Chapter 6

Materials and Methods

6.1 Materials

Unless specified otherwise, chemicals and reagents are purchased in the highest available quality from Sigma, Merck, Fluka, Roche or Serva. $^{15}NH_4Cl$, [U-^{13}C]-glucose and D_2O are from Spectral Isotopes. Enzymes and buffers for cloning are from New England Biolabs (Beverly, MA). Kits for plasmid purification and gel extraction are from Qiagen (Hildesheim, Germany).

6.1.1 Buffers and solutions

Experiment	*Buffer/Solution*	*Components*
His_6-tag purification	Lysis A	20mM TRIS pH 8.0, 150mM NaCl, 10mM imidazole (pH 8.0), 2mM β-mercaptoethanol
	Lysis A+	lysis A + 0.2% (v/v) NP-40 (non-ionic detergent)
	Wash A1	lysis buffer A with 1M NaCl
	Wash A2	lysis buffer A with 25mM imidazole
	Elution A	lysis buffer A with 200mM imidazole
GST-fusion purification	PBS	50mM sodium phosphate buffer pH 7.3, 150mM NaCl
	Lysis G	PBS + 10mM β-mercaptoethanol.
	Elution G	20mM TRIS pH 8.0, 25mM reduced glutathione (GSH)
NMR	NMR-PO_4	20mM $NaPO_4$ pH 6.8, 100mM NaCl, 2mM DTT, 0.02% NaN_3
	NMR-MOPS	20mM MOPS pH 6.8, 100mM NaCl, 2mM DTT, 0.02% NaN_3

Protein lipid overlay assay	PBS-T	PBS + 0.1% (v/v) Tween-20.
	Blocking	PBS-T + 2% (w/v) BSA
DTNB reaction	D	20mM TRIS pH 8.0, 100mM NaCl
Solutions for minimal medium	Trace elements (100x)	5g EDTA, 0.833g $FeCl_3$× 6 H_2O, 84mg $ZnCl_2$, 13mg $CuCl_2$×2 H_2O, 10mg $CoCl_2$×6 H_2O, 10mg H_3BO_3, 1.6g $MnCl_2$×6 H_2O, pH 7.5 H_2O ad 1 litre.
	M9 mineral salts (10x)	60g Na_2HPO_4, 30g KH_2PO_4, 5g NaCl, 5g NH_4Cl ($^{15}NH_4Cl$ for ^{15}N-labelled M9 medium), H_2O ad 1 litre.

6.1.2 Media

- **LB medium** for unlabelled proteins (10 g/L bactotryptone, 5 g/L yeast extract, 10 g/L NaCl, pH 7.4)
- **Minimal medium** for ^{15}N and $^{15}N/^{13}C$ labelled proteins: 100mL 10x M9 medium, 10ml 100x trace elements, 20mL 20% glucose (10mL 20% $^{13}C_6$-glucose for ^{13}C-labelled minimal medium), 1mL 1M $MgSO_4$, 0.3mL 1M $CaCl_2$, 1mL 1mg/mLbiotin, 1mL 1mg/ml thiamin, H_2O ad 1 litre.
- **OD1 rich medium** (Silantes) for triple labelling ($^2H/^{15}N/^{13}C$) of proteins.

6.1.3 Bacterial strains

- *E. coli* DH5α (cloning)
- *E. coli* BL21[DE3] (expression)
- *E. coli* BL21[DE3] pLysS (low basal expression)

6.1.4 Expression vectors

Vector	*Antibiotic*	*Affinity tag*	*Carrier protein*	*Protease*
pGS-N-his	Kan	N-His_6	-	-
pGS-Z	Kan	N-His_6	Z-tag	tev
pGS-GST	Kan	N-His_6/GST	GST	-

All expression vectors are provided by G. Stier. His_6-tagged and TEV cleaved proteins carry the additional N-terminal amino acids $MKH_6P\underline{M}$ and GA$\underline{M}$, respectively. The $\underline{M}$ residue is generated by the NcoI site.

6.1.5 Oligonucleotide primes

Name[1]	***Sequence***[2]	***Restriction site***
Pleck_1s	TTTGCCATGGAACCAAAGCGGATCAGAGAG	NcoI
Pleck_103r	TTTCGGTACC**TTA**TTCAATGCATTTAATGGCCTTATTGA	KpnI/Acc65[3]
Pleck_122s	TTTCCATGGAAACCATTGACTTAGGTGCCT	NcoI
Pleck_243r	TTTGGTACC**TTA**CCCTCGGAATTCTTCTTTCAGAATC	KpnI/Acc65
Pleck_221s	TTCGCCATGGACAGTGGGTCCTTCTGTGAAGAG	NcoI
Pleck_234s	TTCGCCATGGATGTGATTCTGAAAGAAGAATTC	NcoI
Pleck_238s	TTCGCCATGGAATTCCGCGGGGTCATTATCAAGCAGG	NcoI
Pleck_242s	TTTGCCATGGGACGAGGGGTCATTATCAAGCAGGGATG	NcoI
Pleck_350r	TTTCGGTACC**TTA**CTTCCCAGTTCGGGAGGC	KpnI/Acc65

[1] The name indicates the first or last amino acid coded by the oligo depending on whether it is a sense (name ending in "s",e.g. Pleck-1s) or reverse (name ending in "r") primer.
[2] Restriction sites are underlined. The stop codon of reverse primers is in bold.
[3] KpnI and Acc65 are isoschizomers.

6.2 Methods

6.2.1 Cloning

A full-length source clone of human pleckstrin-1 (amino acids 1-350) and the PH domain of PLC-δ are provided by G. Stier. All pleckstrin constructs are generated from the full-length source clone by standard PCR based cloning strategies. Briefly, PCR products are gel purified, digested with appropriate restriction enzymes (NcoI/Acc65), again purified from an agarose gel and ligated into a vector that is opened with the same restriction enzymes 5' dephosphorylated (shrimp alkaline phosphatase). The ligation mixture is transformed into DH5α competent cells. Two clones per ligation are picked and plasmids

are purified following the MiniPrep protocol (Qiagen). The presence of the insert is checked by PCR. Positive plasmids are transformed into an *E. coli* expression strain. Constructs that are used for NMR studies are sequenced from MidiPrep (Qiagen) plasmid preparations.
Detailed descriptions of all cloning procedures can be found on the homepage of the EMBL Heidelberg protein expression and purification unit and the Sattler group homepage. The primers for PCR reactions are given in section 6.1.5 and expression vectors are listed in section 6.1.4.

6.2.2 Mutagenesis

All mutagenesis was carried out by G. Stier. A list of pleckstrin mutants is provided in appendix B.

6.2.3 Recombinant protein expression in E. coli

Transformed bacterial strains are grown overnight at 37° C to saturating cell density and diluted 1:50 the next morning for expression in 250mL to 1L culture medium. Growth at 37° C is monitored by optical density at 600nm (OD_{600}). When OD_{600} reaches 0.4 to 0.8 expression is induced with 0.2mM isopropylthio-β-D-galactoside (IPTG). After induction the cells are either kept at 37° C for another 4 hours or grown at reduced temperature (18-23° C) overnight. Cells are harvested by centrifugation, flash-frozen in liquid nitrogen and pellets stored at -20° C. Cell culture medium is either LB, ^{15}N enriched minimal medium, $^{15}N/^{13}C$ enriched minimal medium or $^{2}H/^{15}N/^{13}C$ rich medium.

6.2.4 Cell breakage

For cell lysis, a combination of lysozyme treatment and sonication is used. Frozen pellets are thawed and resuspended in an appropriate volume (10mL/g pellet) of lysis buffer (lysis buffer A+ for His_6-tagged proteins and lysis buffer G for GST fusion proteins) supplemented with protease inhibitor (Pefabloc SC, Biomol, Hamburg, Germany). The suspension is kept on ice throughout the following procedure. 1 mg/g pellet lysozyme is added to the resuspended cells and incubated for 5 minutes. A few crystals of DNAseI are added to reduce the viscosity of the sample before sonication (2 minutes 50% maximum

power, 50% duty cycle, Branson (Danbury, CT) sonifier 250). After sonication, the homogenous suspension is not viscous anymore and slightly lighter in colour. It is transferred to 2mL Eppendorf tubes and centrifuged for 20 minutes at 20,000g. The supernatant is passed through a 0.22μm filter and loaded onto an appropriate column.

6.2.5 Purification of His_6-tagged proteins

Based on pellet weight and expression levels (SDS-PAGE of raw extract) an appropriate volume of Ni-NTA (Qiagen) agarose is transferred to an Econo chromatography column (BioRad) and equilibrated with lysis buffer A+. Typically between 0.5-1.0mL of column volume is used per 1L culture volume. Batch purification is carried out in the following steps:

(i) Filtered supernatant of the raw extract is passed over the column 3 times.

(ii) The column is washed with 10 column volumes of lysis buffer A+ and 2 times 10 column volumes lysis buffer A (no detergent).

(iii) The column is washed with 5 column volumes of washing buffer A1 (1M NaCl, "salt wash") and re-equilibrated with 5 column volumes of buffer A.

(iv) The column is washed with 5 column volumes of washing buffer A2 ("imidazole wash").

(v) His_6-tagged recombinant protein is eluted in 5 column volumes of elution buffer A.

The 10mM imidazole in all wash buffers is intended to compete with proteins that have a weak intrinsic affinity for the Ni^{2+} resin. In addition, steps (iii) and (iv) remove further impurities. The resin volume is chosen to be limiting, so that it becomes saturated by the recombinant His-tagged protein. The purification is carried out in batch mode, which allows fast parallel processing of several samples. The eluate is usually highly pure (>95%). Protein concentration is determined by Pierce's Coomassie Plus Protein Assay and yields are typically between 5 and 20mg per litre culture volume.

6.2.6 Purification of His_6-Z-tagged proteins

Ni-NTA purification of His_6-Z-tev fusion proteins is the same as of other His_6-tagged proteins. Thereafter, the fusion proteins are digested and the cleaved (untagged) protein of interest is isolated:

(i) The protein is buffer exchanged (PD-10) back into Lysis buffer A.

(ii) TEV digest with 1:100 (w/w) recombinant His_6-tagged TEV protease (produced in-house), 2h at room temperature or overnight at 4°C. The efficiency of TEV cleavage is assessed by SDS-PAGE.

(iii) The 2nd Ni-NTA column is equilibrated with lysis buffer A. The resin of the 1st Ni-NTA column (His_6-fusion protein purification) can be recycled but some fresh resin should be added.

(iv) The TEV digested proteins are applied to the column. The flow-through is collected and passed over the column two more times.

(v) One additional column volume of lysis buffer A is added recover remaining untagged protein from the dead volume of the column.

(vi, optional) The cleaved His_6-Z-tag and uncut fusion protein are eluted from the column in elution buffer A.

The untagged cleaved protein of interest is highly pure in the flow-through. The His_6-tagged carrier protein (Z-tag) and uncleaved fusion protein are retained on the column and only eluted in step (vi).

6.2.7 Purification GST fusion proteins

Based on pellet weight and expression levels (SDS-PAGE of raw extract) an appropriate volume of glutathione sepharose 4B (Amersham Pharmacia Biotech) is transferred to an Econo chromatography column (BioRad) and equilibrated with lysis buffer G. Typically between 0.5-1.0mL of column volume is used per 1L culture volume. Batch purification is carried out in the following steps:

(i) The filtered supernatant of the raw extract is passed over the column 3 times by gravity flow.

(ii) The column is washed with 2 times 10 column volumes of lysis buffer G.

(iii) The GST fusion protein is eluted in 5 column volumes elution buffer G.

GST fusion proteins is moderately pure in the eluate and can be used directly in protein lipid overlay assays. Protein concentration is estimated with Coomassie Protein Plus Reagent (Pierce).

6.2.8 Gel Filtration

Gel filtration is the final purification step in NMR sample preparation. A Superdex-75 16/60 column is equilibrated with phosphate or MOPS NMR buffer. The protein solution is applied to the column through a 1ml loop. Flow-rate is 1mL/min. All protein samples elute in the volume expected for monomers.

6.2.9 NMR sample preparation

Gel filtration peak fractions are pooled and concentrated in Microsep 10K Omega (Pall) or Centriprep YM-10 (Amicon) concentrators. 10% (w/w) D_2O is added to the samples before filling them into NMR sample tubes (normal: 550µl, Shigemi: 250µl). Samples are lyophylised and resuspended in high-purity D_2O (99.99%) where required. Protein solutions in NMR buffers are frozen and stored at -20° C for long periods. Protein concentrations of NMR samples are between 0.1 and 0.5mM, as determined by measuring absorption at 280nm (A_{280}) and applying the Beer-Lambert-law:

$$\mathbf{A_{280}=\varepsilon_{280}.c.l},$$

where $\boldsymbol{\varepsilon_{280}}$ is the extinction coefficient at 280nm (calculated by the ProtPar tool of Swissprot [www.expasy.org/sprot/]), **c** is the protein concentration and **l** is the length of the path.

6.2.10 DTNB reaction

The reaction with Ellman's reagent (5,5'-dithiobis(2-nitrobenzoate, DTNB) is used to determine the number of surface-accessible cystein residues. A 10 fold molar excess of DTNB is added to a 10µM protein sample in buffer D. Formation of the thiolate product is measured at 412nm. The number of accessible cystein residues can then be estimated from a standard curve.

6.2.11 Spin-labelling

Preparation of spin-labelled samples of His_6-tagged single-cystein proteins is achieved by reaction with 3-(2-iodoacetamido)-proxyl (Sigma-Aldrich). The steps of the protocol are:
(i) Purification: standard His_6-tagged protein purification, with the exception of the last three steps (wash, imidazole wash, elution) where buffers without reducing agent (i.e. no β-mercaptoethanol) are used – the spin-label is destroyed by any reducing agent.
(ii) Reaction: addition of a two- to fivefold molar excess of spin-label reagent resuspended in methanol. The reaction is allowed to proceed for 1h at room temperature and is very efficient (~100% by mass spectrometry).
(iii) Removal of reagent: size exclusion chromatography, most easily achieved by buffer exchange over PD-10 columns (Amersham Pharmacia Biotech) directly into NMR phosphate or MOPS buffer without reducing agent (DTT). For efficient removal of the reagent, smaller loading and elution volumes are used: loading = 2ml protein solution + 0.5ml buffer, elution= 3.2ml.
NMR sample preparation is the same as for unmodified samples. To obtain spectra of chemically modified but not paramagnetic samples, spin-labelled samples are reduced by the addition of 10mM ascorbic acid from a 0.5M stock solution.

6.2.12 Protein lipid overlay assays

Protein lipid overlay acids are carried out following the protocol of Dowler et al. with slight modifications. Phosphoinositides are purchased from Echelon (Salt Lake City, UT). The lyophilised lipids are resuspended in 1:1 solution of methanol and chloroform and stored as 1mM stock solutions at -80 °C. 0.2mM solutions are prepared by 5-fold dilution in a 10:5:4 mixture of methanol, chloroform and water and stored at -20 °C. The lipid solutions are kept at -20 °C as much as possible during spotting (cryo-box). 1µl drops are spotted onto HyBond C-extra nitrocellulose membranes (Amersham Pharmacia) while applying a vacuum to minimize spreading of spots and to accelerate drying. Also, serial 1:2 dilutions of the 0.2mM solutions are prepared and spotted in an array. For comparison, PIP strips™ (Echelon) are purchased. Spotted membranes can be stored for several weeks at 4 °C.

The buffer used of the assay is PBS-T unless otherwise stated. The typical working volume of the assay is 5mL. The following steps are carried out:

(i) Membranes are incubated in blocking solution for 1 hour at room temperature.

(ii) 0.1-5μg/mL GST-fusion protein is added to the blocking solution and left for 2 hours at room temperature or overnight at 4 °C.

(iii) The membranes are washed 10 times for 5 minutes with PBS-T.

(iv) α-GST-horseradish peroxidase (HRP)-conjugated antibody (Amersham Pharmacia, 1:5000 dilution) in blocking solution is added and incubated for 1 hour at room temperature.

(v) The membranes are washed 10 times for 5 minutes with PBS-T.

(vi) Application of ECL reagent (Amersham Pharmacia) and detection of the HRP-conjugated antibody.

GST fusions of the PH domains of PLC−δ and TAPP1 are used as positive controls. GST alone is used as negative control.

6.3 NMR spectroscopy

Most NMR spectra are acquired on in-house Bruker DRX500 and DRX600 spectrometers, both equipped with a cryoprobe. Temperature of all experiments is 295K (22°C) unless otherwise indicated. All NMR spectra are processed with the nmrPipe software package (Delaglio et al., 1995) and analysed with NMRView (Johnson and Blevins, 1994).

6.3.1 Protein backbone assignment

Protein backbone assignment is achieved by analysing a standard set of triple-resonance experiments. The experiments are slightly modified when applied to perdeuterated proteins: a TROSY transfer scheme is implemented and ^{2}H is decoupled during carbon frequency labelling periods. Residual ^{1}H is not purged due to its low abundance in the sample. Also, a HNCA with constant time carbon evolution (HNCA-ct) is recorded for perdeuterated samples and the CA-CB transfer delay in the HN(CA)CB and

HN(COCA)CB experiments is set such that all magnetisation is transferred to CB. Backbone assignment is checked and confirmed by a ^{15}N-HSQC-NOESY.

The experiments used for backbone assignment in this thesis are listed in the table below. Details of the backbone assignment of the DEP_C-PH double-domain construct are given in chapter 3, Table 3.1.

Protein	*Experiment*	*Comment*
C-PH$_{234-350}$	HNCA	600MHz
	HNCACB	600MHz
	CBCA(CO)NH	500MHz
	HNCO	500MHz
	^{15}N-HSQC-NOESY	800MHz, 90ms t_{mix}
C-PH$_{234-350}$: Ins(1,3,4)P$_3$ complex	HNCA	600MHz
	HNCACB	600MHz
	^{15}N-HSQC-NOESY	800MHz, 120ms t_{mix}
DEP_C-PH$_{122-350}$	HNCA	600MHz
	HNCA-ct	600MHz
	HN(CA)CB	600MHz
	HN(CO)CA	600MHz
	HN(COCA)CB	600MHz
	^{15}N-HSQC-NOESY	600MHz, 200ms t_{mix}

6.3.2 Protein sidechain assignment

Aliphatic sidechain assignment is achieved with two types of TOCSY experiments: H^N-N detected (H(CCO)NH, (H)C(CO)NH) and H-C detected (HCCH). The former type is too insensitive to obtain complete assignment of long aliphatic sidechains. The latter is recorded in two versions: sensitivity enhanced H(C)CH and (H)CCH with double sensitivity enhancement. Both HCCH TOCSYs are recorded in H_2O buffer. Aromatic sidechain assignment is based on a 2D NOESY in D_2O buffer at 900 MHz. In addition a 3D-^{13}C-HMQC-NOESY where the carrier is centred on the aromatic region for carbon is recorded. Missing aliphatic resonances are also assigned by analysis of NOESY spectra. The experiments used for the assignment of C-PH sidechain resonances are summarised below:

Experiment	*Resonance type*	*Comment*
H(CCO)NH	aliphatic ^{1}H	500MHz
(H)C(CO)NH	aliphatic ^{13}C	500MHz
H(C)CH	aliphatic ^{1}H and ^{13}C	500MHz, sensitivity enhanced
(H)CCH	aliphatic ^{1}H and ^{13}C	500MHz, double sensitivity enhanced
2D NOESY	aromatic ^{1}H	900MHz, D_2O, 80ms t_{mix}
3D-^{13}C-HMQC-NOESY	aromatic ^{1}H and ^{13}C	Carrier centred on aromatic ^{13}C, 600MHz, 90ms t_{mix}

6.3.3 Derivation of structural restraints

NOE restrains are obtained from 2D and 3D spectra recorded at high field. H^N-N RDCs are measured in the ^{1}H dimension of a modified TROSY pulse sequence with α/β spin state selection. Alignment medium is 5% (w/v) hexaethylene glycol monododecyl ether ($C_{12}E_6$)/n-hexanol (molar ratio 0.64) (Ruckert and Otting, 2000). Dihedral angle restraints are obtained from $^{3}J(H_NH_\alpha)$-couplings (Kuboniwa et al., 1994) and from chemical shift using the programme TALOS (Cornilescu et al., 1999). Slowly H_2O/D_2O exchanging amide protons are included as hydrogen bond restraints. Statistics on structural restraints is given in detail in Table 2.1 (chapter 2). Experiments and restraints for the structure calculation of C-PH are summarised briefly below:

Restraint type	*Experiment*	*Number of restraints*	*Comment*
NOE	3D-^{13}C-HMQC-NOESY[1]	2560 (1182/403/261/714)[2]	800MHz, 60ms tmix
	3D-^{15}N-HSQC-NOESY	1164 (408/411/124/221)[2]	800MHz, 90ms tmix
	2D-NOESY[2]	654 (182/95/63/314)[2]	900MHz, 80ms tmix
	total unambiguous	3176 (1310/577/347/942)[2]	
	total ambiguous	115 (29/26/19/41)[2]	
RDC	2D-TROSY (mod.)	33 backbone, 2 indole[4]	H^N-N
Dihedral angle	3D-HNHA (quantitative J)	23 Φ, 23 Ψ	$^{3}J(H_NH_\alpha)$-couplings
	backbone assignment	65 Φ, 65 Ψ	TALOS (chemical shift)
Hydrogen bonds	H_2O/D_2O exchange	29	

[1] Carrier centred on aliphatic carbons

[2] NOE restraints are listed as: total (intraresidue/sequential/ medium/long range).

[3] Only aromatic part of the spectrum is analysed

[4] indole $H^{\varepsilon 1}/N^{\varepsilon 1}$ of tryptophane sidechains.

6.3.4 Structure calculation

The programme Aria1.2 (Linge et al., 2001) is used for structure calculation of C-PH. Standard simulated annealing (SA) molecular dynamics is carried out with 80 structures calculated in the final iteration whereof 10 are subjected to water refinement. The following parameters are changed with respect to the standard protocol:

- qmove=false, qshifts=false
- cool1_steps, cool2_steps, refinesteps are doubled
- RDC restraints are introduced in iteration 3 with a final force constant of 0.3 (1.0) for backbone (indole) H^N-N RDCs.

In the later stages of the structure calculation, hydrogen bonds are not included anymore. The statistics of the structure calculation of C-PH are summarised in Table 2.1 (chapter 2).

6.3.5 Structure validation

Structures are examined in MOLMOL (Koradi et al., 1996) and their quality checked with PROCHECK-NMR (Laskowski et al., 1996). Figures of the structures are prepared with MOLMOL, and structural statistics are computed with MOLMOL and PROCHECK-NMR.

6.3.6 Relaxation

Standard ^{15}N relaxation experiments (R1, R2 and {^{1}H}-^{15}N heteronuclear NOE) are measured. Experiments with C-PH (^{15}N-labelled sample) are recorded at 500MHz. Relaxation delays are varied between 20 and 1600 ms for R1 and between 14.4 and 144 ms for R2.

Experiments with DEP_C-PH are recorded at 800MHz. A triple-labelled sample is used and the pulse sequence is adjusted to decouple carbon in the ^{15}N chemical shift evolution period. Relaxation delays are varied between 20 and 2600 ms for R1 and between 14.4 and 100.8 ms for R2.

All experiments are recorded in an interleaved manner (Kay et al., 1989). R1 and R2 relaxation rates are fitted in the rate analysis module of NMRView. Errors are calculated as described. Figures are produced with the programme Xmgrace.

6.3.7 Chemical shift perturbation

Ligand (Ins(1,3,4)P_3, Ins(1,3,4,5)P_4) is added from a concentrated stock solution (5mM) to a 0.1-0.2 mM ^{15}N-labelled protein sample. ^{1}H,^{15}N-HSQC spectra are recorded. Chemical shift change of backbone amide groups ($\Delta\delta_{HN-N}$) is computed with

$$\Delta\delta_{HN-N}=\sqrt{(\delta_H^2+(\delta_N/10)^2)},$$

where δ_H and δ_N are the changes in ^{1}H and ^{15}N chemical shift, respectively.

If changes in the spectra are observed and the signals are in fast exchange, the dissociation constant (K_D) is calculated by fitting the NMR titration data to the formula:

$$\Delta\delta_{HN-N} = \frac{\delta_b-\delta_f}{2[P_0]}\left([P_0]+[L_0]+K_D-\sqrt{([P_0]+[L_0]+K_D)^2-4[P_0][L_0]}\right),$$

where y is $\Delta\delta$, δ_b-δ_f is the difference in chemical shift between bound and free state (=$\Delta\delta_{max}$), P_0 is the total protein concentration and L_0 is the total ligand concentration. A simple two-state binding model is assumed. Protein concentration is treated as a constant (measured before starting the titration). Effects of increasing volume by ligand addition are neglected because the volumes added are very small relative to the sample volume.

6.3.8 Paramagnetic Relaxation Enhancement

^{1}H,^{15}N-HSQC spectra of spin-labelled proteins (PRE spectrum) and of reduced spin-labelled proteins (reference spectrum) are recorded with sufficient signal to noise. The peak intensities are extracted from each peak using the "jitter" mode in NMRView. The intensity ratio between PRE and reference spectrum of each peak is computed and the data is normalised so that the trimmed mean of the intensity ratios is unity. Peaks are classified as unaffected, mildly affected, strongly affected and bleached, depending on

their peak intensity ratio. Usually, the classes are separated by one standard deviation. The intensity ratio is plotted against the sequence and colour-coded onto the structure.

6.4 References

Cornilescu, G., Delaglio, F., and Bax, A. (1999). Protein backbone angle restraints from searching a database for chemical shift and sequence homology. J Biomol NMR *13*, 289-302.

Delaglio, F., Grzesiek, S., Vuister, G. W., Zhu, G., Pfeifer, J., and Bax, A. (1995). NMRPipe: a multidimensional spectral processing system based on UNIX pipes. J Biomol NMR *6*, 277-293.

Johnson, B. A., and Blevins, R. A. (1994). NMRView: a computer program for the visualization and analysis of NMR data. J Biomol NMR *4*, 603-614.

Kay, L. E., Torchia, D. A., and Bax, A. (1989). Backbone dynamics of proteins as studied by 15N inverse detected heteronuclear NMR spectroscopy: application to staphylococcal nuclease. Biochemistry *28*, 8972-8979.

Koradi, R., Billeter, M., and Wuthrich, K. (1996). MOLMOL: a program for display and analysis of macromolecular structures. J Mol Graph *14*, 51-55, 29-32.

Kuboniwa, H., Grzesiek, S., Delaglio, F., and Bax, A. (1994). Measurement of HN-H alpha J couplings in calcium-free calmodulin using new 2D and 3D water-flip-back methods. J Biomol NMR *4*, 871-878.

Laskowski, R. A., Rullmannn, J. A., MacArthur, M. W., Kaptein, R., and Thornton, J. M. (1996). AQUA and PROCHECK-NMR: programs for checking the quality of protein structures solved by NMR. J Biomol NMR *8*, 477-486.

Linge, J. P., O'Donoghue, S. I., and Nilges, M. (2001). Automated assignment of ambiguous nuclear overhauser effects with ARIA. Methods Enzymol *339*, 71-90.

Ruckert, M., and Otting, G. (2000). Alignment of Biological Macromolecules in Novel Nonionic Crystalline Media for NMR Experiments. JACS *122*, 7793-7797.