Electronic Supplementary Material (ESI) for Analytical Methods.

Electronic Supplementary Information

Site-selective Characterization of Src Homology 3 Domain Molecular Recognition with Cyanophenylalanine Infrared Probes

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Fluorescence Spectroscopy

In addition to an IR probe, *CN*Phe has been utilized as a fluorescent probe of environments in proteins.[1,2,3] Thus, we also characterized the wild-type and *CN*Phe-labeled variants with fluorescence spectroscopy in both unligated SH3^{Sho1} and the Pbs2 peptide-bound states (Fig. S1). SH3^{Sho1} has two Trp, five Tyr, and two Phe residues that also contribute to fluorescence. To help deconvolute any changes due to the single incorporated *CN*Phe residues, we acquired spectra with excitation at both 280 nm, where Trp and Tyr show strong absorption, as well as at 240 nm, where *CN*Phe shows strong absorption,[1] but where the absorption spectra of the natural amino acids show local minima.

All fluorescence spectra show maxima around 350 nm, which primarily reports the fluorescence from the Trp residues. A large, ~1.5-fold increase in fluorescence intensity and small, ~0.5-7.0 nm shifts in the fluorescence maxima occur for all samples upon binding the Pbs2 peptide, regardless of excitation wavelength. A conserved tryptophan in the binding pocket, one of only two in SH3^{Sho1}, interacts with the bound Pbs2 peptide ligand. The blue shift in emission wavelength upon binding has been attributed to the burial of this partially solvent-exposed tryptophan residue, creating a less polar environment.[4] The increase in the fluorescence intensity upon binding the Pbs2 peptide is likely due to reduced quenching by shielding from

solvent.[5] Although small variation in the binding-induced changes were observed among the *CN*Phe variants, the changes were not outside of error.

Clear evidence for increased fluorescence intensity at 295 nm, the maximum emission wavelength of *CN*Phe, is found in the spectra of the *CN*Phe5, *CN*Phe25, and *CN*Phe57 variants, but not *CN*Phe11 when excited at 240 nm. The emission spectrum of *CN*Phe overlaps the excitation spectra of the native Trp and Tyr amino acids and can be quenched by energy transfer. Previous studies of *CN*Phe also found a correlation between greater fluorescence intensity and more polar environments with potential hydrogen bonding partners.[1,2] Neither of these explain the absence of *CN*Phe fluorescence uniquely for *CN*Phe11. The residue is the most distant from the two Trp residues and Tyr residues in the core of the protein (Fig. S2). In fact, the two residues that show greatest 295 nm fluorescence intensity, *CN*Phe57 and *CN*Phe25, appear closest to the Trp residues. *CN*Phe11 is surface residue, but so are *CN*Phe5 and *CN*Phe57. Thus, variations in fluorescence of the *CN*Phe residues do not appear to report in a straightforward manner on the polarity of the environment, extent of hydration, or on their specific location within SH3^{Sho1} with respect to the other aromatic residues.

Table S1. Expression Yields, Dissociation Constants, and Fluorescence Binding Ratios

	yield (mg/L)	K_d	$F_{350,ligated}/F_{350,unligated}$
wild-type	40	2.3 ± 0.7	1.75 ± 0.35
*CN*Phe5	12	1.9 ± 0.3	1.42 ± 0.25
*CN*Phe11	12	3.9 ± 0.6	1.77 ± 0.38
*CN*Phe25	34	2.0 ± 0.7	1.47 ± 0.24
*CN*Phe57	2.5	3.4 ± 1.6	1.32 ± 0.13

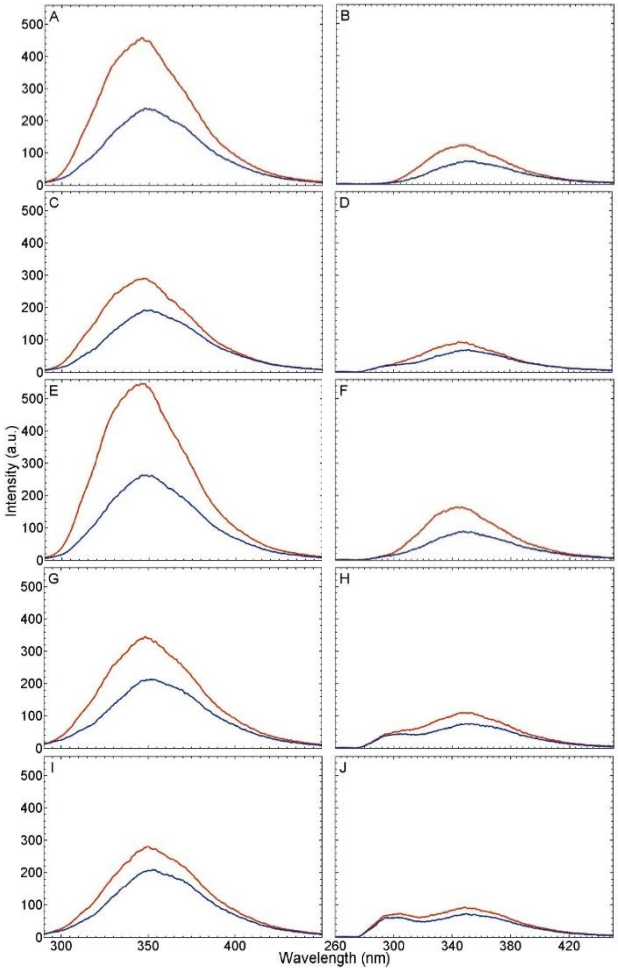

Figure S1. Fluorescence emission spectra of unligated (blue) and Pbs2 peptide-bound (red) SH3^{Sho1} variants with excitation wavelengths at 280 (left) and 240 nm (right): wild type (A and B), *CN*Phe5 (C and D), *CN*Phe11 (E and F), *CN*Phe25 (G and H), and *CN*Phe57 (I and J).

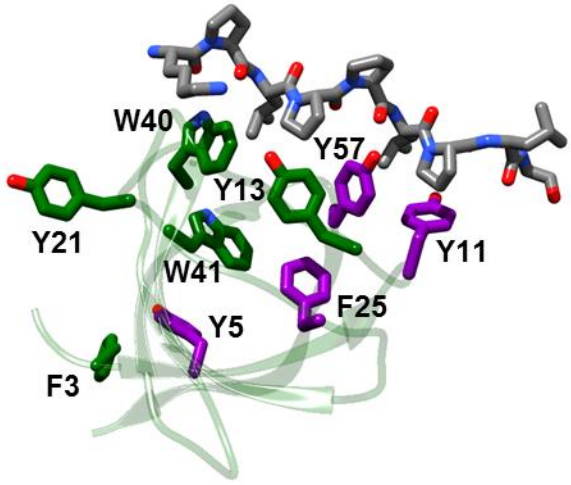

Figure S2. Structure of SH3^{Sho1} ligated to Pbs2-peptide with all aromatic residues shown. Side chains shown in purple are those labelled in this study. Pdb 2VKN. Image generated using UCSF Chimera.

Mass Spectrometry

Trypsin digests were performed at a protein concentration of 0.1 mg/mL of solution with 30:1 mass ratio of trypsin:SH3 with 25 percent acetonitrile. Trypsin was purchased from Thermo Scientific. After overnight digest at 37 °C, samples were desalted with a C4 or C18 Zip Tip (EMD Millipore) before analysis by MALDI-TOF on a Bruker Autoflex III (Billerica, MA).

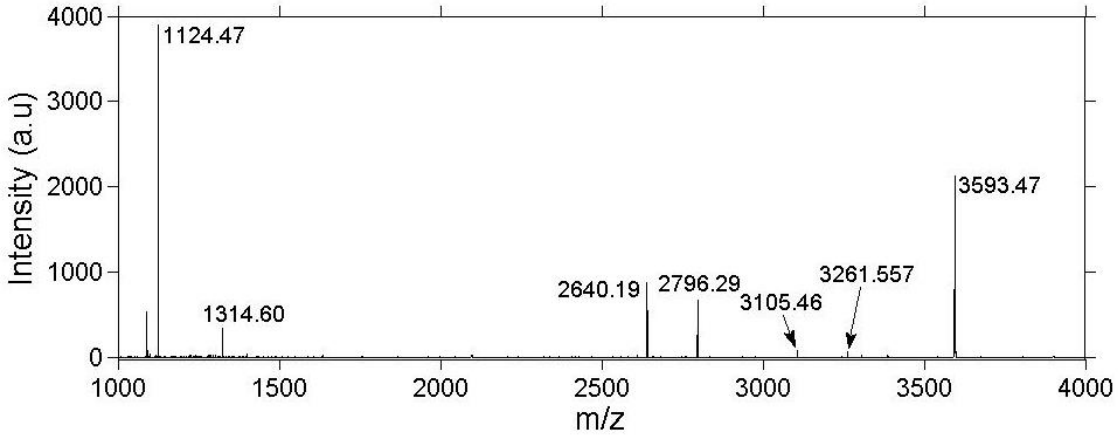

Figure S2. Mass spectrum of a *CN*Phe5 trypsin digest.

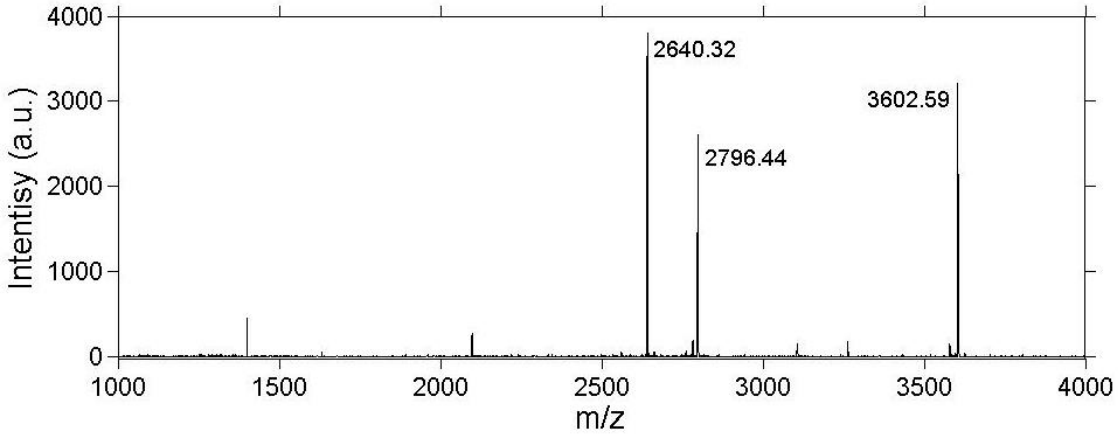

Figure S3. Mass spectrum of a *CN*Phe11 trypsin digest.

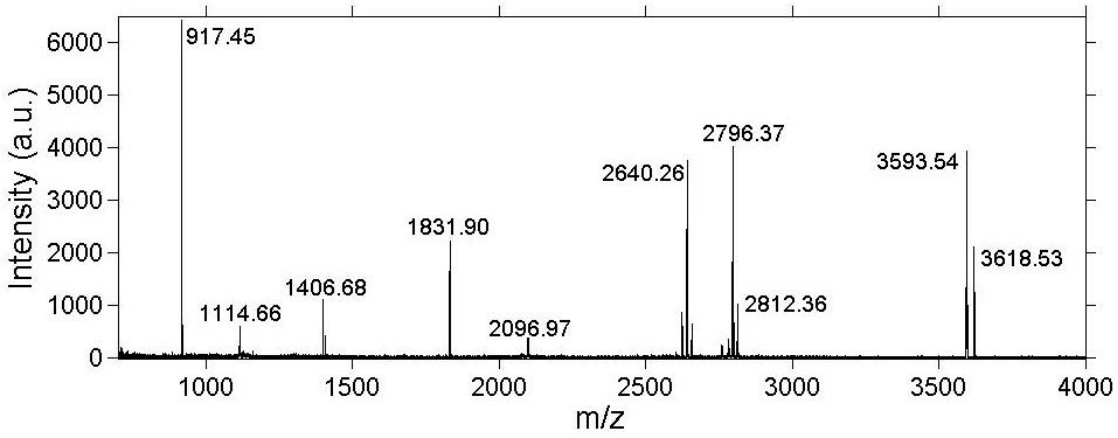

Figure S4. Mass spectrum of a *CN*Phe25 trypsin digest.

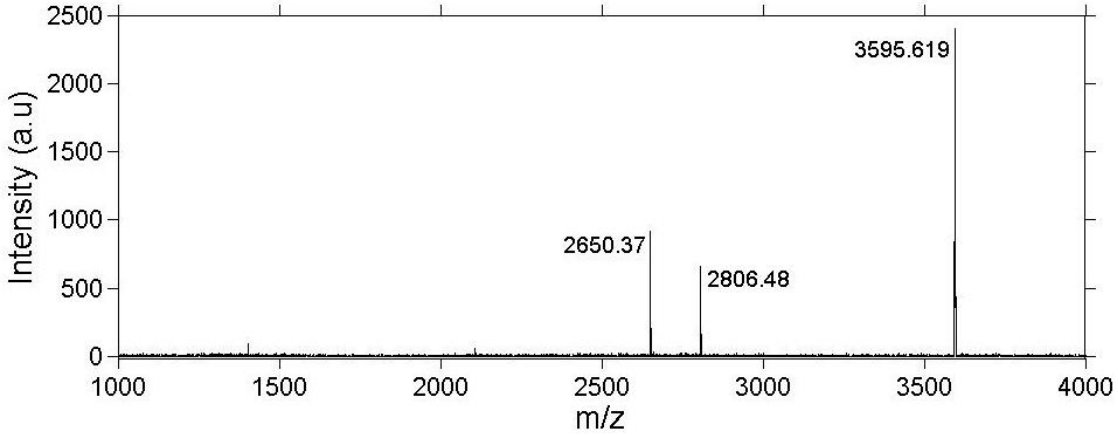

Figure S5. Mass spectrum of a *CN*Phe57 trypsin digest.

Table S2. Summary of tryptic digest results for *CN*Phe-labeled SH3^{Sho1} variants.

Expected Masses for Unlabeled Protein	Sequence	Residue numbers[a]	Modifications	Observed CNPhe5 fragments	Observed CNPhe11 fragments	Observed CNPhe25 fragments	Observed CNPhe57 fragments
1115.50	MGDDGSNFIYK	1-11	1 Met-loss	1124.47**		1114.66	
1304.55	MGDDGSNFIYK	1-11	1 Acetyl 1 Oxidation	1314.60**[b]			
1314.63	MGDDGSNFIYKAK	1-14	1Met-loss	1314.60[b]			
2640.27	ANGETGIIPSNYVQLI DGPEEMHR	51-74		2640.19	2640.32	2640.26	2650.37**
2796.37	RANGETGIIPSNYVQL IDGPEEMHR	50-74		2796.29	2796.44	2796.37	2806.48**
2812.37	RANGETGIIPSNYVQL IDGPEEMHR	50-74	1Oxidation			2812.36, 1406.68	
3105.58	ANGETGIIPSNYVQLI DGPEEMHRLVPR	50-78		3105.46			
3261.68	RANGETGIIPSNYVQL IDGPEEMHRLVPR	50-78		3261.56			
3593.61	ALYPYDADDDDAYEI SFEQNEILQVSDIEGR	14-44		3593.47	3602.59**	3593.54, 3618.53**	3595.62

**Indicates mass shift observed due to incorporation of CNPhe at the desired location.

[a]In this construct, Y5 is at residue number 10, Y11 is at residue number 16, F25 is at residue number 30, and Y57 is at residue number 62.

[b]Mass could be due to the presence of either or both of two fragments, one which exhibits the desired mutation, and one which corresponds to the wild-type mass.

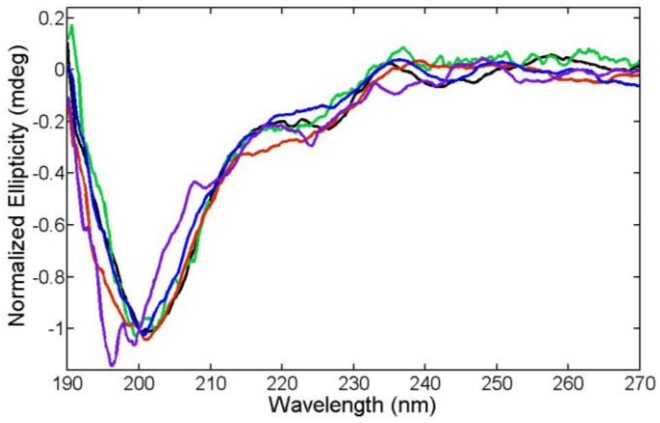

Figure S6. Circular dichroism spectra of *CN*Phe5 (purple), *CN*Phe25 (green), *CN*Phe57 (blue), *CN*Phe11 (red), and wild-type SH3^{Sho1} (black). Spectra were acquired at protein concentrations of 0.01 mM in 10 mM sodium phosphate buffer, pH 7.0 in a 1 mm cuvette. Data have been normalized to 200 nm.

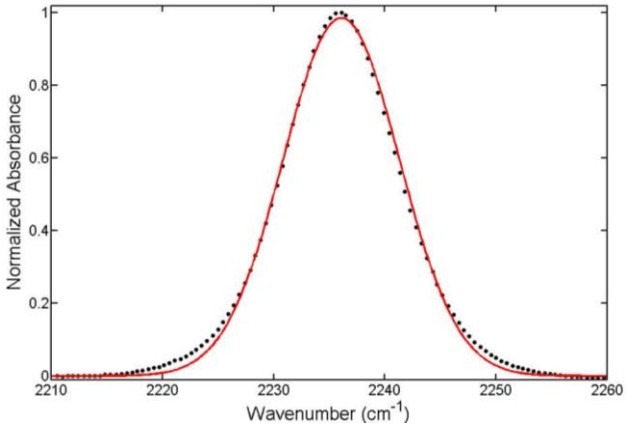

Figure S7. A representative Gaussian fit to FTIR data. Dots show the average of three data sets acquired for Pbs2-bound *CN*Phe11 SH3^{Sho1}. The red line represents the Gaussian fit to the data.

References

1. M. J. Tucker, R. Oyola and F. Gai, A novel fluorescent probe for protein binding and folding studies: p-cyano-phenylalanine, *Biopolymers*, 2006, **83**, 571-576.
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3. R. F. Wissner, S. Batjargal, C. M. Fadzen and E. J. Petersson, Labeling proteins with fluorophore/thioamide Forster resonant energy transfer pairs by combining unnatural amino acid mutagenesis and native chemical ligation, *J. Am. Chem. Soc.*, 2013, **135**, 6529-6540.
4. A. R. Viguera, J. L. R. Arrondo, A. Musacchio, M. Saraste, and L. Serrano, Characterization of the Interaction of Natural Proline-Rich Peptides with Five Different SH3 Domains, *Biochemistry*, 1994, **33**, 10925-10933.
5. W. A. Lim, R. O. Fox, and F. M. Richards, Stability and peptide binding affinity of an SH3 domain from the *Caenorhabditis elegans* signaling protein Sem-5, *Protein Science*, 1994, **3**, 1261-1266.