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Measurements of residual dipolar couplings in peptide inhibitors weakly aligned by transient binding to peptide amyloid fibrils**

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Abstract

In this communication, we suggest that transferred residual dipolar couplings (trRDCs) can be employed to restrain the structure of peptide inhibitors transiently binding to β-amyloid fibrils. The effect is based on the spontaneous alignment of amyloid fibrils with the fibril axis parallel to the magnetic field. This alignment is transferred to the transiently binding peptide inhibitor and is reflected in the size of the trRDCs. We find that the peptide inhibitor adopts a β-sheet conformation with the backbone N-H and C-H dipolar vectors aligned preferentially parallel and perpendicular, respectively, to the fibril axis.

Residual dipolar couplings (RDCs) have been successfully used in the past to restrain the absolute orientation of dipolar vectors with respect to the magnetic field, and to provide critical structural details of biologically important molecules (Tolman et al., 1995; Tjandra & Bax, 1997). As alignment media bicelle type lyotropic liquid crystals (Sanders & Schwonek, 1992), phage particles (Clore et al., 1998b; Hansen et al., 1998), purple membrane fragments (Koenig et al., 1999; Sass et al., 1999) or strained polyacrylamide gels (Sass et al., 2000; Ishii et al., 2001) turned out to be particularly useful (for an overview see Prestegard and Kishore (2001). For most studies unspecific interactions between the alignment medium and the solute are used to obtain structural information. However, similar to transferred NOE (Ni, 1994), transferred cross correlated relaxation (Blommers et al., 1999; Carlomagno et al., 1999; Felli et al., 1999), also specific interactions in the limit of weak binding can be exploited to obtain informations about transiently bound peptides and proteins (Bolon et al., 1999; Koenig et al., 2000, 2002).

In this communication, we suggest to use transferred RDCs (trRDCs) to restrain the structure of peptide inhibitors that bind transiently to amyloid fibrils. The experiments rely on the fact that amyloid fibrils can orient in the magnetic field with the fibril axis parallel to the external field (Worcester, 1978). This property is based on the anisotropic diamagnetic susceptibility of the peptide bond and is employed in X-ray analysis of amyloid fibrils to obtain high resolution diffraction patterns (Fraser et al., 1992; Inouye et al., 1993). As a model fibril, we use a decameric fragment of the amyloid beta-peptide corresponding to residues 14 to 23 ($A\beta^{14-23}$) which is known to form fibrils *in vitro* (Tjernberg et al., 1999). This section of $A\beta^{1-42}$ corresponds to the hydrophobic core of the full-length protein which was identified to be essential for the aggregation behaviour of the peptide (Hilbich et al., 1992). We have chosen the peptide inhibitor $iA\beta5^{inv}$ (DPFFL) which is derived from the iAβ5 (LPFFD) known to interact with amyloid fibrils (Soto et al., 1998). Peptide inhibitors were designed to recognize the hydrophobic core of Aβ. They have a similar hy-

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drophobicity pattern as Aβ, but a low propensity to adopt β-sheet conformation. It was shown that the neurotoxicity of $A\beta^{1-42}$ is decreased upon incubation together with the inhibitor *in vitro* (Soto et al., 1996; Tjernberg et al., 1996; Lowe et al., 2001) and *in vivo* (Soto et al., 1998; Pallitto et al., 1999). It is hypothesized that prolines are an important factor as disruption elements to prevent fibril formation. However, it is not known so far, how iAβ5 can interact with Aβ fibrils at atomic resolution and prevent aggregation and neurotoxicity. Addition of $iA\beta5^{inv}$ to preformed $A\beta^{14-23}$ fibrils yields a significant reduction of the amount of fibrilar structures observed by EM (data not shown). At the same time, the CD spectrum recorded for a mixture of $A\beta^{14-23}$ and $iA\beta5^{inv}$ deviates significantly from the sum spectrum of the individual compounds, indicating that $iA\beta5^{inv}$ interacts with $A\beta^{14-23}$ (data not shown).

$iA\beta5^{inv}$, $A\beta^{14-23}$ were prepared using standard FMOC chemistry. FMOC protected, ^{15}N labeled amino acids were purchased from Senn Chemicals, Switzerland. The buffer has been adjusted to pH 4.0, 10 mM PO_4^{3-}, in order to allow for optimal formation of fibrils (Fraser et al., 1991). Fibrillar $A\beta^{14-23}$ was prepared following the protocol described by Zagorski and co-workers (Zagorski et al., 1999). In this protocol, $A\beta^{14-23}$ is solubilized first in TFA (Trifluoro-acetic acid). After removal of the solvent with dry nitrogen gas, $A\beta^{14-23}$ is re-solubilized in HFIP (Hexafluoro-isopropanol). A small amount of the concentrated stock solution (0.37 mg/50 μl) is transferred to the acqueous buffer (600 μl) to yield a final concentration of 0.5 mM. This protocol for fibril preparation is essential, since simple solubilization of $A\beta^{14-23}$ in buffer yields neither trRDC nor significant intensities in trNOE experiments (data not shown). Inspection by EM indicates that only amorphous aggregates are present in samples that have been obtained from solubilization of $A\beta^{14-23}$ in buffer, whereas high quality fibrils are observed in samples where the protocol described by Zagorski and co-workers was applied. For preparation of the NMR sample, $iA\beta5^{inv}$ (5 mM) was added to preformed $A\beta^{14-23}$ fibrils (incubation time: 2 days). In the experiments described below, a molar ratio of $[iA\beta5^{inv}]:[A\beta^{14-23}] = 10:1$ was employed. Larger amounts of fibrils in the NMR tube induced significant line broadening. The large excess of the peptide inhibitor is in agreement with the reported excess of inhibitor required to prevent neurotoxicity to cultured neuronal cells (Soto et al., 1998). Approximately two weeks after incubation of fibrillar $A\beta^{14-23}$ together with $iA\beta5^{inv}$, neither trNOEs nor trRDCs are observed. This is due to disaggregation of $A\beta^{14-23}$ fibrils by $iA\beta5^{inv}$ which is also reflected in the loss of fibrillar material as observed by EM. All spectra were recorded on a Bruker 750 MHz solution state NMR spectrometer, at 30 °C. No temperature dependence of the observed residual dipolar coupling was observed.

TrNOE experiments have been recorded in order to characterize the binding affinity of $iA\beta5^{inv}$ with respect to fibrillar $A\beta^{14-23}$. Already at a mixing time of $\tau_m = 200$ msec, cross peaks between almost all ^1H resonances are observed (Figure 1). Only spectra recorded at short mixing times avoid spin diffusion effects and allow an unambiguous assignment of longer range correlations. On the other hand, only small cross peaks ($\tau_m = 500$ msec) are present in the sample that contains exclusively $iA\beta5^{inv}$. This observation indicates that $iA\beta5^{inv}$ and the amyloid fibrils interact. At the same time, the diffusion coefficient for water (obtained from DOSY experiments) is comparable for both samples. Therefore, viscosity effects on the correlation time τ_c can be excluded which might affect relative cross peaks intensities in NOESY spectra. NOESY experiments recorded for shorter mixing times.

In the following, we suggest that the diamagnetic anisotropy of β pleated sheets (Lonsdale, 1939; Worcester, 1978) as they are found in the amyloid fibrils can be used to restrain the orientation of small peptides binding to amyloid fibrils. The effect is based on the diamagnetic susceptibility of the peptide bond and can be detected if peptide planes orient synchronously. The largest diamagnetic anisotropy is found in α-helices ($\Delta\chi$ = ca. 4.4×10^{-6} N, where N corresponds to the number of peptide bonds) and is responsible for the orientation of phages and purple membranes. The size of the diamagnetic anisotropy is determined by the angle ϕ between the peptide plane normal and the symmetry axis of the molecular system (neglecting at this point orienting effects of aromatic rings), and is given by $\Delta\chi = \Delta K\,(1 - 3\cos^2\phi)/2$ (where ΔK corresponds to the anisotropic diamagnetic susceptibility of the peptide bond). In similar fashion, spontaneous orientation of purple membranes (Koenig et al., 2000, 2002), nucleic acids, cellulose material, silks, keratins, collagens and muscle fibers is or could be used to obtain the structure of bound ligands.

Successful observation of trRDCs in transiently bound ligands is strongly dependent on the dissoci-

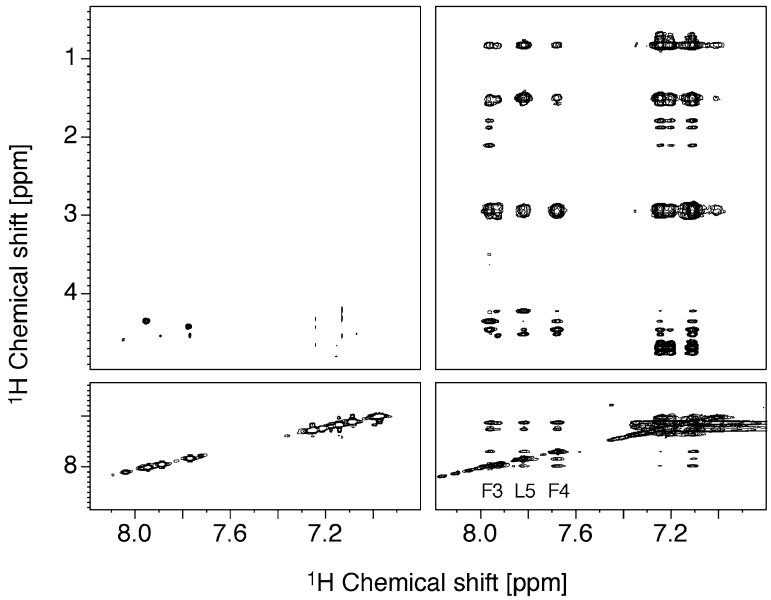

Figure 1. NOESY spectra of iAβ5^{inv} recorded without (left) and with (right) fibrillized Aβ$^{14-23}$. No or very weak cross peaks are observed in the absence of fibrillized Aβ$^{14-23}$. The presence of strong cross peaks in the mixed sample indicates weak interactions between iAβ5^{inv} and fibrillized Aβ$^{14-23}$. A molar ratio of 10:1 for [iAβ5^{inv}]:[Aβ$^{14-23}$] was employed in this experiment, adjusting the concentration of iAβ5^{inv} to 5.0 mM. The mixing time was set to 500 ms and 200 ms for the pure peptide and the mixed sample, respectively. In the mixed sample, strong cross peaks between amide protons and almost all other proton resonances are observed.

ation constant of the complex. In contrast to trNOE, no trRDC might be observable, if the life time of the bound state is too long and binding takes place in the slow exchange regime. As pointed out by Koenig et al. (Koenig et al., 2002), RDCs of transiently bound ligands can be observed if

$$\frac{1}{\tau_f \tau_b} \ll \left[\rho - \left(\frac{1}{\tau_f} - \frac{1}{\tau_b}\right)\right]^2, \qquad (1)$$

where τ_f (= [iAβ^{free}]$^{-1}k_{on}^{-1}$) and τ_b (= k_{off}^{-1}) correspond to the lifetime of the free and the bound ligand, and ρ reflects the transverse relaxation rate in the bound state. A quantitative description can be carried out in the framework of chemical exchange (Cavanagh et al., 1996). The size of the observed dipolar coupling is then given as (Koenig et al., 2002)

$$D_{obs} = \frac{1}{\tau_f \tau_b} \frac{D_b}{\left[\rho - \left(\frac{1}{\tau_f} - \frac{1}{\tau_b}\right)\right]^2 + D_b^2}. \qquad (2)$$

Theoretically, the size of the observed residual dipolar coupling can be adjusted by variation of the concentrations of peptide with respect to the amount of fibrils within the NMR tube. In practice, however, too high a concentration of Aβ$^{14-23}$ (1:1 with respect to iAβ5^{inv}) yields extensive line broadening. Using too low concentrations of Aβ$^{14-23}$ (1:100 with respect to iAβ5^{inv}) prevents the observation of trNOEs. Therefore, we have chosen to use a molar ratio of 1:10 of Aβ$^{14-23}$ with respect to iAβ5^{inv} as the best compromise in the studies presented below. We speculate that due to different affinities of iAβ5 and iAβ5^{inv} with respect to Aβ$^{14-23}$, we could not observe significant trRDCs for iAβ5, but only for iAβ5^{inv}.

Table 1. NH and CH trRDCs for iAβ5^{inv} interacting with Aβ$^{14-23}$ employing a molar ratio of 10:1 for [iAβ5^{inv}]:[Aβ$^{14-23}$]

scaled RDC [Hz]	Asp1	Pro2	Phe3	Phe4	Leu5
N-H^N	–	–	−0.3	−5.5	−1.9
C$^\alpha$-H$^\alpha$	−0.7	−1.1	+3.5	+3.2	+0.9

Figures 2 and 3 represent 1D traces from ^{1}H,^{15}N and ^{1}H,^{13}C correlation spectra that were recorded without ^{1}H decoupling in the indirect dimension, in the presence and absence of fibrillized Aβ$^{14-23}$. ^{13}C HSQC spectra are recorded at ^{13}C natural abundance. ^{13}C,^{13}C homonuclear decoupling was not required in the ^{1}H,^{13}C correlation experiments, since iAβ5^{inv} was

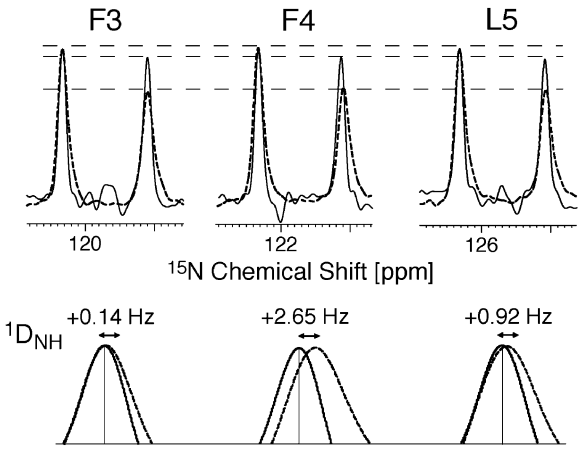

Figure 2. 1D columns of a $^{1}H,^{15}N$ HSQC spectrum of iA$\beta5^{inv}$ with (dashed line) and without (solid line) fibrillized Aβ^{14-23}, recorded without heteronuclear decoupling in the indirect dimension. On the top, intensities are normalized with respect to the low-field multiplet component. On the bottom, only the β multiplet component is represented. The experiment is recorded without phase alternation of the last 90° ^{1}H pulse of the first INEPT.

not enriched in ^{13}C. More sophisticated approaches using the IPAP pulse scheme (Ottiger and Bax, 1998), or a combination of TROSY and semi-TROSY experiments (Lerche et al., 1999) to measure residual dipolar couplings yield quantitatively the same results. These experiments are not required, since resolution in the $^{1}H,^{15}N$ and $^{1}H,^{13}C$ correlation spectra is not compromised by spectral overlap. Upon addition of Aβ^{14-23} fibrils, only a slight increase in ^{15}N line width is observed for the ^{15}N resonances of iA$\beta5^{inv}$. At the same time, the N-H^N dipolar/^{15}N-CSA and the C^α-H^α dipolar/$^{13}C^\alpha$ CSA cross relaxation rate is affected. Especially, differential multiplet intensities are observed for Phe3 and Phe4. Solid-state NMR experiments indicate that the dissociation constant of iA$\beta5^{inv}$ (with respect to Aβ^{14-23}) must reflect a very weak interaction. We subjected a solution-state NMR preparation to ultracentrifugation. The experimental MAS solid-state NMR 1D-^{13}C spectrum of the pellet does not show significant changes compared to the ^{13}C spectrum of a preparation that only contains fibrillized Aβ^{14-23} (data not shown). This is in agreement with surface plasmon resonance experiments which were carried out in the past for various inhibitors interacting with Aβ (Tjernberg et al., 1996; Cairo et al., 2002). The dissociation constant of ligands with respect to Aβ were found to be typically on the order of 0.1 mM.

No concentration dependence – measured for iA$\beta5^{inv}$ alone – of the read-out coupling constant was observed, indicating that iA$\beta5^{inv}$ does not self-orient within the magnetic field. Figure 2 and 3 indicate the values of the one-bond splitting as read out from 1D columns of the respective heteronuclear correlation experiment. In order to relate these values to the absolute orientation of the peptide inhibitor with respect to the magnetic field axis, residual dipolar coupling values have to be normalized (Bax et al., 2001) according to

$$\frac{D_a^{NH}}{D_a^{CH}} = \frac{\gamma_N}{\gamma_C}\frac{\left\langle r_{NH}^{-3}\right\rangle}{\left\langle r_{CH}^{-3}\right\rangle} = -0.48. \quad (3)$$

The sign change in the interpretation of C-H vs. N-H residual dipolar couplings is inferred by the negative sign of the gyromagnetic ratio of ^{15}N (Clore et al., 1998a; Tian et al., 1999; Bax et al., 2001). Values reported in Table 1 correspond to the normalized average trRDC value from two independent experiments. The error for C^α-H^α trRDCs can be estimated to be ± 0.5 Hz, the error for the (unscaled) N-H^N trRDCs is found to be ± 0.2 Hz. In all cases, the sign of the residual dipolar coupling could be reproduced.

Solid state NMR studies (Balbach et al., 2000) suggest an antiparallel organization of Aβ^{14-23}. The polypeptide backbone of an individual molecule is aligned perpendicular to the long axis of the fiber. Fibrils are stabilized by hydrogen bonding between amide nitrogens and carbonyl atoms in subsequent polypeptides. These hydrogen bonds are directed along the fibril axis. With the exception of Asp1 and Pro2, positive residual dipolar couplings for C^α-H^α found throughout the backbone of iA$\beta5^{inv}$ in the presence of fibrillized Aβ^{14-23} indicate that these bonds are oriented perpendicular to the direction of the magnetic field. On the other hand, only negative trRDC are obtained for the case of N-H^N, suggesting a parallel arrangement of the N-H^N bond vectors with respect to B_0. The data are therefore in agreement with a model in which iA$\beta5^{inv}$ is arranged parallel to the individual β-strands of fibrillized Aβ^{14-23}, orthogonal to the fibril axis. We assume that the interaction between iA$\beta5^{inv}$ and fibrillized Aβ^{14-23} is driven by side chain interactions. Hydrophobic side chains might intercalate into amyloid fibrils and weaken hydrophobic Aβ·Aβ interactions which stabilize the fibrillar structure. The projection angle between the vectors N-H^N and C^α-H^α within the peptide bond is a function of the backbone

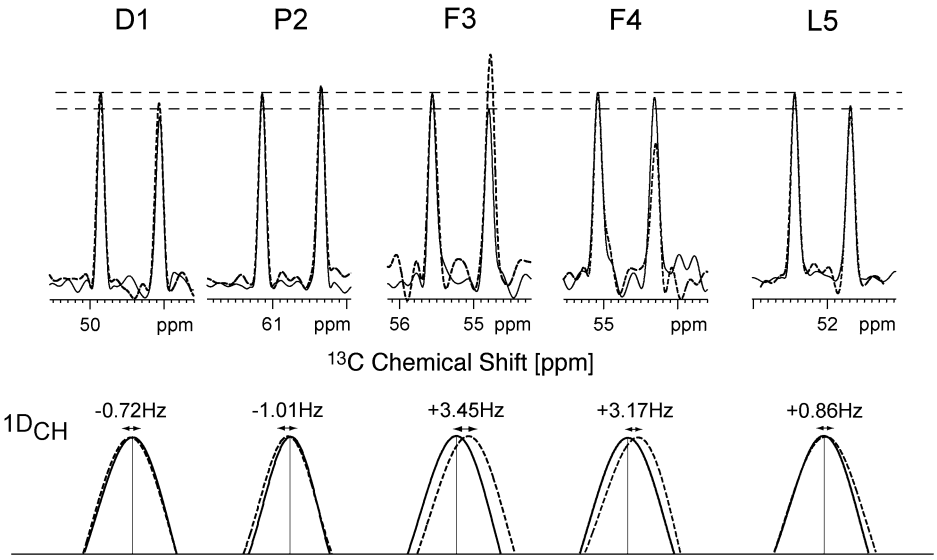

Figure 3. 1D columns of a ^{1}H,^{13}C HSQC correlation spectrum of iAβ5^{inv} with (dashed line) and without (solid line) fibrillized Aβ$^{14-23}$, recorded without heteronuclear scalar decoupling in the indirect dimension. On the bottom, only the β multiplet component is drawn in order to show residual dipolar coupling effects. For this purpose, signals with and without Aβ$^{14-23}$ are represented with the same intensity. The experiment is recorded with phase alternation of the last 90° ^{1}H pulse of the first INEPT in order to suppress intensity artifacts on the doublet components.

torsion angle φ and is given as

$$\cos \angle \left(NH^N, C^\alpha H^\alpha\right) = \\ -0.163 - 0.819 \cos\left(120^\circ + \varphi\right). \quad (4)$$

This equation can be derived in analogy to the derivation given in (Reif et al., 2000). In a β-sheet structure, the (N-H^N,C$^\alpha$-H$^\alpha$) projection angle adopts approximately a value of around 130°–160°. Depending on the exact orientation of the interacting side chains and the planarity of the β-strand, a change of sign for NH^N and C$^\alpha$H$^\alpha$ trRDC values is plausible, if we assume that the N-H^N vectors are arranged not exactly parallel with the fibril axis. The proline induces a turn in the structure and places the charged N-terminus appropriate for binding to Aβ$^{14-23}$. This is reflected in the negative sign of the C$^\alpha$H$^\alpha$ residual dipolar coupling for Asp1 and Pro2. Our experiments do not provide direct information, if iAβ5^{inv} interacts with the long axis or the tips of an amyloid fibril. A detailed analysis of the mode of interaction requires knowledge of side chain torsion angles. Experiments to determine χ_1 are currently being implemented in our laboratory. Large variations for trRDC values are observed along the backbone of iAβ5^{inv}. The largest values are found for Phe3 and Phe4. This would not agree with a flat, but rather a bent β-sheet structure for iAβ5^{inv}. Alternatively, smaller trRDC values could be due to an increased mobility at the C-terminus of iAβ5^{inv}, and could therefore be due to a faster loss of orientation after being dissociated into the unligated state. The same argument is not directly applicable to the N-terminus, since the N-terminus might be stabilized in a turn-type structure. A quantitative interpretation of trRDC values requires the knowledge of the molecular alignment tensor of iAβ5^{inv}. This tensor is, however, difficult to assess experimentally due to the size of the molecule and therefore the few number of RDC values which can be reliably extracted. For this reason, we carry out here only a qualitative analysis of the measured trRDC values.

The asymmetry of the α/β doublet components induced by binding of iAβ5^{inv} to Aβ$^{13-24}$ is due to ^{13}C$^\alpha$–^{1}H$^\alpha$ dipole, ^{13}C$^\alpha$ CSA and ^{15}N-^{1}H^N dipole, ^{15}N CSA cross correlated relaxation (Goldman, 1984; Reif et al., 1997). These differences are directly related to the binding affinity of the respective sites and chemical groups to the amyloid fibrils. Recently, this effect has been suggested to be employed in drug design (Peng, 2003). We also observe differences in the size of the residual dipolar couplings depending on the mode of sample preparation. Almost no changes are observed, if Aβ$^{14-23}$is solubilized in a solution that already contains iAβ5^{inv}. This is most likely due to the fact that fibril formation is abolished under these

conditions, and no preferential orientation of $A\beta^{14-23}$ is possible.

Additional experiments have to be performed in order to restrain the structure of $iA\beta5^{inv}$ in the bound state. For this purpose, we plan on collecting more trRDC (^{1}H-^{13}C, ^{13}C-^{13}C and ^{13}C-^{15}N) in order to be able to evaluate the molecular alignment tensor of $iA\beta5^{inv}$, especially including side chain resonances. For this purpose, synthesis of a u-^{13}C,^{15}N labeled peptide is required. Furthermore, trNOE data have to be carefully interpreted in order to obtain a structure of $iA\beta5^{inv}$ in the bound state, taking into account extensive spin diffusion effects which are present in the experiments.

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