Transferred Cross-Correlated Relaxation Complements Transferred NOE: Structure of an IL-4R-Derived Peptide Bound to STAT-6

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Abstract: A new NMR method is proposed which enables the measurement of projection angles in the bound conformation of a weakly binding ligand complexed to its receptor. The method is based on the cross-correlated relaxation mechanism. In analogy to the transferred NOE experiment (trNOE), cross-correlated relaxation can be transferred and measured at the resonances of the free ligand (trCCR), provided the k_{off} rate is within the time scale of the experiment. The concept is validated by the structure determination of an interleukin-4 (IL-4) receptor-derived partially ^{13}C- and ^{15}N-labeled phosphotyrosine peptide ligated to STAT-6. Distances have been obtained by trNOE experiments, and the torsion angle Pro(ψ) has been determined using trCCR, measuring either cross-correlated $H^N-N/H^\alpha-C^\alpha$ dipole−dipole relaxation or cross-correlated $H^\alpha-C^\alpha$ dipole/CO chemical shift anisotropy relaxation. The resulting structure has been described.

Introduction

Weakly binding ligands are often subjected to transferred NOE (trNOE) studies[1] aimed at determining their structure when bound to the receptor. This NMR method is quite popular, but the interpretation of the experimental data is sometimes difficult: the observed NOE involving two protons of the ligand may be formed indirectly via spin diffusion mediated by protons of the receptor and, thus, might easily lead to misleading conclusions if no special care is taken.[2] At any rate, the observed NOEs provide only distance information, which might not be sufficient for the determination of an accurate and unambiguous structure. *J*-couplings cannot be used since they are equally averaged over the free and bound states of the ligand, with the free state being in large excess. Recently, a new class of NMR experiments was designed to measure angles between bond vectors.[3] It makes use of the cross-correlated relaxation (CCR) mechanism which depends on the projection angle between dipoles. Alternatively, the CCR involving the dipole and chemical shift anisotropy (CSA) tensors[4] depends also on the projection angle between these tensors. Both types of cross-correlated relaxation rates can be measured independently and provide complementary information[4c] (Figure 2).

It will be shown in this article that both types of relaxation mechanisms can be exploited for the study of weakly binding ligands. It will enable the measurement of torsion angles of the ligand in the bound state by transferred cross-correlated relaxation (trCCR).

STAT-6 is a multidomain transcription factor of 96 kDa comprising a DNA-binding domain, SH3 and SH2 domains, and a transcription activation domain at its C terminus.[5] As a member of the signal transduction and activator of transcription family of proteins (STATs 1−6), STAT-6 is activated in response to the cytokines interleukin-4 (IL-4) and IL-13 binding to their cognate receptor on the cell surface: Cytokine binding to the receptor induces receptor oligomerization, thus allowing Jak kinases to phosphorylate two specific tyrosine residues on the IL-4 receptor α chain which serves as a specific binding site for STAT-6. Upon phosphorylation by a second Jak kinase, STAT-6 dimerizes and translocates to the nucleus, whereupon it activates the transcription of a number of genes.

The STAT-6 SH2 domain interacts with the phosphorylated tyrosine residues on the IL-4 receptor α chain. The recent work of Mikita and colleagues[6] has demonstrated that the STAT-6 SH2 domain is most similar to those of the Src family. Although the structures of many SH2 domains have been determined, STAT protein SH2 domains have proven difficult to study because of the difficulty in producing the isolated domain in a soluble active form. Only recently has the crystal structure of the first STAT proteins (STAT-3 and STAT-1 in a dimeric form) been elucidated,[7] and as yet, no structural information regarding STAT-6 is available.

Materials and Methods

STAT-6 Expression and Purification. STAT-6 cDNA with a 6-fold histidine tag was cloned as a 2572 base pair (bp) EcoRI/KpnI fragment

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into the pFastBac transfer vector (Gibo-BRL) and recombinant baculovirus used to infect *Trichoplusia ni* Hi5 insect cells. After a crude lysate preparation, latent STAT-6 was purified as described before.[8]

Peptides. Peptides were synthesized commercially and purchased from either Neosystem (France) or Cambridge Research Biochemicals (UK). Peptide pY[606] (Ac-A-S-S-G–E-E-G-pY-K-P-F-Q-D-L-I-NH_2) and peptide pY[578] (Ac-G-P-P-G-E-A-G-pY-K-A-F-S-S-L-L-NH_2) were derived from the IL-4 receptor α chain.[9] The tetrapeptide Ac-pY-K-P-F-NH_2 is a truncated form of peptide pY[606]. pY indicates a phosphorylated tyrosine residue.

STAT-6 Peptide Binding Assay. Biotinylated peptide pY[578] (150 μL at 0.3 μg/mL) was added to streptavidin-coated 96-well microtiter plates and left to incubate overnight. Plates were washed three times with PBS followed by blocking buffer consisting of 0.1% casein in 0.01 M Tris·Cl pH 8.0, 0.15 M NaCl, 0.2% Tween-20). STAT-6 binding to the plate was detected using europium-labeled anti-STAT-6 and fluorescence measured on a Fluostar plate reader.

NMR Spectroscopy. All experiments have been carried out on a Varian Unityplus 600 spectometer equipped with a ^{1}H–^{13}C–^{15}N triple resonance probe with *z*-gradients. The samples were measured in susceptibility-matched NMR tubes (Shigemi) with a sample volume of 220 μL. Data were processed using the VNMR program. The transferred NOE experiments were run using a spin-lock filter before t_1 of 20 ms to suppress background signals from the protein.[10] Spectra of the free peptide were acquired on an 8 mM solution of peptide in 10 mM phosphate buffer, pH 7.2, 20 mM NaCl at 20 °C. Spectra of the complexed peptide were acquired on a mixture of 0.5 mM tetrapeptide and 40 μM STAT-6 in the same buffer.

The cross-correlated dipole–dipole relaxation experiment was carried out in the implementation of Yang and Kay.[4c] This version of the experiment yields better sensitivity and resolution as compared to the original scheme.[3a] Because of the spectral simplicity (only one amide proton is ^{15}N-labeled in the peptide), the experiment was carried out as a 2D ^{13}C–^{1}H version. The water flip-back pulse was optimized by maximizing the water signal after an additional ^{1}H read pulse. The constant time delay in which cross-correlated relaxation is operative was 16 ms for the free peptide and 12 ms for the complex. The ^{13}C spectral width was set to 1000 Hz. Sixteen complex points were acquired in the ^{13}C dimension for the free peptide and 12 complex points for the complex. The number of transients was 128 for the free peptide and 2304 for the complex. The repetition rate was 1.1 s, resulting in measuring times of 3 h and 36 h, respectively.

The cross-correlated dipole–CSA relaxation experiment[4a] was carried out in the scheme of Yang et al.[4b] The constant time delay in which cross-correlated relaxation is operative was 32 ms for the free peptide and 24 ms for the complex. As above, 2D ^{13}C–^{1}H versions of the experiment were recorded. The ^{13}C spectral width was set to 1000 Hz. Thirty-one and 23 complex ^{13}C points were acquired for the free peptide and the complex, respectively. The numbers of transients were 32 and 1024, respectively. The repetition rate was 1.1 s, resulting in measuring times of 40 min and 16 h, respectively.

The reduction in intensity of the individual signals (cf. Figure 4) are caused by different sources of relaxation including, e.g., relaxation of zero or double quantum coherences. It has been described in detail how to extract the CCR rates Γ_{DD}[4c] and Γ_{DC}[4b]. Shortly, when the six signals in Figure 4 are labeled a–f (from the left to the right)

$$\Gamma_{DD} = \frac{1}{4t}\ln\left(\frac{I_a}{I_b}\frac{I_d}{I_c}\right) \tag{1a}$$

$$\Gamma_{DC} = \frac{1}{2t}\ln\left(\frac{I_e}{I_f}\right) \tag{1b}$$

where t is the total of the constant time delays the CCR is operative and I_i represents the intensities of the subsequent peaks.

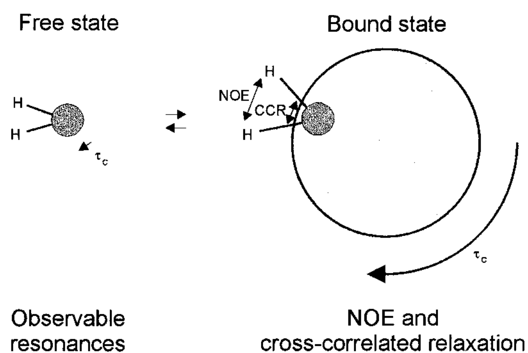

Figure 1. Principle of the experiment. The NOE and cross-correlated relaxation (CCR) are only operative when the ligand is bound, since the ligand experiences a long correlation time (τ_c) only in the bound state. The relaxation is transferred and measured on the resonances of the ligand in the free state. The relaxation thus contains exclusively information on the geometry (projection angles and distances) of the ligand in the bound state.

Structure Calculations. Homology models of the SH2 domain of STAT-6 were generated using InsightII, version 97.2, distributed by MSI. The conformation of the peptide was based on distance geometry calculations using DGII and restrained molecular dynamics calculations using Discover. Forty-five trNOE cross-peaks were classified as strong, medium, and weak and translated to upper bound distances of 2.7, 3.3, and 5 Å, respectively. Distance bounds were smoothed using triangle smoothing. Twenty-five structures were embedded and optimized using a four-dimensional Cartesian space. Subsequently, 10 000 steps of simulated annealing were applied. The final structures were minimized using 2000 steps of conjugate gradient minimization using the cvff force field. Electrostatic interactions were scaled down by 1/4*r*. The models were further analyzed by docking studies on a homology model as well as simulations of transferred NOEs using the program CORCEMA,[11] version 1.5.

Results and Discussion

Theory of Transferred Cross-Correlated Relaxation. The so-called cross-correlated dipole–dipole relaxation, which is operative during certain delays in a heteronuclear NMR experiment, depends on the projection angles between bond vectors as well as on the correlation time (τ_c). Therefore, the cross-correlated relaxation (CCR) is dominant in the bound state and can be transferred to and detected at the resonances of the free ligand in analogy to the trNOE experiment (Figure 1). Also, the cross-correlated relaxation involving the dipole and CSA tensors can be exploited in this respect. It is dominant in the bound state, and will be transferred to the signals of the free ligand, provided that the k_{off} rate is within the time scale of the experiment.

In case of an equilibrium between the bound and free state, which is characterized by a fast k_{off} rate, the two independently measured CCR rates, Γ_{DD} and Γ_{DC}, involving the dipole–dipole and the dipole–CSA interaction, respectively, are proportional to the weighted sum of the contributions from the bound and the free state

$$\Gamma_{DD} = (1-\alpha)\Gamma_{DD}^{free} + \alpha\,\Gamma_{DD}^{bound} \tag{2a}$$

$$\Gamma_{DC} = (1-\alpha)\Gamma_{DC}^{free} + \alpha\,\Gamma_{DC}^{bound} \tag{2b}$$

where α is the fraction of bound ligand. Both relaxation rates[3,4] are dependent on gyromagnetic ratios, γ_i, of the involved nuclei, bond distances, r_{ij}, and other parameters which are considered

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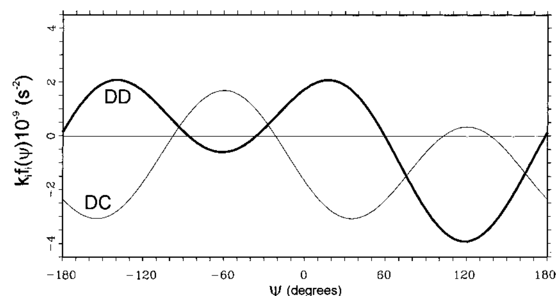

Figure 2. Theoretical curves of $k_i f_i(\psi)$ as a function of the peptide backbone torsion angle ψ (eqs 3−6).

to be constant. When it is assumed that the bound conformation is rigid, the only variables are τ_c and the angle θ between the two interacting tensors

$$\Gamma_{DD} = k_{DD}\tau_c{}^1/_2\,(3\cos^2(\theta^{DD}) - 1) \quad (3a)$$

$$\Gamma_{DC} = k_{DH}\tau_c{}^1/_2\,(3\cos^2(\theta^{DC}) - 1) \quad (3b)$$

Expressions for k_i are[3,4]

$$k_{DD} = {}^2/_5\,\frac{\gamma_H\gamma_N}{\vec{r}^{\,3}_{HN}}\,\frac{\gamma_H\gamma_C}{\vec{r}^{\,3}_{HC}}\hbar^2 \quad (4a)$$

$$k_{DC} = \frac{4}{15}\omega_C\frac{\gamma_H\gamma_C}{\vec{r}^{\,3}_{HC}}\hbar\vec{\sigma} \quad (4b)$$

where ω_c is the ^{13}C angular frequency and σ is the CSA tensor of the carbonyl for which a value determined by solid-state NMR[4d] has been used.

When we consider the triple resonance experiments to be used to determine the backbone torsion angle ψ (N_i−$C\alpha_i$−CO_i−N_{i+1}), the relationships can be simplified using goniometric functions $f_i(\psi)$ (Figure 2) because the torsion angle ψ and θ are related by

$$\cos\theta^{DD} = 0.1628 + 0.8188\cos(\psi - 120°) \quad (5a)$$

$$\cos\theta^{DC}_x = -0.3095 + 0.3531\cos(\psi - 120°)$$

$$\cos\theta^{DC}_y = -0.1250 - 0.8740\cos(\psi - 120°) \quad (5b)$$

$$\cos\theta^{DC}_z = -0.9426\cos(\psi - 120°)$$

The relaxation rates can thus be written as

$$\Gamma_{DD} = k_{DD}(1-\alpha)\tau_c^{free} f_{DD}(\psi^{free}) + k_{DD}\alpha\tau_c^{bound} f_{DD}(\psi^{bound}) \quad (6a)$$

$$\Gamma_{DC} = k_{DC}(1-\alpha)\tau_c^{free} f_{DC}(\psi^{free}) + k_{DC}\alpha\tau_c^{bound} f_{DC}(\psi^{bound}) \quad (6b)$$

Thus, when the two relaxation rates are measured independently and when the relaxation in the bound state is dominant, i.e., $\alpha\tau_c{}^{bound} \gg (1-\alpha)\tau_c{}^{free}$, the torsion angle ψ (as well as $\alpha\tau_c{}^{bound}$) follows from a combination of the two experiments. For ψ:

$$\frac{\Gamma_{DD}}{\Gamma_{DC}} = \frac{k_{DD}}{k_{DC}}\frac{f_{DD}(\psi^{bound})}{f_{DC}(\psi^{bound})} \quad (7)$$

The angle ψ can be extracted by using a graphical approach as explained below, and it should be noted that only one relaxation rate should be used when one of the relaxation rates is close to zero.

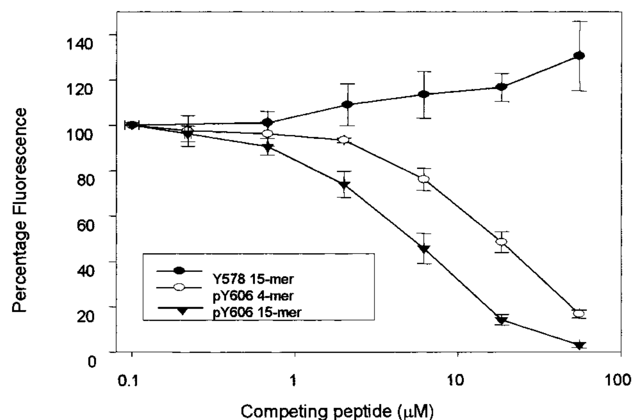

Figure 3. The tetrapeptide Ac-pY-K-F-NH_2 inhibits STAT-6 binding to the 15-mer peptide centered around pY[578] with similar potency to 15-mer peptide centered around pY.[606] Increasing quantities of free peptide were used to compete for binding of STAT-6 to phosphorylated 15-mer-peptide pY.[578] The 15-mer without a phosphorylated tyrosine residue is shown as a negative control.

The herein proposed strategy to determine distances *and* angles in weakly bound ligands is illustrated and validated by the structure determination of a peptide derived from the IL-4 receptor bound to full length STAT-6 protein.[5]

Binding of Peptides to STAT-6. It was demonstrated that upon activation by tyrosine phosphorylation, STAT-6 is able to interact via its SH2 domain with at least two sites on the IL-4 receptor α chain.[9] Each binding site was characterized by a phosphorylated tyrosine residue and a signature motif characterized by conserved residues at the +1 and +3 positions. Two 15-mer peptides, each centered around a single phosphorylated tyrosine, i.e., pY[606] and pY[578], were found to bind STAT-6 in vitro with very similar affinities.[6] In our studies it was found that the 15-mer peptide centered around pY[606] is able to inhibit STAT-6 binding to plate-bound 15-mer peptide centered around pY[578] with an IC_{50} of 5 μM as measured using the assay described in the Materials and Methods section. Experimental data are shown in Figure 3. The tetrapeptide peptide, Ac-pY-K-P-F-NH_2, which is a truncated form of abovementioned pY[606] peptide, was found to bind to the STAT-6 protein relatively similar to that observed for the 15-mer (IC_{50} = 17 μM).

Transferred NOE experiments. The tetrapeptide Ac-pY-K-P-F-NH_2, derived from the IL-4 receptor α chain and found to bind to the STAT-6 protein (see previous section) was chosen for NMR studies, aimed at determining the STAT-6 bound conformation. Whereas no NOE signals were detected for the free peptide in solution, an extensive set of NOE signals was detected when the peptide was measured after addition of STAT-6 protein. The detection of trNOE signals confirms the binding of the peptide. There are a few key NOEs which indicate that the peptide is bound in an extended conformation. The proline Hδ protons show NOEs of roughly equal intensities involving lysine Hα. This implies that the ψ angle is close to 120° and that the peptide bond between Lys and Pro is trans. The NOE intensities involving the HN and Hα protons of the peptide as well as the absence of medium range NOEs indicate that the peptide wraps around the protein with a backbone

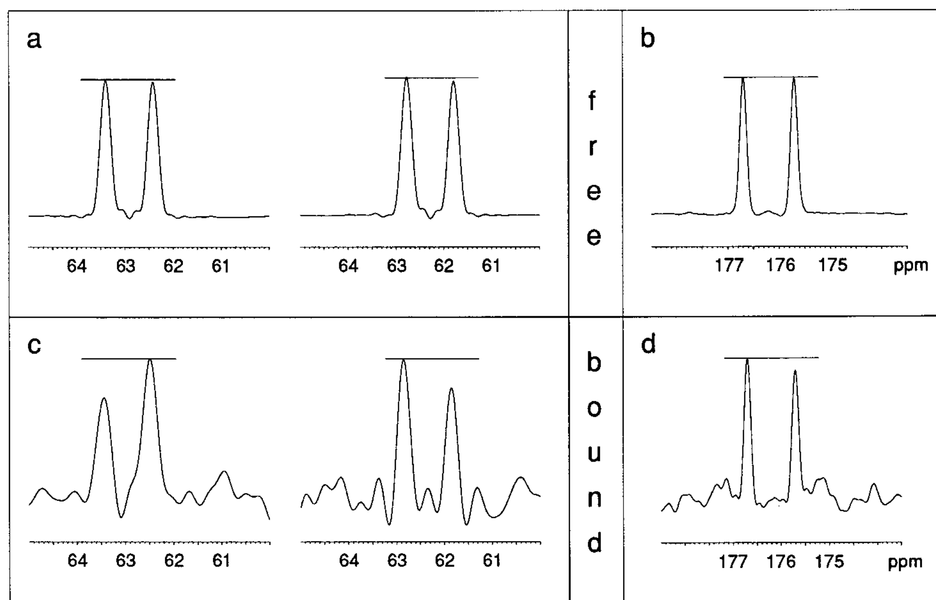

Figure 4. Cross sections through cross-peaks taken from the HN(CO)CA-derived experiments at the Phe ^1H_N frequency. Data are shown for the free tetrapeptide (a, b) and for a mixture of 0.5 mM tetrapeptide and 40 μM STAT-6 (c, d). The doublet signals of the dipole−dipole CCR experiment are shown on the left (a, c). The results of the dipole−CSA experiment are shown on the right (b, d). The horizontal line indicates the intensity of the highest peak. In absence of CCR, each pair of peaks would have equal intensity.

conformation which is close to an extended chain. The absence of medium range distance information makes it especially difficult to obtain a structure of the ligand with reasonable resolution.

In an attempt to obtain more accurate distance information from the trNOE experiment, we measured the NOEs as a function of the mixing time and simulated NOEs by taking into account the relaxation and exchange matrixes using the program CORCEMA[11] and various models of the bound conformation. Best agreement was observed when $k_{off} = 10$ s^{-1} and $\tau_c = 78$ ns was used. The correlation time corresponds to a dimer of STAT-6. Although the resemblance between experimental and simulated NOEs is reasonable, for some NOEs only a qualitative agreement was found, which suggests that the theoretical model is too simple and that protons of STAT-6 could mediate the relaxation. Thus, it was clear that it would be valuable to obtain torsion angle information.

Transferred Cross-Correlated Relaxation Experiments. The amino acids Phe and Pro were isotopically labeled with ^{13}C and ^{15}N in order to measure the trCCR rates, which would yield the backbone torsion angle Pro(ψ) of the ligand in the bound conformation.

The relaxation rates were determined by recording two-dimensional versions of the triple resonance experiments of the peptide in complex with STAT-6 under similar conditions as the trNOE experiment. For both dipole−dipole and dipole−CSA CCR improved pulse sequences[4b,c] were employed, which show significant improvement in sensitivity and resolution, compared to the original versions.[3a,4a] The cross sections from which the rates could be determined are shown in Figure 4. The trCCR rates for the protein−ligand mixture were $\Gamma_{DD} = -8.7$ s^{-1} and $\Gamma_{DC} = 1.5$ s^{-1} (for details, see the Materials and Methods section). The CCR rates for the unligated peptide were $\Gamma_{DD} = -0.38$ s^{-1} and $\Gamma_{DC} = 0.13$ s^{-1}, confirming that the first term in eq 2 can indeed be neglected. The trCCR rates could then be interpreted as follows: in the theoretical curves calculated for both types of trCCR as a function of the torsion angle ψ (Figure 2), one had to look for a region where Γ_{DD} adopts a negative value and Γ_{DC} is positive. When, in addition, the feature $|\Gamma_{DD}| > |\Gamma_{DC}|$ is used, the torsion angle ψ followed unambiguously from these two trCCR experiments to be in the region around 120°. Calculation according to eq 7 results in a value of 108° or 132°.

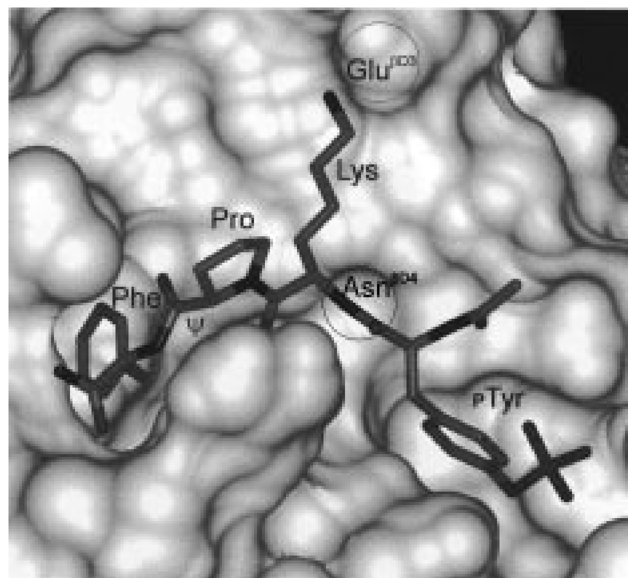

Figure 5. Structure of the IL-4 receptor-derived tetrapeptide Ac-pY-K-F-NH$_2$ ligated to the SH2 domain of STAT-6. The homology model of the SH2 domain is presented by a solid surface. The bound conformation of the peptide (stick model) provides clues about the recognition of the IL-4 receptor and STAT-6 (see text). The torsion angle Pro(ψ) amounts 132°.

Conformation of the Phosphopeptide Bound to the SH2 Domain of STAT-6. Figure 5 shows the resulting structure of the phosphopeptide docked on a homology model generated for the SH2 domain of STAT-6. Homology modeling was done using Src and Grb2 as the template. In analogy with other SH2 domain−phosphopeptide complexes, it was relatively easy to predict the recognition of the phosphotyrosine. pTyr^0 fits nicely in the phosphotyrosine binding pocket, and the phosphorus

moiety is bound via $Lys^{\alpha A2}$, $Arg^{\beta B5}$, $Ser^{\beta B7}$, and $Ser^{\beta C2}$. The earlier proposed residue notation for SH2 domain sequences is used.[12] Then, the amide proton of Lys^{+1} forms a hydrogen bond with the carbonyl of $Asn^{\beta D4}$. Its side chain is directed toward the surface of the protein, and the amino group comes close to $Glu^{\beta D3}$ and $Gln^{\beta D1}$, suggesting the formation of a salt bridge. Interestingly, in other SH2 domains,[13] e.g., Src, the charges of peptide and receptor are reversed: in that case Glu^{+1} interacts with $Lys^{\beta D3}$. Pro^{+2} adjusts the backbone of the peptide, which enables the Phe^{+3} side chain to insert into a deep hydrophobic pocket (Figure 5) flanked by the αB helix, the central β-sheet and several loops.

The model presented here for the interaction of the phosphopeptide and the SH2 domain of STAT-6 has been analyzed using the experimental data obtained by Mikita et al.[6] In this study 17 double mutants have been expressed and analyzed in a peptide binding assay. Nine of the mutants display complete loss of binding of phospopeptides. The loss of binding in the case of six mutants is readily explained by the fact that one of the amino acids involved interacts with the phosphate of $pTyr^{0}$. More interesting is the mutant in which $L^{\alpha B7}$ and $Y^{\alpha B8}$ have been mutated. This mutant LY has retained weak binding properties but has lost specificity of the binding. On the basis of the homology model, this can satisfactorily be explained because $Y^{\alpha B8}$ forms one of the wells of the hydrophobic binding pocket and makes direct contact to Phe^{3+}. The mutants which retain peptide binding have their mutated amino acids relatively far away from the bound peptide.

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Concluding Remarks

It has been shown that transferred cross-correlated relaxation can successfully be used to measure the backbone torsion angle ψ in the bioactive conformation of a weakly binding ligand. These new geometric data are complementing the distance information obtained by the transferred NOE method. It is expected that other projection angles can be obtained as well by using the trCCR mechanism. The value of $\alpha\tau_c$, determined from the present experiments, can be used for the determination of other projection angles, assuming rigid tumbling of the molecule.

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