Chapter 2

Material and Methods

2.1 Expression and Purification of the AF6 PDZ domain

Structural studies require high-level expression systems and efficient purification protocols. *Escherichia Coli* is the one of the most successful host systems for high-level production of both prokaryotic and eukaryotic proteins.[1] These bacterial strains are specifically engineered to expresses the desired protein in large amounts whose DNA is inserted into the cell via specially constructed plasmids. Expression in *E.coli* is often the most favored method because of the ease of expression and isotope labeling (^{15}N, ^{13}C and ^{2}H).

Expression and purification of the AF6 PDZ domain has earlier been described elsewhere.[2,3] The plasmid and the overexpression protocol were kindly provided by Dr. Prisca Boisguerin.

The AF6 PDZ domain was expressed in M9 minimal media,[4] containing 50 mg/l ampicillin, and $^{15}NH_4Cl$ for uniformly ^{15}N labeled samples or $^{15}NH_4Cl/^{13}C$-glucose for uniformly $^{15}N/^{13}C$ labeled samples under identical conditions. For overexpression the cells were grown at 37 ^{0}C till the mid log phase (OD_{600} = 0.5). At this point the expression was induced by addition of IPTG (1.0 mM). The cells were allowed to grow for further 3-4 hours at $28^{0}C$. After harvesting, the cells were lysed and AF6 PDZ domain was purified as a GST fusion protein by affinity chromatography on GST-Sepharose column. Pure AF6 PDZ domain was obtained by cleaving the GST tag by PreScission™ protease followed by size exclusion chromatography. Appropriate amounts of Complete® protease inhibitor cocktail tablets were added at each step to the buffers as suggested by the manufacturer. The expression and purification protocol yielded 20-30 mg of pure AF6 PDZ domain per liter of culture.

Protein concentrations were estimated from the extinction coefficients at 280 nm as calculated using the ProtParam tool (http://expasy.ch/protparam) of the Swiss Institute of Bioinformatics

2.2 NMR instrumentation

All NMR experiments were recorded on Bruker DRX 600 and DRX 750 MHz spectrometers equipped with cryoprobes. Spectra were obtained using a 5 mm single Z-axis gradient probe (600 MHz with a Cryoprobe) and a 5 mm triple axis gradient probe (600 MHz with conventional probehead and 750 MHz with Cryoprobe). Spectral processing was carried out using XWINNMR and was analyzed by Sparky software.

2.3 NMR sample preparation

NMR experiments were performed on 0.05-1.5 mM uniformly ^{15}N and $^{13}C/^{15}N$ labeled samples of AF6 PDZ domain depending on experimental requirements. Samples for sequence specific assignment of resonances and structure determination were prepared in 50 mM NaH_2PO_4/Na_2HPO_4, pH 6.5, 50 mM NaCl, 0.02% (w/v) NaN_3, in 90% H_2O/10% D_2O. Samples for side chain assignments were prepared in the same buffer dissolved in 100% D_2O. For NMR based screening 50 µM protein samples were prepared in 50 mM NaH_2PO_4/Na_2HPO_4, pH 7.0, 50 mM NaCl, 0.02% (w/v) NaN_3, in 90% H_2O/10% d_6-DMSO. Ligands were dissolved in 100% d_6-DMSO at 160 mM concentration.

2.4 General experimental details

All spectra for NMR based screening were recorded at 300^0K. NMR spectra for the resonance assignments and structure determination were acquired at 295^0K. Extensive use was made of the PASTE software package,[5] which provided the majority of required pulse sequences for resonance assignment and structure determination. Water suppression was achieved by application of watergate,[6] jump-return pulse sequences,[7] solvent pre-saturation, or gradient coherence selection.[8,9] Quadrature detection in the indirect dimensions was achieved using

the method of States[10] or States-TPPI.[11] The digital resolution of recorded spectra was enhanced by zero filling to at least twice the number of acquired points, and Lorentzian-to-Gaussian or sine-bell or squared sine-bell window functions applied. Spectra were processed using the program XwinNMR and were analyzed using SPARKY.[12] Sample pH was checked and adjusted if required prior to acquisition.

2.5 NMR based screening of ligands for the AF6 PDZ domain

To identify small molecule modulators of the AF6 PDZ domains a library of 5000 commercially available compounds was screened against the AF6 PDZ domain.

^{15}N labelled AF6 PDZ at 50 µM concentration in a buffer containing 90% H_2O/10% DMSO was used for the screening purposes. Ligand binding was detected at 300^0K by acquiring ^{15}N-^1H heteronuclear single quantum correlation (HSQC) spectra in the presence and absence of compounds. Compounds were initially tested at 400 µM each in mixtures of 16, with subsequent deconvolution to mixtures of 4 at 400 µM each and then to individual compounds. For each screening experiment 128 × 1024 complex points were acquired in the F1 (^{15}N) and F2 (^1H) dimensions respectively, using spectral widths of 49.5 ppm (t1) and 16.6 ppm (t2) with 16 scans per t1 increment on Bruker DRX600 spectrometer equipped with cryoprobe Sample prepration was done automatically by Tecan Genesis RSP 150 pipetting robot. Bruker Sample Track System was used to deliver the samples to the magnet, and IconNMR software package (Bruker) was used for data acquisition. The processing and analysis of the NMR spectra was done manually using XwinNMR and SPARKY[12] respectively. A schematic of NMR based screening is presented in Figure 2.1

Positive “hits” from the screening experiments were identified by comparing the ^1HN-^{15}N chemical shifts of protein alone to those of the protein in presence of ligands.

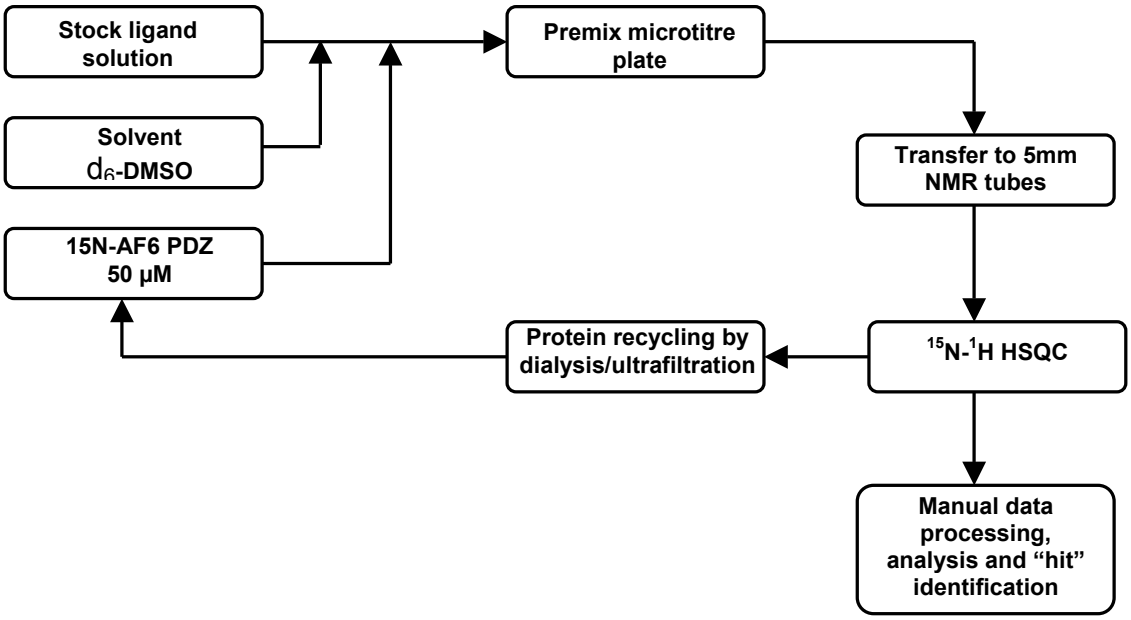


Figure 2.1: Schematic representation of NMR based ligand screening for AF6 PDZ domain.

2.6 Determination of binding constants

Average dissociation constants, Kd, for the binding of AF6 PDZ domain to various organic ligands were calculated from NMR titration data using a non-linear least-squares fitting analysis of the following equation, assuming fast exchange:[13]

$$y(L_o) = a - (a^2 - L_o / P_o)^{0.5} \quad \text{with } a = (P_o + L_o + Kd) / (2 * P_o) \qquad (1)$$

where, $y(L_o)$ is the fraction of bound ligand for each amino acid under observation, P_0 and L_0 give the total concentration of the protein and ligand in the binding assay.

Equation 1 describes a one-site, second order binding model according to the equilibrium :

$$PL \rightleftharpoons P + L, \qquad Kd = \frac{[P][L]}{[PL]} \qquad (2)$$

where, [P] and [L] are the equilibrium concentrations of the AF6 PDZ domain and the organic compounds respectively. Titration of 0.1 mM AF6 PDZ domain upto 1:15 protein to ligand ratio (final concentration of organic compounds 1.5 mM) against selected organic compounds was monitored by following the chemical shift change, d, in standard ^{15}N-^{1}H HSQC spectra, where,

$$d = ((\Delta H)^2 + (\Delta N/5)^2)^{0.5} \qquad (3)$$

For each titration point 128 × 1024 complex points were acquired in the F1 (^{15}N) and F2 (^{1}H) dimensions respectively, using spectral widths of 49.5 ppm (t1) and 16.6 ppm (t2). . Comparing the ^{1}HN-^{15}N chemical shifts of protein alone to those of the protein in presence of ligands, the perturbed Residues were identified during the titration. Control experiments were also performed by titrating the protein against the pure d_6-DMSO was to check the effect of DMSO on the ^{1}HN-^{15}N chemical shifts of the protein.

2.7 Relaxation and Dynamics

2.7.1 ^{15}N T1 measurements

^{15}N T1 measurements at 600 MHz were recorded with relaxation delays of 12, 52, 102, 152, 202, 302, 402, 602, 902, 2002, 5002 ms. Each ^{15}N-^{1}H HSQC CPMG experiment[14] utilized watergate for the suppression of the water signal. 128 × 1024 complex points were acquired in the F1 (^{15}N) and F2 (^{1}H) dimensions respectively, with spectral widths of 49.5 ppm and 16.6 ppm. 12 transient scans were acquired for each t1 point, and the acquisition times for the F1 and F2 dimensions were 171 ms and 85 ms respectively. Data was processed using sine-bell function applied in both dimensions. Data was also zero filled to 1024 (t1) and 2048 (t2) data points. Relaxation times were obtained from an analysis of the exponential decay of peak intensities using the program SPARKY.

2.7.2 ^{15}N T2 measurements

^{15}N T2 measurements at 600 MHz were recorded with relaxation delays of 6, 10, 18,26.34, 42, 82, 122, 162, 202 and 242 ms. Each ^{15}N-^{1}H HSQC CPMG experiment[14] utilized watergate for the suppression of the water signal. 128 × 1024 complex points were acquired in the F1 (^{15}N) and F2 (^{1}H) dimensions respectively, with spectral widths of 49.5 ppm and 16.6 ppm. 16 transient scans were acquired for each t1 point, and the acquisition times for the F1 and F2 dimensions were 171 ms and 85 ms respectively. Data was processed using sine-bell and squared sine-bell filtering functions applied in the indirect and direct dimensions respectively. Data was also zero filled to 1024 (t1) and 2048 (t2) data points. Relaxation times were obtained from an analysis of the exponential decay of peak intensities using the program SPARKY.

2.7.3 ^{1}H -^{15}N Heteronuclear NOE measurements

Heteronuclear ^{1}H -^{15}N NOEs 600 MHz were obtained using a gradient sensitivity enhanced ^{1}H -^{15}N NOE experiment[14], recorded with and without ^{1}H saturation as two interleaved data matrices of 128 × 1024 complex points acquired in the F1 (^{15}N) and F2 (^{1}H) dimensions respectively, and spectral widths of 49.5 ppm and 16.6 ppm. A relaxation delay of 2 s prior to 3 s of ^{1}H saturation was used for the NOE spectra, and a 5 s relaxation delay used for the reference spectra. Data was processed using Lorentzian-to-Gaussian filtering functions applied in both dimensions, and zero filling to 2048 (t1) and 4096 (t2) data points. The heteronuclear enhancement factor NOE600 was calculated from the difference in peak intensities of cross-peaks in the presence (Isat) and absence (I0) of ^{1}H saturation according to:

$$NOE600 = (Isat-I0)/I0, \text{ and}$$

$$SD = \sqrt{[(sdsat \times I0)2 + (sd0 \times Isat)2]/I02},$$

where, the error for each peak is given by the overall standard deviation, SD, of the baseplane noise, and sdsat and sd0 are the standard deviations of the baseplane noise in the saturated and reference spectra respectively. 32 transient scans were acquired for each t1 point, and the acquisition times for the F1 and F2 dimensions were 143 ms and 71 ms respectively.

2.8 Resonance assignment of the AF6 PDZ domain

Sequential assignments of backbone resonances of the AF6 PDZ domain were obtained from inter-residue sequential connectivities from the following pair of J-correlated triple resonance experiments: CBCA(CO)NH[15] and CBCANH.[16] The Hα and Hβ resonances were assigned by HBHA(CO)NH and HBHANH experiments.[17] A solution of 1.5 mM AF6 PDZ domain in standard buffer containing 90% H_2O/10% D_2O was used for this purpose. Suppression of the solvent signals was achieved by using WATERGATE.

The aliphatic side-chain ^{1}H and ^{13}C resonances of AF6 PDZ domain were assigned using 3D H(CCCO)NH , 3D (H)C(CCO)NH experiments [18,19] on the 1.5 mM, $^{13}C/^{15}N$-labelled sample in 90% H_2O/10% D_2O, in order to correlate the ^{15}N and ^{1}HN chemical shifts of residue i with the ^{1}H and ^{13}C chemical shifts of residue i-1 respectively. Assignments of the sidechain resonances were aided by HCCH-TOCSY and HCCH-COSY[20] experiments recorded on a 1.5 mM sample in 100% D_2O. All spectra were folded appropriately to increase the resolution of the indirect ^{13}C and ^{15}N dimensions. To assign the side-chain resonances of the aromatic residues, a combination of 2D ^{1}H -^{1}H DQF-COSY,[21] 2D ^{1}H -^{1}H NOESY[22] 2D ^{13}C-HMQC and 3D ^{13}C-separated NOESY spectra in 100% D2O were used

A summary of the experiments recorded for assignment of backbone and sidechain resonances of the AF6 PDZ domain is presented in Table 2.1

Assignments of the AF6 PDZ in complex with **7i** and the superbinding peptide[2] were carried out in similar manner as described above for the AF6 PDZ domain alone. The samples used for this purpose were either a 1:1 mixture of AF6 PDZ and **7i** or a 1:1 mixture of AF6 PDZ and the superbinding peptide. The assignment of unlabelled superbinding peptide in complex with AF6 PDZ domain was carried out by utilizing isotope filtered NOESY and TOCSY experiments as described below.

Table 2.1: Summary of NMR experiments recorded for the resonance assignment of AF6 PDZ domain and the AF6 PDZ domain complexes.

Experiment	Number of points			Expt. time (hrs)	Correlations observed
	F1	F2	F3		
^{15}N-^{1}H HSQC	512	1024		2	^{1}HN-^{15}N
CBCACONH	128	128	1024	48	$^{13}C^{\beta}_{i-1}$ - $^{13}C^{\alpha}_{i-1}$ - $^{15}N_i$ - $^{1}HN_i$
CBCANH	128	128	1024	48	$^{13}C^{\beta}_{i-1}$-$^{13}C^{\alpha}_{i-1}$-$^{13}C^{\beta}_{i}$ -$^{13}C^{\alpha}_{i}$- $^{15}N_i$- $^{1}HN_i$
HBHACONH	128	128	1024	40	$^{1}H^{\beta}_{i-1}$- $^{1}H^{\alpha}_{i-1}$ -$^{15}N_i$ - $^{1}HN_i$
HBHANH	128	128	1024	42	$^{1}H^{\beta}_{i-1}$- $^{1}H^{\alpha}_{i-1}$- $^{1}H^{\beta}_{i}$ - $^{1}H^{\alpha}_{i}$-$^{15}N_i$-$^{1}HN_i$
(H)CC(CO)NH	128	128	1024	41	$^{13}C^{aliphatic}_{i-1}$-$^{15}N_i$ - $^{1}HN_i$
H(CCCO)NH	128	128	1024	41	$^{1}H^{aliphatic}_{i-1}$- $^{15}N_i$ - $^{1}HN_i$
HCCH-COSY	128	128	1024	60	$^{1}H^{aliphatic}$-$^{13}C^{aliphatic}$
HCCH-TOCSY	128	128	1024	60	$^{1}H^{aliphatic}$-$^{13}C^{aliphatic}$
^{13}C-HMQC	512	1024		2	^{1}H-^{13}C
2D F1-F2 ^{13}C filtered TOCSY [a]	4096	512		15	^{1}H attached to 12C
2D F1-F2 ^{13}C filtered NOESY ("H")[b]	4096	512		24	NOE correlations between ^{1}H attached to ^{12}C

a. for the assignment of AF6-superbinder ($LEGIFV_{COOH}$) in complex with AF6 PDZ domain.

b. for the assignment of **7i** in complex with AF6 PDZ domain.

2.9 NMR experiments for the Structure determination

2.9.1 NMR experiments for intra-molecular NOE restraints

The determination of three-dimensional protein structures in the solution state by NMR spectroscopy relies largely upon the measurement and interpretation of a large number of Nuclear Overhauser Effect (NOE) interactions between the neighboring protons. The interproton distance restraints for the structure determination of free AF6 PDZ domain were obtained from ^{15}N-NOESY-HSQC in H_2O[23] and 3D ^{13}C-NOESY-HSQC in D_2O.[24] All 3D NOESY spectra were recorded at 600 MHz and the spectra were folded appropriately to increase the resolution of the indirect ^{13}C and ^{15}N dimensions respectively.

2.9.2 NMR experiments for inter-molecular NOE restraints

Although the high spatial density of ^{1}H in proteins is an extremely rich source of structural information, this very property makes it difficult to extract structural information from the samples of protein in complex with peptides or small organic ligands. One way to overcome this handicap is to isotopically label the peptide or small molecule ligand, which becomes prohibitively expensive when obtained from non-microbial sources. The second and probably a better option is the use of isotope filtered experiments for selectively observing ligand signals and obtaining spectra that are of suitable quality to extract structural information.

Here we used a combination of half filtered (i.e. filter applied in only one dimension of a 2D spectra) and double half filtered (filter applied in both the dimensions) experiments[25-27] to obtain the resonance assignments of the superbinding peptide as well as intermolecular NOEs between AF6 PDZ domain and the superbinding peptide and between AF6 PDZ domain and **7i**.

2.9.2.1 The filter element

A conventional filter as described earlier was used to filter out the ^{1}H signals arising from ^{1}H-^{13}C spins (Figure 2.2a).[28] In these pulse sequences ^{1}H-X (X=hetero-nucleus) couplings are used to discriminate between the different classes of protons. The fixed delays Δ in the pulse sequence are tuned to $1/2(J_{HX})$, for optimal generation of ^{1}H-X antiphase coherence. This antiphase coherence is then eliminated in favor of the inphase coherence of the protons not coupled to X. This was achieved by applying two consecutive 90^0 pulses on X, which differ in the relative phase of the second pulse. The experiments with x and –x of the final 90^0 X pulse are acquired with invariant receiver phase. To select the protons not coupled to X the collected data are added.

A simple X-half filter experiment can be explained in terms of product operator description as follows:

For the protons I coupled to a hetero-nucleus S,

$$I_z S_z \xrightarrow{90 I_x} -I_y S_z \xrightarrow{\pi J \Delta 2 I_z S_z} -I_y \cos \pi J\Delta + 2I_x S_z \sin \pi J\Delta$$

when $\Delta = 1/2J$

$$2I_x S_z \xrightarrow{180 I_x} -2I_x S_z \xrightarrow{90 S_x} -2I_x S_y \xrightarrow{\pm 90 S_x} \mp 2I_x S_z \xrightarrow{\pi J \Delta 2 I_z S_z} \mp 2I_y S_z$$

and for the protons H not coupled to the hetero-nucleus the net magnetization after the second delay (Δ) will be

$-H_y$

In this experiment when the data collected are added only the protons not attached to the hetero-nucleus are selected.

The actual pulse sequences for the filtered experiments were kindly provided by Dr. Peter Schmieder (FMP, Berlin).

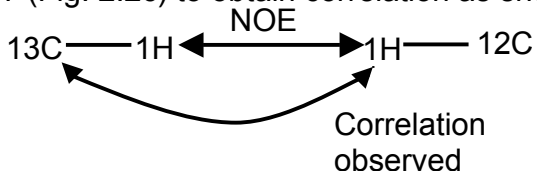
The pulse sequences to obtain intermolecular NOEs between the unlabelled ligand (superbinding peptide and **7i**) and ^{15}N ^{13}C labeled AF6 PDZ domain are described below. All the filtered spectra were recorded on the D_2O sample. Suppression of the residual water signal was achieved by solvent pre-saturation.

2.9.2.2 2D-F2 ^{13}C-filtered NOESY

The 2D-F2 ^{13}C-filtered NOESY is a modified version of a normal ^{1}H-^{1}H NOESY experiment. The filtering element which served to filter out any intra-molecular NOEs was placed after the third 90^0 proton pulse in the pulse sequence (Fig. 2.2b). A composite pulse (90x-180y-90x) was applied on the hetero-nucleus to remove the ^{1}H-^{13}C scalar couplings. Two spectra were recorded, one with the `^{13}C´ carrier frequency placed to optimally suppress the aromatic region of the protein and other to suppress the methyl region.

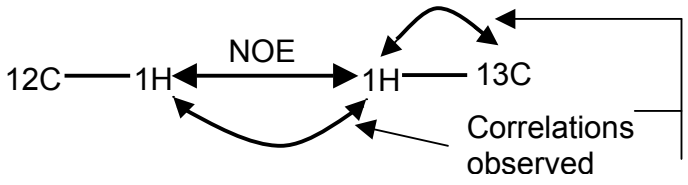
2.9.2.3 2D-F2 ^{13}C-filtered HMQC-NOESY

A combination of the HMQC and the NOESY pulse sequences, in which the NOESY evolution period is omitted leads to a 2D HMQC-NOESY experiment. In this experiment starting from a ^{1}H-^{13}C HMQC cross peak one obtains additional peaks in F1 dimension caused by NOESY transfer. Here we used a 2D-F2 ^{13}C-filtered HMQC-NOESY (Fig. 2.2c) to obtain correlation as shown below,



2.9.2.4 3D-F1 ^{13}C-suppressed NOESY-HMQC

A standard 3D ^{13}C-NOESY-HMQC was modified by incorporating a conventional filter at the beginning if the pulse sequence to create a 3D-F1 ^{13}C-suppressed HMQC-NOESY experiment (Fig. 2.2d). This pulse sequence acquires ^{1}H chemical shift of protons not coupled to the ^{13}C along the F1 dimension. Use of the isotope filter at the beginning of the pulse sequence ensures that only the protons attached to 12C evolve during t1. This is followed by a conventional NOESY mixing time, $\tau_{m,}$ (80 ms). After the NOESY magnetization transfer an HMQC sequence is used to correlate the protons which are "excited" by the NOESY mixing to ^{13}C through their ^{1}H-^{13}C scalar couplings. After the chemical shift evolution of ^{13}C in t2, the protons directly attached to it are detected.



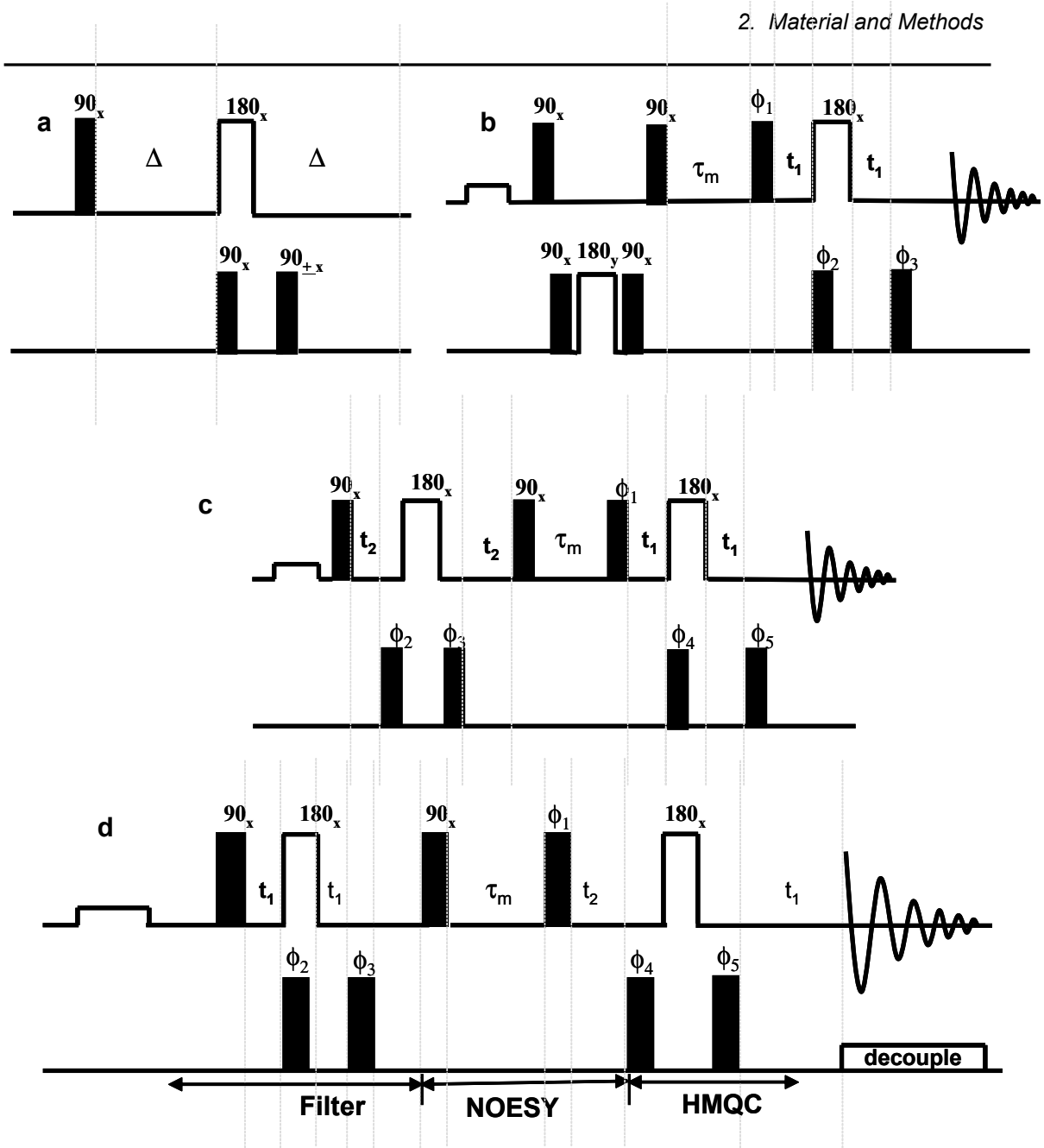


Figure 2.2 Pulse sequences used for the ^{13}C filtered/^{12}C edited experiments
a) A basic X half filter for isotope filtered experiments. For optimal performance the delay Δ is set to $1/2^1J_{HX}$. In the subsequent pulse sequences linear combination of the X half filter is shown **b)** With a 2D NOESY pulse sequence **c)** With 2D-HMQC-NOESY pulse sequence and **d)** With a 3D-NOESY HMQC pulse sequence.
In all sequence the delays were set as follows: t1=, t2=, τ_m=80 msec**.**
Phase cycle: b) ϕ_1= 4(x), 4(y), 4(-x), 4(-y); ϕ2= (x,-x); ϕ3= (x,x,-x,-x)
c) ϕ1= 4(x), 4(y), 4(-x), 4(-y); ϕ2= (x,-x); ϕ3= 8(x), 8(-x); ϕ4= (x,x,-x,-x);
ϕ5= 32(x),32(-x)
d) ϕ1= 4(x), 4(y), 4(-x), 4(-y); ϕ2= (x,-x); ϕ3= 16(x), 16(-x): ϕ4= (x,x,-x,-x);
Receiver= 2(x,x,-x,-x,-y,-y,y,y,-x,-x,x,x,y,y,-y-y)
2(-x,-x,x,x,y,y,-y-y,x,x,-x,-x,-y,-y,y,y)

Table 2.2: Summary of experiments recorded for the structure determination of AF6 PDZ domain and AF6 PDZ complexes.

Experiment	Number of points			Expt. Time (hrs)	Mixing time (ms)
	F1	F2	F3		
^{15}N-NOESY-HSQC	128	128	1024	30	80
^{13}C-NOESY-HMQC	128	128	1024	30	80
2D ^{1}H-^{1}H NOESY	512	1024		3	80
2D-F2 ^{13}C-filtered HMQC-NOESY[b]	512	4096		15	80
2D-F2 ^{13}C-filtered NOESY[b]	512	4096		15	80
3D-F1 ^{13}C-filtered NOESY-HMQC[a]	1024	96	96	36	80
2D F1-F2 ^{13}C filtered NOESY ("H")[b]	4096	512		24	80
2D ^{1}H-^{1}H NOESY[a]	512	1024		4	125
2D ^{1}H-^{1}H NOESY[b]	512	1024		5	300

a. for AF6 PDZ – Superbinder complex.
b. for AF6 PDZ – **7i** complex.

2.10 Automated NOE cross peak assignment using CANDID

Following some initial manual peak assignment of the NOESY spectra of AF6 PDZ domain, several cycles of automated NOE assignment and structure calculation were performed using the CANDID (Combined Automated NOE assignment and structure Determination)[29] algorithm in the program CYANA (Combined assignment and dYnamics Algorithm for NMR Applications)[30]. The input for this protocol requires a complete chemical shift assignment list and volume integrated peak lists for each NOESY spectrum. Chemical shift tolerances for CANDID were set at 0.03 ppm for ^{1}H dimensions and 0.30 ppm for ^{13}C/^{15}N dimension. The default calibration parameters were used for conversion of peak volumes into distance restraints within the CANDID protocol. For the first round of assignment/calculation the spectral data was supplemented with φ/ψ backbone angles derived from the program TALOS.[31]

2.11 Backbone φ and ψ angle determination

Backbone dihedral angles for the initial fold determination, using the CANDID algorithm[29] in the structure calculation program CYANA,[30] were obtained from the program TALOS.[31] All TALOS predictions, from measured HN, ^{15}N and ^{13}C shifts and the corresponding triplet residue sequences in the TALOS database, classified as good were used to generate initial structures. The upper and lower bounds of the TALOS φ and ψ angles were given as the mean value ± 20 standard deviation.

2.12 Structure Calculation

Calibration of interproton distance constraints: The calibration of interproton distance constraints from all NOESY spectra for structures calculated without the CANDID protocol was achieved as follows: NOE peaks were integrated in SPARKY with an initial tolerance of 30%. Resulting NOE volumes were converted into distances using the CALIBA program,[32] with expected distances in secondary structure used as a guide for the distance calibration. An r^{-6} and r^{-4} dependence of peak volumes on distance was used for calibration of NOEs involving backbone atoms, and side chain atoms respectively. The volumes of peaks assigned to pseudo atoms (degenerate methyl, methylene and aromatic protons) were divided by the number of protons represented, and the pseudo atom corrections automatically added by CALIBA were removed. These initial distances were subsequently classified into NOE distance ranges corresponding to very strong (1.8-2.7 Å), strong (1.8-3.3 Å), medium (1.8-4.2 Å), weak (1.8-5.0 Å) and very weak (1.8-6.0 Å) NOEs.

2.12.1 Structure calculation using CYANA

Structures were calculated starting from random dihedral angles using a torsion angle dynamics (TAD) simulated annealing protocol in CYANA.[30] Distances for the CANDID protocol of automated NOE assignment and structure calculation were calibrated using the default parameters based on an expected average distance of 4 Å for all NOEs. Typically, 200 trial structures were generated in each cycle and the 20 structures with the lowest target function values chosen to represent the solution structure (In the case of a CANDID run, a total of 7 cycles of structure calculation is conducted, with the results of the preceding cycle used for further automated assignment and fold refinement in subsequent cycles). A standard simulated annealing protocol was used in which each random conformer is subjected to 4,000 steps of TAD at high temperature (system temperature increased to 8.0 TFU (target function units) per residue), followed by a slow cooling period of 20,000 TAD steps to a final temperature of 0 TFU. A final

conjugate gradient minimization is then conducted (1000 steps). The default force constants on both distance and dihedral angle restraints are maintained at constant values throughout the calculation, while the repel function describing the non-bonded contacts is increased from 0.2 TFU to 2.0 TFU during cooling.

2.13 Docking of 5-(4-trifluoromethylbenzyl)-2-thioxo-4-thiazolidinone (7i) on to the AF6 PDZ domain

2.13.1 Ligand parameterization using Antechamber

Antechamber is a set of auxillary programs within AMBER8, which can recognize atom and bond type, find missing forcefield parameters and give reasonable substitutes for the same and prepare input topology file for LEaP. Antechamber is designed to work with the general amber force field (GAFF),[33] which is compatible with the AMBER force fields and can be used together during a simulation. At present GAFF has 33 basic atom types and 22 special atom types.

For parametrization, a PDB file of **7i** was created in SYBYL6.90. This PDB file was used as input for the antechamber software package. The charge method used for this purpose was AM1-BCC.[34] Atom types assigned by antechamber were carefully inspected and missing atom types, bond length, bond angle and torsion angle parameters were assigned according to Cornell and co-workers.[35]

For creation of the input topology and coordinate files for AMBER docking, **7i** was manually docked onto AF6 PDZ using SYBYL 6.90. The pdb file of the complex (without hydrogen's) was then read into LEaP. The AF6 PDZ **7i** complex was then solvated in a truncated octahedral water box, and an appropriate number of counter ions (Cl^-) was added to neutralize the system. The topology and coordinate files for the solvated complex was exported from LEaP and was used as input for the final molecular dynamics run.

2.13.2 Docking of 5-(4-trifluoromethylbenzyl)-2-thioxo-4-thiazolidinone (7i onto AF6 PDZ domain

The docking procedure was done using the topology and coordinate files of the solvated complex from LEaP. The simulations were divided into 3 parts:

1. Minimization: As LEaP adds hydrogen to the molecule under consideration depending on the geometry specified in the residue database. This "default" geometry may not coincide with the local minima in the forcefield and may result in conflicts and overlap with atoms in other residues. Therefore the complex was first minimized to relax the location of such atoms and remove any bad contacts if present.

2. Equilibration: The next step during the MD simulation was to equilibrate the system. During solvation of the complex, there might be some gaps between the water box and the solutes which may lead to "vacuum bubbles" during the MD simulations and may thus cause instability in the system. To overcome this problem the system was slowly heated from 0 ^{0}K to 300 ^{0}K. The equilibration was carried out at constant pressure using periodic boundaries which allowed the volume of the box to change. This approach allowed the water to equilibrate with the solutes and reach equilibrium density.

3. The Production run: Final docking of **7i** onto AF6 PDZ domain was carried out at constant pressure and temperature (300 ^{0}K). The MD was carried out for 2 ns using 200,000 steps. During the first 1.5 ns the docking of the ligand was driven by the distance restraints obtained from the F2 filtered NOESY experiment. During the final 0.5 ns the system was allowed to relax by gradually removing the restraints applied to the complex to check the viability of the docking procedure.

During the entire process the backbone of the protein was kept rigid and the distance restraints from the last CYANA cycle (cycle 7) were used to preserve the overall structure of the AF6 PDZ domain. The final output coordinate file from

AMBER was converted into PDB file format and was analyzed using Sybyl6.9 and MOLMOL.[36]

As **7i** was enantiomerically impure (~ 1:1 mixture of R and S enantiomers), the docking procedure was performed on both enantiomers under identical conditions.

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