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Induced alignment and measurement of dipolar couplings of an SH2 domain through direct binding with filamentous phage

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Abstract

Large residual ^{15}N-^{1}H dipolar couplings have been measured in a Src homology II domain aligned at Pf1 bacteriophage concentrations an order of magnitude lower than used for induction of a similar degree of alignment of nucleic acids and highly acidic proteins. An increase in ^{1}H and ^{15}N protein linewidths and a decrease in T_2 and $T_1\rho$ relaxation time constants implicates a binding interaction between the protein and phage as the mechanism of alignment. However, the associated increased linewidth does not preclude the accurate measurement of large dipolar couplings in the aligned protein. A good correlation is observed between measured dipolar couplings and predicted values based on the high resolution NMR structure of the SH2 domain. The observation of binding-induced protein alignment promises to broaden the scope of alignment techniques by extending their applicability to proteins that are able to interact weakly with the alignment medium.

Abbreviations: D_{NH}, residual ^{15}N-^{1}H dipolar couplings; HSQC, heteronuclear single quantum correlation; p85, p85αN subunit of phosphatidylinositol 3′ kinase; pI, isoelectric point; rmsd, root mean square deviation; SH2, Src homology II.

Residual dipolar couplings provide an extremely rich source of structural information about biomolecules in solution (Sanders et al., 1994; Salvatore et al., 1996; Bax and Tjandra, 1997; Tjandra and Bax, 1997a; Bolon and Prestegard, 1998; Prestegard, 1998; Prosser et al., 1998; Ramirez and Bax, 1998). While residual dipolar couplings can be measured in some biomacromolecules aligned at very high field strengths (Tolman et al., 1995; Tjandra et al., 1996; Ottiger et al., 1997; Tjandra et al., 1997), the low anisotropic magnetic susceptibility of most molecules precludes the measurement of significant dipolar couplings. However, with the advent of several alignment techniques based upon the addition of a cosolute that aligns in the magnetic field, the accurate measurement of large residual dipolar couplings has become feasible (Bax and Tjandra, 1997; Tjandra and Bax, 1997; Bolon and Prestegard, 1998; Losonczi and Prestegard, 1998; Ottiger and Bax, 1998, 1999; Prosser et al., 1998; Ramirez and Bax, 1998; Wang et al., 1998). Recently, it has been shown that magnetically aligned Pf1 and fd filamentous bacteriophage are versatile tools for inducing steric alignment of highly acidic proteins and nucleic acids at high phage concentrations (20–50 mg/mL) (Clore et al., 1998b; Hansen et al., 1998a,b, 1999). However, many proteins exhibit extreme line broadening at the concentrations of phage used to align highly acidic biomolecules, which would appear to limit the general applicability of the technique (Hansen et al., 1998b). Indeed, these proteins had been previously thought to be unsuitable for study with the phage system. Here, we report the observation of residual dipolar couplings in a Src homology II (SH2) domain (pI = 6.35) aligned with anom-

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alously low concentrations of Pf1 bacteriophage. We propose that a low-affinity binding mechanism leads to enhanced alignment, allowing the observation of structurally important dipolar couplings.

Residual ^{15}N-^{1}H dipolar couplings (D_{NH}) were measured in a uniformly ^{15}N-labeled SH2 domain from the p85αN subunit of phosphatidylinositol 3′-kinase (Otsu et al., 1991; Booker et al., 1992; Hensmann et al., 1994) at low concentrations of Pf1 bacteriophage (Figure 1a). The residual dipolar couplings are linearly dependent on Pf1 concentration, allowing the magnitude of D_{NH} to be readily tuned (Figure 1A,B). At 2.0 mg/mL Pf1, an optimum degree of alignment is achieved, with couplings ranging from +20 to −32 Hz (Figure 2A). Large D_{NH} are observed for the SH2 domain with phage concentrations an order of magnitude less than needed for similar alignment of acidic macromolecules (Clore et al., 1998b; Hansen et al., 1998a,b). Nitrogen linewidths are broadened approximately twofold at this very low phage concentration, and higher phage concentrations result in prohibitively large line broadening. However, at the low phage concentrations required to obtain optimum alignment, the modest line broadening does not interfere with the accurate measurement of large residual dipolar couplings.

A small amount of line broadening is also observed upon the addition of phage to nucleic acids, apocalmodulin and thioredoxin (Hansen et al., 1998b). Since there is no significant change in $T_{1\rho}$ and T_2 relaxation time constants of phage-aligned nucleic acids (Hansen et al., 1998b), the observed broadening is attributed to the presence of additional ^{1}H-^{1}H dipolar couplings rather than a change in correlation time. In contrast to highly acidic proteins and nucleic acids, alignment of the SH2 domain is accompanied by much more pronounced line broadening. To investigate the source of our observed line broadening, and thus probe the mechanism of alignment, the relaxation properties of the aligned protein were determined. ^{15}N T_2 and $T_{1\rho}$ relaxation time constants for the SH2 domain decreased by approximately 45% upon the addition of 2.0 mg/mL Pf1 ($T_{1\rho}$ data are shown in Figure 2B). This observation implies that, in contrast to the steric mechanism proposed to induce alignment of proteins in bicelles (Bax and Tjandra, 1997; Tjandra and Bax, 1997; Prestegard, 1998) and highly acidic molecules in phage (Hansen et al., 1998b), a fast-exchange binding mechanism facilitates alignment in this case. The measured relaxation time constants reflect a population-weighted average of the

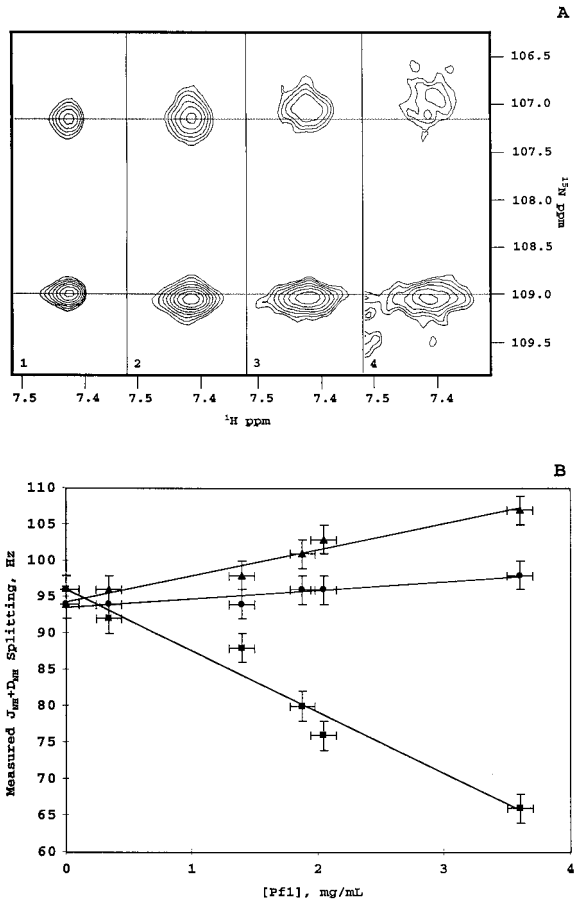

Figure 1. Observation of residual dipolar couplings in a Pf1-aligned Src homology II domain. Uniformly ^{15}N-labeled p85 SH2 domain was expressed in *E. coli* with a pET vector expression system and grown in minimal media containing $^{15}NH_4^+$ as the sole nitrogen source. The SH2 domain was isolated to 99% purity by cation exchange chromatography (Ojennus and Wuttke, unpublished). All NMR samples contained 600 μM ^{15}N-labeled p85 SH2 domain in 10 mM deuterated Tris, pH 7.0, 10 mM NaCl, 0.02% NaN_3, and 10% D_2O. Pf1 phage was expressed and purified as previously reported (Cross and Opella, 1981; Cross et al., 1983; Hansen et al., 1999). (A) Region of the coupled HSQC spectrum depicting J_{NH} + D_{NH} splitting of amide residue 39T at (1) 0.0, (2) 1.4, (3) 2.0, and (4) 3.6 mg/mL Pf1. (B) J_{NH} + D_{NH} couplings measured as a function of Pf1 concentration. The three representative amides depicted (● = I27, ■ = R37 and ▲ = T39) show the linear correlation between the change in splitting due to residual dipolar couplings and Pf1 concentration. J_{NH} + D_{NH} splittings were measured in the indirect nitrogen dimension of an HSQC spectrum by removing the 180° ^{1}H decoupling pulse during the incremented delay period. Measurements were made with Pf1 concentrations of 0.0, 0.3, 1.4, 1.9, 2.0, and 3.6 mg/mL (ε_{270} = 2.25 mL mg^{-1} cm^{-1}). Coupling measurements are accurate to within ±2 Hz; Pf1 concentrations are accurate to ±0.1 mg/mL.

time constants of free and phage-bound states. Using this model, we estimate that >99.9% of the population is in the free state, with a typical solution correlation time (∼9 ns for the Src SH2 domain; Farrow et al., 1994) and that a very small fraction of the population (<0.1%) is bound, with an extremely large correlation time. The observed line broadening is therefore due to both the presence of additional ^{1}H-^{1}H and ^{15}N-^{1}H dipolar couplings (data not shown) and an increase in the averaged rotational correlation time. Overall, the relaxation data and phage concentration dependence of the residual dipolar couplings and the line broadening clearly establish that alignment is occurring via a fast exchange binding mechanism, where the fraction of the protein population bound is very small.

We propose that the weak binding observed between Pf1 and the SH2 domain is due to differences in their surface electrostatics. Filamentous phage are highly acidic (pI = 4.0 and 4.2 for Pf1 and fd, respectively; Zimmermann et al., 1986; Clore et al., 1998b) while the p85 SH2 domain is only slightly acidic at our conditions. A favorable interaction between Pf1 and the SH2 domain leads to low affinity binding and net alignment of the protein. This type of interaction was not observed between bacteriophage and the more highly acidic proteins that presumably align by steric collision (*E. coli* thioredoxin (pI = 4.67), bovine apocalmodulin (pI = 4.09), and streptococcal protein G (pI = 4.55)), likely due to electrostatic repulsion between the phage and protein. In contrast, extreme line broadening and precipitation are observed when phage are added to neutral and basic proteins at the phage concentrations (20–50 mg/mL) needed to facilitate alignment of highly acidic biomolecules by steric collision (Hansen et al., 1998b; Mitton-Fry and Wuttke, unpublished results). The correlation of line broadening and binding with the pI of the protein strongly suggests that the binding interaction is electrostatic in nature. Since most proteins of interest are not highly acidic, we anticipate that the binding mechanism is likely to be the most frequently observed form of protein alignment mediated by phage. Therefore, we set out to ascertain if the dipolar couplings observed by the binding mechanism are consistent with the solution structure.

The observed dipolar couplings in the Pf1-aligned p85 SH2 domain were fit (Bax and Tjandra, 1997; Tjandra and Bax, 1997) to the available solution structure (Booker et al., 1992; Hensmann et al., 1994). Good agreement between measured and calculated dipolar couplings is found for the secondary structural

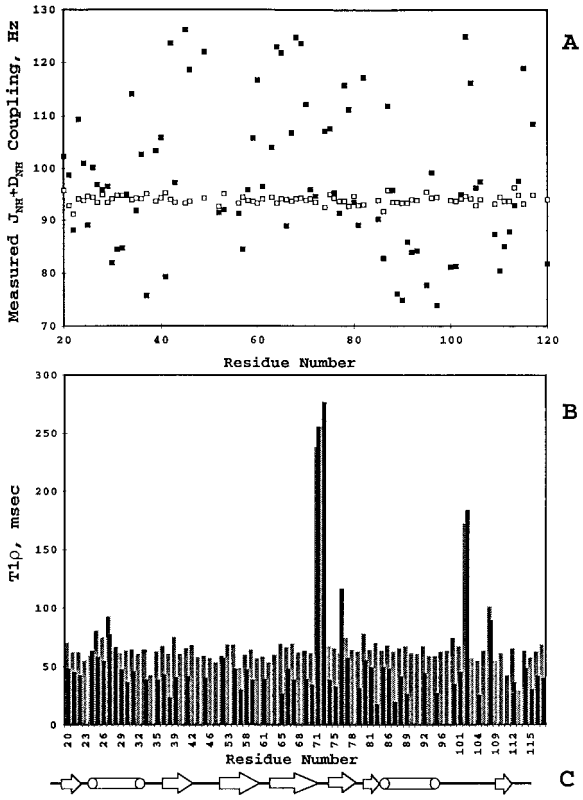

Figure 2. Measured site-specific $J_{NH} + D_{NH}$ splittings and ^{15}N relaxation parameters. (A) $J_{NH} + D_{NH}$ couplings for residues A20–Y120 of the p85 SH2 domain with 2.0 mg/mL Pf1 (□ = no Pf1, ■ = 2.0 mg/mL Pf1). Data were collected on a Varian Unity Inova 500 MHz spectrometer at 25 °C. Couplings were measured with an HSQC-J type experiment (Tjandra et al., 1996; Clore et al., 1998c) using J_{NH} evolution periods of 22.0, 23.0, 23.5, 24.0, 25.0, 26.0, 27.0, 28.0, 29.0, 29.5, and 30.0 ms for the 0.0 mg/mL Pf1 sample and 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 4.5, 5.0, 5.5, 6.5, 7.0, 8.0, 9.0, 9.5, and 11 ms for the 2.0 mg/mL Pf1 sample. $J_{NH} + D_{NH}$ modulation of peak intensity (I) was fit by least squares analysis as previously described (Tjandra et al., 1996; Clore et al., 1998c). (B) ^{15}N relaxation measurements for backbone amides A20–Y120 of the p85 SH2 domain. $T_{1\rho}$ values for the p85 SH2 domain in the presence (black) and absence (grey) of 2.0 mg/mL Pf1. T_2 and $T_{1\rho}$ values were measured by the method of Farrow et al. (1994). (C) Secondary structure of the p85 SH2 domain for residues A20–Y120.

elements of the p85 SH2 domain (Figure 3A). The intervening loops exhibit a poor correlation between measured and predicted dipolar couplings, which is likely due to the inherent flexibility of these regions. Interestingly, the measured dipolar couplings fit better to several available crystal structures of other SH2 domains (Waksman et al., 1993; Lee et al., 1994; Tong et al., 1996, 1998). The structures of these domains are highly conserved, with rmsd values to the p85 SH2 domain mean structure of 1.9–2.1 Å and sequence

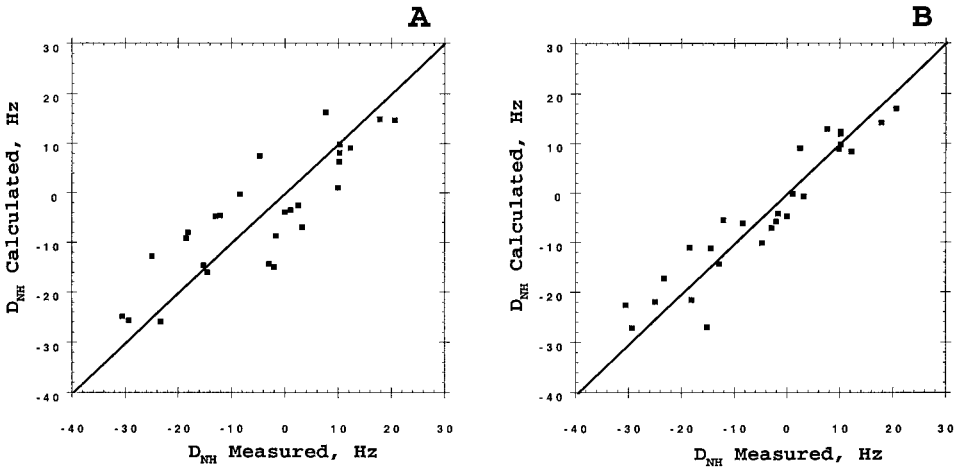

Figure 3. Correlation between measured and calculated D_{NH} couplings for secondary structural elements of (A) the solution structure of p85αN (Booker et al., 1992; Hensmann et al., 1994) and (B) a 2.2 Å resolution crystal structure of Syp (Lee et al., 1994). Predicted values of D_{NH} were calculated based upon the crystal structures for Lck (PDB 1LKK and 1BHH), Src (PDB 1SPR), and Syp (PDB 1AYD) and for the solution structure of p85αN (PDB 2PNB). An alignment tensor was fitted using the equation below as has been previously described (Bax and Tjandra, 1997; Tjandra and Bax, 1997; Tjandra et al., 1997; Clore et al., 1998a,b,c), where $D_{NH}(\theta, \phi)$ is the residual dipolar coupling between coupled nuclei N and H, D_a^{NH} and D_r^{NH} are the axial and rhombic components of the tensor, and R is defined as D_r^{NH}/D_a^{NH}. $D_{NH}(\theta, \phi) = D_a^{NH}\{(3\cos^2\theta - 1) + 3/2(R)(\sin^2\theta\cos 2\phi)\}$ Only measured dipolar couplings with an associated error less than 4 Hz were included in the fits. All loops and the first and last residue of each secondary structural element were eliminated from the fits, with the exception of W22, which is an important residue for defining the Src homology II domains. The distribution of observed couplings indicates that a significant rhombic component is present in the alignment tensor (Clore et al., 1998c). Fit parameters are: $\alpha = -4°$, $\beta = -48°$, $\gamma = -7°$, $D_a^{NH} = -13.5$ Hz, and $D_r^{NH} = -7$ Hz for p85 and $\alpha = -37°$, $\beta = 97°$, $\gamma = 95°$, $D_a^{NH} = -13.1$ Hz, and $D_r^{NH} = -6.6$ Hz for Syp (α, β and γ are angles describing the alignment tensor relative to the PDB-defined structural reference frame for each molecule). Correlation coefficients (R) for the fit of the predicted versus observed couplings to a x = y line: p85, 0.79 (shown); Syp, 0.93 (shown); Lck, 0.91 (not shown); and Src 0.86 (not shown).

identities of 27–29% for the Lck, Syp and Src SH2 domains. The best agreement to the measured dipolar couplings is observed to a 2.2 Å structure of the Syp SH2 domain (Figure 3B; Lee et al., 1994). The structural information provided by dipolar couplings obtained in this manner promises to greatly enhance the refinement of NMR structures.

Our data indicate that the Pf1-induced alignment of the p85 SH2 domain is the result of fast exchange between the free protein in solution and a very small population of phage-bound protein. The very weak affinity between the phage and the protein leads to an enhanced net protein alignment relative to alignment achieved by steric collisions. Two binding-induced alignment mechanisms can be envisioned. The first is that the observed dipolar couplings arise only from a bound state. This bound state may reflect a minor conformation present in solution. Alternatively, a conformational change may occur in the protein upon binding such that the observed couplings no longer are indicative of the predominant solution conformation. Based on our estimate of the fraction of protein bound at the concentration of phage and protein which yields optimal alignment, the K_d for binding is apparently in the millimolar range. Given the very weak affinity of the protein for the phage, it is unlikely that a large conformational rearrangement has occurred. This conclusion is supported by the very good correlation observed between the measured residual dipolar couplings and the domain structure. A second mechanism of Pf1-induced alignment of the p85 SH2 domain can be viewed as an extension of the steric collisional model. In this model, binding leads to alteration of the rotational diffusion properties of the protein via essentially inelastic collisions with phage particles. Thus, the solution population near the phage is no longer completely isotropically averaged, and net alignment is observed. A continuum can be envisioned where macromolecules without affinity for the phage align exclusively by elastic collisions, while macromolecules with a low propensity to interact with the phage align by inelastic collisions. Regardless of the exact mechanism, we have demonstrated that dipolar couplings extracted when alignment is induced by weak phage binding provide useful structural data.

In conclusion, large residual dipolar couplings have been measured in a phage-aligned SH2 domain at very low phage concentrations. Alignment

is attributed to a weak fast-exchange interaction between the protein and the phage. The D_{NH} couplings observed are comparable in magnitude to those determined using other methods of alignment and are readily tunable by adjusting the phage concentration. In contrast to steric collisional alignment, the degree of alignment obtained by binding is not dependent on the shape of the aligned biomolecule, but rather on the binding properties of the biomolecule. The observation of binding-induced protein alignment promises to broaden the scope of alignment techniques by extending their applicability to proteins that are able to interact weakly with the alignment medium.

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Note added in proof

While this paper was under review, two reports appeared describing binding-mediated alignment of ubiquitin with purple membranes (Koenig et al., 1999; Sass et al., 1999). The measured residual dipolar couplings were found to reflect the predominant solution conformation.

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