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Chapter 2

A focal adhesion kinase derived peptide binds the Src SH3 domain in two orientations, as demonstrated using paramagnetic NMR
Abstract

SH3 binding peptides are classified according to their binding orientations, as N-C or C-N. Here we tested the hypothesis that a peptide binds in both orientations but with different populations. A focal adhesion kinase (FAK) derived peptide was tested for its binding orientation on Src SH3 domain. The SH3 domain was mutated at several positions and paramagnetic tags were attached through disulphide bonds. From the paramagnetic relaxation enhancements (PREs) of the amide protons of the SH3 domain distances were derived, to determine the position of paramagnetic centre. Two peptides were synthesized with $^{13}$C enriched Ala or Pro, either at the N-terminal or C-terminal side of the peptide. The distances derived from PREs of the labelled peptide residues were used to determine the peptide orientation on the SH3 domain. The experimental distances were compared with weighed averages of the distances for combinations of the N-C and C-N orientations. We found compelling evidence that the FAK derived peptide bind the SH3 domain in two orientations. The major species observed has the peptide binding to the SH3 domain in the N-C orientation, but about 20% of peptide binds in the C-N orientation.
**Introduction**

Src belongs to the non-receptor tyrosine kinase family. The Src family of kinases is involved in many cellular processes, including cell motility, apoptosis, cell adhesion, cell cycle control, cell differentiation, gene expression and development.\(^{102-105}\) Loss of Src activity leads to uncontrolled cell proliferation, which causes inhibition of apoptosis that leads to tumour growth\(^{106}\), neurodegenerative diseases\(^{107}\), epilepsy\(^{108}\) and metastatic diseases.\(^{109}\) Under normal conditions growth factors or integrins bind to the cell surface receptors and activate them, and the activated receptors engage in elaborate protein-protein interactions in which Src is involved.\(^{103,110,111}\) Src is a modular protein in which several domains have a function in recognition of binding partners. The cellular form of Src (c-Src) was the first identified oncogene that is regulated by tyrosine phosphorylation.\(^{112,113}\)

*Figure 2:1.* Schematic representation of C-Src showing C-terminal regulatory domain, catalytic kinase domain, interacting domains (SH2 and SH3) and N-terminal USrc region composed of SH4 domain and unique region. The figure is adopted from.\(^{114}\)

Src is about 60 kDa in mass and it contains an N-terminal SH4 domain, a unique domain, an SH3 domain, an SH2 domain, a kinase domain and a C-terminal regulatory region (Fig 2.1). Recently, the N-terminal part of Src that includes the SH4 and unique domains were given the name USrc region\(^{115}\). The USrc region is a highly variable, disordered and intrinsically unfolded region in the Src family kinases (SFKs). This USrc region is functionally very important due to its composition of residues that undergo phosphorylation upon binding to their partners. The USrc region is involved in signal transduction and enzyme localization.\(^{116,117}\) It is also predicted to influence the selectivity during the Src interaction with its target proteins.\(^{118}\) In crystal structures of Src the USrc region is not observed due to the fact that it is highly disordered and susceptible to
protease activity.\textsuperscript{119-122} However, NMR characterization has shed some light on this region of Src.\textsuperscript{118} The U\textsubscript{src} is about 84 residues in length. The SH4 domain is myristoylated (attachment of myristic acid) and helps in anchoring Src to membranes, including the plasma membrane and intracellular membranes such as the endoplasmic reticulum.\textsuperscript{123} Approximately 7 residues of the SH4 domain are sufficient for association of Src to the plasma membrane.\textsuperscript{124,125} In some Src family kinases like Src and Blk, in addition to the myristoylation, palmitoylation is also seen.\textsuperscript{126-128} The lipid modification is critical in the activation for effective signal transmission.\textsuperscript{129,130}

The SH3 and SH2 domains of Src are involved in the protein recognition and localization.\textsuperscript{131,132} Mutational studies of these domains showed loss of cellular localization.\textsuperscript{133,134} The three-dimensional structure of the Src SH3 domain comprises five antiparallel \( \beta \)-strands. It also contains a short helix near the C-terminal end and three loops, namely the RT loop (so-called because it generally contains arginine and threonine residues), the n\textsubscript{Src} loop (neuronal isoform) and the distal loop. The Src SH3 domain recognises ‘PXXP’ sequences on partner proteins. The two prolines give the peptide a typical PPII helix structure, which has three residues per turn. The prolines play a major role in the binding, mainly through van der Waals interactions, and the non-proline residues help by correcting the proline position between the conserved aromatic rings found in the SH3 binding site.

The SH2 domain is the largest family of phosphotyrosine recognition domains\textsuperscript{135} and its primary function is to bind the phosphotyrosine (pY) residues on receptors upstream in the signaling pathway. SH2 domains consists of about 100 amino acid residues and the tertiary structure is made up of a central \( \beta \)-sheet, flanked by two \( \alpha \)-helices with two loops connecting them.\textsuperscript{136}

The kinase domain is highly conserved among Src family of kinases. Expression of various deletion constructs showed that the kinase domain itself is sufficient for phosphorylation of substrates. The kinase domain has a typical bilobial structure, with N-terminal and C-terminal lobes. The N-terminal lobe itself consists of a smaller and a
larger lobe. The smaller lobe is made up of antiparallel β-sheets and the larger lobe is α-helical in nature. The ATP binding site is in between these two lobes.\textsuperscript{120,121,137,138}

Several crystal structures have revealed that Src has an inactive and an active conformation. When the kinase Csk phosphorylates Src residue Y529 in the C-terminal region, the SH2 domain binds intramolecularly to this pY residue, resulting in a compact, inactive conformation of Src.\textsuperscript{120,139-145} This conformation is further stabilized by binding of the SH3 domain to the polyproline type II (PPII) helix formed by the linker connecting the SH2 and the kinase domain (Fig 2.2). The SH3 and SH2 domains together down-regulate the kinase activity by pushing the catalytic site of the kinase domain backward in a conformation that is inaccessible for ATP. It shows no binding of phosphotyrosyl peptides, which indicates that the SH2 domain is not accessible. Studies also showed that anti-phosphotyrosine antibodies do not bind to Src in its inactive confirmation.\textsuperscript{146}
Figure 2.2. Schematic representation of inactivated and activated Src. The intramolecular interaction of Src, in which the SH2 domain binds to the C-terminal pY529 with simultaneous binding of the SH3 domain to the PPII motif in the linker region between kinase domain and SH2 domain, results in down-regulation of the kinase activity. The figure is adopted from Yeatman et al.\textsuperscript{147}

Unlike other families of peptide binding proteins, the SH3 domains are quite diverse in their modes of peptide binding. Two classes of peptides have been identified, based on the binding orientation of the peptide to SH3 domains.\textsuperscript{148,149} Peptides that bind to the SH3 domains of PI3K, Abl and Fyn tyrosine kinases bind adopt a so-called N-terminal-C-terminal orientation (N-C), known as class 1 peptides. In other cases, peptides are in an opposite orientation (C-N),\textsuperscript{131,132,149} known as class 2 peptides. The solution structures of c-Src SH3 domain complex with class 1 and class 2 peptides showed that both of these peptides bind to the protein, but in different orientations.\textsuperscript{149} The binding orientation
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depends on an arginine residue that is present either N- or C-terminal of the PXXP sequence, yielding the N-C or the C-N orientation, respectively.

Previous work in our group,\textsuperscript{150} showed that a focal adhesion kinase (FAK) derived peptide was able to bind to the Src SH3 domain. In this study, three peptides were synthesized separately with a paramagnetic TOAC spin label placed at different positions, at C-terminus, N-terminus and in the PXXP sequence. The interactions between the unlabelled peptides and \textsuperscript{15}N labelled Src SH3 domain were studied to determine the dynamics of the protein-peptide interaction. Although the complex between the Src SH3 domain and FAK peptide was weak and transient, it was observed that the peptide binds in a relatively well-defined position on the protein. However, the data also showed that a peptide containing a TOAC spin label at the C-terminus showed paramagnetic effects on residue Asp 93 of the Src SH3 domain, even though this residue was at a considerable distance from the spin label. This observation could be explained if two orientations of peptide binding are possible, with the major orientation being N-C and a small fraction in the alternative C-N orientation. That would suggest that the distinction in two classes of SH3 domains based on peptide orientation is somewhat artifactual and merely a consequence of the static nature of crystal structures.

To test this hypothesis and determine the binding orientation(s) of a class 1 peptide to the Src SH3 domain we applied paramagnetic NMR spectroscopy, which is a powerful tool to detect the presence of minor states in protein-peptide, protein-protein and protein-DNA complexes. The concept of the experiment is shown in Fig 2.3. A paramagnetic label will affect only part of the peptide if bound in a single orientation. If it binds in the opposite orientation a fraction of the time, also the other end of the peptide should experience paramagnetic effects.

Thus, unlike the method mentioned above, in which the protein is enriched in \textsuperscript{15}N and the peptide is paramagnetically tagged, here the peptide is isotope labelled and the protein tagged with a paramagnetic centre.
Figure 2.3. Experimental approach. The SH3 domain is tagged with a paramagnetic centre and the peptide is isotope-labelled and observed in NMR experiments. If the peptide spends a small fraction of the time in the C-N orientation, paramagnetic effects should be detected at both ends of the peptide.

Materials and methods

Cloning
The gene fragment coding for the residues 85-142 of the mouse Src SH3 domain was amplified by PCR using plasmid pUSE-Src-wt (kindly provided by Prof. Dr. B. van de Water) and cloned into the pET28a vector using the NcoI and XhoI restriction sites. This plasmid is called pET28aSH3.

Site-directed mutagenesis
One double and several single cysteine mutations were introduced in the SH3 domain by the whole-plasmid synthesis method (QuikChange, Stratagene La Jolla, CA).
Table 2.1. The primers used to create mutations on SH3 domain. The amino acid code for cysteine is shown in bold.

Expression
The wt or mutant pET28aSH3 plasmid was transformed into E. coli BL21 cells. Fresh transformants were used to inoculate 3 mL LB medium as preculture. After overnight incubation, the preculture was used to inoculate a 1 L culture (2x 500 mL in 2L Erlenmeyers). The ^14N SH3 domain was produced in LB medium, whereas the ^15N-labelled protein was produced in M9 minimal medium with ^15NH4Cl as the sole nitrogen source. The cultures were incubated at 37°C and shaking at 200 rpm. Gene expression was induced by addition of isopropyl β-D-1-thiogalactopyranoside (0.5 mM) when the culture was at OD₆₀₀ 0.6. The cultures were harvested 4 h after induction by centrifugation at 3439 g and the pellets were resuspended in lysis buffer containing 20 mM Tris-HCl, pH 8, 0.5 M NaCl, 10 mM imidazole and 1 mM phenylmethanesulfonyl fluoride. The resuspended pellets were flash frozen using liquid N₂ and stored at -80°C.

Protein purification
Immediately before lysis of the cells, 25 µg/mL DNase was added to the resuspended culture. The cells were lysed by passing the suspension through a French press twice at a pressure of 1000 psi. The lysate was cleared by ultracentrifugation at 40,000 rpm for 40 min. The supernatant was further cleared by using 0.4 µm filter. The cleared supernatant was loaded on a Nickel affinity column (HisTrap HP, GE Healthcare), which had been
pre-equilibrated with a loading buffer containing 20 mM Tris-HCl, pH 8, 0.5 M NaCl, and 10 mM imidazole. The protein was eluted by applying an imidazole gradient from 10 mM – 500 mM. The eluted protein was pooled, concentrated to 5 mL and loaded on a gel filtration column, pre-equilibrated with a buffer containing 20 mM KPi, pH 6.5, 100 mM NaCl (NMR buffer). The purity was checked by using 15% SDS-PAGE to be above 95%. The concentration of the protein was measured at 280 nm, using the theoretical extinction coefficient of 16960 M$^{-1}$cm$^{-1}$.

**Peptide synthesis**

Peptides were kindly provided by Dr. Jan Wouter Drijfhout (Leiden University Medical Centre). Peptides were prepared by normal Fmoc-chemistry using preloaded Tentagel resins, PyBop/NMM for in situ activation and 20% piperidine in NMP for Fmoc removal. Couplings were performed for 75 min. After final Fmoc removal peptides were cleaved with TFA/H2O 19/1 containing additional scavengers when a cysteine or a tryptophan was present in the peptide sequence and isolated by ether/pentane precipitation. The peptides were treated 3 h with 10% ammonia, lyophilized and stored at −20°C until use. Peptides were checked on purity using rpHPLC and on integrity using MALDI-TOF mass spectrometry. Peptide stock solutions were prepared by weighing out the peptide and dissolving it in NMR buffer and adjusting the pH by the addition of small aliquots of NaOH or HCl. Isotope labelled peptides were purchased from sigma chemicals.

**Paramagnetic probe attachment on mutant SH3 domain**

A double cysteine SH3 mutant was used for attachment of a Caged Lanthanide NMR Probe (CLaNP-5), whereas the single cysteine mutants were used to attach MTSL ([(1-Oxyl-2,2,5,5-tetramethylpyrrolidin-3-yl)methyl]-Methanethiosulfonate) spin labels. First, the SH3 mutant protein was incubated with 5 mM DTT for 60 min at room temperature. The DTT was then removed by loading and eluting the protein over a PD10 column (GE Healthcare). Immediately, 10 molar equivalents of CLaNP-5 or spin label were added to the eluted protein and the solution was incubated for 2 h at room temperature. The protein was then loaded on a Superdex G75 gel filtration column to
remove the dimers and oligomers as well as excess probe or spin label. The protein was eluted using NMR buffer. Probe attachment was confirmed by mass spectrometry and the percentage of MTSL attachment was determined by electron paramagnetic resonance (EPR). The double integral of EPR signals measured for all mutants of SH3-MTSL vs. a standard sample of free MTSL indicated that > 95% of the protein was labelled with MTSL. The protein was concentrated and used for further NMR measurements. For the double cysteine mutant, CLaNP-5-Lu was used as the diamagnetic control and CLaNP-5-Gd to observe paramagnetic effects. For the single cysteine mutants MTS ([(1-Acetyl-2,2,5,5-tetramethyl-3-pyrroline-3-methyl) methanethiosulfonate]) was used as the diamagnetic control and MTSL as the paramagnetic spin label.

**NMR experiments**

The interaction of the Src SH3 domain with the peptide was determined using $[^{1}\text{H},^{15}\text{N}]$ and $[^{1}\text{H},^{13}\text{C}]$ constant time HSQC (CT-HSQC) NMR experiments. The CT-$^{1}\text{H}$, $^{13}\text{C}$-HSQC experiments were used in order to improve the resolution and ensure reliable calculations of PREs. The constant time was set to a value of 26 ms in all CT-HSQC experiments. NMR experiments were recorded at 298 K, on a Bruker DMX or Avance III 600 MHz NMR spectrometer, equipped with TCI-Z-GRAD cryoprobe. The $[^{1}\text{H},^{15}\text{N}]$ HSQC spectra were taken with 128 complex points in the indirect dimension and an interscan delay of 1 s. The $[^{1}\text{H},^{13}\text{C}]$ HSQC spectra were acquired with 1024 complex points in the indirect dimension. The length of the constant time delay was 26 ms. The data were processed using nmrPipe software and analysed using CCPN Analysis 2.1.5.

The transverse relaxation rate of resonances in the diamagnetic sample ($R_{\text{dia}}^{H}$) was determined from the peaks after processing with a 2 Hz line-broadening exponential window function. The linewidth at half maximum, $\Delta\nu_{1/2}$, was calculated from a Lorentzian line shape fit using the software FuDA. After correction for the artificial line-broadening, the $R_{\text{dia}}^{H}$ was obtained from equation 2.1.

$$R_{\text{dia}}^{H} = \pi \Delta\nu_{1/2}$$  \hspace{1cm} (2.1)
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The rate of transverse relaxation of protons due to the paramagnetic enhancement effect ($R_{para}^H$) was calculated using equation 1.4\textsuperscript{156} for protein amide protons and from equation 2.2 for peptide protons. This equation is an extension of the equivalent formula given by Battiste and Wagner (equation 1.4\textsuperscript{156}) for PREs determined from $^1$H-$^{13}$C HSQC crosspeaks. In $^1$H-$^{15}$N spectra, the paramagnetic relaxation of $^{15}$N is neglected, because $^{15}$N is 100-fold less sensitive for PRE than protons. For $^{13}$C nuclei this factor is only 16 and, furthermore, in the CT-experiments the $^{13}$C spends a relatively long time in the transverse plane and, thus, the $^{13}$C relaxation contributes significantly to the intensity reduction of the HSQC-peak.

$$\frac{I_{para}}{I_{dia}} = \frac{R_{dia}^H e^{-\left(R_{para}^H \Delta^H + R_{para}^C \Delta^C\right)}}{R_{dia}^H + R_{para}^H} \quad (2.2)$$

where $I_{para}$ and $I_{dia}$ are the paramagnetic and diamagnetic peak intensities, respectively, and $\Delta^H$ and $\Delta^C$ are the times that the $^1$H and $^{13}$C magnetisation coherences are in the transverse plane during the CT-HSQC experiment (14.4 ms and 26 ms, respectively). Distances were derived from $R_{para}^H$ using equation (1.5) The rotational correlation time of the protein-peptide complex was estimated to be 5 ns, using the software hydroNMR\textsuperscript{157} and a structure of chicken Src SH3 bound to a similar peptide (PDB code 1RLQ\textsuperscript{158}).

**Docking calculations**

The PDB file 1RLQ,\textsuperscript{158} containing a structure of chicken Src SH3 bound to the peptide RALPPLPRY was modified by mutating the peptide to the FAK sequence RALPSIPKLA. The sequence of mouse Src SH3 and chicken src SH3 was similar except only one residue Thr 127 in mouse which is Ser in case chicken. However this residue is modified to threonine in the docking calculations. The chicken and mouse To determine the positions of the paramagnetic centres, a pseudoatom was docked on the SH3 protein on the basis of the distances derived from protein PREs using the programme Xplor-
NIH. Based on the ratios of $I_{\text{para}}$ and $I_{\text{dia}}$, the residues were divided into three classes. The residues in class 1 were those which disappeared in the paramagnetic spectrum. The ratio for these residues was set on the basis of the noise level of the paramagnetic spectrum. The residues in class 2 had ratios of $I_{\text{para}}$ and $I_{\text{dia}}$ between 0 to 0.9. The residues in class 3 were those that had ratios of $I_{\text{para}}$ and $I_{\text{dia}} > 0.9$.

**Fit quality**

The agreement between observed and back-calculated NMR observables ($O$) was expressed in a quality factor, $Q$, given in equation 2.3.

$$Q = \frac{\sum_i \left( O_{\text{calc}}(i) - O_{\text{obs}}(i) \right)^2}{\sum_i \left( O_{\text{calc}}(i) + O_{\text{obs}}(i) \right)^2}$$  \hspace{1cm} (2.3)

**Chemical shift perturbation analysis**

CSPs, indicative of peptide binding to the SH3 domain, were analysed using the standard 1:1 binding model (equation 1.3).

**Results**

To study the interaction of the mouse Src SH3 domain with the binding motif RALPSIPKLA of FAK, paramagnetic centres were attached to the SH3 domain. The sites of the introduction of single Cys residues and a cysteine pair, for attachment of MTS detection. The results are shown in Figure 2.4.
Figure 2.4 Mutation sites. A) Amino acid sequence of the mouse Src SH3 domain. The residues coloured red were mutated individually to Cys, except for the T116C/E117C which was a double Cys mutation. The numbering at the bottom is based on Uniprot entry Q80X42 and used throughout. The counting at the top is from PDB entry 1RLQ, the structure used in this study. B) Three dimensional structure of the SH3 domain (green) with a class 1 peptide (magenta) (PDB entry 1RLQ). The cysteines substitutions for the attachment of paramagnetic probes are shown in stick representation in CPK colours.
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The effects of probe attachment on the SH3-peptide interaction.

To verify the influence of paramagnetic probes on the SH3 domain on binding of the FAK peptide, the dissociation constants ($K_d$) for each mutant were determined by NMR SH3 amide chemical shift titrations for the complexes of the peptide and SH3 linked to the diamagnetic control probes Lu-CLaNP 5 or MTS (Fig. 2.4). With the $K_d$ the CSP were extrapolated to 100% bound SH3 and averaged (following the method explained in chapter 1, see Fig. 2.5) and the region of peptide binding was mapped on the SH3 structure for comparison to the wt SH3 binding map (Fig. 2.7). The $K_d$ value and the binding map of the mutant L91C for the peptide were similar to the wt SH3. For mutants T116C/E117C, T98C, and T100C, the $K_d$s were 5, 6, and 3 fold larger, respectively. However, the binding maps for these mutants were almost same as for the wt SH3 (Fig. 2.7). The extrapolated values for the double mutant attached to CLaNP are smaller than for wt, but the binding pattern is similar. Overall, it is concluded that probe attachment has only small effects on peptide binding and does not influence the mode of binding.
Figure 2.5. $^{15}$N chemical shift perturbations of SH3 domain upon FAK peptide binding for selected residues. The fitting of the curves was performed using 1:1 global fit (equation 1.2). The chemical shift perturbations of unassigned peaks are indicted by UA.
Figure 2.6. Average chemical shift perturbations (extrapolated to 100% bound) of wt SH3 and variants linked to diamagnetic control labels upon peptide binding. Missing residues in the variants were either unassigned or broadened upon binding of the peptide.
Figure 2.7. Chemical shift perturbations ($\Delta \delta_{\text{avg}}$) of the SH3 domain upon binding of FAK peptide. The shift changes are extrapolated to 100% bound SH3. The colour pattern represents the size of average chemical shift perturbations. Red: $\Delta \delta_{\text{avg}} \geq 0.25$ ppm; orange: $0.25 > \Delta \delta_{\text{avg}} \geq 0.15$; yellow: $0.15 > \Delta \delta_{\text{avg}} \geq 0.0$; blue: $\Delta \delta_{\text{avg}} < 0.05$. Unassigned residues and residues broadened upon peptide binding are coloured grey.
Localisation of the paramagnetic centres on the SH3 domain.

The positions of the paramagnetic centres relative to the protein were determined. For this purpose, the protein was $^{15}$N labelled and the unlabelled peptide was added in 5-fold access. $[^{15}\text{N}, {^1\text{H}}]$ HSQC spectra were recorded for each SH3 variant of the diamagnetic control samples (Lu-CLaNP-5-SH3-peptide and MTS-SH3-peptide complexes) as well as the paramagnetic samples (Gd-CLaNP-5-SH3-peptide and MTSL-SH3-peptide complexes). The ratios between the intensity of the paramagnetic peak, $I_{\text{para}}$ and that of diamagnetic peak, $I_{\text{dia}}$ of the amide resonances (Fig. 2.8) were used to calculate the distances between each amide proton and the corresponding paramagnetic centre, using equation 1.4. With these distances the paramagnetic centre was docked on the protein (see Methods section for details). The location of the centres is shown in Fig. 2.9, along with the plots of the observed vs. back-calculated distances.

Note that class 1 and 3 restraints only have an upper and lower distance bound, respectively, so they satisfy the data if the calculated distance is lower (higher) than the experimental one. The distances from the metal or oxygen atom to the $\text{C}\alpha$ and $\text{S}\gamma$ atoms of the cysteines are reported in Table 2.1.

The locations of the Gd in SH3 T116C/E117C linked to CLaNP-5 fits well to the observed data. For the MTSL oxygen of SH3 L91C the fit is somewhat poorer, perhaps because fewer class 2 restraints were available. The mutants T98C and T100C are in a flexible loop near the mutation site. The determination of the MTSL positions for these mutants was achieved using a different method, discussed in chapter 3. The location of the paramagnetic centres from the protein surface is reasonable in most cases.
Figure 2.8. The ratios of $I_{\text{para}}$ and $I_{\text{dia}}$ of the backbone amide protons of the mutant SH3 domain. Residues for which the intensity ratio was lower than 0.9 (dotted line) were considered as affected by the spin label or CLaNP-5.
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**Figure 2.9.** Positioning of the paramagnetic centres. Left, the calculated positions of the Gd ion (T116C/E117C) and oxygen atom of MTSL are shown as a blue spheres on the grey surface of the SH3 domain. The peptide is shown in stick model. The Cys positions are shown in cyan. Right, the observed vs. calculated distances from the Gd or oxygen atom of MTSL to backbone amide protons in SH3. The black line represents the perfect correlation and the blue and red lines mark 3 Å error margins. Class 1 and 3 restraints and Q values are indicated (see Methods section for details).
Table 2.2. Distances from experimentally determined paramagnetic centre positions to the Cα and Sγ of respective cysteines.

<table>
<thead>
<tr>
<th>Mutant</th>
<th>Distance centre-Cα (Å)</th>
<th>Distance centre-Sγ (Å)</th>
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<tr>
<td>T116C/E117C</td>
<td>8.6</td>
<td>7.7</td>
</tr>
<tr>
<td>T116C/E117C</td>
<td>8.6</td>
<td>6.0</td>
</tr>
<tr>
<td>T98C</td>
<td>9.8</td>
<td>7.2</td>
</tr>
<tr>
<td>T100C</td>
<td>8.7</td>
<td>6.5</td>
</tr>
<tr>
<td>L91C</td>
<td>7.5</td>
<td>4.9</td>
</tr>
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</table>

**PREs of $^{13}$C-labelled peptide with the complex of SH3 domain**

Two versions of the FAK peptide were synthesized, with a $^{13}$C labelled Ala and Pro residue close to the N-terminus, RALPSIPKLA (Peptide 1) and the C-terminus, RALPSIPKLA (Peptide 2). $^{13}$C-HSQC spectra were recorded for free peptide and in the presence of SH3 domain (SH3 domain:peptide was 1:5), Figure 2.10 and 2.11. Nine major peaks are expected, two from Ala (Hα and Hβ) and seven from Pro (Hα, Hβ1+2, Hγ1+2 and Hδ1+2), as well as some weak natural abundance signals. However, it turned out that the peptide occurs in three forms. In the free form, a major and a minor form are detected, as can be seen for example for the Pro Hδ protons in Fig. 2.9B. Upon binding to SH3, the minor form is not affected. The major form splits into two peaks, one that shifts proportionally with the fraction peptide bound, and another that remains at the position of free peptide. We interpret this observation as follows. Three forms are present and these forms exchange slowly on the NMR timescale. Only one of these is capable of binding to the SH3 domain (which is in the fast exchange regime). From the peak intensities, it was determined that the fraction of peptide capable of binding was about 50%. It is possible that cis-trans isomerisation of the Pro residues is the cause of the presence of multiple resonances. Such isomerisation is known to be slow and could change the conformation of the peptide, thus making it incapable of binding to the SH3 domain.
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Figure 2.10. $^{13}$C, $^1$H] CT-HSQC spectrum of FAK peptide (RALPSIPKLA, $^{13}$C labelled residues in bold). Green contours, free peptide. Blue and red contours, peptide in presence of SH3 domain linked to MTS and MTSL, respectively. A) Full spectrum; B & C) Details.
Figure 2.11. $^{13}$C, $^1$H] CT-HSQC spectrum of FAK peptide (RALPSIPKLA, $^{13}$C labelled residues in bold). Green contours, free peptide. Blue and red contours, peptide in presence of SH3 domain linked to MTS and MTSL, respectively. A) Full spectrum; B & C) Details.
**Figure 2.12.** Peptide PREs. The ratios of $I_{\text{para}}$ and $I_{\text{dia}}$ of the $^{13}$C side chain protons of the peptide 1 (RALPSIPKLA, left) and peptide 2 (RALPSIPKLA, right) are plotted. Nuclei with intensity ratios $< 0.9$ were considered to experience a significant PRE. The numbers in the x-axis correspond to the $^{13}$C side chain protons presented in table 2.2.
<table>
<thead>
<tr>
<th>X-Axis</th>
<th>Peptide1</th>
<th>Peptide2</th>
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<tbody>
<tr>
<td>1</td>
<td>2Ala Hα</td>
<td>10Ala Hα</td>
</tr>
<tr>
<td>2</td>
<td>2Ala Hβ</td>
<td>10Ala Hβ</td>
</tr>
<tr>
<td>3</td>
<td>4Pro Hα</td>
<td>7Pro Hα</td>
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<td>4Pro Hβ1</td>
<td>7Pro Hβ1</td>
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<td>5</td>
<td>4Pro Hβ2</td>
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<td>4Pro Hγ*</td>
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<tr>
<td>8</td>
<td>4Pro Hδ2</td>
<td>7Pro Hδ2</td>
</tr>
</tbody>
</table>

**Table 2.3.** The details of the $^{13}$C side chain protons of the labelled peptide residues which are presented in the x-axis of fig. 2.12 graphs. The Hγ* represents the gamma proton either 1 or 2 (only one peak is appeared in the $^{13}$C HSQC spectra).

As a consequence of the partial overlap between the peaks of the different forms, the PREs of only a subset of signals could be determined. The PREs were calculated from the $I_{\text{para}}/I_{\text{dia}}$ ratios (Fig. 2.12) and converted into distances.

**Fraction of the peptide in the C-N orientation**

From the experimentally calculated positions of the Gd ion in CLaNP-5 and the oxygen atoms of MTSL at various sites on the SH3 domain, the distances to peptide nuclei were calculated for N-C as well as the C-N orientation. For the N-C peptide, PDB entry 1RLQ$^{158}$ was used, with the peptide sequence adjusted to that of the FAK peptide. Since only Pro and Ala residues were used to obtain distances, the nature of the remaining side chains is expected to have only a small influence. For the C-N orientation the peptide was rotated from the N-C to the C-N terminal orientation, ensuring that both prolines still lie in the binding pocket of the SH3 domain. This rotated peptide thus mimics class 2 peptides, which bind in the C-N orientation. The experimental distances were compared with weighed averages of the distances for combinations of the N-C and C-N
orientations. For the range of fractions of the C-N orientation from 0 to 1, the fit to the experimental distances was calculated and expressed as a quality factor $Q$ (Eq. 2.3). The results for the different spin label mutants are shown in Fig. 2.13. The data for the mutant T116CC/E117C clearly show that the fit to the data improves by admixture of the C-N orientation. This is caused by the PREs observed for the C-terminally labelled peptide. The lowest value of $Q$ was found for a combination of 80% N-C orientation and 20% C-N orientation. These results are similar for mutants T98C and T100C. For mutant L91C small but significant PREs were observed for peptide 2, but insignificant effects for peptide 1. The small effects cause considerable uncertainty in the distances and render the $Q$-dependence on the fraction of C-N rather flat, but the data do not disagree with a contribution to the PREs from the C-N orientation of about 20%.
Figure 2.13. Determination of the C-N fraction. The graphs on the left represent the correlation between experimental distances derived from PREs and calculated distances from the structure models of the peptide bound to the SH3 domain in the C-N and N-C orientations. The solid black line represents the perfect correlation and the blue and red lines a 3 Å error margin. The graphs on the right show the quality of the fit to the experimental distances expressed as the Q value (equation 2.3) as a function of the fraction of the C-N orientation.
Discussion

The peptide derived from the FAK binding motif of the Src SH3 domain belongs to the class 1 sequence (N-C orientation). PREs derived from the paramagnetic centres on the mutants T116C/E117C, T98C and T100C suggest that the peptide binds for 80% of the time in the N-C and 20% in C-N orientation. For these mutants the fit between the experimental and average back-calculated distances was better for the combination of the two orientations than for N-C orientation alone. For all three variants the best fit was for 20-30% C-N contribution (Fig. 2.13). The PREs of peptide nuclei from the MTSL linked to SH3 L91C were small and did not disagree with a significant fraction of C-N orientation. Although the majority of the data suggest that the N-C conformation is not populated 100% of the time, and the C-N conformation contributes significantly, some caution in the data interpretation is necessary. The model for the C-N orientation is crude, because it is not known how the FAK peptide binds exactly to the Src SH3 domain in that orientation. It is likely, though, that the nuclei that were labelled, in particular the Pro residues, are modelled in approximately the correct position, because the location of the Pro residues in the C-N orientation mirrors that in the N-C orientation.

The PRE data on the protein enable reliable positioning of the paramagnetic centres. The observed positions for T116C/E117C, T98C and T100C deviated from the expected ones (see also chapter 3). Apparently, probe attachment to the small protein domain SH3 can easily affect its structure, and thus its interaction with the peptide. The binding maps are similar but the $K_d$ values for the peptide for these mutants was somewhat larger than for wt SH3. Thus, it cannot be excluded that the introduction of the paramagnetic centre influences the fraction of the peptide that binds in the C-N orientation.

If the peptide can indeed bind to the SH3 domains in two orientations, this could imply that the classification into N-C and C-N orientations is a gradual rather than absolute one and it would be important to perform similar experiments on other peptide-SH3 complexes. Crystal structures are expected to consist of one of the two forms, but it would be worth re-evaluating NMR spectra to see whether minor NOEs between the SH3 domain and the peptide in the minor orientation can be detected.