Binding Orientation of Proline-Rich Peptides in Solution: Polarity of the Profilin−Ligand Interaction

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The recognition of proline-rich sequences is a common strategy for the assembly and regulation of macromolecular complexes responsible for biological function.[1] The modules that bind proline-rich sequences, including SH3,[2] WW,[3] and EVH1[4] domains and profilin,[5] all utilize a series of conserved aromatic side chains to recognize the unique features of the left-handed type II poly-L-proline helix (PPII) present in their binding partners. The PPII helix is characterized by a 3 Å rise and 120° rotation per residue, and possesses strong pseudo-2-fold symmetry that preserves similar van der Waals and hydrogen bonding interactions with the binding modules, regardless of peptide orientation. These properties are responsible for the well-documented ability of SH3 modules to accommodate proline-rich ligands in either of two opposite polarities.

Profilin is a small (∼15 kD) actin regulatory protein that participates in site specific actin filament assembly by binding to highly proline-rich sequences in its target proteins.[5] We recently demonstrated that (i) eight consecutive proline residues are sufficient to span the binding surface and (ii) this sequence represents the core target sequence present in a number of profilin binding partners.[5] Furthermore, the use of N- and C-terminally tagged proline oligomers provided direct crystallographic evidence that profilin, like SH3 domains, can bind proline-rich peptides in either of two backbone orientations.[5] As has been previously proposed for SH3-related functions,[2] the ability of profilin to bind ligands in multiple orientations may control the organization of multicomponent signaling complexes, and provides a mechanism for the modulation of actin filament assembly. Identifying the mechanisms that control binding polarity will be essential for understanding profilin function *in vivo*.

We describe an NMR approach involving spin labeled peptides that circumvents potential crystallization artifacts and allows for the rapid determination of peptide binding orientation in solution. Paramagnetic spin labels increase nuclear relaxation rates in a distance-dependent manner ($1/r^6$), resulting in significant broadening of the NMR resonances of atoms in close proximity (<15−20 Å) to the free radical.[6] These relaxation effects provide a simple and direct means to map the location of specific peptide residues with respect to the protein binding surfaces (Figure 1).

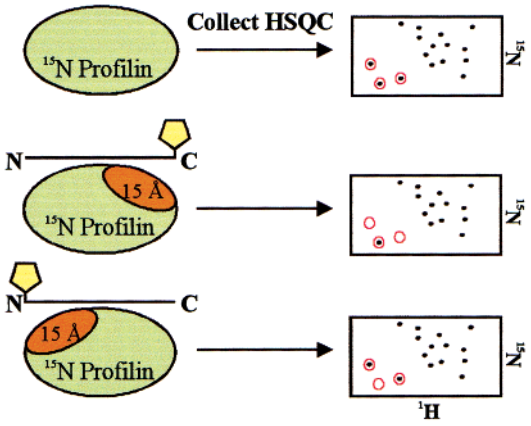

Figure 1. Strategy for NMR experiments. ^{15}N-labeled human profilin is used to generate 2D HSQC spectra in the absence (top) and presence (middle and bottom) of spin-labeled ligands. Each cross-peak represents an individual nitrogen-bound proton. The protein residues within ∼15 Å of the spin label (yellow) nitroxide moiety are shown in orange. Red circles indicate the resonances from residues at the poly-L-proline binding site that are subject to broadening. The pattern of affected residues reflects the location of the spin label and consequently the orientation of the bound peptide.

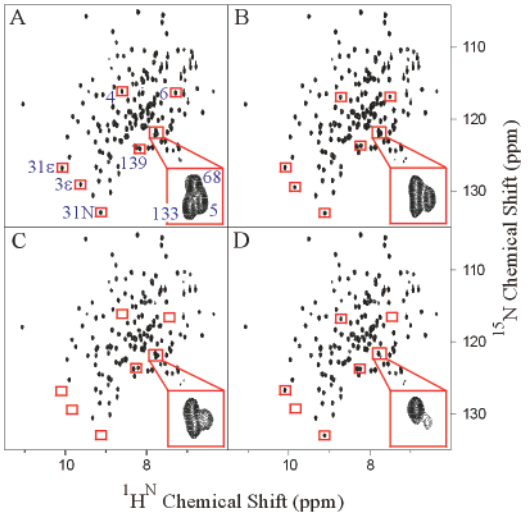

Figure 2. Effect of spin labeled peptides on human profilin. Cross-peaks corresponding to residues constituting, or in close proximity to the poly-L-proline binding site, including residues W3, Y6, W31, H133, and Y139, are highlighted (red boxes). (a) HSQC spectrum of ^{15}N-profilin. (b) HSQC spectrum of ^{15}N-profilin bound to underivatized Ac-$(Pro)_8$-Cys peptide. (c) HSQC spectrum of ^{15}N-profilin bound to Proxyl-Cys-$(Pro)_8$ peptide. (d) HSQC spectrum of ^{15}N-profilin bound to $(Pro)_8$-Cys-Proxyl peptide. All NMR samples contained 540 μM ^{15}N-profilin. Peptides were used in 1.0−1.1 molar ratios. NMR spectra were acquired at 293 K on a Bruker DRX-600 spectrometer. Gradient enhanced HSQC spectra[10] were processed using nmrPipe.[11]

A series of (^{1}H,^{15}N) HSQC spectra were obtained from ^{15}N-labeled human profilin[7] bound to either underivatized or 3-maleimido-Proxyl derivatized Cys-$(Pro)_8$ and $(Pro)_8$-Cys peptides[8] (Figure 2). HSQC spectra of ^{15}*N*-profilin were consistent with those published previously,[9] allowing for unambiguous identification of all cross-peaks. The two underivatized peptides induce

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(7) ^{15}N-labeled human profilin was purified by poly-L-proline affinity chromatography as previously described[5] and dialyzed against 10 mM KPO_4 and 25 mM KCl, pH 6.4, for NMR.^{15}N-labeling was accomplished by providing 1 g/L of $(^{15}NH_4)_2SO_4$ as the sole nitrogen source during cell growth.

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Table 1. Cross-Peak Intensities and Calculated Nitroxide−Amide Proton Distances for Human Profilin

	$CysPro_8$				Pro_8Cys			
residue	$\Delta\nu_{1/2}$ (Hz)[a]	amplitude ratio[b]	calcd distance[c] (Å)	model distance[d] (Å)	$\Delta\nu_{1/2}$ (Hz)[a]	amplitude ratio[b]	calcd distance[c] (Å)	model distance[d] (Å)
Trp31ϵ	77.3	<0.01	5.6	8.6	24.4	0.05	8.4	15.4
Asn4	28.6	0.04	8.1	9.3	18.3	0.59	15.4	12.3
Ala5	22.4	0.61	15.0	12.5	18.3	0.17	11.1	9.8
Tyr6	48.8	<0.01	6.0	11.1	33.6	0.02	6.7	9.4
Ile7	26.5	0.81	17.4	14.1	20.3	0.29	12.3	10.7
Asp13	16.2	1.05	>20	25.0	16.3	0.19	11.6	13.2
Trp31N	28.6	0.23	10.9	10.5	28.5	1.01	>20	19.1
Trp31ϵ	14.3	0.01	7.4	9.4	16.3	0.82	19.0	21.1
His133	20.2	0.96	24.4	22.9	20.4	0.13	10.3	12.6
Tyr139	30.5	1.04	>20	19.4	28.5	0.88	18.9	15.6

[a] The amide proton line width at half-height in the control sample. [b] HSQC intensity in the presence of spin-labeled peptides divided by the intensity with unlabeled peptides. Spectra were normalized with a scale factor calculated from resonances of residues distant from the binding site. [c] Distances between the nitroxide and amide protons were calculated as described by Yu et al.,[12] using a $\tau_c = 1.06 \times 10^{-08}$ obtained from ^{15}N relaxation data (not shown). [d] A model of the Pro_8 ligand proxylated at both termini was docked to the profilin structure by simulated annealing using XPLOR 3.851.[13] The profilin coordinates were fixed, the proline residues of the peptide were constrained to the conformation observed in the crystal structure. Calculated distances between the proxyl-maleimido-Cys moiety at each end of the peptide and profilin protons were used as restraints. Simulated annealing and final energy minimization resulted in a peptide orientation consistent with the Pro_{15} crystal structure, regardless of the initial orientation of the peptide model.

nearly identical shifts for residues at the poly-L-proline binding site, consistent with previous studies.[9] In the presence of peptides bearing spin labels, the cross-peaks corresponding to residues at or near the poly-L-proline binding site are significantly reduced in intensity. Reduction of the spin labels with 5 mM ascorbate confirms that the nitroxide moiety is responsible for the loss of intensity (data not shown). Importantly, two different patterns of affected resonances are observed, depending upon whether the spin label resides at the N- or C-terminus of the peptide (Table 1 and Figure 2). These observations show that the two spin labels localize to different regions of the profilin molecule for the Proxyl-Cys-$(Pro)_8$ or $(Pro)_8$-Cys-Proxyl peptides, a situation that is only fulfilled when the amide backbone orientation is identical for both bound peptides. The specific pattern of altered cross-peak intensities, including the nearly complete elimination of the cross-peak corresponding to Trp31ϵ with the Proxyl-Cys-$(Pro)_8$ peptide and complete elimination of His133 with the $(Pro)_8$-Cys-Proxyl peptide, indicates that the N-termini of these peptides contact profilin near Trp31, while the C-termini are located near His133. These two residues, Trp31 and His133, reside at the extremes of the peptide binding site and, owing to the $1/r^6$ distance dependence of the broadening effect, are the most diagnostic for peptide orientation. All other effects are consistent with the assigned polarity (Table 1).

Although the difference in binding energies between the two orientations is predicted to be small, these results show that the L-Pro_8 sequence present in a number of profilin binding partners exhibits a preferred binding mode in solution. The orientation observed in solution is consistent with the crystal structure of the complex between profilin and Pro_{15} tagged at the N-terminus with hydroxymethylcoumarin, but contrasts with the orientation observed in the crystal structure of profilin complexed with Pro_{10} tagged at the C-terminus with iodo-tyrosine.[5] The observation of only a single binding mode in the solution could be due to several factors. The sensitivity of the NMR experiments would preclude the observation of a small population in which the peptide is bound in an alternate orientation (i.e., less than 10%). Additionally, the chemical tags required to identify the peptide termini in the crystallographic studies could bias binding polarity. Regardless of the mechanism, the observation of two orientations in the crystalline state highlights the energetic similarity of the two binding modes, and suggests that binding orientation could readily be controlled by determinants in the primary target peptide sequence or through interactions with other signaling and regulatory proteins.

The determinants that regulate the profilin−peptide interaction are central to profilin function, as binding polarity should have significant implications for the assembly and organization of the multicomponent complexes that regulate actin filament assembly *in vivo*. The observation of binding degeneracy in both profilin and SH3 domains suggests that this property may be a general feature of modules that bind proline-rich ligands, including WW and EVH1 domains. The NMR method described here provides a general approach to determine the binding orientation for a wide range of ligands in solution, and is directly applicable to examining the effects of peptide length and composition on binding to these modules. Once the backbone NMR resonances of a particular protein module have been assigned, a series of HSQC spectra allows the binding orientation to be determined rapidly, without requiring a complete structure determination.

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(8) The N-terminally blocked peptides, Ac-Cys-$(Pro)_8$ and Ac-$(Pro)_8$-Cys (940 M_r), were coupled to 3-maleimido-Proxyl (2,2,5,5-tetramethyl-1-pyrrolidinyloxy) in 100 mM MOPS buffer, pH 7.0, at room temperature for >10 h. The reaction mixture was fractionated by reverse phase HPLC, and the resulting derivatized peptides were characterized by mass spectrometry and quantitative amino acid analysis.

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