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Stability and peptide binding affinity of an SH3 domain from the *Caenorhabditis elegans* signaling protein Sem-5

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Abstract

We have determined the thermodynamic stability and peptide binding affinity of the carboxy-terminal Src homology 3 (SH3) domain from the *Caenorhabditis elegans* signal-transduction protein Sem-5. Despite its small size (62 residues) and lack of disulfide bonds, this domain is highly stable to thermal denaturation—at pH 7.3, the protein has a T_m of 73.1 °C. Interestingly, the protein is not maximally stable at neutral pH, but reaches a maximum at around pH 4.7 ($T_m \cong 80$ °C). Increasing ionic strength also stabilizes the protein, suggesting that 1 or more carboxylate ions are involved in a destabilizing electrostatic interaction. By guanidine hydrochloride denaturation, the protein is calculated to have a free energy of unfolding of 4.1 kcal/mol at 25 °C. We have also characterized binding of the domain to 2 different length proline-rich peptides from the guanine nucleotide exchange factor, Sos, one of Sem-5's likely physiological ligands in cytoplasmic signal transduction. Upon binding, these peptides cause about a 2-fold increase in fluorescence intensity. Both bind with only modest affinities ($K_d \cong 30\ \mu$M), lower than some previous estimates for SH3 domains. By fluorescence, the domain also appears to associate with the homopolymer poly-L-proline in a similar fashion.

Keywords: chemical denaturation; poly-L-proline II helix; proline-rich peptide binding; SH3 domain; thermal denaturation

The signal transduction pathways that regulate cell growth and differentiation are characterized by a cascade of specific protein–protein recognition events that occur upon extracellular stimulation. Proteins in many different pathways are found to contain a number of related, small, modular domains that are responsible for mediating specific classes of protein–protein interactions (Cantley et al., 1991). Two examples are the Src homology 2 and 3 (SH2 and SH3) domains, first identified in the Src oncogene (Kuriyan & Cowburn, 1993; Pawson & Schlessinger, 1993). SH2 domains are approximately 100 residues in length and mediate recognition of specific protein sequences bearing a phosphotyrosine. SH3 domains are approximately 60 residues in length and mediate recognition of specific proline-rich protein sequences.

The *Caenorhabditis elegans* protein, Sem-5 (Clark et al., 1992), and its mammalian and *Drosophila* homologues, Grb-2 (Lowenstein et al., 1992) and Drk (Olivier et al., 1993; Simon et al., 1993), make up a family of small adaptor proteins composed solely of an SH2 domain and 2 SH3 domains. At least 1 function of these proteins is to link receptor protein tyrosine kinase (PTK) activation to the signaling by the G protein Ras. In current models, upon stimulation, a receptor PTK undergoes autophosphorylation of specific cytoplasmic domain tyrosines. The SH2 domains of this family specifically bind these phosphotyrosine sequences, and the SH3 domains specifically bind the guanine nucleotide exchange factor Sos, via several repeats of the sequence PPPXPPR near the Sos C-terminus. Localization of Sos to the membrane via these adaptor proteins may then allow the protein to subsequently activate membrane-associated Ras by catalyzing the exchange of bound GDP for GTP (Egan et al., 1993; Li et al., 1993; Rozakis-Adcock et al., 1993).

Although the structures of a number of SH3 domains have been solved (Musacchio et al., 1992; Yu et al., 1992; Booker et al., 1993; Kohda et al., 1993; Koyama et al., 1993; Noble et al., 1993), little is known about the biophysical properties of these domains. How stable are these extremely small domains? With what affinity do they recognize their proline-rich peptide ligands? To begin answering such questions, we have overexpressed and purified the C-terminal SH3 domain from Sem-5. We have determined the stability of this domain to both heat

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and chemical denaturation and measured its affinity to 2 peptides derived from Sos. The stability of another SH3 domain, from the protein spectrin, has been analyzed in a related study by Viguera et al. (1994).

Results

Stability

The results from thermal and chemical denaturation experiments are summarized in Table 1. Upon thermal denaturation, this domain shows a marked change in its CD spectrum (Fig. 1A). There is a significant decrease in ellipticity near 220 nm. This change in ellipticity may correspond to a loss of β-turn structure in this all β-sheet domain because Chang et al. (1978) have reported that β-turns yield a positive ellipticity signal at this wavelength. By monitoring ellipticity at 222 nm, cooperative unfolding of the protein, consistent with a 2-state transition, can be detected (Fig. 1B). The protein was found to be very stable to heat denaturation, with an unfolding transition midpoint of 73 °C near neutral pH. The pH dependence of thermal stability, unlike that of most proteins, shows an increase in stability as the pH is lowered (Fig. 2). The stability maximum, with a T_m of 79 °C, occurs near pH 4.7. At pH values lower than 4, however, the stability decreases again. At pH 7.3 in the presence of 500 mM NaCl, the stability was also found to increase (T_m = 77 °C), suggesting an ionic strength effect.

The protein also unfolds cooperatively with increasing chemical denaturant (Fig. 3), showing approximately a 2-fold decrease in fluorescence intensity at 340 nm upon unfolding. By linear extrapolation of the guanidine hydrochloride (GuHCl) denaturation results, the protein is calculated to have a stability of about 4.1 kcal/mol at 25 °C at 0 M GuHCl. Thus, despite its resistance

Table 1. *Stability of the C-terminal SH3 domain from Sem-5*[a]

A. Thermal denaturation

Buffer	T_m (°C)	$\Delta H_{van't\ Hoff}$ (kcal/mol)	$\Delta\Delta G$ (kcal/mol)
pH 3.7	73.0	64.0	−0.14
pH 4.5	78.8	69.2	0.97
pH 4.9	79.1	64.8	0.90
pH 6.1	74.2	62.9	0.12
pH 7.3	73.4	64.6	–
pH 7.3, 500 mM NaCl	77.0	66.4	0.57

B. GuHCl denaturation

c_m = 2.36 M m = 1.7 kcal/mol·M ΔG_{H_2O} = 4.1 kcal/mol

[a] Buffer compositions are given in the Materials and methods. $\Delta\Delta G$ is given relative to the free energy of unfolding at 73 °C in the pH 7.3, 20 mM NaCl buffer. c_m is the GuHCl denaturation midpoint concentration. Errors are estimated to be less than: ±1 °C for T_m, ±10 kcal/mol for $\Delta H_{van't\ Hoff}$, ±0.07 kcal/mol for $\Delta\Delta G$, ±0.02 M for c_m, ±0.1 kcal/mol·M for m, and ±0.5 for ΔG_{H_2O}.

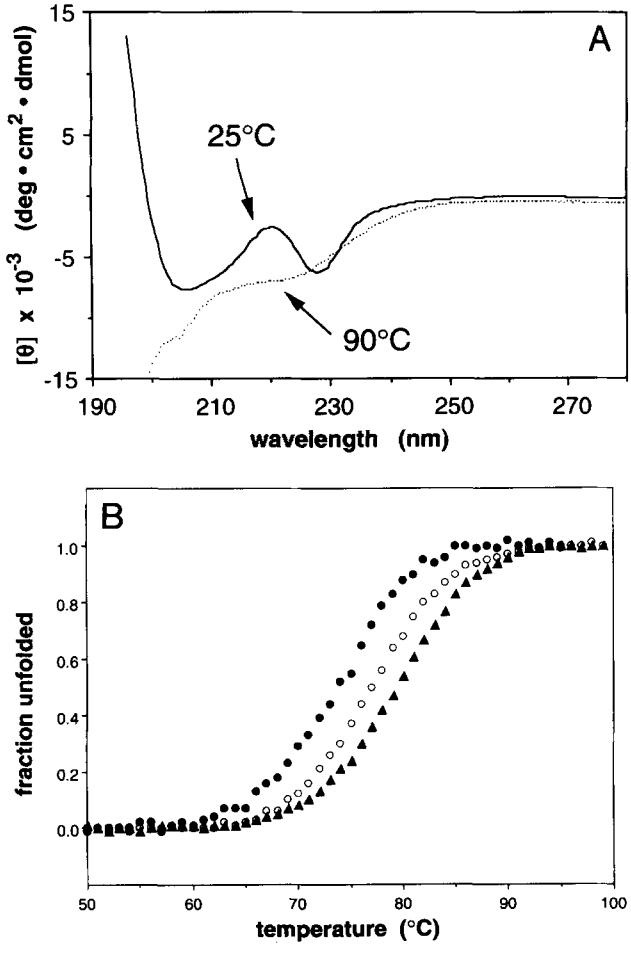

Fig. 1. A: Change in circular dichroism spectrum of the Sem-5 C-terminal SH3 domain upon thermal denaturation. Spectra were taken in 50 mM Na phosphate (pH 7.3), 20 mM NaCl. The solid line is the spectrum obtained at 25 °C and the dotted line is the spectrum obtained at 90 °C. **B:** Thermal unfolding curve of the SH3 domain under 3 different conditions. Unfolding was monitored by change in ellipticity at 222 nm. The filled circles are in 50 mM Na phosphate (pH 7.3), 20 mM NaCl; the open circles are in 50 mM Na phosphate (pH 7.3), 500 mM NaCl; the filled triangles are in 50 mM Na acetate (pH 4.9), 20 mM NaCl.

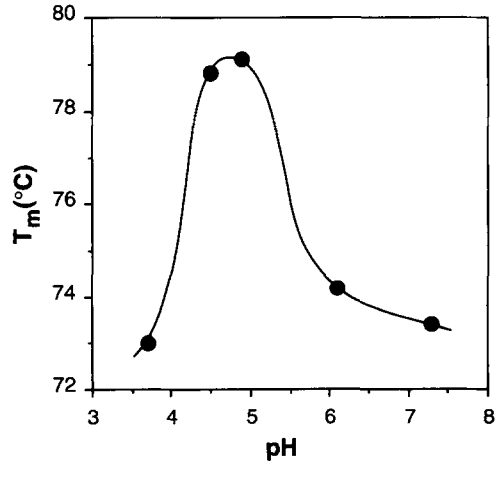

Fig. 2. pH dependence of the SH3 domain thermal stability. The curve is drawn by inspection. Buffers used are described in the Materials and methods.

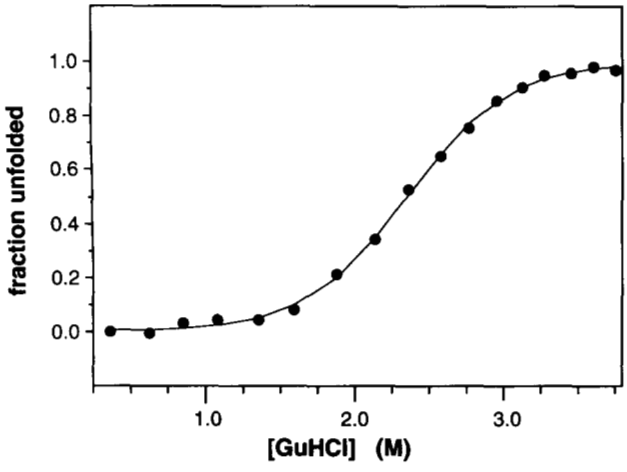

Fig. 3. GuHCl unfolding curve of the SH3 domain. The curve indicates the results of a nonlinear least-squares fitting to Equations 1 and 2.

to heat denaturation, the domain is not unusually stable at ambient temperatures.

Peptide binding affinities

The results of peptide binding assays are summarized in Table 2. Upon binding of either of the 2 Sos-derived proline-rich peptides, the protein shows about a 2-fold maximal increase in fluorescence intensity at 340 nm (Fig. 4A). This increase may occur because the peptide is known to bind at a surface consisting of several aromatic residues, and binding may shield these residues from solvent quenching (Yu et al., 1992; Lim & Richards, 1994). Peptide binding isotherms could be obtained by monitoring change in fluorescence as a function of peptide ligand concentration (Fig. 4B). At 25 °C, both peptides are calculated to bind with dissociation constants (K_d) of 30–40 μM. Titration with poly-L-proline was also found to cause an increase in fluorescence intensity (Fig. 5), although in this case, the maximal increase is about 1.6-fold.

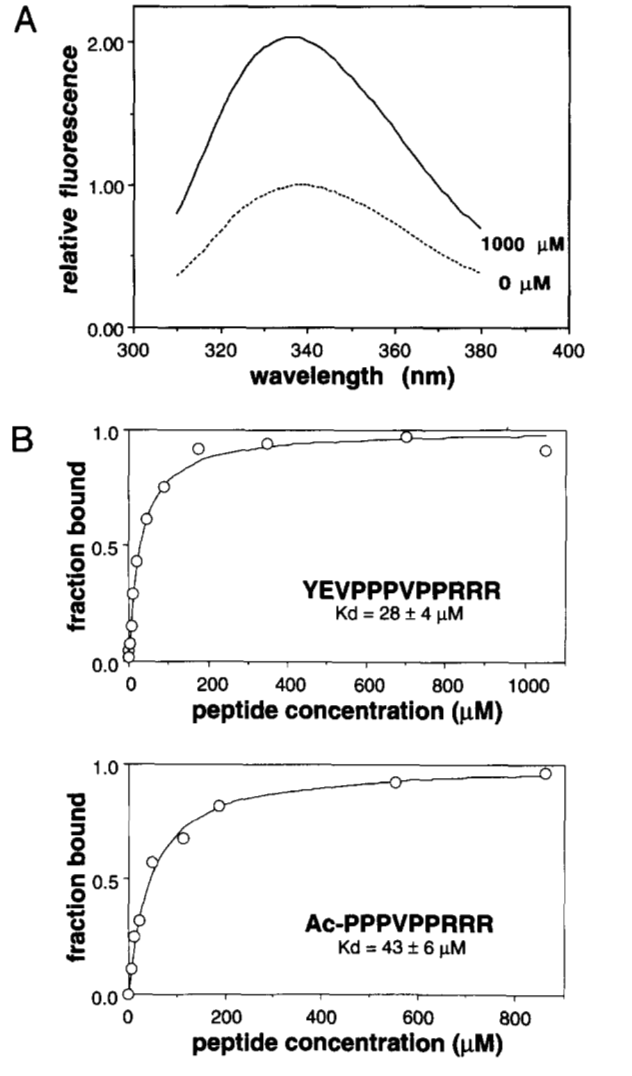

Fig. 4. A: Change in fluorescence emission spectrum upon binding of the peptide Ac-PPPVPPRRR. Excitation is at 280 nm and spectra are scaled to the intensity at 340 nm for the protein alone. **B:** Binding isotherms of the SH3 domain with a Sos-derived 9-mer (AC-PPPVPPRRR) and 12-mer (YEVPPPVPPRRR), assayed at 25 °C. Curves indicate the best fit to Equation 3 by nonlinear least-squares analysis.

Discussion

Stability of the SH3 domain

SH3 domains are small (~60 residues) independently folding domains responsible for mediating a number of specific protein–protein interactions in cytoplasmic signaling pathways. These domains closely approach the lower size limit of about 50 residues postulated to be required for the formation of a stable folded unit (Privalov, 1979). Our denaturation studies show that despite their small size and lack of disulfide bonds, these domains are extremely thermostable. The C-terminal SH3 domain from Sem-5, studied here, has a thermal denaturation midpoint of 73 °C. Near pH 4.7, the domain is even more stable, with a T_m of almost 80 °C. Nonetheless, as measured by GuHCl denaturation, this domain is not unusually stable at ambient temperatures—the domain has a calculated ΔG in water of 4.1 kcal/mol. This behavior, i.e., extraordinary thermal stability but normal stability at ambient temperatures, is similar to that observed for another very small, independently folding unit, the 56-residue B1 IgG-binding domain of protein G, studied by Alexander et al. (1992). This domain has a T_m of 90 °C but a stability at 37 °C of about 6 kcal/mol. These authors argue that

Table 2. *Peptide binding affinity of the C-terminal SH3 domain from Sem-5*[a]

Peptide	K_d (μM)	I_∞/I_0
Ac-PPPVPPRRR	43	2.1
YEVPPPVPPRRR	28	1.9

[a] K_d is the dissociation constant. I_∞/I_0 is the relative change in fluorescence intensity at 340 nm, at saturation. Errors are estimated to be less than ±6 μM for K_d, and less than ±0.2 for I_∞/I_0.

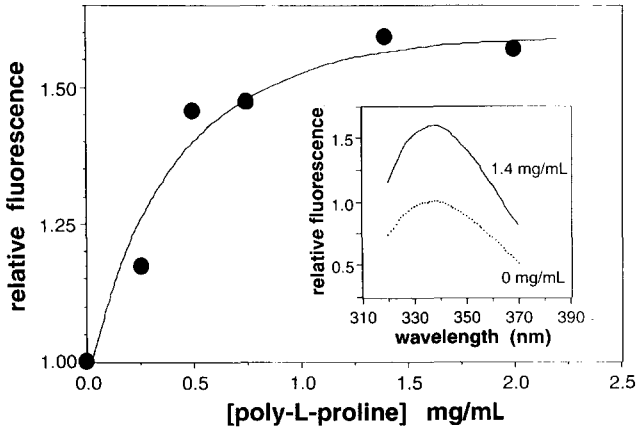

Fig. 5. Binding of the SH3 domain to poly-L-proline. Insert shows the change in relative fluorescence, with spectra scaled to intensity at 340 nm for the protein alone. Outer plot shows the change in relative fluorescence with poly-L-proline concentration. The curve is drawn by inspection.

such behavior is typical of small domains because such domains tend to have a relatively small ΔS and ΔC_p of unfolding. Low values of ΔS and ΔC_p lead to a shallow stability profile (ΔG vs. temperature). Thus, even if such a domain has a high T_m, its stability at ambient temperature would not be higher than that of most larger proteins. The value of ΔC_p is often directly associated with the degree of hydrophobic stabilization of a protein. Alexander et al. (1992), however, point out that for small domains, a low ΔC_p does not mean that hydrophobic forces play an insignificant role, because on a per residue basis, ΔC_p is not any lower than for larger proteins. All known SH3 structures have a compact hydrophobic core of substantial volume, and the burial of these core residues probably does make a significant contribution to the stability.

This SH3 domain is apparently considerably more stable than the SH3 domain from spectrin, studied by Viguera et al. (1994). The spectrin SH3 domain has a T_m of approximately 63 °C and a ΔG of unfolding at 25 °C of slightly greater than 3 kcal/mol. It is noteworthy, however, that the same pattern of a relatively high thermal stability, considering the protein's low stability at ambient temperatures, is observed for the spectrin domain.

pH dependence of thermal stability: A potential destabilizing electrostatic interaction

This SH3 domain has a very unusual pH dependence of stability (Fig. 2)—the T_m actually increases as the pH drops below neutrality, reaches a maximum at around pH 4.7, and decreases as the pH drops below 4. These data suggest that 1 or more carboxyl groups that titrate between pH 5 and 5.5 are involved in a destabilizing interaction when charged, or a stabilizing one when protonated. Two possible explanations are that such a carboxyl group is either partially buried or involved in a destabilizing electrostatic interaction. To test these hypotheses, we also determined the effect on thermal stability of increasing ionic strength, which would shield and reduce electrostatic interactions. At pH 7.3 in the presence of 500 mM NaCl, the T_m is also found to increase to 77 °C. This result is therefore consistent with a model in which a carboxylate anion is involved in a destabilizing electrostatic interaction. Consistent with this model is the observation that, in the folded structure, this particular SH3 domain is expected to have a relatively high negative charge density on the binding face. Electrostatic repulsion between a closely spaced set of acidic residues could be destabilizing. Several acidic residues are implicated in interacting with the positively charged arginine residues of the ligand (Lim & Richards, 1994). Thus, the presence of a high negative charge density may enhance function, i.e., binding of the positively charged peptide ligand, at the cost of reduced stability of the domain.

Sos peptide binding affinities

The function of SH3 domains, in general, appears to be the recognition of specific proline-rich peptides, an activity that is utilized in the assembly of cytoplasmic signaling protein complexes. We were interested in determining the affinity of such domains for their peptide ligands. Li et al. (1993) have reported that, as measured using the BIAcore system (a solid-phase system), the Sem-5 homologue, Grb-2, binds the peptide PPPVPPRRR with an affinity of 25 nM. Chen et al. (1993), however, have reported affinities of 5–40 μM for a number of SH3 domains and their ligands. Our solution affinity measurements are closer in magnitude to the lower affinities reported by Chen et al. (1993). The C-terminal SH3 domain of Sem-5 binds to 2 different length peptides derived from the protein Sos with affinities of about 30–40 μM (Table 2). The 12-amino acid peptide binds with a slightly, but not dramatically, higher affinity than the 9-amino acid peptide, indicating that there may be a modest length dependence on affinity.

Such affinities are quite low compared to many other well-characterized, biological protein-peptide interactions, such as antibody-peptide interactions, which have affinities in the nanomolar range (Janin & Chothia, 1990). A modest affinity, however, may be necessary for a signaling molecule. A very high affinity, and the correspondingly long complex lifetimes, could lead to constitutive signaling. A modest affinity would not lead to these problems, and the effective affinity in a large signaling complex could be easily increased through cooperative or multivalent interactions.

Binding to poly-L-proline—Implications for mechanism of recognition

Based on mutational evidence, we have noted that SH3 domains present a critically shaped surface, which is complementary to a poly-proline II (PPII) helix (Lim & Richards, 1994). We have therefore proposed a model for recognition in which at least part of the proline-rich peptide ligands bind in the left-handed PPII conformation. If this model were correct, we postulated that SH3 domains might be able to, at least weakly, bind homopolymeric poly-L-proline, which in aqueous solution is known to adopt an all-PPII conformation (Creighton, 1984). Titration of this SH3 domain with poly-L-proline in fact leads to a saturating change in aromatic residue fluorescence, which is similar, though not identical to that observed when titrated with Sos peptide (Fig. 5). Thus, even in the absence of interactions with specifically recognized peptide polar residues, the domain appears capable of weakly binding poly-L-proline, most likely at the same aromatic face used for peptide recognition.

Materials and methods

Protein expression and purification

The C-terminal SH3 domain of Sem-5 (residues 155–214) was amplified and cloned into the vector pET19b (Novagen), as described previously (Lim & Richards, 1994). This vector expresses the domain with a 10-histidine leader, which allows for single-step purification on a metal-chelate resin. This construct was expressed in the *Escherichia coli* strain BL21 (DE3) pLysS (Studier & Moffat, 1986). Three liters of LB media containing 200 μg/mL of ampicillin was inoculated with 60 mL of a saturated culture and grown at 37 °C for 2 h. Protein expression was induced by addition of 300 mg isopropyl-β-D-thiogalactopyranoside, and the culture was grown for an additional 5 h. Cells were harvested and sonicated in 30 mL of 50 mM Na phosphate (pH 7.8), 300 mM NaCl. The centrifuged sonicate was mixed batchwise with 5 mL of a nickel-NTA resin (Qiagen) and the bound protein purified as recommended by the manufacturer. The material eluted in 50 mM Na phosphate (pH 4), 300 mM NaCl, 10% glycerol, was about 95% pure. The protein (now at approximately 1 mg/mL) was then dialyzed in 50 mM Na acetate (pH 4.8), 10 mM $CaCl_2$, and digested with 150 units/mL of porcine enterokinase (Biozyme) overnight at 37 °C. This process removes the histidine leader, which was separated from the domain by an enterokinase recognition site. The leader and uncleaved protein were removed by flowing the digest over fast flow SP-sepharose (Pharmacia). The resulting protein was greater than 99% pure as assayed by analytical reverse-phase HPLC.

Circular dichroism spectroscopy

Measurements were taken on an AVIV 62DS spectropolarimeter with protein at a concentration of 40 μM in 50 mM Na phosphate (pH 7.3), 20 mM NaCl, in a 2-mm-pathlength cell. Scans were repeated 4 times, between 195 and 290 nm using a 0.5-nm stepsize and a 1-s averaging time.

Fluorescence spectroscopy

Emission spectra were obtained on a SPEX Fluoromax fluorimeter with protein at a concentration of 0.5–1 μM in 20 mM Hepes (pH 7.3), 50 mM NaCl, in a 1-cm × 1-cm cell. Samples were excited at either 280 or 295 nm and spectra taken between 380 and 310 nm using a 1-nm stepsize and a 0.5-s integration time.

Thermal denaturation

Thermal unfolding of the protein was monitored by measuring CD signal at 222 from 30 to 100 °C. Measurements were taken every 1 °C, with a 1-min equilibration time and a 30-s averaging time. All samples contained 40 μM protein. For pH dependence experiments, all samples contained 20 mM NaCl as well as 50 mM of the following buffers: Na citrate (pH 3.7), Na citrate (pH 4.5), Na acetate (pH 4.9), Na citrate (pH 6.1), or Na phosphate (pH 7.3). pH readings were taken directly on the sample at 25 °C. Thermal denaturation was also measured in 50 mM Na phosphate (pH 7.3), 500 mM NaCl. Unfolding was completely reversible under all conditions, except at pH 3.5, where there was a 5% loss. Van't Hoff enthalpy at T_m ($\Delta H_{\text{van't Hoff}}$) was calculated from the dependence of the folding equilibrium constant on temperature. Stability is not expected to be dependent on protein concentration, especially in the micromolar range used in these studies, because SH3 domains are monomeric.

Guanidine hydrochloride denaturation

Unfolding was monitored by the decrease in fluorescence emission intensity at 340 nm after excitation at 280 nm in 50 mM Hepes (pH 7.3), 20 mM NaCl. Unfolding experiments were performed at 25 °C using the method of Shortle and Meeker (1986), except that both starting sample and stock denaturant contained 1 μM protein. Measured intensities (I) were fit to a 2-state model by nonlinear least-squares analysis using the program SigmaPlot (Jandel Scientific). The equation used for fitting was

$$I = [(a_N[\text{GuHCl}] + b_N) + (a_D[\text{GuHCl}] + b_D) \times \exp(-\Delta G'/RT)]/[1 + \exp(-\Delta G'/RT)], \quad (1)$$

where b_N is the intensity of the native state at 0 M GuHCl, a_N is the slope of the native baseline, b_D is the (extrapolated) intensity of the denatured state at 0 M GuHCl, a_D is the slope of the denatured state baseline, and $\Delta G'$ is the the free energy change upon unfolding at the given concentration of GuHCl. $\Delta G'$ is assumed to be a linear function of denaturant concentration, as described by the equation

$$\Delta G' = \Delta G_{H_2O} - m[\text{GuHCl}], \quad (2)$$

where ΔG_{H_2O} is the free energy of unfolding at 0 M GuHCl calculated by extrapolation (Schellman, 1978; Santoro & Bolens, 1988).

Peptide binding

Peptides of sequence Ac-PPPVPPRRR and YEVPPPVPPRRR, derived from the guanine nucleotide exchange factor Sos, were synthesized and purified by the Keck Center for Biotechnology, Yale University. The N-terminal 2 residues in the longer peptide are not from Sos, but were added to allow for more accurate concentration determination (Y) and higher solubility (E). Assay samples contained a constant concentration of protein (between 0.5 and 1 μM) and between 0 and 1,100 μM peptide, in 20 mM Na Hepes (pH 7.3), 50 mM NaCl. Samples were allowed to equilibrate for several hours. For the shorter peptide, spectra were obtained by exciting at 280 nm. For the longer tyrosine-containing peptide, spectra were obtained by exciting at 295 nm, in order to minimize excitation of the ligand tyrosine. Under these conditions, the ligand alone still gave a small, linear, concentration-dependent fluorescence signal, and experimental intensities were corrected for this component. Corrected emission intensities at 340 nm (I) were fit, by nonlinear least-squares analysis, to the equation

$$I = [I_0 + I_\infty ([\text{L}]/K_d)]/[1 + ([\text{L}]/K_d)], \quad (3)$$

where I_0 is intensity in the absence of ligand, I_∞ is intensity at saturation, K_d is the dissociation constant, and [L] is peptide ligand concentration (Connors, 1987). Peptide concentrations

were determined either by tyrosine absorbance, for the 12-mer, or quantitative amino acid analysis.

Poly-L-proline binding

Binding was also monitored by change in fluorescence intensity as described above. Assay samples contained 1 μM protein and between 0 and 2 mg/mL poly-L-proline (1,000–10,000 MW, from Sigma) in 20 mM Na Hepes (pH 7.3), 50 mM NaCl.

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