placed in a teflon tube of 1.7 mm diameter. After the polymerization was completed, the gels were washed at least five times for several hours with a ∼100–1000-fold excess of deionized (MilliQ) water. During the washing, the polyelectrolyte gels undergo very pronounced electroosmotic swelling which leads to a volume increase of up to a hundredfold (see below and Figure 1A,B).

This effect was used to prepare NMR samples of biomacromolecules dissolved in an anisotropic acrylamide/acrylate gel matrix. To this end, a thin glass capillary (diameter ∼0.3 mm) drawn from a glass pipette was stuck through the center of the cylindrical swollen gels (Figure 1B). The gels were then dried in a drying oven at 50 °C on a non-adhesive support such as PVDC (polyvinylidene chloride) household wrapping foil. Drying of the swollen gel was complete within two days. Apparently, the drying and the adhesion to the glass capillary result in an asymmetric stretching of the dried gel in the direction of the glass capillary (Figure 1D). The glass capillary with the dried gel was then placed into a normal 5 mm NMR tube (Figure 1E) and the desired sample solution was added. After the gel had reswollen within the NMR tube, the glass capillary was pulled out (Figure 1F), and the sample was ready for NMR measurements.

As an alternative to this stretch-drying method for achieving anisotropy, the conventional compression method (Tycko et al., 2000; Sass et al., 2000; Chou et al., 2001) was also investigated. Due to lower mechanical stability at high acrylate concentrations (see below), this method was only practical for charged gels with a relative acrylate content of less than 40%. For this conventional method, the gels were dried on a non-adhesive support without the glass capillary. The dried cylindrical gels (Figure 1C) were placed into a

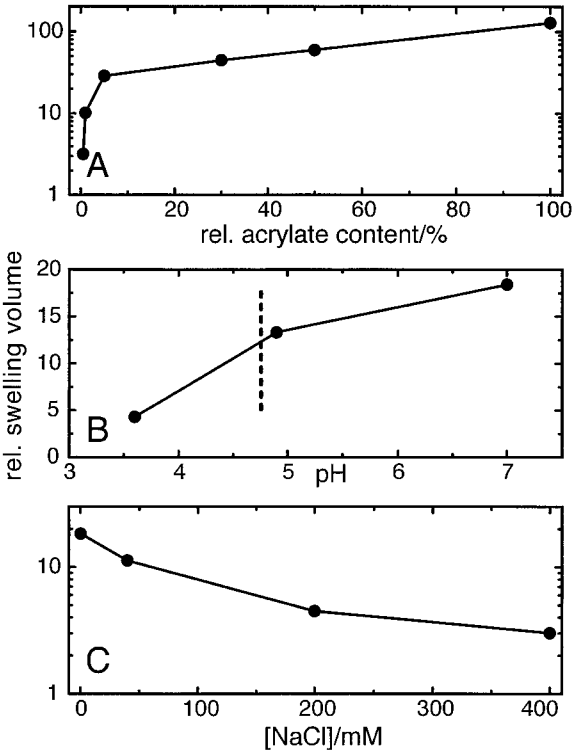

Figure 2. Swelling behavior of a 7% (w/v) acrylate/acrylamide copolymer gel. The relative swelling volume is calculated as the volume of the swollen gel divided by the original polymerization volume. A: Dependence on the relative acrylate content of the gel. Swelling was performed in deionized water. B: Dependence on pH. Swelling was performed in 10 mM sodium phosphate (pH 7.0 and pH 4.9) or ammonium acetate (pH 3.6) buffer solutions. The dashed line indicates the pK_a of polyacrylic acid. C: Dependence on salt content. Swelling was performed in 10 mM phosphate, pH 7.0 with varying amounts of NaCl.

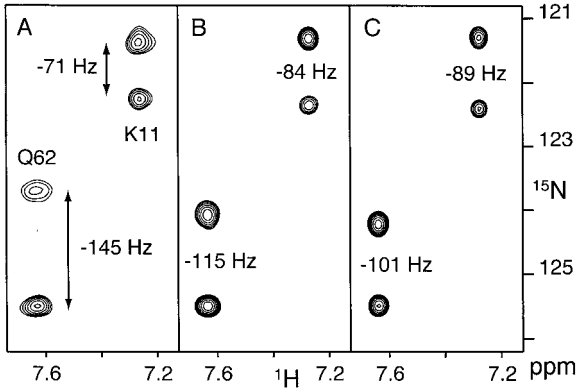

Figure 3. Ionic strength dependence of ubiquitin alignment in stretch-dried 50% acrylate/50% acrylamide copolymer gel. Sample conditions: 0.15 mM ^{15}N-labelled ubiquitin, pH 6.5, final gel concentration 2% (w/v), 298 K. NaCl concentrations are 0 (A), 60 (B) and 240 mM (C), respectively. Amplitudes A_{zz} and rhombicities η of the fitted alignment tensors were: -60.8, -24.1, -12.0 Hz and 0.16, 0.28, 0.39 for 0, 60, and 240 mM NaCl, respectively. The dipolar coupling D is calculated from A_{zz} and η as $D = A_{zz}\left(3\cos^2\theta - 1 + \eta\sin^2\theta\cos 2\phi\right)/2$ where θ and ϕ are the polar angles of the internuclear distance vector in the principal axis system of the orientation tensor.

5-mm NMR Shigemi tube and sample solution added. Compression was achieved by pressure applied via the plunger of the Shigemi tube as described (Tycko et al., 2000; Sass et al., 2000).

The swelling of the charged acrylate/acrylamide copolymers is caused by electroosmotic flow of water into the polymer matrix. Such swelling has been well described for various polyelectrolytes (Vermaas and Hermans, 1948; Katchalsky et al., 1951; Flory, 1953; Horkay et al., 2000). In brief, the concentration of mobile ions is always larger inside the gel than in an exterior solution because of the attraction by the fixed charges. In consequence, the osmotic pressure in the gel is larger than in the external solution, resulting in a net influx of solvent and swelling (Flory, 1953). Figure 2A depicts the relative volume increase from swelling in deionized water for an initial 7% (w/v) polyacrylate/acrylamide gel as a function of the relative acrylate content. For a 50% relative acrylate concentration, a more than 60-fold volume increase is observed. At this swelling ratio, corresponding to a final concentration of about 1 g gel per 1000 g water, the gels are still stable enough such that they can be pierced by the glass capillary or transferred onto another support while retaining their shape. At larger than 50% relative acrylate concentration, the volume further increases. However, the gels rapidly loose their mechanical stability. Presumably, this decrease in stability is caused by a lesser degree of polymerization or by a breaking of covalent bonds due to the increased osmotic pressure. Clearly, the swelling depends on the concentration of the fixed charges of the polyelectrolyte and therefore also on the ionization state (Flory, 1953) of the acrylic acid. A strong decrease of volume is observed for pH values lower than about 5, i.e., when the carboxylate of polyacrylate is titrated to neutrality ($pK_a \sim 4.8$, Figure 2B). Obviously, the ionic strength of the solvent also influences the electroosmotic swelling (Flory, 1953; Horkay et al., 2000; Horkay et al., 2001). For a dilute polymer and intermediate salt concentrations, the swelling ratio q is expected to be proportional to $S^{-3/5}$, where S is the ionic strength of the external solution (Flory, 1953). This behavior is observed approximately in Figure 2C

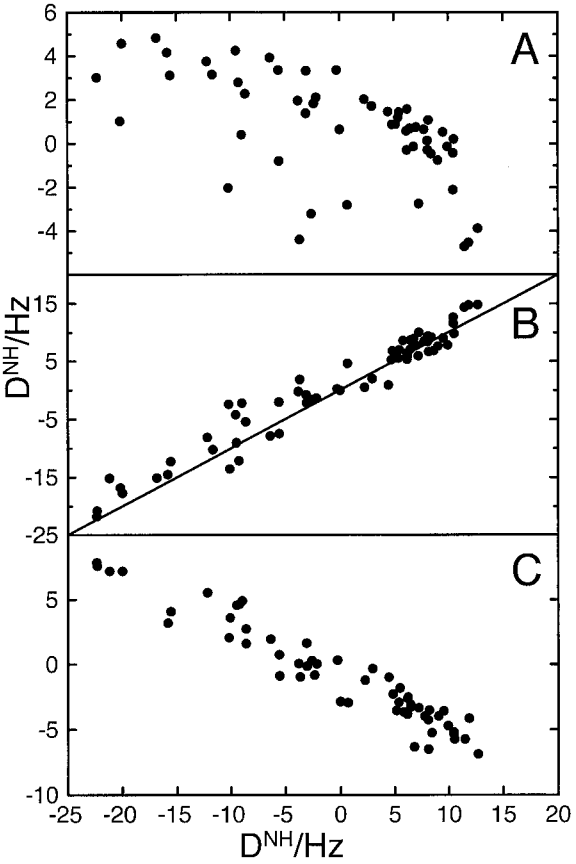

Figure 4. Correlation of RDCs determined for ubiquitin in different media. Horizontal axis: RDCs determined for 0.15 mM ubiquitin, 60 mM NaCl, pH 6.5, in stretched-dried 50% acrylate/50% acrylamide copolymer, final gel concentration 2%. A: Correlation to RDCs determined in compressed uncharged polyacrylamide gel (vertical axis, data are taken from Sass et al., 2000). Correlation coefficient $r = -0.59$. B: Correlation to RDCs (vertical axis) calculated from ubiquitin's crystal structure (1ubi) and a linear fit of the alignment tensor (Sass et al., 1999), $r = 0.97$. NMR quality factor $Q = 0.28$, where Q is defined as the ratio of the rmsd between observed and calculated couplings and the rms of the observed couplings (Cornilescu et al., 1998). C: Correlation to RDCs (vertical axis) measured for ubiquitin in a conventionally (longitudinal) compressed 35% acrylate/65% acrylamide copolymer, final gel concentration 7% (w/v), 0.15 mM ubiquitin, pH 6.5. $r = -0.96$.

for the swelling of a 50% acrylate/acrylamide gel (7% w/v, pH 7) as a function of external NaCl concentration. A fit to the data points (not shown) yields approximately $q \sim S^{-0.50}$. Clearly, at NaCl concentrations of several hundred millimolar, the electroosmotic swelling collapses and the behavior reverts to that of an uncharged gel. For divalent cations, this collapse is reported for concentrations as low as a few millimolar (Horkay et al., 2000, 2001). Therefore, the application of anisotropic strain by electroosmotic swelling is probably limited to cases where cations of higher valency are only required at low concentrations.

The use of the anisotropic polyacrylate/acrylamide gel as an alignment medium for protein NMR was demonstrated on human ubiquitin as well as on TipAS, the 17 kDa antibiotic binding domain of the thiostrepton induced protein A from *streptomyces lividans* (Holmes et al., 1993). Figure 3 depicts small sections of a ^{1}H-coupled ^{1}H-^{15}N HSQC recorded on ubiquitin at pH 6.5 dissolved in a stretch-dried 50% acrylate/acrylamide gel of a final concentration of 2% (w/v). Without the addition of salt (Figure 3A), ^{15}N-splittings of -145 Hz and -71 Hz are observed for amino acids Q62 and K11, corresponding to a residual dipolar coupling (RDC) contribution of -52 and $+22$ Hz, respectively. Upon addition of salt (60 and 240 mM NaCl, Figure 3B,C), the dipolar contribution gradually diminishes to -8 and $+4$ Hz for both amino acids. This dependency confirms the electrostatic character of the residual alignment in the charged gels and proves that this method can still be used at relatively high ionic strengths. As expected, the observed dipolar couplings only show a weak correlation to couplings obtained from the alignment in mechanically strained, neutral polyacrylamide gels (Figure 4A), but correlate well (Figure 4B) with dipolar couplings predicted from ubiquitin's crystal structure (Vijay-Kumar et al., 1987). A negative correlation is observed between couplings determined in the stretch-dried charged gels and in the conventionally compressed charged gels (Figure 4C), thus indicating that the orientation tensor simply inverts when changing the direction of the applied mechanical strain. Table 1 quantitates the correlations between the charged gel alignment tensors for ubiquitin and other alignment tensors obtained in various media. The listed correlation coefficients present a measure for the linear dependence of the alignment tensors (Sass et al., 1999). It is evident that the orientation tensor for the stretch-dried charged gel in the absence of salt has a high correlation (-0.91) to the orientation tensor obtained with the negatively charged purple membranes (Sass et al., 1999), but has only weak correlation to uncharged DMPC/DHPC bicelles (0.21). Therefore, information obtained from uncharged bicelle and charged gel alignment is very complementary. An intermediate correlation (-0.55) is obtained to compressed polyacrylamide gels. Upon increasing the salt concentration, the correlation to the purple membrane orientation decreases, whereas the correlations to the uncharged acrylamide and the uncharged DMPC/DHPC bicelles increase. This clearly

Table 1. Correlation coefficients[a] between ubiquitin alignment tensors in different media

Sample	Charged gel, 0 mM NaCl[b]	Charged gel, 60 mM NaCl[b]	Charged gel, 240 mM NaCl[b]	Compressed charged gel[c]	Uncharged acrylamide gel[d]	DMPC/DHPC bicelles[e]	Purple membrane[e]
Charged gel, 0 mM NaCl	1.00	0.99	0.90	−0.98	−0.55	0.21	−0.91
Charged gel, 60 mM NaCl		1.00	0.95	−0.99	−0.58	0.25	−0.86
Charged gel, 240 mM NaCl			1.00	−0.96	−0.69	0.40	−0.80
Compressed charged gel				1.00	0.56	−0.22	0.87
Uncharged acrylamide gel					1.00	−0.92	0.63
DMPC/DHPC bicelles						1.00	−0.34
Purple membrane							1.00

[a]The correlation coefficient is calculated as the normalized scalar product of the irreducible components of two orientation tensors $<A^1|A^2>/(<A^1|A^1>^{1/2}<A^2|A^2>^{1/2})$ with $<A^1|A^2> := \Sigma_{m=-2,2}\ A^1_m A^{2*}_m$ (Sass et al., 1999).

[b]Further sample conditions are indicated in the legend to Figure 3.

[c]Anisotropy was achieved by conventional, longitudinal compression via the plunger of the Shigemi tube. Sample consisted of 35% acrylate/65% acrylamide copolymer, final gel concentration 7% (w/v), 0.15 mM ubiquitin, pH 6.5, 298 K. Amplitude A_{zz} and rhombicity η of the fitted alignment tensor were 9.7 Hz and 0.11, respectively.

[d]Data taken from (Sass et al., 2000).

[e]Data taken from (Sass et al., 1999).

indicates that the orientation mechanism changes gradually from mainly electrostatic to steric.

The rather strong alignment observed within the charged gels and their relatively high mechanical stability makes it possible to reduce the gel concentrations well below what is practical for orientation by strained uncharged polyacrylamide gels. The same method of stretch-drying on a glass capillary and reswelling in the sample tube could also be used on gels cast originally from a volume of 50 µl with a diameter of 1.7 mm. After reswelling in a 5-mm Shigemi tube, final gel concentrations of approximately 0.9% (w/v) were reached for 50% and 100% relative acrylate content. Under these conditions, alignment with maximal ^{1}H-^{15}N RDCs of about 10 Hz was still observed (data not shown) for ubiquitin at pH 6.5 with no additional salt. These couplings corresponded to an amplitude A_{zz} of the alignment tensor of −8.5 Hz (50% acrylate) and −11.4 Hz (100% acrylate).

The alignment by the stretched 50% acrylate/50% acrylamide copolymer was further tested on the 17 kDa antibiotic domain TipAS in its apo form. Figure 5A shows the ^{15}N downfield component of a doublet-separated sensitivity-enhanced HSQC (Cordier et al., 1999) for this protein in the copolymer at a final gel concentration of 3% (w/v). The quality of this spectrum is comparable to spectra of TipAS in normal aqueous solution. Note that the large number of relatively weak peaks in mostly random coil positions results from the fact that the first 56 amino acids of this 144 amino acid long protein do not adopt a stable conformation in the absence of antibiotic. For the remaining 86 non-proline residues 72 RDCs could be determined, which give an NMR quality factor Q (Cornilescu et al., 1998) of 0.39 relative to an NMR structure that was determined without the use of these RDCs (Figure 5B).

The spectral quality within the charged gel was further assessed by ^{15}N transverse relaxation times. Average T_2 values for the structured part of the negatively charged TipAS at pH 5.9 (pI = 4.6) were 74 ms both within the acrylate/acrylamide copolymer and in normal aqueous buffer. This indicates that within the error limits the rotational diffusion was not hindered by the interaction with the charged gel. For electrically neutral ubiquitin at pH 6.5 (pI = 6.5) and low ionic strength, a reduction in $^1H^N$ T_2s of about 20% was observed in the presence of 50% acrylate/50% acrylamide, final gel concentration 4% (w/v). Apparently rotational diffusion is already slightly hindered by the strong electrostatic interactions under these conditions. It should be noted that larger reductions in T_2 are observed for ubiquitin under identical conditions in the presence of negatively charged filamentous phages Pf1 (Hansen et al., 1998) or purple membranes (S.M., unpublished observations).

In summary, we have shown that negative charges can be introduced easily into mechanically strained acrylamide gels by copolymerization with acrylic acid. The resulting alignment is electrostatic in nature and deviates strongly from the alignment achieved by mechanically strained polyacrylamide gels. The electroosmotic swelling of the charged gels can be used advantageously to achieve mechanically induced

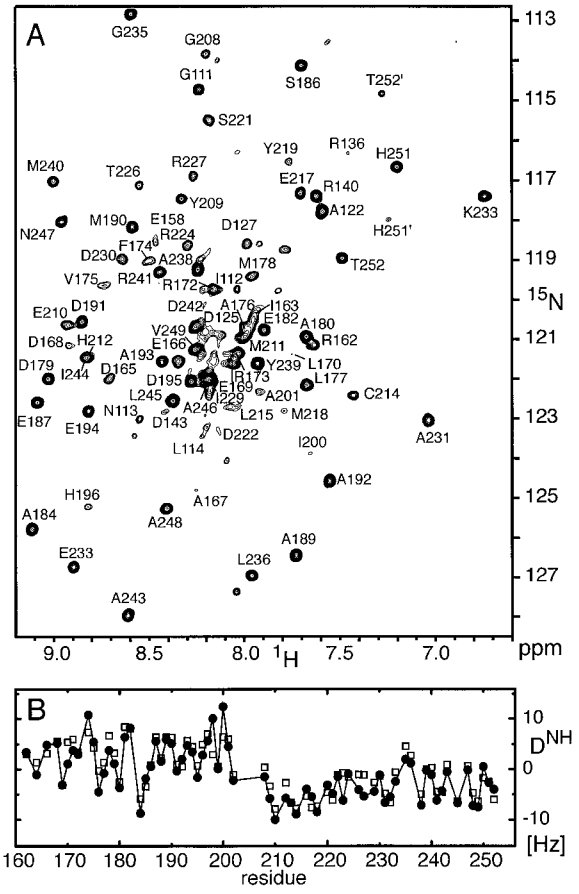

Figure 5. Use of anisotropic polyacrylate/acrylamide gel for determination of RDCs in TipAS. A: Downfield component of a ^{15}N-DSSE spectrum (18.7 T) recorded on 0.2 mM TipAS (0.2 mM), 10 mM phosphate, pH 5.9, in stretch-dried 50% acrylate/50% acrylamide copolymer, final gel concentration 3% (w/v), 298 K. B: Comparison of measured (filled circles) and theoretical (open squares) RDCs calculated from the NMR structure of the folded C-terminal part of TipAS. The NMR structure was determined without use of RDCs of the charged gel. The structure is all-helical leading to a characteristic periodicity in the dipolar couplings for many parts of the sequence.

anisotropy especially at low gel concentrations. Various possibilities are currently under investigation to copolymerize positively charged groups into the polyacrylamide matrix.

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