A Thermostable 35-Residue Subdomain within Villin Headpiece

C. James McKnight¹,²*, Don S. Doering², Paul T. Matsudaira² and Peter S. Kim¹,²

The actin-bundling protein villin contains, at its extreme C terminus, a compact f-actin binding domain called “headpiece”. This 76-amino acid domain from chicken is highly thermostable. Here, we show that the stable folded structure in headpiece is localized to a subdomain formed by the C-terminal 35 residues. The subdomain, denoted HP-35, is monomeric and retains high thermostability, with a $T_m$ of 70(±1) °C at pH 7.0. There are no cysteine residues in HP-35 and its folding is not dependent on the binding of metals or other ligands. HP-35 is not a molten globule, but instead, has properties expected for a fully folded protein with a unique structure. In particular, the slowly exchanging amide protons in HP-35 have protection factors that are slightly larger than those predicted if exchange occurred only from globally unfolded molecules. NMR studies indicate that the headpiece subdomain contains three short $\alpha$-helices, and that these same helices are present in the corresponding regions of intact headpiece. HP-35 is the smallest monomeric polypeptide characterized consisting of only naturally occurring amino acids that autonomously folds into a unique and thermostable structure without disulfide bonds or ligand binding.

Subdomains that fold independently are important tools for solving the protein folding problem (Oas & Kim, 1988; Rose, 1979). Subdomains are

units of folded structure larger than an isolated helix or sheet, but smaller than an entire domain. The shorter amino acid sequences of subdomains reduce complexity, relative to intact proteins. This relative simplicity facilitates experimental studies and also comparison between experiments and theoretical calculations. The stable structures formed by subdomains may also be important intermediates or initiation sites for protein folding. Here, we describe the identification of a short, monomeric subdomain from chicken villin that folds without disulfide bonds or ligand binding.

The f-actin bundling protein villin is implicated in the formation of microvilli in the absorptive epithelium of the gut and the proximal tube of the kidney (Bretscher & Weber, 1979; Finidori et al., 1992). Villin is composed of two domains defined by partial proteolysis: a “core” domain of 84 kDa, and a small, 8 kDa carboxy-terminal domain called “headpiece” (Bazari et al., 1988; Glenny et al., 1981). Each of these domains contains an f-actin binding site, enabling villin to crosslink actin filaments into structural bundles that support the microvillus membrane (Glenny et al., 1981).
Headpiece binds f-actin in the absence of the core domain (Glenny et al., 1981; Pope et al., 1994). The modular nature of the 76-amino acid headpiece domain is demonstrated by its presence in the f-actin bundling protein dematin (band 4.9), which has a core domain that is unrelated to villin (Rana et al., 1993). The amino acid sequence of headpiece (Bazari et al., 1988) is not similar to other known actin-binding motifs (Vanderkerckhove, 1990). The sequence of the chicken villin headpiece is: VFTATTTLVPTKLETFPLDVVLVTAAEDLPRGDPSRKENHLSDEDKAVFGMTSASAFANLPLWKEQNNLKEKGLF. Residue 1 of headpiece corresponds to residue 750 of intact chicken villin. The underlined residues correspond to the 35-residue subdomain described in this study.

Table 1. Backbone resonance assignments at pH 5.0, 30°C

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NMR data were collected on a Bruker AMX-500 spectrometer at 30°C. 15N-HP-45 and 15N-HP-36 were expressed and purified as described in the legend to Figures 1 and 2, except that cells were grown in M9T minimal media supplemented with 1 g/l 15N-ammonium sulfate (Muchmore et al., 1989). The NMR samples used for assignments were 6.6 mM for 15N-headpiece, 4.0 mM for 15N-HP-45, and 3.2 mM for 15N-HP-36. Samples contained 5% 2H2O and the pH was adjusted using a glass electrode with no correction for the effect of 2H2O on the measured pH (Bundi & Wuthrich, 1979).

Partial NMR assignments of 15N-enriched headpiece were made at pH 5.0, 30°C by examination of spectra from 3D (1H, 1H, 15N) NOESY-HSQC (Bodenhausen & Ruben, 1980; Marion et al., 1989), 3D (1H, 1H, 15N) TOCSY-HSQC (Bodenhausen & Ruben, 1980; Griesinger et al., 1988), and HSQC (Bodenhausen & Ruben, 1980) experiments. NMR assignments for 15N-HP-36 at pH 3.7, and 15N-HP-45 at pH 5.0 (not shown), were based on standard 2D homonuclear methods using DQF-COSY, NOESY and TOCSY spectra (Wuthrich, 1986). Heteronuclear 2D (1H, 15N) spectra collected to aid resonance assignments included HMQC-COSY (Gronenborn et al., 1989), HSQC-TOCSY (Bodenhausen & Ruben, 1980; Griesinger et al., 1988; Gronenborn et al., 1989), and HSMQC-NOESY (Gronenborn et al., 1989; Zuiderweg, 1990). The backbone assignments of 15N-HP-36 were transferred from pH 3.7 to pH 5.0 by comparison of the HSQC spectra and then verified with HSMQC-NOESY. HMQC-COSY spectra were recorded to extract Hα proton assignments of 15N-HP-35 at pH 5.0.

* Chemical shifts (p.p.m.) are relative to 3-(trimethylsilyl)-1-propanesulfonic acid (−0.01 p.p.m.) for 1H, and NH4Cl (24.93 p.p.m.) for 15N. Unassigned resonances are left blank.
Identification of the subdomain

Only partial NMR assignments of headpiece can be obtained readily (Table 1). Resonances from the amino-terminal half of headpiece are split into multiple peaks and/or are in intermediate exchange, indicating the presence of multiple interconverting conformations that may arise, at least in part, from cis–trans isomerization about the three proline residues at positions 17, 30 and 35. Sequential resonance assignments are possible for the C-terminal residues of headpiece, starting at Asp44 and continuing through to Phe76, with breaks at residues 54 and 71 due to ambiguities (Table 1). These NMR data suggest the presence of a C-terminal subdomain in headpiece.

To delineate the subdomain, headpiece was subjected to limited proteolysis by trypsin. Trypsinolysis was carried out in 1 M guanidine hydrochloride (GuHCl) to destabilize any residual folded structure that might exist in the N-terminal region of headpiece (Riviere et al., 1991). A 45-amino acid fragment, denoted HP-45 and corresponding to residues 32 to 76 of headpiece, is the most populated digestion product, accumulating to 14% of the total sample absorbance at 229 nm by 90 minutes (Figure 1). At later time points, the population of HP-45 decreases, while several smaller C-terminal fragments become populated at low levels. The relative resistance to trypsin digestion of HP-45, which contains eight potential trypsin cleavage sites, argues strongly for the presence of stably folded structure at the C terminus of headpiece.

The thermal unfolding of headpiece, as monitored by circular dichroism (CD), is reversible, with a transition midpoint (Tm) of 74°C (Doering, 1992). This Tm value is the same in the presence of the metal chelator ethylenediaminetetraacetic acid (EDTA) (Figure 2a), indicating that the thermostable structure of headpiece is not dependent on metal binding. The Tm value for the unfolding transition of HP-45 is 72°C, only 2°C lower than for intact headpiece (Figure 2a). Thus, the 31 N-terminal residues of headpiece are not required for the thermostability of HP-45. However, the differences in the slopes in the transition regions of headpiece compared to HP-45 in the thermal unfolding experiment may indicate a significantly higher molar enthalpy for headpiece unfolding than for HP-45. This may indicate that there is a larger difference in

Aliquots were removed at given time points, quenched by addition of glacial acetic acid to a final concentration of 5%, and frozen in liquid nitrogen. The quenched samples were analyzed by reverse-phase HPLC using a C18 column (Vydac, 0.46 cm × 30 cm) and a 1% min⁻¹ water/acetonitrile gradient containing 0.1% (v/v) trifluoroacetic acid (TFA). Peaks were monitored by absorbance at 229 nm. The molecular masses of the fragments were determined by laser desorption mass spectrometry (Finnigan MAT). Reported peak identities were confirmed by N-terminal sequence analysis (model 470A, Applied Biosystems, Inc.).
Figure 2. CD measurements of headpiece and C-terminal peptides. 

a, Thermal unfolding monitored by CD. For ease of comparison, the data have been normalized to fraction unfolded after subtraction of the folded and unfolded baselines. Protein concentrations were 20 μM in 10 mM phosphate (pH 7.0), with 150 mM NaCl and 500 μM EDTA. 
b, CD spectra of headpiece and headpiece fragments at 25°C. Protein concentrations were 20 μM in 10 mM phosphate (pH 7.0). 
c, Guanidine hydrochloride denaturation of HP-36 in H2O. The samples were 20 μM HP-36 in 150 mM NaCl, 25 mM acetate (pH 5.0), at 4°C. The line is the best fit to the equation described by Santoro & Bolen (1988). For this fit ΔG = 3.3 kcal mol⁻¹, and the m value was 800 cal mol⁻¹ M⁻¹. 

Methods: The expression plasmid for HP-45, pVHP32-76, was constructed by PCR from the parent headpiece expression plasmid pDDHP10 (Doering, 1992). Expressed HP-35 retains the additional N-terminal initiator methionine residue, so the resultant peptide is 36 residues long and is referred to as HP-36. An initial expression plasmid for HP-36, pVHP42-76, was constructed by single-stranded mutagenesis of pVHP32-76, deleting the first ten codons after the initiator methionine codon. This plasmid expressed poorly, so the coding region was recloned into the pDDHP10 plasmid backbone (pVHP42-76b). The pVHP42-76b plasmid was found to express the HP-36 fragment at levels sufficient for purification. 

Recombinant DNA techniques were based on standard protocols (Sambrook et al., 1989). Constructs were confirmed by DNA sequencing (Sanger et al., 1977).

HP-45 and HP-36 were expressed and purified as described in the legend to Figure 1, except that buffer A was replaced by 50 mM phosphate buffer (pH 7.0), and the ACA34 column was replaced by a Sephadex G-50 column (1000 x 2.5 cm) run in 50 mM phosphate. The synthetic peptide HP-35 was synthesized using standard solid phase Fmoc peptide synthesis protocols on an Applied Biosystems model 430A automated peptide synthesizer. The peptide was cleaved from the resin with TFA and purified by HPLC as described in the legend to Figure 1.

CD spectra were recorded on an AVIV 60DS or 62DS spectrometer equipped with a thermoelectric sample temperature controller. Samples for wavelength spectra contained 20 μM peptide in 10 mM phosphate buffer (pH 7.0). The cell path length was 0.1 cm. The spectra were collected as the average of six scans, using a three-second integration time at 1.0 nm wavelength increments. Spectra were baseline-corrected against the cuvet with buffer alone.

Samples for the thermal unfolding experiments were 20 μM peptide in 150 mM NaCl, 500 μM EDTA, 10 mM phosphate buffer (pH 7.0). A 1.0 cm path-length cell was used with continuous stirring. Data points were collected at one-degree intervals with an integration time of 20 seconds after a one-minute equilibration period at the desired temperature. The thermal unfolding curves were baseline-corrected by subtraction of a buffer only control experiment. In all cases the thermal unfolding was over 95% reversible and the Tm values did not change after repeated thermal
ΔG value between headpiece and the HP-45 fragment than that indicated by the small difference in Tm. Accurate determination of the ΔG value for intact headpiece by CD denaturant unfolding experiments is complicated by difficulties establishing a folded baseline.

From the magnitude of the CD signal at 222 nm (Figure 2b), we estimate that expressed HP-45 contains approximately the same number of residues in a helical conformation as intact headpiece (~18 residues compared with ~19 for intact headpiece). Helix content was estimated from the CD signal by dividing the mean residue ellipticity at 222 nm by the value expected for 100% helix formation by short helices, ~30,000 deg.cm².dmøl⁻¹ (Chen et al., 1974).

The N-terminal 14 residues of HP-45 have rapid amide-proton exchange rates (Figure 3a) and do not give rise to significant non-sequential NOE peaks (not shown), suggesting that this region is unfolded. A peptide (HP-35) was synthesized to test whether the N-terminal ten residues were required for the folding of HP-45. We began HP-35 at Leu42, since the backbone carbonyl oxygen of unfolding (not shown). The Tm values were determined by examination of the derivative of the [θ]222 values with respect to the reciprocal of the temperature (Cantor & Schimmel, 1980). The error in estimation of Tm is ±1°C.

Denaturation of HP-36 by GuHCl was performed in H₂O and ²H₂O under conditions similar to those of the amide exchange measurements (pH 5.0, 4°C). Seven stock solutions spanning 0 to ~8 M GuHCl, in H₂O or ²H₂O, were prepared containing 20μM HP-36, 150 mM NaCl and 25 mM acetate and then adjusted to pH 5.0. No corrections were made for the effect of ²H₂O on the measured pH (Bündi & Wüthrich, 1979). The GuHCl concentration of each stock was determined by refractive index (Pace, 1986). Linear combinations of the stock solutions were used to prepare samples at intermediate GuHCl concentrations. The samples were equilibrated at 4°C overnight. CD data were collected in kinetics mode for five minutes, with a five second averaging time, at 222 nm, in a 0.5 cm path-length cell thermostated at 4°C. The average of the data points for the last 100 seconds of collection was taken for the final value. Selected samples in the transition region of the GuHCl denaturation experiment in H₂O were remeasured after an additional 24 hours of equilibration at 4°C and were superimposable with those at 12 hours, indicating that the samples were fully equilibrated (not shown). In addition to linear extrapolation, data were fit to a six-parameter equation to determine the slopes and intercepts of the folded and unfolded baselines, the m value, and ΔG⁺₅⁺₅°C (Santoro & Bolen, 1988).
Figure 3. NMR characterization of headpiece, HP-45, and HP-36. a, Hydrogen-exchange protection factors for HP-45 and HP-36. Amide groups that exchanged too fast to measure are shown with protection factors of 1 for reference only. Open squares, HP-45; open triangles, HP-36. Exchange was measured at pH 5.0, 30°C for HP-45, and 4°C for HP-36. HSQC spectra were recorded at various times after addition of ²H₂O to previously lyophilized H₂O samples. The volumes of the peaks were fit to exponentials to determine the exchange rates, and then converted to protection factors after calculation of the intrinsic, random-coil exchange rates (Bai et al., 1993). b, NMR assignment summaries of villin headpiece and HP-36. Bars under the sequence show unambiguous sequential (α,N(ᵢ₊₁) and N,N(ᵢ₊₁)) and helical (α,N(ᵢ₊₃)) NOE connectivities. Filled circles indicate residues with greater than 1000-fold amide exchange protection. The assignments were made at pH 5.0, 30°C for headpiece and at pH 3.7, 30°C for HP-36. c, HSQC spectra of headpiece and HP-36 at pH 5.0 and 30°C. Indicated assignments are for HP-36. Headpiece spectrum is in green, HP-36 is in red.

this residue is expected to be the hydrogen bond acceptor for the amide proton of Asp46, the most N-terminal amide proton protected from exchange in HP-45.

The CD spectrum of HP-35 (Figure 2b) indicates that it folds, and that ~16 residues are in a helical conformation. Most importantly, the $T_m$ for the unfolding of HP-35 (70°C) is only 4°C less than intact headpiece (Figure 2a). Therefore, the thermostable folded structure of headpiece is located within the C-terminal 35 residues.
The HP subdomain is monomeric and has a fixed structure

Figure 4 shows a sample of the sedimentation equilibrium data collected on HP-35 to determine its oligomeric state. Over a tenfold range of peptide concentration (10, 30, and 100 μM), the average experimental molecular mass of HP-35 is 3890(±130) (s.d.), as determined by analytical ultracentrifugation (Laue et al., 1992). This value is in good agreement with the calculated molecular weight of 4059, indicating that HP-35 is monomeric in solution.

The 35-residue subdomain was expressed in Escherichia coli to facilitate further studies. The expressed peptide, HP-36, retains the additional N-terminal initiator methionine. Both the CD spectrum and thermal unfolding of HP-36 are similar to that of HP-35 (Figure 2a and b). The only significant difference is a slightly elevated thermal unfolding temperature (T_m = 72°C) for the longer, recombinant HP-36.

Linear extrapolation of the GuHCl denaturation of HP-36 to 0 M GuHCl (Figure 2c) yields a \( \Delta G_{\text{pH}5,\text{VC}} \) value of 3.3(±0.4) kcal mol\(^{-1}\) (1 kcal = 4.184 Joules). In \( ^2\text{H}_2\text{O} \), \( \Delta G_{\text{pH}5,\text{VC}} \) is 4.1(±0.3) kcal mol\(^{-1}\), with an m value of 1040 cal mol\(^{-1}\) M\(^{-1}\) (not shown). These values are likely to be the lower limits of \( \Delta G \) because of the simple linear extrapolation fitting procedure used (Pace, 1986; Santoro & Bolen, 1988).

If the villin C-terminal subdomain has a unique, well-defined structure, it should contain slowly exchanging amide protons, with protection factors equal to, or greater than, those predicted from the global stability determined by chemical denaturation (Lumb & Kim, 1995). For HP-36, this would correspond to amide-exchange protection factors in excess of \( \sim 2000 \) (using \( \Delta G = -RT \ln K \), and \( \Delta G = 4.1 \) kcal mol\(^{-1}\), in \( ^2\text{H}_2\text{O} \)). HP-35 contains five amides with protection factors between 20,000 and 50,000 in \( ^2\text{H}_2\text{O} \) (Figure 3a). Thus, HP-36 exhibits the amide exchange behavior expected of a protein in a fixed, unique conformation.

In the C-terminal region of headpiece, the pattern of \( z,N_{\text{H}(i-3)} \) NOEs, characteristic of \( z \)-helices, indicates the presence of three short helical segments (Figure 3b). This same pattern is also seen in HP-36, indicating that it contains the same three helical segments. Further support for the similarity in the structure between HP-36 and the corresponding residues in headpiece is provided by the close correspondence of the respective backbone chemical shifts (Figure 3c and Table 1).

**Conclusions**

The 35-residue subdomain from villin headpiece is fully folded in a single, discrete conformation and is not a molten globule. Unlike molten globules (Kuwajima et al., 1976; Ptitsyn, 1992), HP-35 undergoes a cooperative thermal unfolding transition and has an NMR spectrum with substantial chemical shift dispersion. Proteins that fold into a unique conformation generally contain amide protons with hydrogen-exchange protection factors that are an order of magnitude greater than expected if exchange occurred only from globally unfolded molecules (Bai et al., 1994; Loh et al., 1993; Marmorino et al., 1993; Mayo & Baldwin, 1993; O’Shea et al., 1993; Roder, 1989). Conversely, molten globules and most designed proteins or peptides have amide-proton exchange rates that are substantially faster than that predicted from a global unfolding exchange mechanism, suggesting that ill-defined, fluctuating structures are formed (see Discussion in Lumb & Kim, 1995). As expected for a folded protein with a unique, fixed structure, HP-36 contains amides with hydrogen-exchange protection factors in excess of those predicted from the global stability of the subdomain determined by chemical denaturation.

Although some short monomeric helical peptides are marginally thermostable (Marqusee et al., 1989), peptides of length comparable to HP-35 generally
require oligomerization, ligand binding, co-solvents, or disulfide-bond formation for stable folding. In contrast, HP-35 folds independently and autonomously in the absence of these additional stabilizing mechanisms. Recently, the NMR structure of a monomeric, 23-residue synthetic polypeptide corresponding to a zinc finger has been reported (Struthers et al., 1996). However, in order to fold in the absence of zinc, the peptide requires one unusual amino acid (d-proline) and one non-naturally occurring amino acid (3-(1,10-phenanthrol-2-yl)-l-alanine).

After HP-35, the shortest sequence of naturally occurring amino acids that folds autonomously into a stable structure, of which we are aware, is the 43-residue peripheral subunit-binding domain of dihydrolipoamide acetyltransferase from pyruvate dehydrogenase (Kalia et al., 1993). Although the NMR structure of that subdomain contains a 33-amino acid core, it has not been determined whether the 33-amino acid core can fold to form a stable structure, in isolation. Indeed, residues at both the N and C-terminal regions, outside the 33-amino acid core, contain amides that are protected from hydrogen exchange (Kalia et al., 1993), suggesting that, while less ordered in the NMR structure, these additional residues contribute to the folding and/or stabilization of the subdomain.

Like HP-35, the acetyltransferase subdomain folds to form three helices. However, the lack of sequence similarity, coupled with the longer sequence length and the wider gap between the central and C-terminal helices in acetyltransferase, suggests that the folds of these two subdomains may differ.

Without a detailed three-dimensional structure of the HP-35 subdomain, it is difficult to account for the high thermostability of this short, three-helix motif. The native-like amide exchange data and chemical-shift dispersion strongly suggest, however, that these helices fold to form a tightly packed hydrophobic core.

Finally, our NMR results indicate that the corresponding residues in intact headpiece fold into the same overall three-helix conformation found in the HP-35 subdomain. It is of interest that all of the residues identified as essential for actin binding (Doering, 1992; Friederich et al., 1992) are in the C-terminal helix of the HP-35 subdomain. HP-35 should be a useful subdomain for experimental and theoretical studies of protein folding, and for the design of small actin-binding proteins.

Acknowledgements

We thank Lawrence McIntosh for assistance with the NMR studies, Mike Burgess for peptide synthesis, and Kevin Lumb and Rheba Rutkowski for their careful reading of the manuscript. We acknowledge financial support from the American Cancer Society (C.J.M.), the National Institutes of Health (P.T.M.), and the Howard Hughes Medical Institute (P.S.K.).

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Edited by P. E. Wright

(Received 2 January 1996; received in revised form 4 April 1996; accepted 18 April 1996)