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# 4-Fluoroproline derivative peptides: effect on PPII conformation and SH3 affinity

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**Abstract:** Eukaryotic signal transduction involves the assembly of transient protein–protein complexes mediated by modular interaction domains. Specific Pro-rich sequences with the consensus core motif PxxP adopt the PPII helix conformation upon binding to SH3 domains. For short Pro-rich peptides, little or no ordered secondary structure is usually observed before binding interactions. The association of a Pro-rich peptide with the SH3 domain involves unfavorable binding entropy due to the loss of rotational freedom on forming the PPII helix. With the aim of stabilizing the PPII helix conformation in the Pro-rich HPK1 decapeptide PPPLPPKPKF (P2), a series of P2 analogues was prepared, in which specific Pro positions were alternatively occupied by 4(*S*)- or 4(*R*)-4-fluoro-L-proline. The interactions of these peptides with the SH3 domain of the HPK1-binding partner HS1 were quantitatively analyzed by the NILIA-CD approach. A CD thermal analysis of the P2 analogues was performed to assess their propensity to adopt the PPII helix conformation. Contrary to our expectations, the $K_d$ values of the analogues were lower than that of the parent peptide P2. These results clearly show that the induction of a stable PPII helix conformation in short Pro-rich peptides is not sufficient to increase their affinity toward the SH3 domain and that the effect of 4-fluoroproline strongly depends on the position of this residue in the sequence and the chirality of the substituent in the pyrrolidine ring. Copyright © 2006 European Peptide Society and John Wiley & Sons, Ltd.

**Keywords:** 4-fluoroproline; HS1; NILIA-CD; protein–peptide interactions; SH3 domain

## INTRODUCTION

SH3 domains are probably the most abundant molecular-recognition modules of the proteome [1]. They are present in a large variety of functionally different proteins that participate in the intracellular signaling networks, cytoskeleton organization and membrane traffic [2]. Their role is to establish protein complexes inside the cell and/or to regulate enzyme activities through protein intramolecular interactions [3,4].

These domains share a completely conserved folding that consists of two antiparallel $\beta$-sheets packed against each other at an approximate right angle. These domains contain three variable loops connecting strands, which are named RT, *n*-Src and distal loops; the first two participate in the creation of the ligand-binding surface [5]. A common feature of SH3 domains is their interaction with ligand proteins containing Pro-rich sequences. Crystal (X-ray diffraction) and solution (NMR) structures of SH3 domain complexes with Pro-rich peptides show that the peptide ligands are bound over a range of up to seven residues in a poly-$[Pro]_n$ type-II (PPII) helix conformation [6]. In addition, a specific non-PPII region can be found at one of the ends [7].

SH3 domains are present in oncoproteins and proteins over-expressed in deregulated signaling pathways during cancer development and are also intimately involved in the pathogenesis of other diseases such as Alzheimer's syndrome and muscular dystrophy [8]. The finding that the inhibitors of the interactions occurring between SH3 domains and their partners are considered promising therapeutic agents [9] validates these domains as attractive targets for drug design.

The binding between SH3 domains and synthetic ligands is relatively weak, with $K_d$ values in the range of 1–100 μM [10]. The $K_d$ values of the full-length proteins that interact with SH3 domains are significantly lower than those found with peptides; e.g. the HIV Nef protein

Abbreviations: DCM, dichloromethane; DIEA, *N*,*N*-diisopropylethylamine; DMF, *N*,*N*-dimethylformamide; Fmoc, 9-fluorenylmethoxycarbonyl; FPro, 4-fluoroproline; GST, glutathione-*S*-transferase; HBTU, *N*-[(1H-benzotriazol-1-yl)(dimethylamino) methylene]-*N*-methylmethanaminium hexafluorophosphate N-oxide; HOBt, 1-hydroxy-1,2,3-benzotriazole; HPK1, hematopoietic progenitor kinase 1; HS1, hematopoietic-lineage cell-specific protein 1; NMP, *N*-methyl pyrrolidone; OSu, 1-oxysuccinimido; PPII, poly-$[Pro]_n$ type-II; TFA, trifluoroacetic acid; TFFH, fluoro-*N*, *N*, *N*′, *N*′-tetramethylformamidinium hexafluorophosphate.

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‡ Deceased, 21 November 2005. This paper is dedicated to the memory of Dr Gianfranco Borin, an outstanding and enthusiastic mentor, a dear friend and colleague.

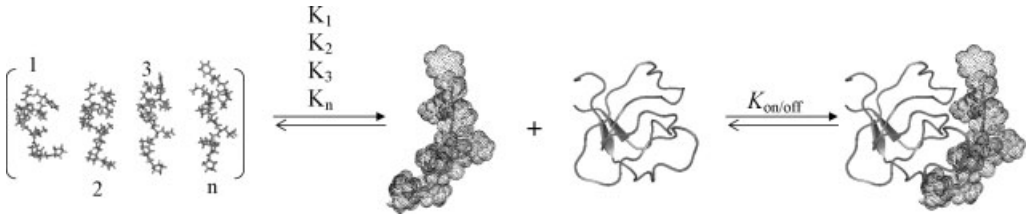


**Figure 1** Binding equilibrium involving a conformational manifold of the HPK1 peptide **P2**. The association process consists of the redistribution of the conformational ensemble from the binding-incompetent (in brackets) to the binding-competent (PPII) species, and then of the interaction of the latter with the SH3 domain.

**Table 1** Peptide sequences of the Pro-rich **P2** peptide and its FPro-derivative analogues

| Peptide | Pro replacement | Sequence |
|---|---|---|
| P2 | — | H-Pro-Pro-Pro-Leu-Pro-Pro-Lys-Pro-Lys-Phe-OH |
| R2F | 4(*R*)-FPro$^{3,6}$ | H-Pro-Pro-**FPro**-Leu-Pro-**FPro**-Lys-Pro-Lys-Phe-OH |
| R3F | 4(*R*)-FPro$^{2,5,8}$ | H-Pro-**FPro**-Pro-Leu-**FPro**-Pro-Lys-**FPro**-Lys-Phe-OH |
| R5F | 4(*R*)-FPro$^{2,3,5,6,8}$ | H-Pro-**FPro**-**FPro**-Leu-**FPro**-**FPro**-Lys-**FPro**-Lys-Phe-OH |
| S2F | 4(*S*)-FPro$^{3,6}$ | H-Pro-Pro-**FPro**-Leu-Pro-**FPro**-Lys-Pro-Lys-Phe-OH |
| S3F | 4(*S*)-FPro$^{2,5,8}$ | H-Pro-**FPro**-Pro-Leu-**FPro**-Pro-Lys-**FPro**-Lys-Phe-OH |
| S5F | 4(*S*)-FPro$^{2,3,5,6,8}$ | H-Pro-**FPro**-**FPro**-Leu-**FPro**-**FPro**-Lys-**FPro**-Lys-Phe-OH |

binds to the Hck SH3 domain with a $K_d$ of ~250 nM [11]. Consequently, the development of high-affinity peptide ligands for these domains has been proved to be a difficult task. Classical approaches such as synthetic library screening and mutational analysis were not very successful [12], although some encouraging results have been reported with libraries of peptoids (peptides based on N-substituted residues), which can bind the Abl SH3 domain with $K_d$ values in the nanomolar range [13]. A rationalization of the design of SH3 domain ligands is much required, especially considering their ubiquitous nature and their key role in cell-cycle regulation and disease development.

The two features that highly influence the affinity of a protein for its ligand are (i) specific ligand/protein interactions and (ii) change in conformational freedom of the two components in the complex. In general, the former feature favors binding, while the latter hampers it. Indeed, there is always a large entropic cost associated with the binding due to the loss of the conformational freedom of the peptide in the complex (Figure 1) [14]. Therefore, an increase of the SH3 affinity for the peptide ligand could be obtained by stabilizing the PPII helix conformation of the peptide.

We propose here the introduction of 4(*S*)- or 4(*R*)-4-fluoroproline (FPro) instead of Pro residues into a suitable peptide sequence to obtain a stable peptide scaffold able to induce and stabilize the PPII conformation, thus reducing the entropic cost of the ligand–protein interaction. The ability of FPro residues to induce a stable PPII helix conformation and a collagen structure into model peptides has been already shown [15–17]. We synthesized a series of analogues of the Pro-rich peptide corresponding to the sequence 394–403 of hematopoietic progenitor kinase 1 (HPK1), named **P2**, replacing Pro residues at different positions with their 4(*R*)- or 4(*S*)-FPro derivatives (Table 1).

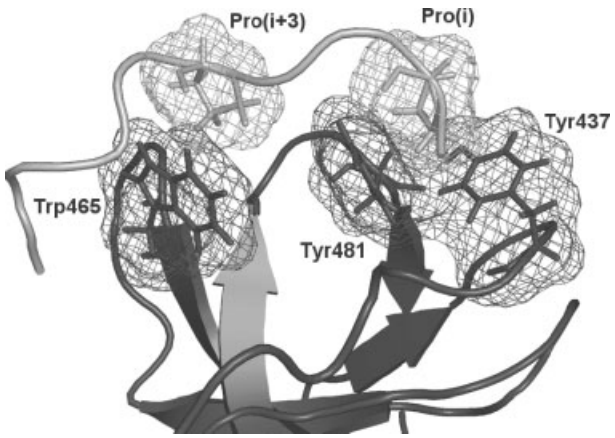


**Figure 2** Ribbon representation of the *in silico* structure of the HS1 SH3 domain bound to the HPK1 peptide **P2**. The peptide/$SH3_{HS1}$ complex was modeled on the structure of the murine Mona/Gads SH3C domain in complex with the peptide encompassing the 465–480 sequence of mouse HPK1 (PDB code 1UTI). An energy minimization of the SH3-peptide complex was performed with the program OPTIMIZE (TINKER Package) and Amber 98 force field until the energy reached a minimum. The figure was drawn using the PYMOL program (K. L. DeLano, www.pymol.org). The residues implicated in the interaction between the Pro-rich peptide **P2** (light gray) and the HS1 SH3 domain (dark gray) are shown.

Here, we use the NILIA-CD approach [18] to investigate the binding between the *C*-terminal SH3 domain of the human hematopoietic-lineage cell-specific protein 1 (HS1) and the peptides derived from the recognition sequence of its binding partner HPK1 protein (**P2** peptide) described above [19].

Common features of SH3 domain binding sites are the Trp and Tyr residues that clamp two Pro residues of the ligand in a zip-type fashion (Figure 2). Upon peptide addition, the binding was clearly monitored by the CD changes of the Trp side-chain chromophore of the SH3 domain. The dissociation constant $K_d$ was determined by analyzing the CD data measured at 294 nm using a nonlinear regression method [18].

A comparative analysis of the binding properties of this set of closely related peptides provides striking results that confirm the complexity of the SH3 recruitment and points to an important role of additional interactions.

## MATERIALS AND METHODS

### General Methods for Peptide Synthesis and Purification

9-Fluorenylmethoxycarbonyl (Fmoc)-protected amino acids and pre-loaded Wang resins were purchased from Calbiochem-Novabiochem (Läufelfingen, Switzerland). Fmoc-4(*R*)-FPro-OH was purchased from Bachem (Bubendorf, Switzerland), while the 4(*S*)-enantiomer was synthesized starting from 4(*R*)-4-hydroxyproline methyl ester (Chem-Impex, Wood Dale, IL) according to the method described by Demange *et al.* [20] and then converted into the Fmoc-derivative by treatment of Fmoc-1-oxysuccinimide (OSu) [21]. *N*-[(1H-benzotriazol-1-yl)(dimethylamino) methylene]-*N*-methylmethanaminium hexafluorophosphate *N*-oxide (HBTU), 1-hydroxy-1,2,3-benzotriazole (HOBt), *N*,*N*-diisopropylethy-lamine (DIEA) and *N*-methyl pyrrolidone (NMP) were obtained from Chem-Impex, whereas fluoro-$N, N, N', N'$ -tetramethylformamidinium hexafluorophosphate (TFFH) was purchased from PerSeptive Biosystems (Foster City, CA).

Peptides were synthesized by the manual solid-phase method using Fmoc chemistry in 0.05 mmolar scales. HBTU/HOBt activation employed a three-fold molar excess (0.2 mmol) of Fmoc-amino acids in *N*,*N*-dimethylformamide (DMF) solution for each coupling cycle, unless otherwise stated. Coupling to the secondary amino group of 4FPro was performed in DMF using TFFH (5 eq) in the presence of the carboxyl component (5 eq) and DIEA (10 eq). Coupling time was 40 min. Deprotection was performed with 20% piperidine. Coupling yields were monitored on aliquots of peptide-resin either by the Kaiser test for the amino groups or by evaluation of Fmoc displacement [22]. Cleavage from the resin and deprotection were performed by treatment with trifluoroacetic acid (TFA)-anisole-triisopropylsilane-$H_2O$ (95 : 2.5 : 2.0 : 0.5 v/v) (45 min).

Peptides were purified by preparative reversed-phase HPLC using a Shimadzu model LC-8 (Shimadzu, Kyoto, Japan) system with a Vydac 218TP1022 column (10 μ, 250 × 22 mm) (The Separation Group, Hesperia, CA). The column was perfused at a flow rate of 12 ml/min with a mobile phase containing solvent A (0.05% TFA in water) and a linear gradient from 18 to 25% of solvent B (0.05% TFA in acetonitrile/water, 9 : 1) for 30 min. The fractions containing the desired product were collected and lyophilized to constant weight.

HPLC analyses were performed on a Shimadzu liquid chromatography model LC-10 instrument fitted with a Jupiter C18 column (10 μ, 250 × 4.6 mm) (Phenomenex, Torrance, CA) using the above solvent system (solvents A and B), flow rate of 1 ml/min, and detection at 216 nm. All peptides showed less than 1% of impurities.

Molecular weights of compounds were determined by ESI-MS on a Mariner mass spectrometer instrument (PerSeptive Biosystem). The mass was assigned using angiotensin II (1046.2 a.m.u.) as the external standard. In addition, to confirm the correct amino acid composition of the synthetic peptides, acid hydrolyses were carried out in azeotropic hydrochloric acid containing 0.25% phenol for 22 h at 110 °C in sealed evacuated vials. The amino acid compositions of the hydrolysates were determined with a Carlo Erba model 3A30 amino acid analyzer.

### Circular Dichroism

CD spectra were recorded using a nitrogen-flushed Jasco spectropolarimeter model J715 (Tokyo, Japan) using a 4 s time constant, 10 nm $min^{-1}$ scan speed, and 2 nm spectral bandwidth. A 0.5 cm path-length cell was used for the near-UV CD region (245–360 nm), a 0.02 cm cell for the far-UV CD region (190–260 nm), and a 0.1 cm cell for the far-UV region of Pro-rich peptides. All spectra were recorded in the 25 mM Tris-HCl, pH 7.0 buffer. Each spectrum was the average of six scans, with the background of the buffer subtracted. Temperature was controlled by a Haake model F3 temperature controller.

The glutathione-S-transferase (GST)-$SH3_{HS1}$ titration was performed at room temperature by the addition of a small amount of peptide stock solution. CD data were analyzed using the Origin program [23] assuming a 1 : 1 stoichiometry for the ligand : SH3 domain complex.

Protein concentration was determined by absorption spectroscopy (abs. 0.1% = 1749 at 280 nm). Peptide concentration was determined by weight, using a microbalance Mettler Toledo (Columbus, OH) model AT21 Comparator (sensitivity ±1 μg).

### Preparation and Expression of the GST-$SH3_{HS1}$ Domain

The SH3 domain used in this work corresponds to residue 428–486 of the HS1 protein. The domain was expressed fused with GST.

The oligonucleotides 5′-CGT GGG ATC CCC GGG ATC TCA TCA GCT-3′ and 5′-GAC CCG GGA ATT CTC CAG AAG CTT GAC-3′ were used to amplify the region encoding the SH3 domain (amino acids 430–486) from pTcrHis HS1 by polymerase chain reaction [24]. The introduced BamHI and EcoRI restriction sites were exploited to directionally clone the insert in the expression vector pGEX-5X-3 (Amersham Pharmacia Biosciences, Piscataway, NJ). GST and GST-SH3 fusion protein were expressed in *Escherichia coli* BL21 and affinity purified on glutathione-agarose (Sigma-Aldrich,

St. Louis, MO) according to the manufacturer's instructions. The GST-SH3 domain concentration was determined either by the method of Bradford [25] or by absorption spectroscopy (abs 0.1% = 1749 at 280 nm).

## RESULTS AND DISCUSSION

### Peptide Design

The binding of Pro-rich peptides to SH3 domains takes place through the formation of a PPII helix, which involves at least half of the peptide length, the stacking of the Pro residues with aromatic rings in the domain and the specific interactions occurring between non-Pro residues and the *n*-Src and RT-loops of the SH3 domains [5,7]. Our goal was to enhance the affinity of the HPK1 binding peptide **P2** for HS1-SH3 domain. To achieve this goal, we increased the propensity of the **P2** peptide to adopt a PPII conformation by replacing some of the Pro residues with FPro derivatives.

Despite minor differences in the van der Waals radius, particularly in the length and dipole moment of the C–F bond, fluorine is generally considered to be isosteric to the hydrogen atom that it replaces [26]. The substitution of Pro by 4(*R*)-FPro in synthetic collagen peptides was found to enhance the stability of the triple helical fold. In contrast, the replacement of the residue at the same position with the diastereoisomeric 4(*S*)-FPro greatly decreases the PPII folding [15]. Remarkably, other authors [27] found that the stereoelectronic preference at the *n*-1 residue position is opposite to that quoted above, as 4(*S*)-, but not 4(*R*)-FPro, endows hyperstability. This effect is attributed to the inductive effect of the fluorine atom, which enhances the strength of the C=O bond, thus weakening the double-bond character of the Xaa-Pro amide bond [28]. The resulting pyramidization of the nitrogen atom of the Pro residue leads to an increase of the *cis*/*trans* isomerization. The large difference in the *cis*/*trans* isomer ratios of the amide bond involving a 4(*S*)- or a 4(*R*)-FPro is related to the different puckering of the Pro ring: $\gamma$-*exo*- for 4(*R*)-FPro and $\gamma$-*endo*- for 4(*S*)-FPro, as assigned by NMR [29] and X-ray crystallography [30]. The puckering preference of the $\gamma$-substituted Pro rings can be explained by invoking the 'gauche effect' [15], i.e. the tendency of the molecule to adopt the conformation which displays the highest number of gauche interactions between adjacent polar bonds (Figure 3).

To obtain a more complete conformational picture, one must include the $\Phi$ and $\Psi$ backbone torsion angles, which correlate with the ring pucker. The *exo* ring pucker has a smaller $\Psi$ and a less negative $\Phi$ than the *endo* ring pucker [31]. Thus, the pucker of the pyrrolidine ring in a $\gamma$-substituted Pro residue, its $\Phi$ and $\Psi$ torsion angles, and its peptide bond *cis*/*trans* ratio are interdependent parameters. In this context, it

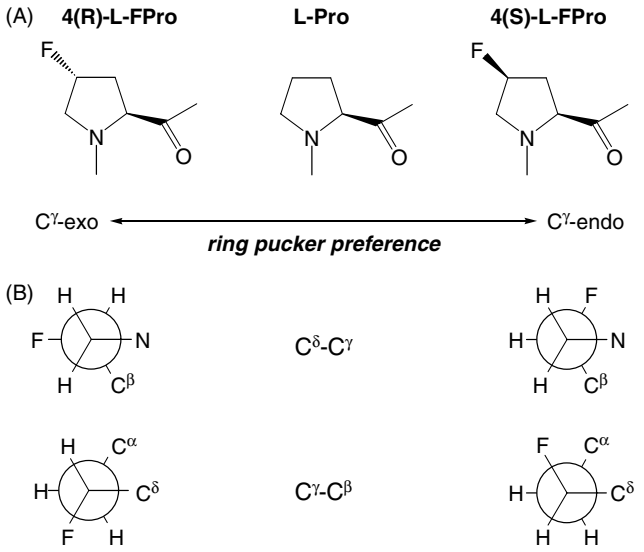


**Figure 3** (A) Effect of the stereochemistry of the electron-withdrawing substituent in the control of the pucker of the L-Pro pyrrolidine ring. (B) Newman projections about the $C^{\delta}-C^{\gamma}$ and $C^{\gamma}-C^{\beta}$ bonds illustrating the stereochemistry of the FPro residue. For simplicity, each dihedral angle is shown in its fully staggered conformation rather than in its optimized geometry.

was found that 4(*S*)-FPro stabilizes the *cis* form of a Xaa-Pro bond, while the 4-(*R*)- diastereoisomer stabilizes the *trans* form, the latter greatly increasing the propensity of the peptide to adopt the PPII conformation.

### Peptide Conformation in Aqueous Solution

CD spectroscopy is the technique of choice to detect the left-handed PPII helix conformation in solution [32]. The presence of a positive CD band at about 217 nm is diagnostic of the PPII conformation [33]. For Pro-rich peptides, the positive CD band is generally red-shifted toward 227 nm because of the increased content of tertiary amide chromophores [34]. By contrast, truly unordered peptides with a dynamic structure that samples all the available conformational space, exhibit a similar but somewhat distinct shape. Their hallmark is a negative band at about 225 nm accompanied by a stronger negative band at 200 nm [35]. Thus, both shape and magnitude of the CD spectra differentiate the unordered polypeptides from those displaying a preference for a PPII-helical structure.

The tendency of the **P2** peptide to adopt the PPII helix conformation was assessed by varying the temperature from 5 to 45 °C. In Figure 4 the CD spectra show that by lowering the temperature a weak positive band at about 225 nm is emerging, consistent with an increased PPII contribution. The presence of an isosbestic point at about 210 nm is also indicative of an equilibrium between two forms: the PPII conformation and the irregular (the so-called random coil) structure.

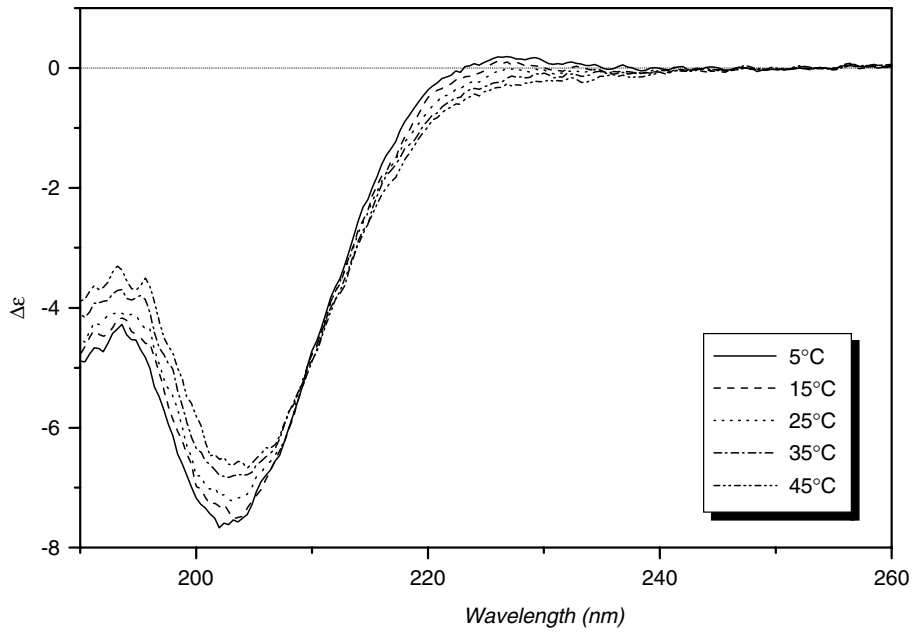


**Figure 4** Far-UV CD spectra of the HPK1 **P2** peptide in buffer solution, pH 7.0, as a function of temperature.

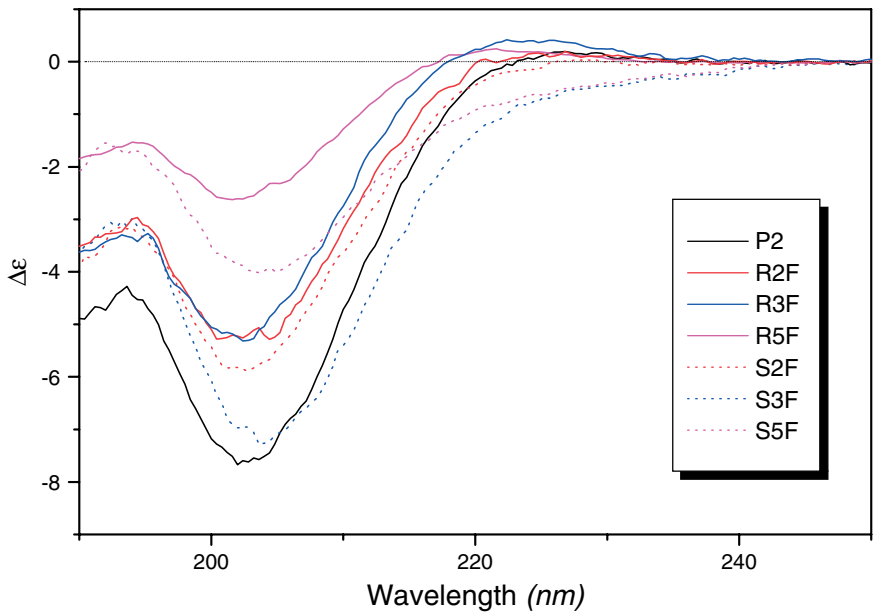


**Figure 5** Far-UV CD spectra of the **P2** peptide and its FPro analogues in buffer solution, pH 7.0, at 5 °C.

Figure 5 illustrates the effects of the FPro residues on the shape of the CD spectra of the **P2** analogues. A blue-shifting of the positive CD band was observed by increasing the number of 4(*R*)-FPro residues in the peptide sequence. The electronic effect of the fluorine atom appears to perturb the $n-\pi^*$ (~225 nm) transition more than the $\pi-\pi^*$ (~205 nm) transition of the amide chromophore. The peptide **R3F** shows the most intense positive CD band, indicative of the highest content of PPII conformation (followed by **R5F** and **R2F**).

The replacement of the Pro residue by 4(*S*)-FPro is characterized by the disappearance of the positive CD band, which suggests a lower content or the absence of the PPII conformation. Nevertheless, the presence of 4(*S*)-FPro at positions 3 and 6 (peptide **S2F**) is less detrimental to the PPII conformation than the substitution at positions 2,5,8 (peptide **S3F**) and 2,3,5,6,8 (peptide **S5F**). We conclude that the ability of FPro to stabilize the PPII helix conformation depends on several factors: the configuration of the $\gamma$-substituent, the number of FPro residues, and their positions in the sequence. On the other hand, the 4(*R*)-FPro residues at positions 2, 5 and 8 (peptide **R3F**), each corresponding to the $i+1$ position of the $(i \rightarrow (i+3))$ PPII turn, were

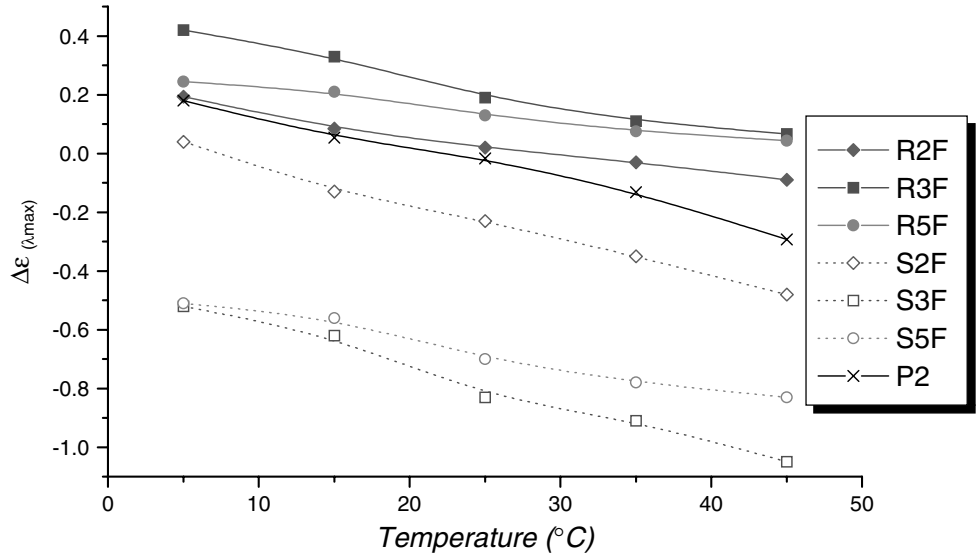


**Figure 6** Effect of temperature on the intensity of the positive CD band at $\lambda_{max}$ for the **P2** peptide and its FPro analogues in buffer solution, pH 7.0.

found to promote a higher content of PPII helix than at the $i+2$ position (residues 3 and 6, peptide **R2F**) and at both $i+1$ and $i+2$ positions (residues 2, 3, 5, 6 and 8, peptide **R5F**).

It is noteworthy that the effect promoted by 4(*S*)-FPro seems to be opposite to that promoted by 4(*R*)-FPro. Indeed, the 4(*S*)-FPro residue tends to destabilize the PPII conformation. This property is summarized in the plot of the $\Delta\varepsilon$ intensity at the maximum wavelength of the positive CD band associated with the PPII conformation, or at 225 nm for the 4(*S*)-FPro containing peptides, *versus* temperature that reveals qualitatively similar trends for all peptides (Figure 6). The relative PPII content of all peptides increases at low temperatures and decreases at high temperatures. In terms of absolute $\Delta\varepsilon$ intensity, however, the 4(*R*)-FPro peptide analogues show a higher tendency to adopt the PPII conformation than the parent peptide **P2**, while the 4(*S*)-FPro peptide analogues display a lower propensity. The observed rank order is as follows: **R3F**>**R5F**>**R2F**=**P2**>**S2F**≫**S5F**>**S3F**. It is interesting to note that the curves in Figure 6 clearly reflect the peptide conformational behavior as a function of the configuration, number, and positions of the FPro residues.

## Determination of the $K_d$ Values for the HS1-SH3 Domain

HS1 is a protein, the expression of which is limited to hematopoietic and lymphoid cells [36]. The amino acid sequence of HS1 contains a variety of structurally significant motifs: (i) an *N*-terminal region responsible for the binding of the mitochondrial protein HAX-1 [37], (ii) four 37 amino-acid repeats that can form a helix-turn-helix structure frequently found in the DNA binding domain of various transcription factors [36], (iii) a Pro-rich region located at the *C*-terminus of the central segment containing a stretch of Pro-Glu repeats, and (iv) an SH3 domain located at the *C*-terminus.

Recently, Nagata *et al.* [19] found that the HS1 protein is associated with HPK1 in hematopoietic cells by interaction of its *C*-terminal SH3 domain with the core Pro-rich motifs of HPK1. We have previously demonstrated that the recombinant HS1-SH3 domain fused to glutathione-*S*-transferase (GST-$SH3_{HS1}$) binds with high affinity to the peptide corresponding to the sequence 394–403 of HPK1 kinase (**P2**) and that the HS1 whole protein and the isolated HS1-SH3 domain show comparable peptide binding affinities [38].

The near-UV CD spectra of the free- and complexed GST-$SH3_{HS1}$ fusion protein provide evidence for the binding of Pro-rich peptides to the SH3 domain. The binding of peptides induces a variation of the CD pattern in the aromatic region. As an example, the near-UV CD spectra of the GST-$SH3_{HS1}$ domain at increasing **S2F** peptide concentration are reported in Figure 7.

The SH3 domain interactions with ligands are mediated through the stacking of aromatic rings from the Trp, Tyr and Phe residues with the pyrrolidine rings of the Pro residues located in the binding motif. These interactions modify the environment of the aromatic rings, generating an excellent *in situ* molecular probe for the protein–peptide interaction. Binding of the Pro-rich peptide induces CD changes in the near-UV region, which are ascribed to the local tertiary structure of the Trp side-chain affected by the peptide ligand. The Trp indole chromophore shows a complex CD spectrum in the UV region, characterized by two distinct electronic transitions, named $L_b$ and $L_a$ bands [39]. The $L_b$ band is relatively weak but is characterized by a well-developed fine structure. The $L_a$ band is considerably more intense

 

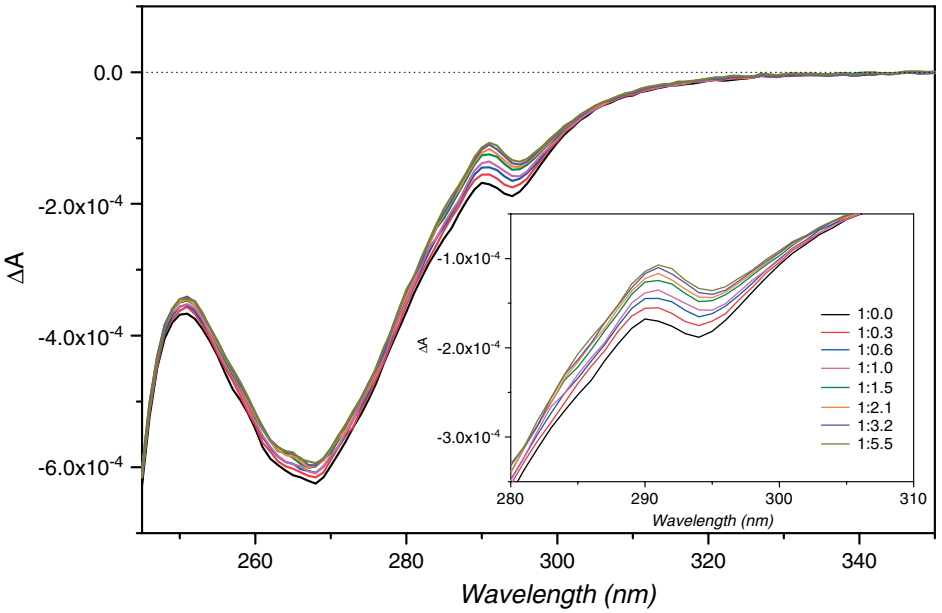


**Figure 7** Near-UV CD spectra for the binding of **P2** to the GST-SH3$_{HS1}$ domain in buffer solution, pH 7.0, at room temperature. The inset highlights the Trp CD region. The ΔA values were measured as a function of increasing the SH3/peptide molar ratio.

and nearly devoid of fine structure. Because of the large change in dipole moment upon excitation of the $L_a$ state, the $L_a$ band is especially sensitive to environment [39].

Previous works showed that the changes in the far-UV CD spectra of the SH3/peptide complexes are essentially due to a variation of peptide–ligand conformation rather than a change of the SH3 domain conformation [40]. Starting from this information, we made a comparative analysis of the far-UV CD spectra of the free GST-SH3$_{HS1}$ domain and their complexes with peptides to analyze the possible conformational changes following peptide–ligand interactions. The overlapping of the CD spectrum of the 1 : 1 GST-SH3$_{HS1}$/**R3F** complex and the sum of the individual spectra of GST-SH3$_{HS1}$ and **R3F** are similar (Figure 8(A)). On the other hand, measured and calculated CD spectra of other 1 : 1 GST-SH3$_{HS1}$/peptide complexes are not identical (as an example, the corresponding CD spectra of the **P2** complex are reported in Figure 8(B)). These results confirm that the interaction occurring between the SH3 domain and the peptide ligand induces a conformational change and that this change is higher when the content of PPII helix conformation in the ligand is lower.

The titration of the isolated GST protein with **P2** analogues does not induce any CD changes in both the near- and far-UV regions (data not shown), confirming the absence of nonspecific peptide–GST interactions, and therefore validating the use of the GST-SH3 fusion protein instead of the isolated SH3 domain in the binding studies.

The $K_d$ values of the peptide/GST-SH3 complexes (Table 2) were determined by analyzing the CD data at a single wavelength (294 nm) by nonlinear regression analysis (16). This band corresponds to the 0–0 transition of the indole chromophore of the Trp residues, which generally lies between 290 and 295 nm [39] (Figure 7). Upon addition of the peptide to the SH3 domain, the intensity of the aromatic side chain-derived signal increases, indicative of a mobility reduction and a local environmental change at the binding site (Figure 7). This change in intensity following the addition of the peptide to GST-SH3 enabled the determination of the dissociation constant, $K_d$. The contribution of the Phe aromatic side-chain present in the ligand peptides, at a given concentration, was subtracted from the CD spectrum of the corresponding complex.

The CD data were analyzed using the Origin program assuming a 1 : 1 stoichiometry of peptide: GST-SH3$_{HS1}$ protein. This assumption was confirmed by the titration curves of the GST-SH3$_{HS1}$ fusion protein with the synthetic peptides monitored at 294 nm. As none of

**Table 2** Calculated $K_d$ values for the GST-SH3$_{HS1}$/peptide complexes

| Peptide | $K_d$(μM)[a] |
|---|---|
| P2 | 1.4 |
| R2F | 10.0 |
| R3F | 29.2 |
| R5F | 10.0 |
| S2F | 17.0 |
| S3F | 26.9 |
| S5F | 45.3 |

[a] The standard errors are less than 10%.

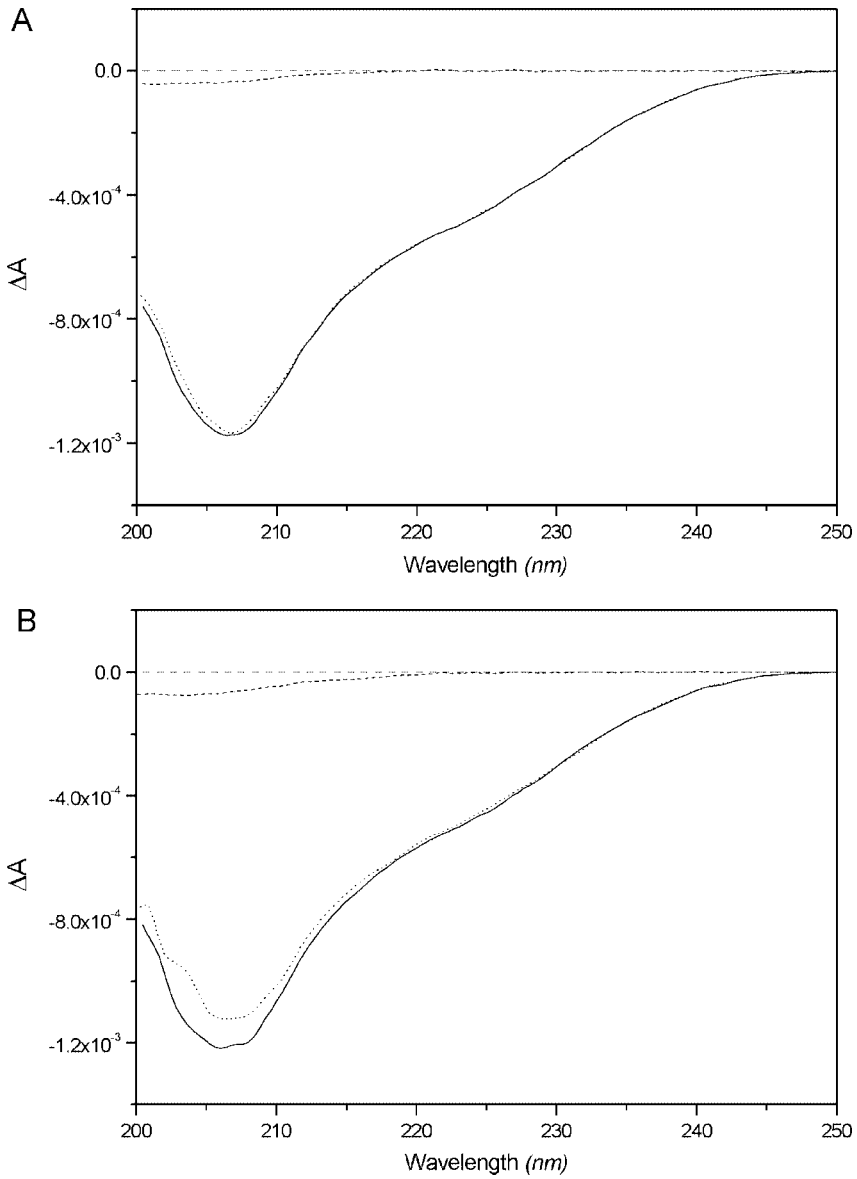


**Figure 8** Far-UV CD spectra in buffer solution, pH 7.0, of GST-$SH3_{HS1}$ alone and in complex with the peptide ligands **R3F** (A) and **P2** (B). CD spectra of the free peptide (- - - - ), and the calculated (———) and measured ( ····· ) 1 : 1 GST-$SH3_{HS1}$/peptide complex.

the curves shows discontinuities, each curve may be fitted with one binding constant, indicating that one protein molecule binds only one peptide (data not shown).

The introduction of 4(*S*)-FPro residues into the **P2** peptide chain decreases the affinity for the SH3 domain (Table 2), as expected from the conformational properties of the synthetic peptides (contents of PPII helix) previously described. The 4(*S*)-FPro peptides are less structured than the **P2** peptide and consequently these analogues undergo a change from almost nonstructured to partially structured conformation in a PPII helix. This process is likely to produce a significant entropic penalty and undoubtedly accounts for some of the unfavorable contributions to the observed $K_d$ values. Indeed, the $K_d$ value determined for the peptide **S2F** (17.0 μM) is 12-folds higher than that determined for the parent peptide **P2** (1.4 μM), while peptides **S3F** and **S5F**, characterized by the absence of PPII helix also at low temperature (5 °C, Figure 5), display high $K_d$ values (26.9 and 45.3 μM, respectively).

Surprisingly, the peptides containing the 4(*R*)-FPro residue and adopting a PPII helix conformation are also characterized by $K_d$ values that are higher than that of the parent peptide **P2** (see Table 2). These data suggest that the binding affinity of the Pro-rich peptides to the HS1-SH3 domain is the result of a delicate balance of mutually compensating contributions. An apparently simple interaction based on the stacking of hydrophobic residues shows a complex behavior, which

suggests that different factors play a role in the binding energetics.

## CONCLUSIONS

Most of the ligands identified so far for protein SH3 domains are rich in Pro residues. This is because they can fit into the aromatic pocket of SH3 domains and readily adopt a PPII conformation in solution without a significant loss in conformational entropy. This PPII conformation seems to fit the steric and hydrogen-bonding pattern of the SH3 ligand surface best [6].

We introduced 4(*S*)- or 4(*R*)-FPro instead of Pro residues into a suitable SH3 binding sequence with the aim of stabilizing the PPII helix conformation in short peptides and consequently increasing the binding affinity of short Pro-rich peptides toward the SH3 domains. The conformation of FPro-containing peptides and their interaction with the SH3 domain of the HS1 protein were investigated using the CD spectroscopic method.

The results presented in this paper provide two different conclusions: (i) The replacement of Pro residues by 4(*R*)- or 4(*S*)-FPro analogues confirms that the ability of FPro to stabilize the PPII-like conformation is strictly related to the residue position as well as to the configuration of the $\gamma$-substitution. (ii) The stabilization of the peptide conformation is not sufficient to increase the binding affinity.

4(*R*)-FPro stabilizes the PPII helix more efficiently than its 4(*S*) analogue owing to the $\gamma$-fluorine effect on the puckering of the pyrrolidine ring, the $\Phi$ and $\Psi$ backbone torsion angles, and the peptide bond *cis*/*trans* ratio. This effect is strongly position-dependent: the replacement of the residue at the $i+1$ position of the PPII helix (amino acids 2, 5 and 8 of the native peptide sequence) with 4(*R*)-FPro increases the helix stability more than replacing the Pro residue at the $i+2$ position (amino acids 3 and 6) or at both $i+1$ and $i+2$ positions (amino acids 2, 3, 5, 6 and 8). Remarkably, the effect induced by 4(*S*)-FPro is opposite to that of its 4(*R*) analogue. Indeed, it promotes helix stability at the $i+2$ (but not at the $i+1$) position.

Introduction of the FPro residue into the peptide sequence does not improve the $K_d$ value of SH3/peptide complexes (Table 2). It is important to point out that, while the increase of $K_d$ values as a result of the introduction of 4(*S*)-FPro residues is likely to be explained by the significant entropy penalty correlated to the effect of this residue on peptide conformation, the low affinity of **R2F**, **R3F** and **R5F** peptides confirms the occurrence and the importance of other factors in the binding energetics that might be affected by the presence of the $\gamma$-fluorine substituent. As previously reported, the fluorine atom is considered isosteric to the hydrogen atom that it replaces [18]. Therefore, the electronic properties of the fluorine atom should be considered to explain the increase of the $K_d$ values.

The fluorine electronic effect influences the puckering of the pyrrolidine ring as well as the strength of the C=O bond. The effect on the puckering of the Pro residue explains the low affinity correlated with the entropic effect. Indeed, the *exo*-puckering, but not the *endo*-puckering, of the Pro residue stabilizes the PPII helix and consequently it docks the hydrophobic groove formed by $Tyr^{437}$ and $Tyr^{481}$ (Figure 2), stabilizing the peptide binding. On the other hand, the increased C=O strength may influence the capability of the carbonyl oxygens of the FPro residues to form hydrogen bonds with the HS1-SH3 domain (the most significant being that between the carbonyl oxygen essential is that from the carbonyl oxygen of $Pro^{3}$ and the hydroxyl group of the $Tyr^{481}$ side chain of the $SH3_{HS1}$ domain) resulting in unfavorable enthalpic effect that counteracts the favorable entropic effect derived from the stabilized PPII helix conformation in 4(*R*)-FPro peptides.

These data support our hypothesis that the introduction of 4-(*R*)-FPro residues into Pro-rich short peptide stabilizes the PPII helix conformation in aqueous solution. This effect is important when the PPII helix structure is the relevant biological binding conformation required to trigger the signal transduction mediated by ligand interactions. In addition, these data confirm the importance of interactions other than hydrophobicity on the stabilization of the SH3 peptide-protein complexes that must be rationalized to obtain highly selective peptides.

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