Amino Acids (2013) 44:607–614
DOI 10.1007/s00726-012-1383-y

ORIGINAL ARTICLE

The impact of either 4-R-hydroxyproline or 4-R-fluoroproline on the conformation and $SH3_{m\text{-}cort}$ binding of HPK1 proline-rich peptide

Andrea Borgogno · Paolo Ruzza

Received: 9 March 2012 / Accepted: 30 July 2012 / Published online: 14 September 2012
© Springer-Verlag 2012

Abstract SH3 domains are probably the most abundant molecular-recognition modules of the proteome. A common feature of these domains is their interaction with ligand proteins containing Pro-rich sequences. Crystal and NMR structures of SH3 domains complexes with Pro-rich peptides show that the peptide ligands are bound over a range of up to seven residues in a PPII helix conformation. Short proline-rich peptides usually adopt little or no ordered secondary structure before binding interactions, and consequently their association with the SH3 domain is characterized by unfavorable binding entropy due to a loss of rotational freedom on forming the PPII helix. With the aim to stabilize the PPII helix conformation into the proline-rich decapeptide PPPLPPKPKF (**P2**), we replaced some proline residues either with the 4(R)-4-fluoro-L-proline (FPro) or the 4(R)-4-hydroxy-L-proline (Hyp). The interactions of **P2** analogues with the SH3 domain of cortactin ($SH3_{m\text{-}cort}$) were analyzed by circular dichroism spectroscopy, while CD thermal transition experiments have been used to determine their propensity to adopt a PPII helix conformation. Results show that the introduction of three residues of Hyp efficiently stabilizes the PPII helix conformation, while it does not improve the affinity towards the SH3 domain, suggesting that additional forces, e.g., electrostatic interactions, are involved in the $SH3_{m\text{-}cort}$ substrate recognition.

Keywords Cortactin · Pro-rich peptides · 4-R-Fluoroproline · 4-R-Hydroxyproline · Protein–protein interaction

Electronic supplementary material The online version of this article (doi:10.1007/s00726-012-1383-y) contains supplementary material, which is available to authorized users.

A. Borgogno
Department of Chemical Sciences, University of Padova, 35131 Padua, Italy

P. Ruzza (✉)
Institute of Biomolecular Chemistry of CNR, Padova Unit, 35131 Padua, Italy
e-mail: paolo.ruzza@cnr.it

Introduction

SH3 domains are one of the most frequently occurring protein–protein interaction modules in eukaryotic cells, which are involved in a wide variety of cell processes (Cohen et al. 1995; Mayer 2001; Marchiani et al. 2009). These domains typically bind to proline-rich sequences containing the PxxP motif, which adopt a left-handed polyproline type II (PPII) helix conformation when they are bound to the SH3 domain (Feng et al. 1994). The position of a critical basic residue identifies the two major binding classes of peptide SH3 ligands, named class I or class II, respectively (Lim et al. 1994). Recently, different authors demonstrated the significant role of residues outside the core consensus in determining both affinity and specificity; this allows defining two distinct binding regions in the structure of many SH3 domains, referred to as Surface I and Surface II, respectively (Kim et al. 2008). Surface I has been extensively described and corresponds to the region which interacts with the PxxP motif (Li 2005; Zarrinpar et al. 2003). On the other hand, Surface II, referred to as the "specificity pocket" in some studies (Li 2005; Zarrinpar et al. 2003), is much broader and contains less conserved residues, located both in the RT or N-Src loops and in the strands c and d of the SH3 domain (Larson and Davidson 2000).

Previously, we demonstrated that the SH3 domain of murine cortactin ($SH3_{m\text{-}cort}$) interacts with peptides derived from proline-rich region of the HPK1 kinase (Rubini et al.

2010). Using the near-UV CD analysis, we determined that the peptide reproducing the region 394–403 of HPK1, named **P2**, interacted with high affinity at the $SH3_{m\text{-}cort}$ domain.

PPII helix is a left-handed helix with all the amide bonds in the *trans* conformation. It has been well demonstrated that the stereoelectronic effects of the 4-substituent to the proline residue play a major role in determining the pucker of the proline ring and hence the *cis/trans* conformer ratio of the Xaa-Pro amide bond, influencing the PPII helical stability (Kuemin et al. 2010).

With the aim to enhance the tendency of the **P2** peptide to adopt a PPII helix conformation in aqueous solution, with a consequent reduction of the entropic cost of the ligand–protein interaction, we synthesized a series of **P2** analogues replacing Pro residues with either 4R-fluoroproline (FPro) or 4R-hydroxyproline (Hyp) (Table 1).

The interactions of the $SH3_{m\text{-}cort}$ domain with the aforementioned Pro-rich peptides were analyzed by CD spectroscopy, monitoring the CD changes of the Trp side-chain chromophore of the SH3 domain. The dissociation constants, K_d, were determined by analyzing the CD data measured at 294 nm using a non-linear regression method (Siligardi et al. 2012; Siligardi and Hussain 1998). Additionally, CD thermal transitions of the **P2** analogues were measured to correlate their propensity to adopt a PPII helix with their binding affinity for SH3. The comparative analysis of the binding properties of this set of closely related peptides confirms the complexity of the SH3 recruitment, supporting the role of additional interactions outside the region that interacts with the PxxP motif in the substrate recognition by the $SH3_{m\text{-}cort}$ domain as previously indicated (Rubini et al. 2010).

Experimental procedures

Peptide synthesis

Fmoc-protected amino acids and preloaded Wang resin were purchased from Calbiochem-Novabiochem (Läufelfingen, Switzerland). Fmoc-FPro-OH was obtained from Bachem (Weil am Rhein, D). HBTU, HOBt, DIEA and DMF were obtained from Iris Biotech (Marktredwitz, D), whereas TFFH was purchased from PerSeptive Biosystems (Foster City, CA). Peptides were assembled using the Fmoc/HBTU chemistry in 0.06 mmol scale by manual solid-phase synthesis. HBTU/HOBt activation employed a threefold molar excess (0.24 mmol) of Fmoc-amino acids in DMF solution for each coupling cycle unless otherwise stated. Coupling to the secondary amino group of 4-fluoroproline was performed using TFFH as coupling reagent (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 Kaiser test or by evaluation of Fmoc displacement (Wellings and Atherton 1997). Peptides were side chain-deprotected and removed from the resin by TFA treatment in the presence of 2.5 % TIS, 2.0 % anisole and 0.5 % water, and then precipitated by addition of diethyl ether.

Crude peptides were purified by preparative reversed-phase HPLC using a Shimadzu LC-8 (Shimazdu, Kyoto, Japan) system with a Vydac 218TP1022, 10 µm, 250 × 22 mm column (Grace Davison Discovery Sciences, Deerfield, IL). 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 15 to 35 % of solvent B (0.05 % TFA in acetonitrile/water, 9:1 by vol.) in 40 min. The fractions containing the desired product were collected and lyophilized to constant weight in the presence of 0.01 N HCl. Analytical HPLC analyses were performed on a Shimadzu LC-10 instrument, fitted with a Jupiter C18, 10 µm, 250 × 4.6 mm column (Phenomenex, Torrance, CA) using the described solvent system (solvents A and B), with a flow rate of 1 mL/min, and detection at 216 nm. All peptides showed less than 1 % impurities. Molecular weights of compounds were determined by ESI–MS on a Mariner (PerSeptive Biosystem) mass spectrometer instrument. The mass was assigned

Table 1 Sequences of **P2** proline-rich peptide analogues

Peptide	Sequence									
	3	2	1	0	−1	−2	−3	−4	−5	−6
Class II		P	x	x	P	x	R/K			
P2	Pro	Pro	Pro	Leu	Pro	Pro	Lys	Pro	Lys	Phe
F2	Pro	Pro	**FPro**	Leu	Pro	**FPro**	Lys	Pro	Lys	Phe
F3	Pro	**FPro**	Pro	Leu	**FPro**	Pro	Lys	**FPro**	Lys	Phe
F5	Pro	**FPro**	**FPro**	Leu	**FPro**	**FPro**	Lys	**FPro**	Lys	Phe
H2	Pro	Pro	**Hyp**	Leu	Pro	**Hyp**	Lys	Pro	Lys	Phe
H3	Pro	**Hyp**	Pro	Leu	**Hyp**	Pro	Lys	**Hyp**	Lys	Phe
H5	Pro	**Hyp**	**Hyp**	Leu	**Hyp**	**Hyp**	Lys	**Hyp**	Lys	Phe

Residue position nomenclature according to Lim et al. (1994). Abbreviations in bold indicate the substitution of Pro residues for FPro and Hyp residues

using a mixture of neurotensin, angiotensin and bradykinin, at a concentration of 1 pmol/μL, as external standard.

Expression and purification of GST-SH3$_{m\text{-}cort}$

GST-SH3$_{m\text{-}cort}$ fusion protein was kindly provided by Chiara Rubini (Rubini et al. 2010). The GST-SH3$_{m\text{-}cort}$ concentration was determined by absorption spectroscopy ($\varepsilon = 57{,}420\ M^{-1}\ cm^{-1}$ at 280 nm).

Circular dichroism and GST-SH3$_{m\text{-}cort}$ titration

All measurements were obtained using a nitrogen flushed Jasco J-715 spectropolarimeters (Tokyo, Japan) and a 0.5 cm or a 1.0 cm quartz cells for the near-UV region and a 0.1 cm quartz cell for the far-UV region. Each titration was performed in aqueous buffer (20 mM Tris–HCl, pH 7.5, in presence of 1 mM dithiothreitol) at 25 °C by addition of small aliquots of peptide stock solution in the same buffer as previously described (Ruzza et al. 2006). The contribution of the phenylalanine aromatic side chain present in the ligand-peptides, at a given concentration, was subtracted from the CD spectra of the domain in complex with each peptide. Peptide concentration was determined by weight using a Mettler Toledo microbalance (Columbus, OH) model AT21 Comparator (sensitivity ± 1 μg). The dissociation constants K_d of the different complexes were determined by analyzing the CD data at a single wavelength by non-linear regression analysis as previously described (Siligardi and Hussain 1998).

Far-UV CD spectra of proline-rich peptides were obtained in aqueous buffer (20 mM Tris–HCl, pH 7.5) by varying the temperature from 5° to 45 °C, and in *n*-propanol/20 mM Tris–HCl buffer, pH 7.5, (96:4, v/v) solution at room temperature.

Estimates of PPII helix content can be obtained using

$$\%PPII = \frac{[\Theta]_{max} + 6,100}{13,700} \times 100$$

where $[\Theta]_{max}$ is the molecular ellipticity at the characteristic maxima. The upper (100 %) and lower (0 %) limits were determined from CD spectra for the polyproline peptide in an 8.4 M guanidine hydrochloride solution and for a peptide model for completely disordered proteins, respectively (Kelly et al. 2001).

Results

Peptide design and synthesis

Our goal is to enhance the tendency of the **P2** peptide to adopt a PPII helix conformation in aqueous solution, stabilizing the *trans*-conformers in Xaa-Pro amide bonds. This effect should decrease the entropic cost of the peptide-SH3$_{m\text{-}cort}$ interaction, increasing the affinity of peptide towards the SH3 domain. Proline derivatives with a substituent in the γ-position (C4) have proven to be useful for the tuning of the *cis/trans* conformer ratio (Renner et al. 2001; Kotch et al. 2008; Shoulders et al. 2006). Proline can adopt either a Cγ-exo ring pucker, in which Cγ is puckered toward the Cα proton, or a Cγ-endo ring pucker, in which Cγ is puckered toward the carbonyl group (Fig. 1). Conformational studies show that both the nature of the substituent at γ position and the absolute configuration at this center influence the pyrrolidine ring pucker and consequently the *cis/trans* conformer ratio of the Xaa-Pro amide bond. An exo pucker, and consequently a *trans* Xaa-Pro amide bond, is favored when an electron-withdrawing group is in the 4R position while an endo pucker is preferred when the substituent is in the 4S position. This has been attributed to the reduction of the bond order of the C–N linkage by the increased N-pyramidalization due to the electron-withdrawing group (Panasik et al. 1994; Eberhardt et al. 1996), and to an $n \rightarrow \pi^*$ electrostatic interaction between the carbonyl groups of the *i-1* and *i* residues present when the peptide bond adopts a trans conformation (Bretscher et al. 2001; Hinderaker and Raines 2003).

In the case of Hyp, the strong preference for a γ-exo pucker has been attributed also to the gauche effect (Wolfe 1972): in the γ-exo conformation, a *gauche* orientation of the 4-OH group and the pyrrolidine nitrogen, relative to the Cγ-Cδ bond axis, is possible (Taylor et al. 2005). Moreover, recent theoretical studies suggest that the driving force to adopt the γ-exo conformation is hyperconjugative interaction which involved the donation from the $\sigma(C\beta\text{-}H)_{ax}$ and $\sigma(C\delta\text{-}H)_{ax}$ bonding orbitals into the $\sigma^*(C\gamma\text{-}O)$ antibonding orbital (Alabugin and Zeidan 2002; Improta et al. 2001).

The proline ring pucker constrains the φ, ψ, and ω main chain torsion angles, and thus, a selective control of the ring pucker provides the possibility of preorganizing the

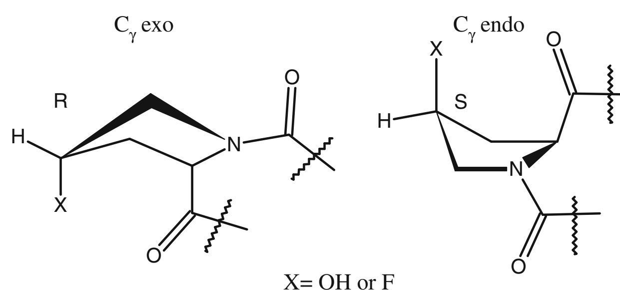

Fig. 1 Ring conformation of 4-substituted Pro derivatives. The ring conformational preferences depend on the stereochemistry and electronic effects of the substitution

peptide backbone conformation and modulating the peptide stability (Zheng et al. 2010; DeRider et al. 2002).

Peptides were synthesized by the solid phase method on a Wang resin according standard Fmoc/HBTU protocol. To overcome low yields and subsequent side reactions, the coupling to the secondary amino group of 4R-fluoroproline residues was performed using TFFH as coupling reagent (Ruzza et al. 2006). Following the removal of the Fmoc group, side chain protecting groups were left on during cleavage from the resin by TFA standard treatment (see Experimental Section). Crude products were purified by preparative reversed-phase chromatography. The correct composition of the synthetic peptides was confirmed by ESI–MS analysis.

Peptide conformation in aqueous solution

CD spectroscopy is the technique of choice to detect PPII helices conformation in solution. The presence of a positive CD band at about 217 nm and a negative band between 200 and 210 nm are diagnostic of the PPII helix conformation (Siligardi and Drake 1995; Drake et al. 1988). The positive CD band is generally red-shifted towards 227 nm in proline-rich peptides due to the increased content of tertiary amide chromophore (Venugopal et al. 1994) and its intensity is proportional to the PPII helical content (Kelly et al. 2001). 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 stronger negative band at about 200 nm accompanied by a negative band at about 225–227 nm (Woody 1992). Thus, both shape and magnitude of the CD spectra differentiate the unordered polypeptides from those displaying a preference for a PPII-helical structure.

The conformation of **P2** and its analogues in the temperature range from 5° to 45 °C was assessed by far-UV CD spectroscopy. As an example, the far-UV CD spectra of **H3** registered at different temperature have been reported in Fig. 2. Upon heating, the positive CD band at about 228 nm, hallmark of the PPII helix, decreased, and the negative band at about 200 nm slightly shift towards a higher wavelength, according to the results described by different authors (see Woody 2010, for a compressive Review). An isodichroic point at about 210 nm was observed in temperature dependence CD spectra of **H3,** as well as in the CD spectra of other compounds (Figure 1S in Supplementary Material), indicative of an equilibrium between two forms: the PPII helix conformation and the irregular structure (the so-called extended). The position of positive maximum will vary slightly from the 228 nm, depending on the ratio of tertiary to secondary amides in each peptide, as well as upon the nature of other structure adopted.

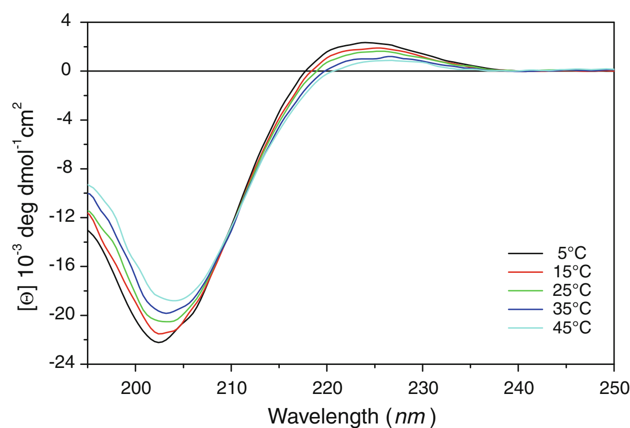

Fig. 2 Far-UV CD spectra of the **H3** peptide (0.1 mM) in 20 mM Tris–HCl, pH 7.5, buffer solution as a function of temperature

The plot of the molar ellipticity values of the positive band in the CD spectra versus temperature revealed that all peptides showed qualitatively similar trends (Fig. 3). In terms of absolute molar ellipticity intensity, however, the **H3** analogue showed the highest values, and consequently the highest PPII helical content (Table 2). On the contrary, the parent **P2** peptide displayed the lowest propensity to adopt a PPII helix conformation on the all range of tested temperature. **H2** and **F2** peptides were characterized by a very similar behavior to **P2** peptide. The intensity of the positive band nm at 25 °C, and consequently the PPII helical content (Table 2), is seen to decrease in the order **H3** > **H5** ≈ **F3** > **F5** > **F2** ≥ **H2** ≈ **P2**.

The CD spectra of peptides were also registered in *n*-propanol/20 mM Tris–HCl buffer, pH 7.5, (96:4, v/v) solution, condition which favors the right-handed poly-proline I helix (PPI), after a week's incubation, due to the slow conversion of amide bond from *trans* to *cis* form. The shape of the CD spectra of the **P2** analogues (data not

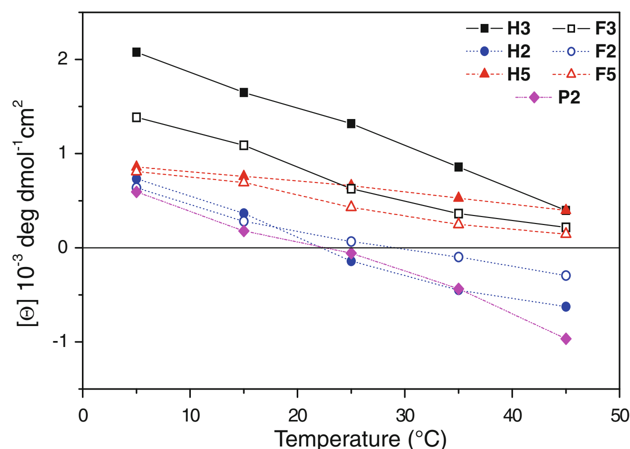

Fig. 3 Temperature dependence on the maxima in the CD spectra for the **P2** peptide and its Hyp or FPro analogues in 20 mM Tris–HCl, pH 7.5, buffer solution

Table 2 Positions and heights of maxima in CD spectra of peptides at 5 and 25 °C in 20 mM Tris–HCl, pH 7.5, estimated PPII helix content, and values of peptide binding affinity to GST-SH3$_{m\text{-}cort}$ domain

Peptide	Wavelength of maximum (nm)		[Θ] (10^{-3}deg dmol^{-1} cm^{2})		% PPII		K_d (μM)
	5 °C	25 °C	5 °C	25 °C	5 °C	25 °C	
P2	227.4	227.4	0.593	−0.056	48.9	*n.d.*	2.5 ± 1.3
F2	224.8	225.0	0.637	0.066	49.2	45.0	8.1 ± 1.8
F3	225.0	223.4	1.385	0.626	54.6	49.1	8.3 ± 1.6
F5	221.6	222.8	0.808	0.428	50.4	47.6	6.6 ± 2.0
H2	227.2	226.6	0.732	−0.141	49.9	*n.d.*	2.6 ± 0.7
H3	223.4	224.8	2.078	1.319	59.7	54.2	2.9 ± 0.4
H5	221.8	222.4	0.857	0.659	50.8	49.3	13.2 ± 1.0

Experimental errors are SD

shown) is lacking in the characteristic hallmark of the PPI helix (positive band at about 210–215 nm), but also of that of PPII helix, indicating that an electron-withdrawing group on the 4R position substantially disfavors the formation of PPI conformation (Horng and Raines 2006; Kümin et al. 2007).

Determination of the K_d values for GST-SH3$_{m\text{-}cort}$ domain

The interaction occurring between the P2 analogues and GST-SH3$_{m\text{-}cort}$ fusion protein was analyzed using the near-UV CD spectroscopy. The interactions of the SH3 domain with ligands are mediated through the stacking of aromatic amino rings from residues like Trp, Tyr and Phe with the pyrrolidine rings of the prolines located in the binding motif. These interactions modify the environment of the aromatic ring, generating an excellent in situ molecular probe of the protein-peptide interaction. The binding of the peptides to GST-SH3$_{m\text{-}cort}$ fusion protein can be clearly determined by the spectral changes of the Trp side chain band at approximately 290 and 295 nm (Fig. 4). The values of the apparent dissociation constant K_d are determined by the non-linear regression analysis of the ΔA values of the CD spectra at 294 nm with increasing SH3/peptide molar ratios (Table 2; Fig. 5).

The titration of the isolated GST protein with **P2** does not induce any CD changes in both the near and far-UV regions (Figure 2S), thus confirming the absence of non-specific peptide-GST interaction. Moreover, since GST protein might undergo dimerization (Dourado et al. 2008), parallel titrations using either free SH3$_{m\text{-}cort}$ domain or GST-SH3$_{m\text{-}cort}$ fusion protein and a suitable proline-rich peptide were previously performed (Rubini et al. 2010). The K_d values determined for the proline-rich peptide were very similar, supporting the validity of using the recombinant fusion protein instead of the free SH3$_{m\text{-}cort}$ domain in CD spectroscopy analysis.

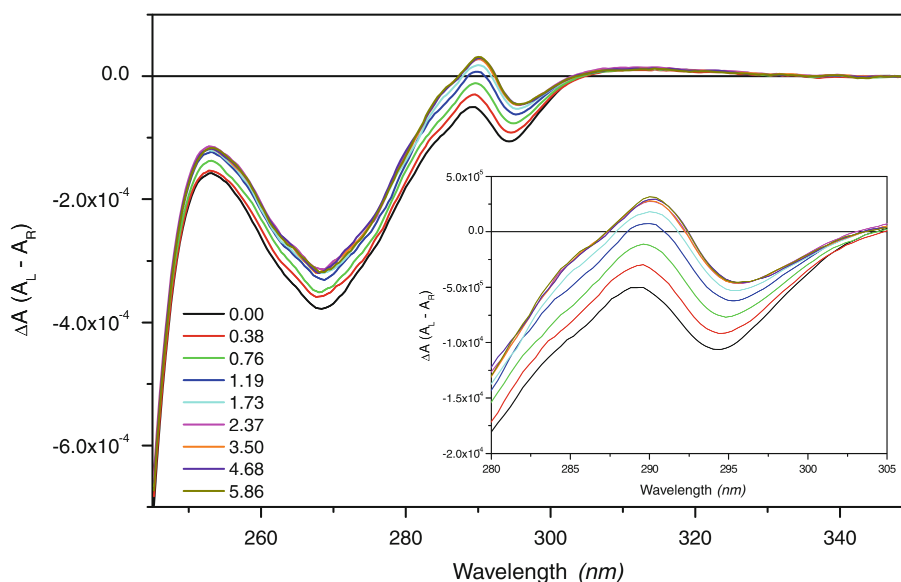

Fig. 4 Near-UV CD spectra of GST-SH3$_{m\text{-}cort}$ in the presence of increasing amount of **H3** peptide. GST-SH3$_{m\text{-}cort}$ was 53.4 μM in 20 mM Tris–HCl buffer, pH 7.5; **H3** peptide was 2.574 mM in 20 mM Tris–HCl buffer, pH 7.5. Spectra were recorded at 25 °C. The ΔA values were measured as a function of the increasing peptide/GST-SH3$_{m\text{-}cort}$ molar ratios (indicated)

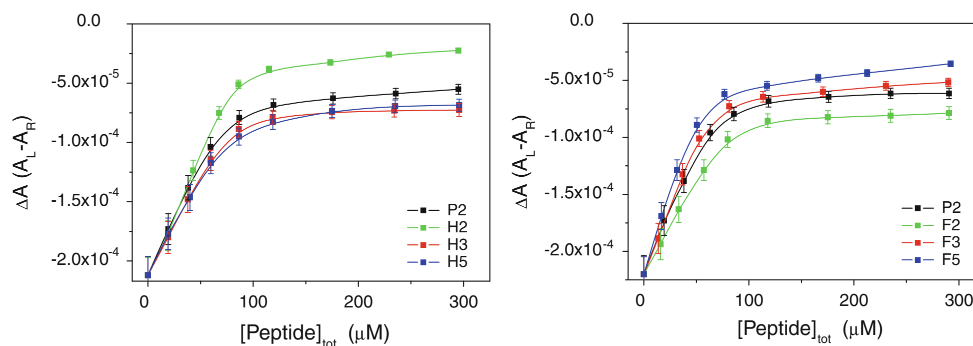

Fig. 5 Determination of the K_d values by CD spectroscopy analyses. The ΔA values at 294 nm, measured as a function of different SH3/peptide molar ratios, are plotted versus the peptide concentration

Table 2 shows that **P2** peptide interacts with the GST-SH3$_{m\text{-}cort}$ fusion protein with a very favorable K_d value (2.5 ± 1.3 μM). The substitution of Hyp for Pro residues into both **H2** and **H3** peptides does not have a significant effect on the K_d values, while **H5** binds with an affinity about sixfold lower (K_d = 13.2 ± 1.0 μM) than that **P2**.

Surprisingly, the replacement of Pro residues into the **P2** sequence with FPro residues is a negative determinant in the binding process. Peptides containing the FPro residues are characterized by higher K_d values with respect to **P2** peptide (see Table 2).

Discussion

Many of the so far identified SH3 domain ligands are rich in Pro residues; this could be due to the fact that they readily adopt a PPII helix conformation in solution (Lim et al. 1994). PPII is highly extended with a helical pitch of 9.3 Å/turn and 3.0 residues/turn that seem to fit best steric and hydrogen bonding pattern of SH3 ligand surface, without a significant loss in conformational entropy.

Artificial induction of PPII helices has been reported using modified proline residues either by non-covalent stabilization (Tuchscherer et al. 2001; Ruzza et al. 2006; Jacquot et al. 2007; Flemer et al. 2008) or by ring closing metathesis macrocyclization (Liu et al. 2010, 2011).

The introduction of an electron-withdrawing group in the 4 position of the pyrrolidine ring of Pro residue influences both proline ring pucker and the *cis/trans* conformer ratio of the amide Xaa-Pro bond. The substitution of either Hyp or FPro for Pro residues into the **P2** sequence has been used to stabilize the proline γ-exo pucker, and thus, the PPII helix conformation. In addition to the configuration of the γ-substituent, the ability of these residues to stabilize the PPII helix conformation is strictly related to their position into the peptide sequence.

As shown in Fig. 3, the introduction of Hyp residues at 2, −1 and −4 positions (peptide **H3**), involving the two canonical positions occupied by Pro residues in class II binding motif (2 and −1 positions, see Table 1), was found to promote the higher content of PPII helix either with respect to other positions or the presence of the FPro residues (Table 2). Indeed, the substitution of Hyp for Pro stabilizes the PPII helix more efficiently than the introduction of FPro analogue, although the hydroxyl group is less electronegative than the fluorine atom. This may be due to the capability of the hydroxyl group to perform additional non-covalent interaction between the γ-substituent of the Pro residue and the amide backbone, stabilizing the *trans* conformer in Xaa-Pro bond (Kuemin et al. 2010). Moreover, the capability of the hydroxyl group of Hyp to realize strong interactions with the aqueous solvent could efficiently stabilize the PPII helix conformation. Conformational studies have been suggested that peptide–solvent interaction is a major driving force in PPII helix formation (Shi et al. 2002; Rucker et al. 2003).

These results support our hypothesis that the introduction of a 4-(R)-electron-withdrawing group into proline residue belonging to a proline-rich peptide stabilizes the PPII helix conformation in aqueous solution. This is important when the PPII structure is the relevant biological binding conformation required to trigger the signal transduction mediated by ligand-binding interactions, particularly when peptides mimic a portion of protein structure where the intramolecular interactions, characteristic of the protein structure, are lost.

Unexpectedly, the induction of a stable PPII helix conformation in ligands does not improve the K_d values of GST-SH3$_{m\text{-}cort}$/peptide complexes (Table 2). The lack of improved affinity may be ascribed to two different factors: the unfavorable placement of the puckers of the Pro residue involved in the interactions with aromatic SH3 residue, and/or the difficulty to optimize interactions outside the classical Surface I binding site.

Previously we demonstrated that the Pro2 and Pro-1 residues into **P2** sequence interacted with the SH3$_{m\text{-}cort}$ Tyr541 and Trp525 residues, respectively (Rubini et al. 2010). The

pyrrolidine ring of the Pro-1 residue adopts almost an un-puckering conformation which can make an edge-face arrangement to form a strong aromatic-proline interaction (Bhattacharyya and Chakrabarti 2003) with the indole ring of Trp525. Thus a strongly preorganized exo ring pucker would push the Cγ atom and the pyrrolidine ring away from Trp525 weakening the aromatic-proline interaction, which cannot be compensated by the increase in the C–H$\cdots\cdot\pi$ interaction due to the inductive effect imposed by the γ-substituent (F or OH). Recent studies using conformationally biased proline derivative showed that a preorganized ring pucker matching the native proline conformation would stabilize the protein structure; otherwise, a destabilizing effect would be observed when residues are involved in protein interaction (Shoulders et al. 2010; Zheng et al. 2010).

Additionally, our data also provide the evidence that the $SH3_{m\text{-}cort}$ domain recognizes proline-rich sequences, which contain basic residues outside the PxxPxK core. In particular, a Lys residue at the −5 position of the peptide-ligand is markedly effective in promoting the binding to the $SH3_{m\text{-}cort}$ domain by the interaction with Asp505 belonging to Surface II binding site (Rubini et al. 2010). The absence of increased affinity towards the $SH3_{m\text{-}cort}$ domain after the induction of a stable PPII helical conformation in the peptide-ligand suggests that a rigid scaffold is not suited for the ligand-binding surface, more likely due to a decrease of the peptide flexibility necessary for an optimal interaction with both Surface I and Surface II of the $SH3_{m\text{-}cort}$ domain.

Therefore, the binding affinity of the proline-rich peptides to the $SH3_{m\text{-}cort}$ domain is the result of a delicate balance of mutually compensating contributions that play a role in the binding energy: the PPII helix conformation that promotes aromatic-proline interactions, and the ionic interaction that involves the Lys-5 residue outside the classical class II binding motif.

Conclusions

An increasing body of evidence highlights the importance of SH3-mediated protein interactions in critical signaling processes that result in cancers and other pathological conditions. Therefore, several attempts have been performed aimed at generating drugs that can interfere with the SH3-mediated processes. However, the promiscuity and restrictions characterizing the SH3/ligand interactions are challenges for rational drug design. In this respect, the finding that the critical step in the $SH3_{m\text{-}cort}$/ligand interaction involves both aromatic-proline interaction and ionic interaction outside classical class II binding motif allows the design of specific inhibitors of biological pathways, where cortactin-signaling has been demonstrated to be involved in malignancy.

Acknowledgments We are very grateful to Dr. D. Montagner for helpful discussions we had.

Conflict of interest The authors declare that they have no conflict of interest.

References

Alabugin IV, Zeidan TA (2002) Stereoelectronic effects and general trends in hyperconjugative acceptor ability of sigma bonds. J Am Chem Soc 124:3175–3185

Bhattacharyya R, Chakrabarti P (2003) Stereospecific interactions of proline residues in protein structures and complexes. J Mol Biol 331:925–940

Bretscher LE, Jenkins CL, Taylor KM, DeRider ML, Raines RT (2001) Conformational stability of collagen relies on a stereoelectronic effect. J Am Chem Soc 123:777–778

Cohen GB, Ren RB, Baltimore D (1995) Modular binding domains in signal-transduction proteins. Cell 80:237–248

DeRider ML, Wilkens SJ, Waddell MJ, Bretscher LE, Weinhold F, Raines RT, Markley JL (2002) Collagen stability: insights from NMR spectroscopic and hybrid density functional computational investigations of the effect of electronegative substituents on prolyl ring conformations. J Am Chem Soc 124:2497–2505

Dourado D, Fernandes PA, Ramos MJ (2008) Mammalian cytosolic glutathione transferases. Curr Protein Pept Sci 9:325–337

Drake AF, Siligardi G, Gibbons WA (1988) Reassessment of the electronic circular dichroism criteria for random coil conformations of poly(L-lysine) and the implications for protein folding and denaturation studies. Biophys Chem 31:143–146

Eberhardt ES, Panasik N, Raines RT (1996) Inductive effects on the energetics of prolyl peptide bond isomerization: implications for collagen folding and stability. J Am Chem Soc 118:12261–12266

Feng SB, Chen JK, Yu HT, Simon JA, Schreiber SL (1994) Two binding orientations for peptides to the Src SH3 domain—development of a general-model for SH3–ligand interactions. Science 266:1241–1247

Flemer S, Wurthmann A, Mamai A, Madalengoitia JS (2008) Strategies for the solid-phase diversification of poly-L-proline-type II peptide mimic scaffolds and peptide scaffolds through guanidinylation. J Org Chem 73:7593–7602

Hinderaker MP, Raines RT (2003) An electronic effect on protein structure. Protein Sci 12:1188–1194

Horng J-C, Raines RT (2006) Stereoelectronic effects on polyproline conformation. Protein Sci 15:74–83

Improta R, Benzi C, Barone V (2001) Understanding the role of stereoelectronic effects in determining collagen stability. 2. A quantum mechanical study of proline, hydroxyproline, and fluoroproline dipeptide analogues in aqueous solution. J Am Chem Soc 123:12568–12577

Jacquot Y, Broutin I, Miclet E, Nicaise M, Lequin O, Goasdoué N, Joss C, Karoyan P, Desmadril M, Ducruix A, Lavielle S (2007) High affinity Grb2-SH3 domain ligand incorporating Cβ-substituted prolines in a Sos-derived decapeptide. Bioorg Med Chem 15:1439–1447

Kelly MA, Chellgren BW, Rucker AL, Troutman JM, Fried MG, Miller AF, Creamer TP (2001) Host-guest study of left-handed polyproline II helix formation. Biochemistry 40:14376–14383

Kim JM, Lee CD, Rath A, Davidson AR (2008) Recognition of non-canonical peptides by the yeast Fus1p SH3 domain: elucidation of a common mechanism for diverse SH3 domain specificities. J Mol Biol 377:889–901

Kotch FW, Guzei IA, Raines RT (2008) Stabilization of the collagen triple helix by O-methylation of hydroxyproline residues. J Am Chem Soc 130:2952–2953

Kuemin M, Nagel YA, Schweizer S, Monnard FW, Ochsenfeld C, Wennemers H (2010) Tuning the *cis*/*trans* conformer ratio of Xaa-Pro amide bonds by intramolecular hydrogen bonds: the effect on PPII helix stability. Angew Chem-Int Edit 49:6324–6327

Kümin M, Sonntag LS, Wennemers H (2007) Azidoproline containing helices: stabilization of the polyproline II structure by a functionalizable group. J Am Chem Soc 129:466–467

Larson SM, Davidson AR (2000) The identification of conserved interactions within the SH3 domain by alignment of sequences and structures. Protein Sci 9:2170–2180

Li SSC (2005) Specificity and versatility of SH3 and other proline-recognition domains: structural basis and implications for cellular signal transduction. Biochem J 390:641–653

Lim WA, Richards FM, Fox RO (1994) Structural determinants of peptide-binding orientation and of sequence specificity in SH3 domains. Nature 372:375–379

Liu F, Stephen AG, Waheed AA, Freed EO, Fisher RJ, Burke TR Jr (2010) Application of ring-closing metathesis macrocyclization to the development of Tsg101-binding antagonists. Bioorg Med Chem Lett 20:318–321

Liu F, Giubellino A, Simister PC, Qian W, Giano MC, Feller SM, Bottaro DP, Burke TR (2011) Application of ring-closing metathesis to Grb2 SH3 domain-binding peptides. Biopolymers 96:780–788

Marchiani A, Antolini N, Calderan A, Ruzza P (2009) Atypical SH3 domain binding motifs: features and properties. Curr Top Pept Protein Res 10:45–56

Mayer BJ (2001) SH3 domains: complexity in moderation. J Cell Sci 114:1253–1263

Panasik N Jr, Eberhardt ES, Edison AS, Powell DR, Raines RT (1994) Inductive effects on the structure of proline residues. Int J Pept Protein Res 44:262–269

Renner C, Alefelder S, Bae JH, Budisa N, Huber R, Moroder L (2001) Fluoroprolines as tools for protein design and engineering. Angew Chem Int Ed 40:923–925

Rubini C, Ruzza P, Spaller MR, Siligardi G, Hussain R, Udugamasooriya DG, Bellanda M, Mammi S, Borgogno A, Calderan A, Cesaro L, Brunati AM, Donella-Deana A (2010) Recognition of lysine-rich peptide ligands by murine cortactin SH3 domain: CD, ITC, and NMR studies. Biopolymers 94:298–306

Rucker AL, Pager CT, Campbell MN, Qualls JE, Creamer TP (2003) Host-guest scale of left-handed polyproline II helix formation. Proteins 53:68–75

Ruzza P, Siligardi G, Donella-Deana A, Calderan A, Hussain R, Rubini C, Cesaro L, Osler A, Guiotto A, Pinna LA, Borin G (2006) 4-Fluoroproline derivative peptides: effect on PPII conformation and SH3 affinity. J Pept Sci 12:462–471

Shi ZS, Olson CA, Rose GD, Baldwin RL, Kallenbach NR (2002) Polyproline II structure in a sequence of seven alanine residues. Pro Natl Acad Sci USA 99:9190–9195

Shoulders MD, Hodges JA, Raines RT (2006) Reciprocity of steric and stereoelectronic effects in the collagen triple helix. J Am Chem Soc 128:8112–8113

Shoulders MD, Satyshur KA, Forest KT, Raines RT (2010) Stereoelectronic and steric effects in side chains preorganize a protein main chain. Proc Natl Acad Sci USA 107:559–564

Siligardi G, Drake AF (1995) The importance of extended conformations and, in particular, the PII conformation for the molecular recognition of peptides. Biopolymers 37:281–292

Siligardi G, Hussain R (1998) Biomolecules interactions and competitions by non-immobilised ligand interaction assay by circular dichroism. Enantiomer 3:77–85

Siligardi G, Ruzza P, Hussain R, Cesaro L, Brunati AM, Pinna LA, Donella-Deana A (2012) The SH3 domain of HS1 protein recognizes lysine-rich polyproline motifs. Amino Acids 42:1361–1370

Taylor CM, Hardre R, Edwards PJB (2005) The impact of pyrrolidine hydroxylation on the conformation of proline-containing peptides. J Org Chem 70:1306–1315

Tuchscherer G, Grell D, Tatsu Y, Durieux P, Fernandez-Carneado J, Hengst B, Kardinal C, Feller S (2001) Targeting molecular recognition: exploring the dual role of functional pseudoprolines in the design of SH3 ligands. Angew Chem Int Ed 40:2844–2848

Venugopal MG, Ramshaw JAM, Braswell E, Zhu D, Brodsky B (1994) Electrostatic interactions in collagen-like triple-helical peptides. Biochemistry 33:7948–7956

Wellings DA, Atherton E (1997) Standard Fmoc protocols. In: Fields GB (ed) Solid-phase peptide synthesis, vol 289. Academic Press, New York, pp 44–67

Wolfe S (1972) Gauche effect. Stereochemical consequences of adjacent electron pairs and polar bonds. Acc Chem Res 5:102–111

Woody RW (1992) Circular dichroism and conformation of unordered polypeptides. Adv Biophys Chem 2:37–79

Woody RW (2010) Circular dichroism of intrinsically disordered proteins. In: Uversky V, Longhi S (eds) Instrumental analysis of intrinsically disordered proteins: assessing structure and conformation. Wiley, pp 303–321

Zarrinpar A, Bhattacharyya RP, Lim WA (2003) Sci STKE: RE8.

Zheng TY, Lin YJ, Horng JC (2010) Thermodynamic consequences of incorporating 4-substituted proline derivatives into a small helical protein. Biochemistry 49:4255–4263