Structural Requirements and Thermodynamics of the Interaction of Proline Peptides with Profilin†

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ABSTRACT: The binding to poly(L-proline) is used for the affinity purification of profilins, but little is known about the structural and thermodynamic aspects of the interaction. We used changes in the intrinsic fluorescence of profilin, CD spectroscopy, and isothermal titration calorimetry to assess how the size and composition of synthetic proline-rich peptides influence binding to *Acanthamoeba* and human profilins. Although a 6 residue type II poly(L-proline) helix can span the binding site, highest affinity binding is achieved by proline oligomers ≥10 residues. Binding is stereospecific since $(D\text{-proline})_{11}$ does not bind. In 75 mM KCl the dissociation equilibrium constant for poly(L-proline) is about 10 μM proline decamer units for amoeba profilin and 20−30 μM for human profilin. Consistent with a significant hydrophobic component of the interaction, ΔC_p is negative and higher salt concentrations enhance the affinity. No protons dissociate or bind during the interaction. Binding of poly(L-proline) is favored both entropically and enthalpically. Substitution of glycine in proline undecamers reduces affinity by about 1 kcal mol^{-1} for each substitution due to increased rotational freedom of the free peptides. Substitution of alanine has a similar effect. Disorder in the free peptides imparts an unfavorable entropic cost for immobilizing the substituted peptides on the binding site on profilin.

Profilins from many sources interact with actin, polyphosphoinositides, and poly(L-proline) (Machesky & Pollard, 1993). Profilin binding to actin inhibits the nucleation and elongation phases of actin polymerization, but profilin can also deliver actin to the barbed end of actin filaments (Pollard & Cooper, 1986; Pantaloni & Carlier, 1993). Even at substoichiometric concentrations, profilin also accelerates the exchange of nucleotide bound to actin (Mockrin & Korn, 1980; Goldschmidt-Clermont *et al.*, 1991a). Profilin bound to phosphatidylinositol 4,5-bisphosphate (PIP_2) inhibits the production of the second messengers IP_3 and diacylglycerol by unphosphorylated, but not phosphorylated phospholipase C-γ1 from bovine brain (Goldschmidt-Clermont *et al.*, 1991b). Release of profilin from membrane binding sites by PIP_2 hydrolysis may link signaling pathways and the cytoskeleton. Profilin also binds a macromolecular complex consisting of actin-related protein 2 (Arp2), Arp3, and five novel proteins localized in the cortex of *Acanthamoeba* (Machesky *et al.*, 1994a; Kelleher *et al.*, 1995).

Profilin binds to a poly(L-proline) affinity column used to purify prolyl hydroxylase (Tuderman *et al.*, 1975; Tanaka & Shibata, 1985). This column provides a convenient method to purify profilin from diverse sources (Tanaka & Shibata, 1985; Lindberg *et al.*, 1988; Kaiser *et al.*, 1989; Janmey, 1991). NMR studies provided the data to map the poly(L-proline) binding sites of amoeba (Archer *et al.*, 1994) and human profilin (Metzler *et al.*, 1994). Most of the residues perturbed by poly(L-proline) binding lie in a shallow groove between the N- and C-terminal helices and the underlying β-sheet. Mutations or deletions of aromatic residues exposed to the solvent in this region confirm their importance for poly(L-proline) binding (Björkegren *et al.*, 1993; Kaiser & Pollard, 1996; Vinson and Pollard, unpublished).

In water, poly(L-proline) forms a left-handed helix with trans peptide bonds and 3 residues per turn (type II), while in organic solvents such as 1-propanol it forms a right-handed cis helix (type I) (Cantor & Schimmel, 1980; Rabanal *et al.*, 1993). Under physiological conditions, sequences of ≥3 consecutive prolines may form type II helices (Brandts & Lin, 1979; Winklmair *et al.*, 1971).

The physiological significance of profilin binding to poly(L-proline) is unknown. Many proteins contain sequences of 4−8 consecutive prolines (Williamson, 1994; Metzler *et al.*, 1994), but only VASP (vasodilator-stimulated phosphoprotein) is known to bind the poly(L-proline) site of profilin (Reinhard *et al.*, 1995). Other candidate proteins, such as vinculin (Coutu *et al.*, 1987), calcineurin (Guerni & Klee, 1989), ActA from *Listeria monocytogenes* (Kocks *et al.*, 1992), and cyclase-associated protein (Field *et al.*, 1990) still must be tested in detail.

We report the effects of size and composition of type II helical peptides on their affinity for profilin. The interaction is stereospecific and requires about ten residues of L-proline. The stability of the helical conformation of the free peptide is the major determinant of the affinity.

EXPERIMENTAL PROCEDURES

Peptides. Proline peptides $L\text{-}Pro_6$, $L\text{-}Pro_{10}$, and $L\text{-}Pro_{15}$ were made on a MilliGen/Biosearch 9050 peptide synthesizer (Millipore, Inc.) using *N*-[(fluorenylmethoxy)carbonyl] (Fmoc) amino acids. The synthesis utilized Fmoc-L-proline-OH starting resin, an 8-fold molar excess of Fmoc-L-proline-OH,

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diisopropylethylamine (DIPEA) base in 2-fold molar excess over activator (*O*-(7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate, (HATU)), 30 min acylation time, and 1 h cycle time. Peptides were cleaved from the resin by treatment with 100% trifluoroacetic acid (TFA) for 30 min. The cleavage product was collected and dried down under a stream of nitrogen, resuspended in a small volume of glacial acetic acid, and lyophilized. The lyophilized crude peptide was resuspended in a small volume of distilled deionized water/0.1% TFA and purified by reversed-phase high-performance liquid chromatography (HPLC) on a C_{18} column utilizing CH_3CN/water gradients. 0.1% TFA was routinely included in HPLC solvents.

We obtained L-Pro_8, L-Pro_{11}, L-P_5GP_5, L-P_5AP_5, L-$(P_3G)_2P_3$, L-$(P_2G)_3P_2$, D-Pro_{11}, and additional L-Pro_6 from PeptidoGenic, Inc. (Livermore, CA). These peptides were >90% pure by HPLC and electrospray ionization mass spectrometry performed by the supplier. The commercial peptides contained substantial amounts of nonvolatile acid and minor fluorescent contaminants which we removed by gel filtration on a 0.7 × 25 cm column of Bio-Gel P2 (Bio-Rad) equilibrated with deionized H_2O. Sample load was 100 μL, and 830 μL fractions were collected at a linear flow rate of 5 cm h^{-1} and checked for the presence of peptide by absorption at 204 nm. L-Proline-proline-glycine (L-$[PPG]_5$) and L-proline-hydroxyproline-glycine ($[P{\cdot}P^{OH}{\cdot}G]_5$) pentadecamers were a generous gift of Dr. Jürgen Engel, Universität Basel, Switzerland. Tim Worral of the Mid-Atlantic Mass Spectrometry Facilty at Johns Hopkins University assessed the purity of L-Pro_{10}, L-Pro_{15}, L-$(PPG)_5$, and L-$(P{\cdot}P^{OH}{\cdot}G)_5$ by matrix-assisted laser desorption/ionization mass spectrometry. For all peptides, a single molecular mass peak dominated the spectrum.

Molar extinction coefficients (ϵ_{204}, $\times 10^{-4}$ M^{-1} cm^{-1}) for purchased peptides were determined gravimetrically (L-Pro_6 = 2.84 , L-Pro_8 = 3.65, L-Pro_{11} and D-Pro_{11} = 5.63, L-P_5GP_5 = 4.88, L-P_5AP_5 = 5.20, L-$(P_3G)_2P_3$ = 4 .42, L-$(P_2G)_3P_2$ = 4.59) and for L-Pro_{10} (4.98) and L-Pro_{15} (7.82) by interpolation/extrapolation from a standard curve of ϵ_{204} vs homooligomer length.

Sigma Chemical Co. prepared 4.9 and 8 kDa poly(L-proline) by base-initiated polymerization of the *N*-carboxyanhydride of L-proline and purified a weight-average sized polymer by gel filtration. The supplier determines weight-average molecular weight by solution viscosity and static low angle laser light scattering. We refer to these polymers as L-Pro_{50} and L-Pro_{82}. The absorption maximum of proline polymers is 204 nm, and the extinction coefficient per mole of proline residues is $\epsilon_{204} = 5.63 \times 10^3$ M^{-1} cm^{-1}.

Proteins. We purified *Acanthamoeba castellanii* profilin I and profilin II (Kaiser *et al.*, 1989), recombinant amoeba profilins (Vinson *et al.*, 1993), human platelet profilin (Goldschmidt-Clermont *et al.*, 1991a), and recombinant human profilin I (Federov *et al.*, 1994a) by poly(L-proline) affinity chromatography and determined their concentrations by absorbance at 280 nm using ϵ_{280} of 1.4×10^4 M^{-1} cm^{-1} (Tseng *et al.*, 1984).

Circular Dichroism Spectroscopy. Circular dichroism (CD) measurements were performed on an Aviv 60DS spectrometer utilizing a 0.1 mm path length demountable quartz cuvette (Hellma). The instrument was purged with nitrogen. A water-jacketed cuvette holder maintained the sample temperature at 20 °C. Spectra represent an average of five repetitive scans made at 0.5 nm intervals, 1 s signal integration time, and 1.5 nm bandwidth. The averaged spectra were corrected by subtraction of averaged buffer baseline scans and smoothed using a third-order polynomial with a ±2-point averaging algorithm. Each of the corrected, smoothed spectra were *y*-axis offset-adjusted such that ellipticity at 260 nm is equal to zero. Data are plotted as the mean residue ellipticity ($[\theta]_\lambda$) calculated from the relationship:

$$[\theta]_\lambda = \frac{M\theta_\lambda}{10dc}$$

where M is the mean amino acid residue molecular weight in g/mol, θ_λ is the observed ellipticity in deg, d is the cuvette path length in cm, and c is the concentration of peptide in g mL^{-1}.

Fluorescence Spectroscopy. Equilibrium binding of proline peptides to profilin was quantitated using a change in the intrinsic fluorescence of profilin. To consider solely tryptophan fluorescence, the excitation wavelength used routinely was 295 nm. Data were collected at 22 °C with a PTI AlphaScan Model A1010 spectrofluorometer (Photon Technologies, Inc.) with monochrometer slitwidths set at 2 nm. Fluorescence titrations were performed by incremental additions of concentrated peptide in 5 μM profilin to 5 μM profilin in 75 mM KCl, 10 mM Tris, pH 7.5, in a 1 × 0.1 cm quartz cuvette (Hellma, Inc.). When titrant solutions did not contain profilin, data were corrected for profilin dilution. Emission spectra were collected from 300 to 400 nm at 0.5 nm intervals with a 0.25 s integration time. Spectra were corrected by subtraction of background buffer scans, but were not smoothed. Alternatively, titration data were collected by recording the magnitude of the emission at a fixed wavelength of 318 nm.

We used the global target data analysis program Spectrabind (Toptygin & Brand, 1995) to analyze the families of fluorescence spectra generated by peptide/profilin titrations. This program determines the spectroscopic characteristics and concentrations of the individual species in the binding reaction using model equilibrium equations with different stoichiometries as constraints and derives binding constants using nonlinear least-squares procedures. For titrations performed by recording emission at 318 nm, data were fit directly to a rectangular hyperbola by the nonlinear least squares fitting programs KFIT (Neil C. Millar) or Origin (MicroCal Software) assuming ligand binding to identical noninteracting sites.

The biochemical standard state free energy change of binding ($\Delta G^{\circ\prime}_{bind}$) was calculated from the dissociation equilibrium constant (K_d) using the relationship

$$\Delta G^{\circ\prime}_{bind} = -RT \ln(1/K_d)$$

where R is the gas constant (1.987 cal mol^{-1} K^{-1}) and T is temperature in K.

For collisional quenching experiments 3 mL of 5 μM *Acanthamoeba* profilin I was titrated with quenchers from 3 M stock solutions (except for nitromethane, which was 0.5 M). The excitation wavelength was 295 nm, and emission was observed at 330 nm for free profilin I and 325 nm for profilin saturated with 1 mM L-Pro_{50}. Fluorescence readings were corrected for dilution. The Stern−Volmer quenching constant (K_{sv}) is the slope of a plot of F_0/F versus the quencher concentration, where F_0 is the fluorescence without

quencher and F is the fluorescence at a particular quencher concentration (Lakowicz, 1983).

Calorimetry. Isothermal titration calorimetery (ITC) was performed with an Omega titration calorimeter (MicroCal, Inc.) equipped with a 1.4 mL reaction cell. Titrations were performed by injecting peptide solutions, 5 or 10 μL × 20−45 injections, into a profilin solution. The concentration of reactants was such that the experimental c value was ≥1, where

$$c = K_a M_{tot} n$$

and K_a is the association equilibrium constant, M_{tot} is the total macromolecule concentration in the cell at the start of the experiment, and n is the stoichiometry. These conditions allow for accurate determination of the biochemical standard state enthalpy change upon binding ($\Delta H^{\circ\prime}_{bind}$), K_a, and n. Both protein and peptide were in 75 mM KCl, 3.1 mM NaN_3, and either 10 mM Tris or potassium phosphate, pH 7.5. Titrations were conducted at 25, 28, 30, or 35 °C. The raw data correspond to the power, per unit time, required to maintain a constant temperature of the reaction cell after each injection. Software supplied with the calorimeter (Origin for ITC, MicroCal Software) determined the peak areas. Control injections of peptide into buffer were performed to correct for heat effects not directly related to the binding reaction. For most titrations, the heats of dilution for control injections were averaged, and the average subtracted from each injection heat for the binding experiment. Where quantities of peptide were not limiting, the full heat of dilution control titration was performed and subtracted from the experiment.

Data were analyzed using Origin for ITC (MicroCal Software). The program generates binding isotherms by plotting heats of reaction per mole of peptide injected versus the ratio of total peptide to total protein (X_{tot}/M_{tot}) per injection. Binding isotherms were analyzed directly utilizing the Marquardt (Bevington, 1969) nonlinear least squares fitting routine. The equations used for fitting take into account the volume displaced by each injection. For titrations performed under ideal conditions, *i.e.*, 5 < c value < 500, we allowed Origin for ITC to float all three parameters, $\Delta H^{\circ\prime}_{bind}$, K_a, and n, whereas for weaker binding peptides where titrations were performed at or near the lower limit of c, we fixed n at 1:1.

To determine whether protonation/deprotonation is involved in the binding of poly(L-proline) to profilin, we performed calorimetric titrations at constant pH in buffers with different heats of ionization ($\Delta H^{\circ\prime}_{ion}$). $\Delta H^{\circ\prime}_{bind}$ is the sum of the change in reaction enthalpy (which is independent of the buffer) and $\Delta H^{\circ\prime}_{ion}$ times the number of protons bound or released by the buffer. The relationship between $\Delta H^{\circ\prime}_{bind}$ versus $\Delta H^{\circ\prime}_{ion}$ yields the number of protons released by the buffer. No difference in $\Delta H^{\circ\prime}_{bind}$ between buffers indicates that binding does not involve protonation/deprotonation.

We determined the heat capacity change (ΔC_p) for the interaction of profilin and poly(L-proline) by performing calorimetric titrations at different temperatures. ΔC_p is given by the slope of a plot of $\Delta H^{\circ\prime}_{bind}$ versus temperature. Using the derived ΔC_p for the interaction of profilin with poly(L-proline), we adjusted the values of $\Delta G^{\circ\prime}_{bind}$ for peptides obtained from fluorescence measurements at 22 °C to 28 °C, the temperature at which the calorimetry experiments on peptides were performed, using the Gibbs−Helmholtz equation (Stenesh, 1993):

$$\left(\frac{(\Delta G)_{T_2}}{T_2}\right) - \left(\frac{(\Delta G)_{T_1}}{T_1}\right) = -\int_{T_1}^{T_2} \left(\frac{(\Delta H)_T}{T^2}\right) dT$$

where T is temperature in K, ΔG (at T_1) and ΔG (at T_2) are biochemical standard state free energy changes at T_1 and T_2, and ΔH (at T) is the enthalpy change at a given temperature. This form of the equation assumes a temperature dependence of ΔH and constant pressure.

The overall entropy change ($\Delta S^{\circ\prime}$) for the binding of proline peptides to profilin was calculated from $\Delta G^{\circ\prime}_{bind}$ and $\Delta H^{\circ\prime}_{bind}$ using the relationship

$$\Delta G^{\circ\prime}_{bind} = \Delta H^{\circ\prime}_{bind} - T\Delta S^{\circ\prime}$$

RESULTS

Conformation of Proline Peptides and Polymers. L-Pro_{82} has a circular dichroism spectrum typical of a type II poly(L-proline) (PPII) helix (Rabanal *et al.*, 1993; Sreerama & Woody, 1994) with a large negative band (-5.5×10^4 deg cm^2 dmol^{-1}) at 204.5 nm and a small positive band (2163 deg cm^2 dmol^{-1}) at 228 nm (Figure 1A). The shorter L-proline oligomers, L-Pro_6 to L-Pro_{15}, have qualitatively similar CD spectra, as expected from previous infrared (Isemura *et al.*, 1968), NMR (Rothe *et al.*, 1976), and CD (Isemura *et al.*, 1968; Rothe *et al.*, 1976) spectroscopic studies showing that oligomers of ≥4 prolines form PPII helices. For peptides of <15 prolines, the ellipticity per residue and the wavelength of the peaks decline with peptide length as reported earlier (Okabayashi *et al.*, 1968; Rothe *et al.*, 1976). The length dependence of the ellipticity is due to the proportion of end effects, both electronic and conformational. The ellipticity per residue is approximately the same for all proline homopolymers, if the equivalent of two residues at each end of the polymer are assumed to contribute nothing to the signal. D-Pro_{11} has a CD spectrum of equal magnitude but opposite sign to that of L-Pro_{11} (Figure 1A).

Substitution of glycine for proline reduces the rotational strength of proline undecamers (Figure 1B). A single symmetrically positioned glycine (L-P_5GP_5) reduces the ellipticity of the negative band from -3.4×10^4 (L-Pro_{11}) to -2.0×10^4 deg cm^2 dmol^{-1} and blue shifts the peaks of both positive and negative bands by 0.5 nm. The CD spectrum of a peptide with a single symmetrically positioned alanine (L-P_5AP_5) is similar (data not shown). Undecamers with two or three symmetrical glycines had no positive CD band and a small amplitude negative band shifted to 201 nm for L-$(P_3G)_2P_3$ and 199.5 nm for L-$(P_2G)_3P_2$. The CD spectra of pentadecamers L-$(PPG)_5$ and L-$(P{\cdot}P^{OH}{\cdot}G)_5$ are very similar to L-$(P_2G)_3P_2$, blue shifted and lower in amplitude than the corresponding homooligomer (Figure 1C). The magnitude and position of the minima are -2.2×10^4 deg cm^2 dmol^{-1} (L-$(PPG)_5$) and -2.5×10^4 deg cm^2 dmol^{-1} (L-$(P{\cdot}P^{OH}{\cdot}G)_5$), both at 199.5 nm, compared to -4.1×10^4 deg cm^2 dmol^{-1} at 205 nm for L-Pro_{15}. Glycine imparts rotational freedom to the conformationally constrained PPII helix and shifts the CD spectrum toward that of a less ordered polypeptide.

Analysis of Profilin−Poly(L-Proline) Binding by Fluorescence Spectroscopy. Binding poly(L-proline) enhances the intrinsic tryptophan fluorescence of profilin, increasing the intensity and blue shifting the emission peak (Machesky,

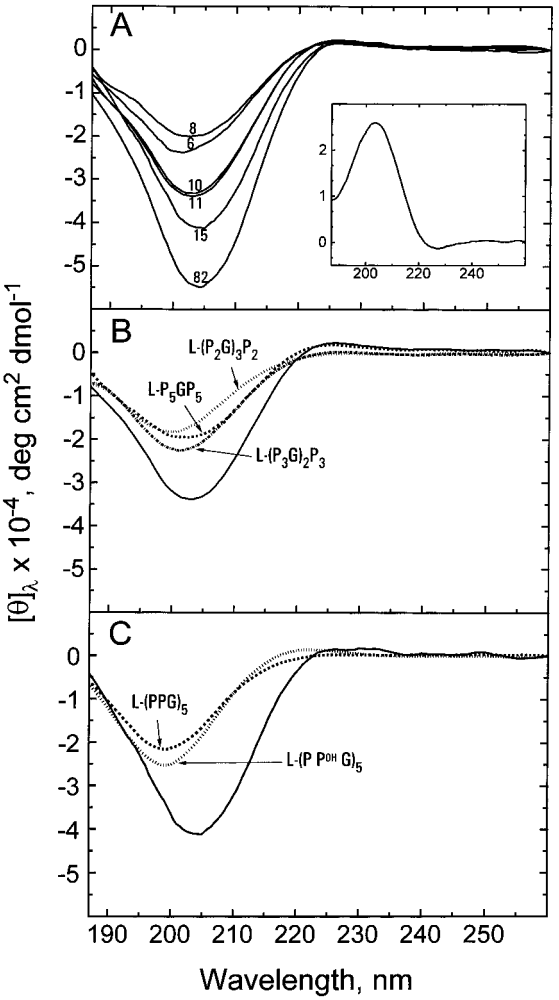

FIGURE 1: Circular dichroism spectra of proline-containing peptides. Spectra are plotted as the mean residue ellipticity ($[\theta]_\lambda$). Conditions: Peptide concentrations were 333 μM (except for L-Pro$_{10}$ (376 μM), L-Pro$_{15}$ (82 μM), L-Pro$_{82}$ (20 μM)) in 10 mM potassium phosphate, pH 7.0. (A) L-Proline homopolymers ranging in size from 6 to 82 proline residues. (A, inset) D-Pro$_{11}$. (B) Peptide undecamers: L-Pro$_{11}$ (solid line), L-P$_5$GP$_5$, L-(P$_3$G)$_2$P$_3$, and L-(P$_2$G)$_3$P$_2$. (C) Peptide pentadecamers: L-Pro$_{15}$ (solid line), L-(PPG)$_5$, and L-(P·P^{OH}·G)$_5$.

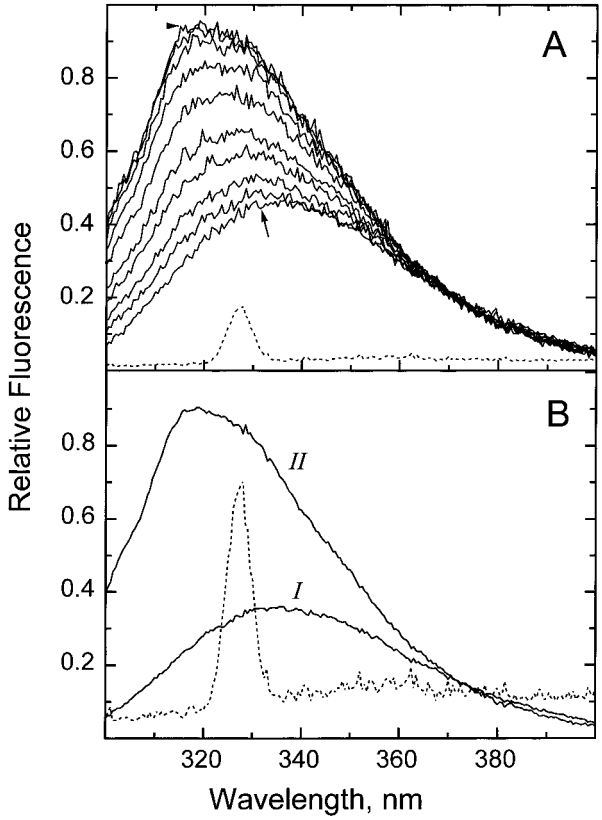

FIGURE 2: Effect of poly(L-proline) on the intrinsic fluorescence of profilin. Conditions: 5 μM *Acanthamoeba* profilin I in 10 mM Tris (pH 7.5), 75 mM KCl, and 3.1 mM NaN_3, 22 °C. Excitation wavelength = 295 nm. (A) A representative experiment showing the family of spectra obtained by titrating with L-proline decamer from a 5.5 mM stock solution. The arrow indicates the spectrum of profilin alone. The end point of the titration (arrowhead) corresponds to an L-Pro$_{10}$ concentration of 205 μM. For clarity, only 11 of the 20 spectra collected are shown. The spectrum of the buffer (dashed line) is subtracted from all other spectra. As L-Pro$_{10}$ binds, the emission maximum shifts from 333 to 318 nm and the fluorescence intensity increases >2-fold. (B) Spectroscopic characteristics of free (I) and fully liganded (II) profilin obtained by global analysis of all 20 spectra with the program Spectrabind and assuming 1:1 profilin:peptide stoichiometry. The model which produces the best fit to the data yields a K_d of 433 μM (proline residues). The spectroscopic characteristic of the artificial species "background" displays the sharp Raman H_2O peak (dashed line).

1993; Perelroizen *et al.*, 1994; Metzler *et al.*, 1994). Free *Acanthamoeba* profilin (Figure 2A, arrow) has an emission maximum at 333 nm, indicating that the tryptophans are partially exposed to solvent. Saturating concentrations of L-Pro$_{10}$ increase the emission intensity 2-fold and shift the maximum to 318 nm. Poly(L-proline) did not appreciably affect the quantum yield of free tryptophan in solution, so poly(L-proline) must change the environment of either or both of the tryptophans when it binds profilin.

We used global analysis of these data with the program Spectrabind to test models for the interaction (Figure 2B). The program calculates the emission spectrum for each of the spectroscopically detectable species in the binding reaction and fits models of the interaction to the full family of spectra. Curves I and II are the spectra of free and fully liganded profilin. The sharp Raman line of H_2O is evident in the spectrum of the artificial species "background" (dashed line). Fitting the whole family of curves with a model with 1:1 stoichiometry gave a dissociation equilibrium constant (K_d) for profilin and L-Pro$_{10}$ of 433 μM proline residues (confidence interval, [426, 439], $\chi^2 = 0.97$). Other stoichiometries (*e.g.*, 2:1) gave poorer fits to the family of curves. Global analysis of similar titrations with other peptides also gave the best fits with 1:1 stoichiometry and the K_d's summarized in Table 1.

The fluorescence change upon binding poly(L-proline) to profilin is saturable (Figure 3A), stereospecific (Figure 3B), and enhanced by salt (Figures 3C and 4). We fit the concentration dependence of the fluorescence at 318 nm by a nonlinear least squares method to determine K_d's for binding of profilin to different poly(L-prolines) (Table 1). The interaction is stereospecific, since D-Pro$_{11}$ produces no fluorescence change up to a concentration of 3.1 mM. In the absence of salt, the affinity of poly(L-proline) for profilin is diminished by 50% (Figure 4). Highest affinities are obtained with a concentration of 4 M NaCl. Measurements were not made above this concentration due to the insolubility of poly(L-proline) in high salt. By NMR, *Acanthamoeba* profilin I bound L-Pro$_{10}$ weakly (K_d >1000 μM proline or 100 μM decamer) in distilled water (Archer *et al.*, 1994). The low salt concentration and/or the purity of the peptide are most likely to account for this difference with the current work.

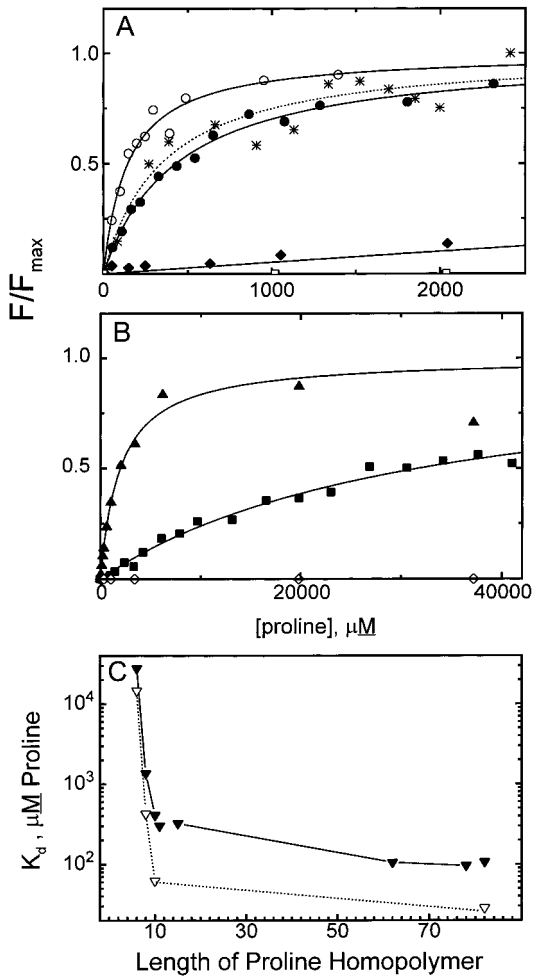

FIGURE 3: Length and ionic strength dependence of L-proline oligomers binding to profilin. Conditions: 5 μM *Acanthamoeba* profilin I in 10 mM Tris (pH 7.5), 75 mM KCl, and 3.1 mM NaN_3, 22 °C. Excitation 295 nm, emission intensity 318 nm. Titrations were performed by adding peptide from concentrated stock solutions. All data sets are normalized (F/F_{max}) to their respective maximum fluorescence. Equilibrium dissociation contants (K_d) were obtained by fitting data directly using nonlinear least squares analysis. (A) Binding isotherms for L-Pro_{10} (●), K_d = 433 μM proline; L-Pro_{15} (*), K_d = 344 μM proline; L-Pro_{82} (○), 120 μM proline; and L-$(PPG)_5$ (◆), K_d = 34.1 mM proline; and L-$(P{\cdot}P^{OH}{\cdot}G)_5$ (□). (B) Binding isotherms for L-Pro_6 (■), K_d = 28 mM proline; L-Pro_8 (▲), K_d = 2.0 mM; and D-Pro_{11} (◇). One outlier point at 37 mM proline in the L-Pro_8 titration is not included in the fit. (C) Dependence of affinity on L-proline polymer length and KCl concentration. (▼), 75 mM KCl. (▽), 2 M KCl.

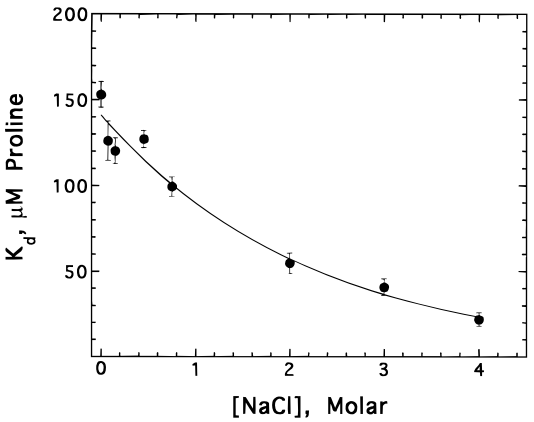

FIGURE 4: The effect of ionic strength on binding of poly(L-proline) to profilin. Conditions: 5 μM *Acanthamoeba* profilin I in 10 mM Tris (pH 7.5), 22 °C. Excitation 295 nm, emission intensity 318 nm. Error bars represent the confidence interval associated with the nonlinear least squares determination of K_d, for each experiment. The line was generated by fitting the data to a single exponential.

Table 1: Dissociation Equilibrium Constants for the Interaction of Profilin with Proline Oligomers[a]

profilin type:	K_d (μM proline residues)	n[b]
Profilin Interaction with L-Pro_{82}		
native *Acanthamoeba* profilin I	107 ± 9	6
native *Acanthamoeba* profilin II	107 ± 10	3
recombinant *Acanthamoeba* profilin I	112 ± 5	3
recombinant *Acanthamoeba* profilin II	102 ± 18	3
native human platelet profilin I	232 ± 35	3
recombinant human profilin I	359 ± 67	9
Interaction of Native *Acanthamoeba* Profilin I with Peptides		
peptide:		
L-Pro_6	30 700 ± 6076	4
L-Pro_8	1471 ± 225	3
L-Pro_{10}	382 ± 43	5
L-Pro_{11}	396 ± 9[c]	1
D-Pro_{11}	no binding	1
L-Pro_{15}	254[d]	2
L-P_5GP_5	2754 ± 185[c]	1
L-P_5AP_5	2269 ± 249[c]	1
L-$P_3GP_3GP_3$	35 127 ± 4321[c]	1
L-$P_2GP_2GP_2GP_2$	54 220 ± 16 508[c]	1
L-$(PPG)_5$	31 367 ± 3535	3
L-$(P{\cdot}P^{OH}{\cdot}G)_5$	no binding	2

[a] K_d's determined by fluorescence titrations and direct fitting of binding isotherms by nonlinear methods as described in Experimental Procedures. Conditions: 75 mM KCl, 10 mM Tris, pH 7.5, 3.1 mM NaN_3; 24 °C; excitation 295 nm, emission 318 nm. [b] Number of determinations. [c] Error associated with the derivation of K_d by nonlinear least squares analysis. [d] Average of two determinations.

We used charged and neutral collisional quenchers to monitor the environment around the profilin tryptophans when poly(L-proline) binds. For free profilin, Stern−Volmer plots for charged quenchers (KI, CsCl) are linear, indicating that both tryptophans are equally accessible to solvent (Figure 5). Negatively charged iodide quenches tryptophan fluorescence more efficiently than positively charged cesium, as previously documented (Eftink & Ghiron, 1981). The profilin tryptophans are more accessible to the neutral quenchers acrylamide and nitromethane, and their Stern−Volmer plots curve upward (Figure 5B), typical of a mixture of dynamic and static quenching (Lakowicz, 1983). Saturation of profilin with 1 mM L-Pro_{50} reduces the accessibility of the tryptophans to all quenchers, as indicated by lower Stern−Volmer constants (K_{sv}) (Figure 5, Table 2). The charged quenchers should not dissociate poly(L-proline) from the binding site on profilin (Figure 4). Acrylamide reduces the affinity slightly (K_d is 180 μM proline residues in 300 mM acrylamide), but the binding site should remain saturated with 1 mM L-Pro_{50} in the sample. With poly(L-proline) bound to profilin, Stern−Volmer plots for iodide and cesium curve downward (Figure 5A), indicating that the two tryptophans have different solvent accessibilities (Lakowicz, 1983).

Amoeba and human profilin differ in their affinities for poly(L-proline) (Table 1). Both the acidic (profilin I) and basic (profilin II) *Acanthamoeba* isoforms (Kaiser *et al.*, 1986), whether purified from amoeba or purified from *E. coli* as a recombinant protein (lacking posttranslational modifications at the N-terminus and K103), bind to poly(L-proline) with a K_d between 102 and 112 μM proline residues. Human platelet profilin (K_d = 232 μM proline residues) and recombinant human profilin I (K_d = 359 μM proline residues) have lower affinities for poly(L-proline).

Table 2: Effect of Poly(L-proline) Binding on Profilin Tryptophan Solvent Accessibility[a]

	quencher:							
	KI		CsCl		acrylamide[d]		CH_3NO_2	
	K_{sv}[b]	RA[c]	K_{sv}	RA	K_{sv}	RA	K_{sv}	RA
profilin	0.8	0.049	0.3	0.064	4.1 ± 0.3	0.223	24.3	0.338
profilin + 1 mM L-Pro_{50}	0.7	0.043	0.1	0.026	3.2 ± 0.7	0.174	22.6	0.315

[a] Conditions: 5 μM profilin in 75 mM KCl, 10 mM Tris, pH 7.5, 3.1 mM NaN_3; 22 °C; excitation 295 nm; emission 330 nm (profilin), 325 nm (profilin + L-Pro_{50}). [Poly(L-proline)] = 1000 μM. [b] Stern−Volmer constants, M^{-1}. [c] Relative accessibilities expressed as fraction of K_{sv} for free L-tryptophan. [d] Average of three determinations.

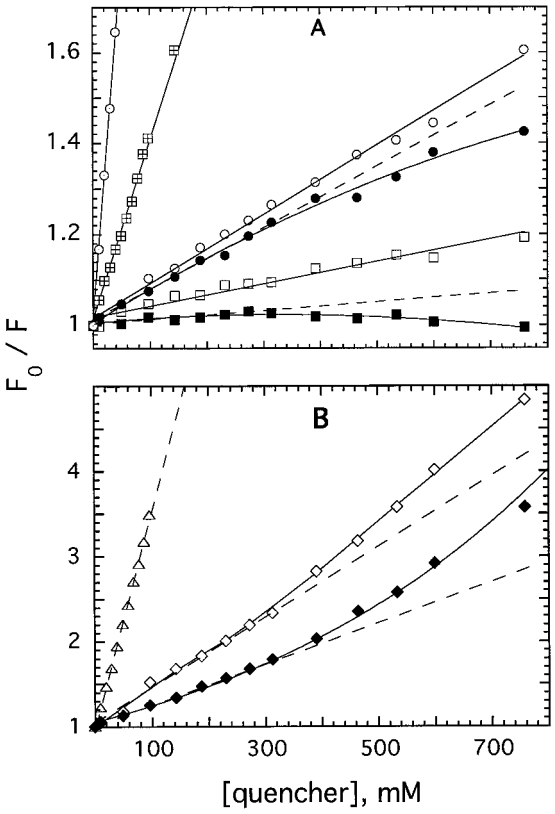

FIGURE 5: The effect of collisional quenchers on profilin intrinsic fluorescence. Shown are Stern−Volmer plots generated by incremental addition of quenching agent to either free or poly(L-proline)-bound *Acanthamoeba* profilin I or to L-tryptophan. Stern−Volmer constants (K_{sv}) for each quencher are derived from the slope of the Stern−Volmer plot (see Table 2). Conditions: 5 μM *Acanthamoeba* profilin I or 10 μM free L-tryptophan in 10 mM Tris (pH 7.5), 22 °C. Excitation 295 nm, emission intensity 350 nm (L-tryptophan), 330 nm (profilin), and 325 nm (profilin + poly(L-proline)). (A) Potassium iodide quenching of free L-tryptophan (⊙), profilin (○), and profilin + 1 mM L-Pro_{50} (●). Cesium chloride quenching of free L-tryptophan (□ with +), profilin (□), and profilin + 1 mM L-Pro_{50} (■). Downward curvature of plots for poly(L-proline)-bound profilin indicates different environments for the two tryptophans. (B) Acrylamide quenching of free L-tryptophan (△), profilin (◇), and profilin + 1 mM L-Pro_{50} (♦). Upward curvature indicates a combination of static and dynamic quenching mechanisms. Straight lines were generated by linear least squares fit of data below 300 mM quencher concentrations.

As observed previously (Perelroizen *et al.*, 1994), the affinity for profilin depends on the length of the poly(L-proline) peptide. L-Proline homopolymers of ten or more residues bind to profilin with similar affinities (Figure 3C). Shorter oligomers have dramatically lower affinities (Figure 3B, Table 1).

The affinity of profilin for proline-rich peptides, presumed to be PPII helices, depends on their composition (Figures 3 and 6). Pure L-proline peptides have a higher affinity than any substituted peptide that we tested. For undecamers, each

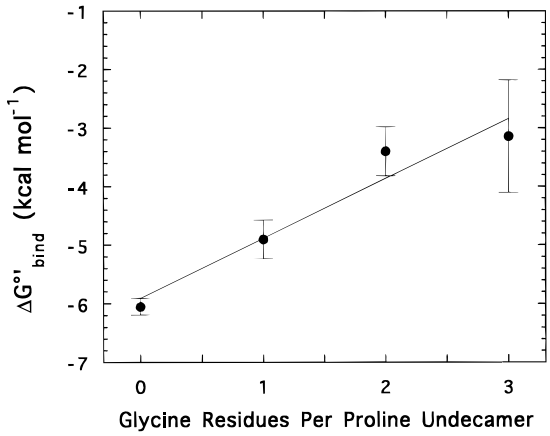

FIGURE 6: The effect of glycine content on the free energy of binding of L-proline undecamers to profilin. The free energy of binding ($\Delta G°'_{bind} = -RT \ln(1/K_d)$) for four proline undecamers L-Pro_{11}, L-P_5GP_5, L-$(P_3G)_2P_3$, and L-$(P_2G)_3P_2$ to *Acanthamoeba* profilin I was derived from the K_d's obtained from fluorescence titrations. Error bars represent the error associated with the nonlinear least squares determination of $\Delta G°'_{bind}$ for each experiment.

symmetrically spaced substitution of a glycine for a proline reduced the binding energy by approximately 1 kcal mol^{-1} (Figure 6). For pentadecamers, substitution of glycine at every third position (L-$(PPG)_5$) reduces the affinity 100-fold and substitution of glycine and hydroxyproline (L-$(P{\cdot}P^{OH}{\cdot}G)_5$) abrogates binding (Figure 3, Table 1).

Energetics of the Profilin−Poly(L-proline) Interaction. Using isothermal titration calorimetry, we characterized the thermodynamics of the interaction of profilin with proline-rich peptides. Binding of L-proline peptides to profilin is an exothermic reaction which can be titrated to saturation, whereupon no additional heat is evolved (Figure 7A, I). Each injection was corrected for the small endothermic heat of dilution of peptides into buffer (Figure 7A, II). Plots of the accumulated heats of injections normalized to molar quantities of reactants yielded binding isotherms which were analyzed directly by nonlinear least squares fitting to derive the $\Delta H°'_{bind}$, K_d, and stoichiometry (n, peptide:protein) for each titration. For the interaction of L-Pro_{11} with *Acanthamoeba* profilin I (Figure 7), $\Delta H°'_{bind}$ is −5.1 kcal mol^{-1}, n is 0.94, and K_d is 37.2 μM, in excellent agreement with the K_d of 36 μM determined by fluorescence titration.

A van't Hoff plot of the temperature dependence of the K_a for the binding of L-Pro_{50} to *Acanthamoeba* profilin I is linear between 4 and 55 °C ($R^2 = 0.97$) (data not shown). The K_d is 50 μM at 4 °C and 288 μM at 55 °C. The $\Delta H°'_{bind}$ calculated from these data is −5.4 kcal mol^{-1}, which agrees well with the isothermal titration calorimetry (−5.1 kcal mol^{-1}) with L-Pro_{11}.

$\Delta H°'_{bind}$ for L-Pro_{82} binding to *Acanthamoeba* profilin I depends linearly on temperature (Figure 8). ΔC_p, given by the slope of the line, is negative with a value of −113 cal

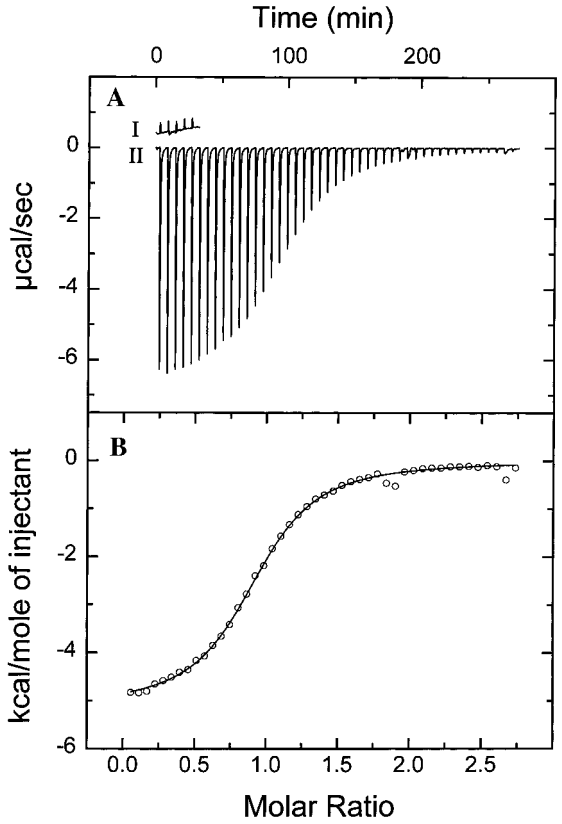

FIGURE 7: Isothermal titration calorimetry of profilin with proline undecamer. Titration was performed by injecting 45×5 μL of 10 mM peptide solution at 5 min intervals into 1.37 mL of 651 μM *Acanthamoeba* profilin I at 28 °C, both in 10 mM potassium phosphate (pH 7.5), 75 mM KCl, and 3.1 mM NaN_3. (A, trace II) Titration of *Acanthamoeba* profilin I with L-Pro_{11}. Each peak represents the heat of binding following one injection. (A, trace I) Control injections (5×5 μL) of peptide into buffer, used to correct for heat of dilution. (B) Binding isotherm corresponding to the experiment in (A). Each point represents the normalized heat change for each injection. The line represents the best fit to the data. The 3 outlying points were not included in the fit. For this experiment, $n = 0.94$, $K_d = 37.2$ μM peptide, and $\Delta H^{\circ\prime}_{bind} = -5.1$ kcal mol^{-1}.

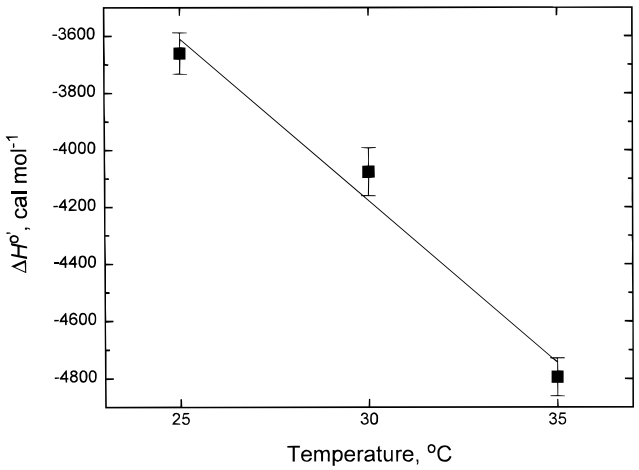

FIGURE 8: Temperature dependence of $\Delta H^{\circ\prime}_{bind}$ for the binding of poly(L-proline) to profilin. $\Delta H^{\circ\prime}_{bind}$ was determined by isothermal titration calorimetry as described in Figure 7. Error bars represent the error associated with the nonlinear least squares determination of $\Delta H^{\circ\prime}_{bind}$ for each experiment.

K^{-1} mol^{-1}. By comparison, the interaction of the octapeptide hormone angiotensin II with an antibody Fab fragment has a ΔC_p of -240 cal K^{-1} mol^{-1} (Murphy *et al.*, 1993).

A comparison of $\Delta H^{\circ\prime}_{bind}$ in Tris and phosphate buffers showed that no protons are released to the buffer during the binding of L-Pro_{82} to *Acanthamoeba* profilin I. Calorimetric titrations of profilin with L-Pro_{82} in Tris ($\Delta H^{\circ\prime}_{ion,30°C} = 11.0$ kcal mol^{-1}) and phosphate ($\Delta H^{\circ\prime}_{ion,30°C} = 1.0$ kcal mol^{-1}) yielded the same $\Delta H^{\circ\prime}_{bind}$, -3.8 and -3.7 kcal mol^{-1}.

From $\Delta G^{\circ\prime}_{bind}$ determined by fluorescence, adjusted to 28 °C, and $\Delta H^{\circ\prime}_{bind}$ determined calorimetrically, we calculated the value of $\Delta S^{\circ\prime}$ for the binding of four peptides to profilin I (Table 3). The binding of L-Pro_{11} to profilin is favored entropically ($\Delta S^{\circ\prime} = 1.1$ cal mol^{-1} K^{-1}) and enthalpically ($\Delta H^{\circ\prime}_{bind} = -5.1$ kcal mol^{-1}), whereas the entropy change is unfavorable for binding of L-Pro_8 ($\Delta S^{\circ\prime} = -13.0$ cal mol^{-1} K^{-1}) and L-P_5GP_5 ($\Delta S^{\circ\prime} = -5.7$ cal mol^{-1} K^{-1}). L-P_5AP_5 binding is entropically unfavorable albeit to a lesser extent ($\Delta S^{\circ\prime} = -2.3$ cal mol^{-1} K^{-1}). $\Delta H^{\circ\prime}_{bind}$ for L-Pro_{11} and L-P_5AP_5 are identical, so the difference in the overall binding energy for these peptides can be attributed predominantly to the entropy cost of binding the more flexible substituted peptide. Conversely, the affinities of L-Pro_8, L-P_5GP_5, and L-P_5AP_5 for profilin are similar so that the unfavorable entropy changes are compensated for by the more favorable $\Delta H^{\circ\prime}_{bind}$ values. The low affinity of L-Pro_6, L-$(P_3G)_2P_3$, and L-$(P_2G)_3P_2$ for profilin precluded a full calorimetric analysis of these peptides.

Table 3: Thermodynamic Parameters for the Interaction of Profilin with Proline-Rich Peptides[a]

peptide	K_d (μM)[b]	$\Delta G^{\circ\prime}_{bind}$ (kcal mol^{-1})[c]	$\Delta H^{\circ\prime}_{bind}$ (kcal mol^{-1})[d]	$\Delta S^{\circ\prime}$ (cal mol^{-1} K^{-1})[e]
L-Pro_8	250 ± 24.6[g]	-4.3	-8.2 ± 0.20[f]	-13.0
L-Pro_{11}	36 ± 0.8	-5.4	-5.1 ± 0.03	1.1
L-P_5GP_5	250 ± 16.8	-4.3	-6.0 ± 0.11	-5.7
L-P_5AP_5	206 ± 22.6	-4.4	-5.1 ± 0.05	-2.3

[a] Conditions: 75 mM KCl, 10 mM potassium phosphate, pH 7.5, 3.1 mM NaN_3, 28 °C (ITC); 75 mM KCl, 10 mM Tris pH 7.5, 3.1 mM NaN_3, 22 °C (fluorescence). [b] K_d, expressed in μM peptide, determined by fluorescence titration. [c] Calculated using K_d obtained from fluorescence titration and adjusted to 28 °C. [d] Determined by isothermal titration calorimetry. [e] Calculated from $\Delta G^{\circ\prime}_{bind}$ and $\Delta H^{\circ\prime}_{bind}$. [f] Error associated with the derivation of $\Delta H^{\circ\prime}_{bind}$. [g] Error associated with the derivation of K_d.

DISCUSSION

Poly(L-proline) binds rapidly and reversibly (Archer *et al.*, 1994) to all profilins except vaccinia virus profilin (Machesky *et al.*, 1994b). Many cellular proteins contain contiguous stretches of proline and/or PPII secondary structure (Adzhubei & Sternberg, 1993; Williamson, 1994; Metzler *et al.*, 1994), but to date only one proline-rich protein, VASP, is known to bind profilin *in vitro* (Reinhard *et al.*, 1995). We used poly(L-proline) and proline peptides of varied composition and length to define the structural requirements and thermodynamics of the interaction of profilins with poly(L-proline).

Archer *et al.* (1994) and Metzler *et al.* (1994) mapped the binding site for poly(L-proline) by NMR spectroscopy. Poly(L-proline) substantially changes the chemical shifts of two tryptophans and 15 other residues located on and near the N- and C-terminal helices. The tryptophans and two other aromatic side chains are exposed to solvent and are in close proximity to other hydrophobic residues. These residues are generally conserved across phyla (Pollard & Rimm, 1991). Substitution of human profilin tryptophan-3 with glutamine abolishes poly(L-proline) binding (Björkegren *et al.*, 1993). Substitution of the *Acanthamoeba* profilin phenylalanine-

125 (Kaiser & Pollard, 1996) or tryptophans-2 or −29 (Vinson and Pollard, unpublished) abrogates or substantially diminishes poly(L-proline) binding. Metzler *et al.* (1994) estimated the length of the interaction surface on human profilin to be 1.7 nm, the length of a proline hexamer. Our measurements show that the interaction surface on *Acanthamoeba* profilin is similar.

Poly(L-proline) enhances the tryptophan fluorescence of profilin, without inducing significant changes in secondary structure or conformational changes in the protein backbone (Archer *et al.*, 1994; Metzler *et al.*, 1994). Presumably, the bound peptide simply changes the environment of one or both of the tryptophan residues. Exclusion of water and/or contact with the hydrophobic proline pyrrolidine ring would create a less polar environment and account for the observed blue shift and increase in emission intensity.

Collisional quenching experiments confirm that both profilin tryptophans are exposed and equally accessible to solvent, as expected from the atomic structure (Vinson *et al.*, 1993; Federov *et al.*, 1994b). With poly(L-proline) bound, both tryptophans are less accessible to collisional quenchers and each is in a different environment, as indicated by the downward curvature of the Stern−Volmer plots (Lakowicz, 1983). The tryptophans are considerably more accessible to acrylamide and nitromethane than the charged quenchers, perhaps due to the hydrophobic environment of the tryptophans.

If six prolines in a type II helix really span the binding site on profilin, why do short proline oligomers bind so weakly (Table 1)? CD measurements show that all proline oligomers form PPII helices, but rotational strength decreases with decreasing length (this study; and Okabayashi *et al.*, 1968; Rothe *et al.*, 1976). Disorder at the ends (fraying) of the peptides could contribute to decreased rotational strength. In fact, when theoretical and experimental CD spectra for a proline decamer are compared, small deviations in peptide backbone geometry produce large-scale changes in CD spectra (Manning & Woody, 1991). If fraying is the dominant determinant of decreased rotational strength for shorter peptides, then it is possible that a proline peptide of ten or more residues is required to provide an ordered hexamer to bind profilin. When immobilized on the surface of a protein two turns of PPII helix, six residues, could suffice for binding to profilin. Alternatively, electronic and electrostatic end effects may cause the decreased rotational strength for shorter peptides without any significant fraying. If this were the case, then binding to profilin may require at least three turns of a PPII helix, or nine residues. An additional three residues could serve to increase the affinity by displacing solvent (more favorable solvent entropy, discussed below) in and around the binding site and/or provide heretofore unidentified contacts within the binding site.

A favorable enthalpy change ($\Delta H^{\circ\prime}_{\text{bind}}$) contributes to the free energy change when proline homopolymers bind profilin. $\Delta H^{\circ\prime}_{\text{bind}}$ represents the sum of intermolecular interactions upon complexation (favorable) and the breaking of solvent hydrogen bonds (unfavorable) in the ordered water surrounding both the peptide and nonpolar groups in the binding site. Hydrogen bonding and van der Waals and hydrophobic interactions are all likely to contribute to the favorable $\Delta H^{\circ\prime}_{\text{bind}}$. Although proline polymers cannot act as hydrogen bond donors due to cyclization of the side chain onto the amide nitrogen, the carbonyl oxygen is more electronegative than that in a secondary amide and a strong hydrogen bond acceptor (Veis & Nawrot, 1970; Lilley, 1992). This explains the high water solubility of proline homopolymers and free proline. Hydrogen bonds stabilize the interaction of poly(L-proline) with the phenolic hydroxyls of tannins (Hagerman & Butler, 1981). Proline-rich peptides also accept hydrogen bonds from their binding sites in at least three different SH3 domains (Lim *et al.*, 1994; Musacchio *et al.*, 1994; Terasawa *et al.*, 1994; Wittekind *et al.*, 1994; Feng *et al.*, 1994). Of the amino acids in the poly(L-proline) binding site of *Acanthamoeba* profilin, W2, W29, Y5, Y119, and N9 can donate hydrogen bonds.

Several lines of evidence show that hydrophobic interactions occur between the conserved aromatic residues in the profilin poly(L-proline) binding site and proline peptides. First, high salt favors binding. Second, the negative ΔC_p for the interaction indicates a favorable change in the solvent entropy ($\Delta S_{\text{solvent}}$) which results from the release of solvent from the binding site and from the peptide, the hallmark of hydrophobic interactions. Finally, the hydrophobic character of the proline pyrrolidine ring is ideal for formation of van der Waals contacts with the binding surface. The structure of a cocrystal should reveal the details of the interactions.

A positive entropy change favors the binding of proline homopolymers to profilin. The overall entropic contribution ($\Delta S^{\circ\prime}$) to $\Delta G^{\circ\prime}_{\text{bind}}$ is the sum of $\Delta S_{\text{solvent}}$ and the change in the configurational entropy (ΔS_{config}) of the peptide (Lee *et al.*, 1994). The configurational entropy of unbound proline homooligomers is relatively low, owing to the constrained conformation of the PPII helix. The small loss of configurational entropy for immobilizing poly(L-proline) on the surface of profilin favors binding. As expected, imparting configurational freedom to unbound proline peptides reduces the affinity for profilin, because of the greater entropy loss upon binding. For example, the affinity of L-P_5AP_5 is lower than L-Pro_{11} entirely due to the unfavorable $\Delta S^{\circ\prime}$ since $\Delta H^{\circ\prime}_{\text{bind}}$ is the same. Similarly, each glycine symmetrically placed within proline undecamers reduces $\Delta G^{\circ\prime}_{\text{bind}}$ by approximately 1 kcal mol^{-1}. The largest entropic penalty exists for the binding of short proline peptides like L-Pro_8, most likely due to a less favorable $\Delta S_{\text{solvent}}$ resulting from less exclusion of solvent from the binding site.

Our experiments with synthetic proline peptides have established that the interaction of profilin with proline-rich ligands is stereospecific for type II L-proline helices, but the effects of substitutions on the entropy of the unbound peptides precluded further insights about the sequence specificity. Simply due to its rigidity, pure poly(L-proline) binds profilin better than any substituted peptide that we tested. Peptide rigidity may not be an issue when a proline-rich sequence is part of the folded protein, so non-proline residues in a PPII helix may contribute to the affinity and specificity of the interaction with profilin. This is true for the PPII helices that bind to SH3 domains (Saraste & Musacchio, 1994). Some SH3 domains are very selective for their proline-rich ligands (Abl-SH3) while others are promiscuous (Fyn-SH3) (Viguera *et al.*, 1994). None of them binds pure poly(L-proline) as well as profilin, and even the best peptide ligands bind their SH3 receptors with affinities like those of profilin and poly(L-proline).

Six prolines, two turns of a PPII helix, may be sufficient to mediate binding to profilin. In the context of a folded protein, conformational constraints and non-proline residues may provide enhanced affinity. In analogy with SH3

domains, the interaction with poly(L-proline) motifs in other proteins may allow profilin to link proteins together in the cytoplasm (Schutt *et al.*, 1993). For example, profilin might serve as an adaptor to link VASP to actin, the Arp2/3 complex, or membrane lipids in the cortex of the cell.

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