Published in final edited form as:

J Mol Biol. 2006 November 24; 364(2): 225–240.

Partially Unfolded Forms and Non-Two-State Folding of a β -Sandwich: FHA Domain from Arabidopsis Receptor Kinase-Associated Protein Phosphatase

Xiangyang Liang, Gui-in Lee[†], and Steven R. Van Doren^{*}

Department of Biochemistry, 117 Schweitzer Hall, University of Missouri, Columbia, MO 65211 USA

Abstract

FHA domains adopt a β -sandwich fold with 11 strands. The first evidence of partially unfolded forms of a β -sandwich is derived from native-state hydrogen exchange (NHX) of the FHA domain from Kinase-Associated Protein Phosphatase from Arabidopsis. The folding kinetics of this FHA domain indicate that EX2 behavior prevails at pH 6.3. In the chevron plot, rollover in the folding arm and bends in the unfolding arm suggest folding intermediates. NHX of this FHA domain suggests a core of six most stable β -strands and two loops, characterized by rare global unfolding events. Flanking this stable core are β -strands and recognition loops with less stability, termed subglobal motifs. These suggest partially unfolded forms (near-native intermediates) with two levels of stability. The spatial separation of the subglobal motifs on the flanks suggests possible parallelism in their folding as additional β -strands align with the stable core of six strands. Intermediates may contribute to differences in stabilities and m -values suggested by NHX or kinetics relative to chemical denaturation. Residual structure in the unfolded regime is suggested by superprotection of β -strand 6 and by GdmCl-dependence of adjustments in amide NMR spectra and residual optical signal. The global folding stability depends strongly on pH, with at least 3 kcal/mol more stability at pH 7.3 than at pH 6.3. This FHA domain is hypothesized to fold progressively with initial hydrophobic collapse of its stable 6-stranded core followed by addition of less stable flanking β -strands and ordering of recognition loops.

Introduction

FHA domains share with SMAD MH2 domains and interferon regulatory factor (IRF) transactivation domains the following: a β -sandwich fold with 11 strands^{1; 2}, a set of hydrophobic folding core residues³, and the ability to recognize proteins phosphorylated on threonine or serine. However, these proteins diverge greatly in sequence and signaling contexts. Among these widely important modules, the Kinase-Interacting FHA domain (KI-FHA) from Arabidopsis is the only one for which both detailed structure and solution dynamics have been described, in addition to hydrodynamics and phosphopeptide binding effects^{3; 4}. As such, KI-FHA is an attractive representative of these protein families for further biophysical and protein folding studies. The sole report of folding measurements of an FHA domain or SMAD MH2 domain is the stability to chemical or thermal denaturation of about 3 kcal/mol of the FHA domain from kinesin subfamily UNC104⁵.

^{*}To whom correspondence should be addressed. E-mail: vandorens@missouri.edu., Phone: 1 (573) 882-5113, FAX: 1 (573) 884-4812

[†]Current address: Department of Chemistry, 225 Prospect St., Yale University, New Haven, CT 06520 USA

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

KI-FHA is the phosphoThr recognition module from Kinase-Associated Protein Phosphatase (KAPP) in plants ^{6; 7}. KAPP attenuates some receptor-like kinase (RLK) signaling pathways by dephosphorylating activated RLKs in plant development, environmental response, and defense mechanisms ⁸. KI-FHA and its protein superfamily are formed of a sandwich of a 5-stranded mixed β -sheet with a 6-stranded anti-parallel β -sheet. The phosphoprotein recognition surface is formed by the 3/4, 4/5, 6/7, 8/9, and 10/11 loops ³. Consistent with the principle of N-to C-terminal contact ⁹, β -strands 1 and 11 are adjacent, bringing the N- and C-termini of the domain close together on the edge of the sandwich opposite the phosphoprotein-binding surface. A dozen key side chains from the two β -sheet pack in the proposed folding core of this protein superfamily ³.

In the early stages of protein folding, global hydrophobic collapse forms clusters of non-polar groups, reducing the conformational possibilities and guiding the folding pathway ¹⁰. These compact intermediates can form rapidly (in microseconds) ¹¹ and contain extensive secondary structure. Intermediates have been thought to lack the fixed and near crystalline tertiary side chain contacts characteristic of the native state. However, the equilibrium atomic structures of folding intermediates or partially unfolded forms were determined for Rd-apocytochrome b₅₆₂ ^{12; 13}.

Native-state hydrogen exchange (NHX), detected at residue-specific amide groups by NMR, enables exploration of the equilibrium energy landscape between native, intermediate and unfolded states. NHX exploits the dependence on low denaturant concentrations. NHX is distinctive among protein folding techniques in that it can detect rarely populated intermediates, known as partially unfolded forms (PUFs) visited transiently from the native state ¹⁴. This unique ability results from the transient intermediate states being populated significantly relative to the rare, higher energy unfolded state U that is open for hydrogen exchange ¹⁵. Since NHX identifies minutely populated intermediates at *equilibrium*, it does not rely upon special arrangements of activation barriers required for kinetic trapping of folding intermediates ¹⁴. NHX evidence has identified PUFs in the folding of cytochrome c ^{14; 16}, RNase H ¹⁷, T4 lysozyme ¹⁸, CD2 ¹⁹, redesigned apocytochrome b₅₆₂ ²⁰, OspA ²¹, barnase ²² and PDZ-3 from PSD-95 ²³. The ability of NHX to identify folding intermediates had been disputed ²⁴. Failures of NHX to identify PUFs were since attributed to the lower stability and insufficient dynamic range of those proteins ^{14; 25}. PUFs have been demonstrated to correspond to kinetic folding intermediates in seminal studies of cytochrome c ^{16; 25} and RNase H ^{17; 26}, and subsequently in other proteins listed above.

The Linderstrøm-Lang model states that a hydrogen-bonded “closed” state equilibrates with the “open” state where amide hydrogen exchange occurs with characteristics of a free amide ^{27; 28; 29; 30}:

where k_{op} is the rate constant for the opening step, k_{cl} is the rate constant for the closing step, and k_{rc} is the intrinsic rate of chemical exchange of the exposed amide hydrogen from the random coil state. To represent each amide in its random coil state, k_{rc} is calculated from systematic results for model peptides ^{31; 32}. Salient aspects of obtaining thermodynamics of protein stability from HX ³³ are repeated here. Under native-state conditions, $k_{cl} \gg k_{op}$, the experimental exchange rate k_{ex} is given by equation 2:

$$k_{ex} = \frac{k_{op}k_{rc}}{k_{cl} + k_{rc}} \quad (2)$$

There are two limits for this kinetic exchange. In the EX2 limit, the closing step is much faster than the exchange step ($k_{cl} \gg k_{rc}$), making the intrinsic hydrogen exchange rate k_{rc} the rate-limiting step. In EX2, equation 2 can be simplified:

$$k_{ex} = K_{op}k_{rc} = \frac{k_{op}}{k_{cl}}k_{rc} \quad (3)$$

where K_{op} is the equilibrium constant for the reaction between the “closed” and “open” states. The EX1 limit describes unfolding under conditions far from physiological, such as high pH, high temperature or high denaturant concentration. In the EX1 limit, $k_{cl} \ll k_{rc}$, making the opening step the rate-limiting step. In the EX2 limit, the free energy of exchange of the amide protons can be calculated from the equation:

$$\Delta G_{HX} = -RT \ln(k_{ex}/k_{rc}) \quad (4)$$

The most stable among a protein's ΔG_{HX} values, with a proline correction, usually provide a global stability estimate in excellent agreement with traditional unfolding methods³⁴.

Two processes for opening and exchange, i.e. rare global unfolding events and frequent local fluctuations, became widely accepted since their proposal^{35; 36}. The frequency of the global unfolding events is increased by denaturant-induced shifts of the unfolding equilibrium. Local fluctuations are scarcely sensitive to the presence of denaturant. In order to distinguish global from local fluctuations, NHX measures ΔG_{HX} as a function of low denaturant concentration well below the unfolding transition^{16; 37}.

Observation of a stable core of six most hydrogen-exchange-protected β -strands, among 11, led us to investigate whether there could be a progression in the folding of KI-FHA. NHX indicates that KI-FHA samples two partially unfolded forms in which the subglobal motifs comprise β -strands and loops that flank the stable core of six strands of the β -sandwich. The equilibrium energy landscape of the PUFs and location of the subglobal motifs suggests a folding progression hypothesis in which events occur in parallel on opposite ends of the β -sandwich. Differences in apparent stabilities between NHX and denaturant melts are shown and discussed. Three lines of evidence suggest subtle residual structure in the unfolded regime of KI-FHA.

Results

Evidence for EX2 hydrogen exchange regime

The native-state hydrogen exchange approach used requires EX2 behavior. We tested whether the hydrogen exchange kinetics of KI-FHA lie in the EX2 regime. A pH-dependence in the stability of KI-FHA described below interfered in testing EX2 by changing pH. EX2 can be recognized when residues of equivalent ΔG_{HX} , calculated by equation 4, have widely varying intrinsic random coil exchange rates k_{rc} ³⁸. This diagnostic suggests that EX2 prevails for KI-FHA at pH 6.3 and 7.3. For example, at pH 6.3, residues with $\Delta G_{HX} = 6.0$ kcal/mol show 17-fold variation in k_{rc} . At pH 7.3, residues with $\Delta G_{HX} = 6.57$ kcal/mol show 11-fold variation in k_{rc} . This approach suggests EX2 behavior persists to 0.425 M GdmCl at pH 6.3 where 18.5-fold variation in k_{rc} is observed at a stability of ~5.4 kcal/mol and 35-fold variation in k_{rc} is observed at about ~5.1 kcal/mol. The EX2 property of variation of k_{rc} at shared stability can be illustrated by log-log plotting of k_{ex} vs. k_{rc} for residues of similar stability²¹. Such plots are general enough to reveal EX1 behavior as well. EX2 behavior has a slope of one in these log-log plots. The k_{rc} -independence of EX1 gives a slope of zero²¹. For KI-FHA at pH 6.3 in the absence and presence of 0.425 M GdmCl, lines with slope of 1.0 on the log-log scale

(EX2) fit the log-log scatter plots much better than lines with slope of 0.0 (EX1); see figure in Supplementary Data. This is the case both for the majority of residues sharing similar global stability and for a smaller group of residues sharing less stability. We tested further the hypothesis of an EX2 regime.

Kinetics of folding and unfolding of the FHA domain

We estimated k_{cl} as folding rate k_f from kinetics of intrinsic tryptophan fluorescence changes after denaturant jump using a stopped-flow fluorimeter. This established that k_f and k_{cl} exceed 85 s^{-1} at pH 6.3 from zero through 425 mM GdmCl at 20 °C in D₂O (Figure 1). These are the conditions used for NHX measurements described below. Clearly, $k_{cl} \gg k_{rc}$ globally, even for the fastest intrinsic exchange rates k_{rc} of $\sim 3 \text{ s}^{-1}$ at pH 6.3 for His196, Ser248 and Gly251. Consequently, KI-FHA folding appears to lie far into the EX2 regime at pH 6.3.

Most of the folding arm is linear. The slope of the linear phase, $m_f^\ddagger$, is $-1.7 \text{ kcal}\cdot\text{mol}^{-1}\cdot\text{M}^{-1}$, using the relationship $k_{ij} = k_{ij,\text{H}_2\text{O}} \cdot \exp(m_{ij}^\ddagger \cdot D/RT)$ ³⁹. Extrapolating the linear phase from 0.25 to 0.7 M GdmCl back to 0 M would suggest $k_{f,\text{H}_2\text{O}}$ of about 318 s^{-1} at 0 M. However, rollover is present at $< 0.25 \text{ M}$ GdmCl (Figure 1). We examined whether transient aggregation could account for this rollover, by looking for a dependence of folding kinetics on protein concentration^{40; 41}. At final [GdmCl] of 0.2 and 0.35 M, we observed no dependence of apparent folding rate on protein concentrations varying from 0.5 to 20 μM . This implies that the rollover in the folding arm results from a near-native intermediate, but without identifying it as on- or off-pathway³⁹. The folding and unfolding kinetics of KI-FHA suggest behavior more complex than two states.

The unfolding arm has a steep phase between 0.7 and 0.85 M GdmCl. The unfolding rate fitted to the steep phase in Figure 1 is based on supposing $k_u = k_{f,\text{H}_2\text{O}} \cdot \exp(-\Delta G_{g,\text{NHX}}/RT)$, using the estimate of $\Delta G_{\text{NHX},g}$ reported below (see Discussion). Rough estimates of the slope $m_{u,\text{steep}}^\ddagger$ of the steep unfolding phase range from $8.4 \text{ kcal}\cdot\text{mol}^{-1}\cdot\text{M}^{-1}$ for the fit shown down to $7 \text{ kcal}\cdot\text{mol}^{-1}\cdot\text{M}^{-1}$. The steep phase in the unfolding arm is followed by a pronounced bend around 0.85 to 0.9 M, a more gradual linear slope from 1 to 1.75 M and a flat phase from about 1.75 M to about 2.75 M (Figure 1). Another gradual slope in the unfolding arm extends from about 3.25 M to about 5.5 M GdmCl; this coincides with a region of subtle changes in equilibrium spectra shown below. The m -values for the two shallow unfolding phases are 0.14 and $0.15 \text{ kcal}\cdot\text{mol}^{-1}\cdot\text{M}^{-1}$. A shift of the transition state ensemble can cause broad curvature in just the unfolding arm of a chevron plot⁴²; this might be relevant to the gradual bend around 3 M GdmCl. However, a shift across a broad transition state appears inadequate to explain the sharpness of the bend in the unfolding arm at 0.85 M GdmCl (Figure 1). This abrupt change in the unfolding arm is better explained by a multi-state model in which the rate-limiting barrier shifts among the barriers as a function of denaturant concentration, as illustrated for Arc repressor and cyt c_2 ^{43; 44}. Those authors pointed out that the shallowness of the denaturant-dependence of unfolding rate k_u at high [GdmCl] in those cases suggests the shift to a rate-limiting transition state that is compact and near the native state. Such a rationale suggests a compact, near-native state of KI-FHA, as does native-state hydrogen exchange data presented below.

The folding and unfolding kinetics of KI-FHA suggest both near-native and partly folded intermediates (Figure 1), but are not enough to conclude whether the intermediates are on- or off-pathway. Four-state folding with a near-native intermediate and partially folded intermediates were proposed as a linear, on-pathway scheme for *Rhodobacter* cyt c_2 ⁴⁴. In like manner, we propose at least four states:

U represents the unfolded state and *N* the native state. *I* represents the intermediate that spectroscopy at equilibrium suggests to be an ensemble with only residual structure (see below). Whether placement of *I* on-pathway is correct is speculative. *C* represents a compact, near-native intermediate. Placement of *C* on-pathway is consistent with NHX evidence presented below. Linear fit to the shallow phase of the unfolding arm from 1 to 1.75 M GdmCl suggests the rate of unfolding of *N* to *C*, k_{NC} ^{43; 44} to be around 110 sec⁻¹, by extrapolation to 0 M GdmCl (Figure 1).

pH-dependence of stability

Based on the EX2 behavior of KI-FHA, ΔG_{HX} was calculated from k_{ex} and k_{rc} using equation 4³³ for several GdmCl concentrations below the unfolding transition at pH 6.3. β -strands 1, 5, 6, 10 and 11 plus the 9/10 loop show the most stability (Figure 2). EX2 is likely to prevail also at pH 7.3 where KI-FHA is more stable by chemical denaturation. (Even in the unlikely event k_{cl} were 10-fold slower at pH 7.3, k_{cl} would still be rapid enough to exceed most 10-fold faster k_{rc} values at pH 7.3 to establish EX2). Estimating ΔG_{HX} at pH 7.3 is then valid and meaningful. KI-FHA's greater stability at pH 7.3 is evident from its decreased k_{ex} values at its most stable residues, coupled with the 10-fold increase in k_{rc} values from stepping up 1 pH unit. Why ΔG_{HX} values are systematically higher at pH 7.3 (Figure 2) remains unknown. This could result either from higher k_f or lower k_u . A protein's global stability can be estimated by averaging ΔG_{HX} of the three most stably protected sites. This is corrected for proline isomerization that is too slow to occur in the transiently open, unfolded state accessible from the native state (at low denaturant)^{34; 38}. The small correction for the five *trans* prolines in the folded domain of KI-FHA (see 1mzk.pdb) is ~0.38 kcal/mol at pH 6.3. Subtracting the correction, the global stability estimates for KI-FHA at 20 °C are 7.0 kcal/mol at pH 6.3 and 10.9 kcal/mol at pH 7.3.

Native state hydrogen exchange of KI-FHA

We explored the nature of compact folding intermediate *C* further using the NHX approach to near-native intermediates^{14; 16}. We used pH 6.3 where k_{rc} random coil exchange rates are slower and where the NMR structure³ and dynamics⁴ are available, though pH 7.3 has a greater dynamic range of stability. The exchange rates of 44 backbone amide hydrogens were measured at 20 °C at pH 6.3 using¹⁵N TROSY spectra. Among the most stably protected amide groups in or near β -strands 1, 5, 6, 10 and 11 (Figure 2) are 23 residues with a very slow hydrogen exchange rate of $7.8 \times 10^{-5} \text{ min}^{-1} \leq k_{ex} < 3.5 \times 10^{-4} \text{ min}^{-1}$, in the absence of denaturant. Among these 23, their protection factors or $\log_{10}P = \log(k_{ex}/k_{rc})$ values are at least 4.6 for residues 182 – 184, 186 and 188 at β -strand 1; for 230 - 233 from $\beta 5$; for 240, 242 and 243 from $\beta 6$; for 275, 276 and 278 from the 9/10 loop; for 279 – 282 from $\beta 10$ and for 288 – 291 from $\beta 11$. Among the 21 less protected residues, the exchange rates k_{ex} vary between $3.4 \times 10^{-4} \text{ min}^{-1}$ and $2.1 \times 10^{-2} \text{ min}^{-1}$ in absence of denaturant. Sites in β -strands 2, 3, 6, 7 and 9 as well as 6/7 and 10/11 phosphoprotein recognition loops are protected with intermediate degrees of stability, with $\log_{10}P$ varying from 4 to 4.5 (Figure 2). Sites in the 4/5 and 8/9 phosphoprotein recognition loops, 1/2 loop, and distal 2/3 and 5/6 loops are protected more modestly with $\log_{10}P$ varying from 3 to 4 (Figure 2). Most of the other amide NMR peaks of KI-FHA are not observed after several minutes in D₂O due to their rapid exchange with solvent due to their high exposure in loops or at the edge of the β -sandwich.

GdmCl concentration was varied from 0 to 0.425 M, below the unfolding transition (see below). TROSY spectra indicate that KI-FHA retains its native structure up to 0.425 M GdmCl but denatures from around 0.5 M GdmCl upward. ΔG_{HX} as a function of [GdmCl] for the 44 protected residues is displayed in Figure 3 and listed in the table in Supplementary Data. The acceleration of amide hydrogen exchange rates with low [GdmCl] in the native state is evident in Figure 2 and 3. Most of the residual amide proton signals exchange away within 2 to 8 days.

Three types of exchange behavior of amide protons can be observed: from global, subglobal and local opening events¹⁶. Local openings lack any denaturant-dependent slope. The largest group of amide protons exhibits NHX with a phase of steep dependence on [GdmCl], attributable to rare opening events of global unfolding that expose substantial non-polar surface area. The 27 most stable residues with steepest denaturant dependence are fit best by an expression with global and local terms (equation 6B and Table 1). The majority of these cluster around an average global stability ΔG_g of 7.53 ± 0.44 kcal/mol and m -value m_g of -5.5 ± 0.76 kcal/mol/M (Figure 3D); these are colored blue in Figures 3 and 4. (The m -value is the denaturant-dependence of the free energy.)

For residues with intermediate stability, NHX is sensitive not only to global fluctuations, but also to subglobal fluctuations. Lower m -values obtained from these curves represent exchange during a partial opening reaction exposing a small nonpolar surface of the subglobal motif¹⁶. The subglobal behavior of KI-FHA can be partitioned into two groups. The residues of intermediate stability are fit best by an expression with a subglobal term (equations 6C and 6D, Table 1). Thirteen of these residues cluster thermodynamically to form the largest subglobal motif averaging ΔG_{sg} of 5.62 ± 0.34 kcal/mol with m_{sg} of -2.01 ± 0.91 kcal/mol/M (green in Figure 3C,D and 4). The small subglobal motif of lowest stability averages ΔG_{sg} of 4.16 ± 0.11 kcal/mol with m_{sg} of -1.00 ± 0.14 kcal/mol/M for these three residues colored red in Figures 3C,D and 4. Either one or both of the partially unfolded forms suggested by these subglobal motifs could correspond to the kinetic evidence for a compact intermediate C.

Table 2 and Figure 4 display the stability of the elements of secondary structure of KI-FHA. The β -strands characterized by rare global unfolding fluctuations are β -strands 5 and 6 from the 5-stranded sheet (Figure 3A) and β -strands 1, 10 and 11 from the 6-stranded sheet (Figure 3B). β -strand 9, the 9/10 loop and the 1/2 loop are nearly as stable. Flanking the most stable core, β -strands 2, 3 and 7 as well as the 6/7 and 10/11 recognition loops display an intermediate level of stability (Table 2 and Figure 4). β -strands 4 and 8, which lie on the edges of opposite ends of the β -sandwich, lack HX protection on a time scale of several minutes, rendering their stability too low to estimate. Combining the thermodynamic clustering of Figure 3D with the structural mapping of Figure 4B locates the residues marked by collective subglobal or global opening events. The least stable subglobal group is red and comprises Val225 of the 4/5 recognition loop, Val254 of $\beta 7$ and Leu264 of the 8/9 recognition loop (Figure 4). The former two are hydrophobic positions shared throughout the superfamily of FHA domains and SMAD MH2 domains³. The small m_{sg} of -1 kcal/mol/M is consistent with limited extent of opening of either the 4/5 loop or the segment extending from the end of $\beta 7$ through the 8/9 loop. We label the partially unfolded form with these limited openings as PUF1. Collective behavior is more evident in the green subglobal motifs. The packing of His196 of $\beta 2$ with Val208 of $\beta 3$ suggests the pair of β -strands 2 and 3 forms a foldon. Collective unfolding fluctuations of the $\beta 2/\beta 3$ pair at the edge of the β -sandwich may account for its m_{sg} of around -1.8 kcal/mol/M, judging from the correlation of m -value with ΔASA and number of residues⁴⁵. The adjoining 6/7 and 10/11 phosphoprotein recognition loops show the green level of stability (top in Figure 4B). The neighboring 6/7 and 10/11 loops may form another foldon at this green level of stability. PUF2 is our label for the rare forms with subglobal unfolding of the $\beta 2/3$ hairpin or the pair of 6/7 and 10/11 recognition loops.

In contrast to the structural separation of groups of FHA domain residues with similar subglobal stability, the residues in blue reporting the rare global unfolding events form a single cohesive structural core. β -hairpins are known to fold quickly⁴⁶ and the heart of the blue foldon is the $\beta 5/6$ hairpin sandwiched directly against the $\beta 10/11$ hairpin (Figure 4B). Joining the $\beta 10/11$ hairpin to complete the most stable core are $\beta 1$ on one side and the 9/10 loop and end of $\beta 9$ on the opposite side (foreground in Figure 4B). Glu242 and Leu243 of β -strand 6 (colored black in Figure 3A,D and 4) stand out as having notably higher apparent global stability and m_g

(Figure 3D and Tables 1 and 2), albeit with greater uncertainty. This raises the question of whether the center of β -strand 6 experiences residual hydrogen-bonding and residual structure in the unfolded state accessed rarely from the native state.

Stability upon GdmCl denaturation: pH-dependence and intermediate

We further explored the nature of intermediate *I* and the extra high HX protection in β strand 6 using chemical denaturation. Addition of GdmCl shifts the negative CD peak at 216 nm to shorter wavelength and diminishes its amplitude, consistent with increased random coil contribution at the expense of the β -structure. After the predominant unfolding transition at comparatively low denaturant concentration, a subtle transition of much smaller amplitude is observed by CD at higher GdmCl concentrations. The near-native intermediate *C* observed kinetically was not resolved in equilibrium melts. Consequently, the unfolding data were fitted to a three-state equilibrium expression among states *N*, *I* and *U* described in equation 7. These non-linear fits of ΔG and *m*-value of the main transition equal those from the linear extrapolation method. At pH 6.3 in D₂O at 20 °C, the midpoint of the predominant *N* to *I* transition (~90% of GdmCl-dependent optical change) is at 0.84 M GdmCl. The coincidence of the denaturation and renaturation curves (Figure 5) indicates the reversibility of the equilibrium. Each of five individual CD scans acquired at each point were consistently virtually identical, indicating that the 2.5 minutes of mixing and settling sufficed for reaching equilibrium. In H₂O at pH 6.3, the mid-point of this transition is at 0.95 M GdmCl. The stability and *m*-value are similar between D₂O and H₂O (Table 3). At pH 7.3 in D₂O at 20 °C, the midpoint of the main transition (~90% of the optical change) lies at 1.58 M GdmCl (Figure 5). Whether monitored by CD or intrinsic tryptophan fluorescence, the main transition at pH 6.3 displays an apparent ΔG_{NI} of about 2.9 kcal/mol and *m*-value of -3.4 kcal/(mol·M) (Table 3). The main transition at pH 7.3 shows clearly higher ΔG_{NI} of about 6.2 kcal/mol and *m*-value of -3.9 kcal/mol/M (Table 3). The lower stability at pH 6.3 may correlate with low solubility of KI-FHA at pH 6.0 and below.

About 10% of the denaturant-dependent optical signal change is attributed to a melting transition between *I* and *U*. The subtlety of the transition is subject to lower signal-to-noise, resulting in greater uncertainty in ΔG_{IU} (Table 3). The *I*-*U* transition has clearly smaller *m*-value than the lower melting main transition (Table 3). This indicates a smaller exposure of surface area in melting the residual structure of *I* than in the main *N*-*I* transition. The small amplitude of the optical transition (Figure 5) and relatively small size of *m*_{IU} suggest *I* to be mostly unfolded.

NMR investigation of residual structure of GdmCl-unfolded KI-FHA ensemble

¹⁵N TROSY spectra in H₂O were acquired to monitor [GdmCl] effects on residual structure of intermediate *I*. From 1.5 M GdmCl upward where intermediate *I* is populated, ¹⁵N TROSY spectra show all KI-FHA peaks to lie at essentially random coil positions, as evident in all panels of Figure 6. The random coil chemical shifts of *I* give the appearance of an unfolded ensemble of states. At 1.5 M GdmCl, line broadening is evident (Figure 6A). This line broadening is attributed to chemical exchange with the native state, populated to about 3 to 4% of the intermediate state population at 1.5 M, judging from the CD-detected GdmCl unfolding curve in H₂O (Table 3). By 1.75 M GdmCl (not shown), this line broadening is gone and the NMR lines continue to be sharp up to 3 M GdmCl (Figure 6B), consistent with essentially 100% population of the *I* state according to the CD-detected melt. The amide line widths broaden at 3.5 M GdmCl (Figure 6C) and are sharp again from 4 M through 5 M GdmCl (Figure 6D), consistent with chemical exchange between *I* and *U* states suggested by the slight transition in the CD-detected GdmCl melt (c.f. Figure 5). Shifts in relative amide peak positions, as [GdmCl] is raised from 1.5 M through 5.5 M, are more pronounced for part of the denatured KI-FHA peaks (Figure 6). This is consistent with effects on locales of residual

equilibrium structure. A locale suggested to have residual structure by NHX residues at and around β -strand 6.

Discussion

The folding of KI-FHA is clearly more complex than two states. Subtle equilibrium evidence suggests a non-compact intermediate *I* with minimal structure. The chevron plot (Figure 1) is consistent with the presence of *I*. Rollover in the folding arm at low denaturant concentration cannot be attributed to aggregation and suggest a compact, near-native intermediate *C*. Native-state hydrogen exchange suggests two partially unfolded forms near the native state. Intermediate *C* from kinetics might correspond to one or both of these PUFs from NHX.

Apparent discrepancy of stability by NHX and kinetics from chemical denaturation

For KI-FHA at pH 6.3, the average $\Delta G_{g,NHX}$ of 7.53 ± 0.44 kcal/mol and $m_{g,NHX}$ of -5.5 ± 0.76 kcal/mol/M (Table 2) exceed $\Delta G_{NI,GdmCl}$ of 2.8 kcal/mol and $m_{NI,GdmCl} = -3.4$ kcal/mol/M of the predominant optically-detected transition in GdmCl (Table 3). The degree of agreement between stabilities of KI-FHA suggested by NHX studies and the chevron plot (Figure 1) can be evaluated from the kinetics of the main folding transition as $\Delta G = RT \ln(k_f/k_u)$. Suppose ΔG equals the $\Delta G_{g,NHX}$ of 7.53 kcal/mol (without proline correction), based on the blue residues of Figure 3 and Table 2. Suppose $k_{f,H2O}$ is about 318 s^{-1} when extrapolating the folding arm of the chevron plot to 0 M GdmCl (Figure 1). These estimates with a two-state assumption then suggest a $k_{u,H2O}$ of 7.7×10^{-4} at 0 M GdmCl. This estimate of $k_{u,H2O}$ gives an acceptable fit ($R^2 = 0.94$) of the principal steep phase of the unfolding arm of the chevron plot (Figure 1). This estimate also falls in the middle of the range of possible values of $k_{u,H2O}$ that could fit this main phase of the unfolding arm. This implies agreement between that stability estimated from NHX and the chevron plot. By contrast, a similar comparison of stability by optical melt and chevron plot yields a seemingly unacceptable fit. Suppose that ΔG instead equals the ΔG_{NI} of 2.8 kcal/mol of the main optical transition in equilibrium melts at pH 6.3 (Table 3). This assumption and a k_f of 318 s^{-1} imply a $k_{u,H2O}$ of 2.6 s^{-1} . Such a large k_u results in only a poor fit ($R^2 = 0.45$) of the steep, main phase of the unfolding arm. The overall m -value suggested by the stopped-flow kinetic data can be estimated by summing the $m_{ij}^\ddagger$ -values for the individual phases⁴⁴. Estimates of overall kinetic m -value range from 7.0 to $5.6 \text{ kcal} \cdot \text{mol}^{-1} \cdot \text{M}^{-1}$, depending on the estimate of $m_{u,steep}^\ddagger$ used (see Results). Like stability, the kinetic m -value also agrees better with estimates of global m -value from NHX (Table 2) than m -value from chemical denaturation (Table 3).

Why might the NHX and kinetics agree in supporting a somewhat higher stability than fitted to the main optical transition of equilibrium melts? We entertain two possible contributions to the discrepancy. First, a leading consideration is the evidence for near-native, compact intermediates. These are termed PUF1 and PUF2 based on NHX, or *C* based on kinetics. We omitted *C* from our fits of the main equilibrium melting transition since *C* does not appear to be resolved at equilibrium (Figure 5). RNase A and cytochrome c were also reported to have significantly greater stability by NHX than by denaturation^{33; 47}. Particularly relevant is that cytochrome c has global stability by NHX (proline corrected) of 12.7 kcal/mol with m_g of -4.6 kcal/mol/M. Optical detection and two-state fitting of denaturant melting of cyt c indicates $\Delta G_{NU,GdmCl}$ of 10.0 kcal/mol with m -value of -3.6 kcal/mol/M⁴⁷. Partially unfolded forms of cytochrome c were proposed to populate up to 20% at the midpoint of its melting transition, accounting for underestimation of both m -value and free energy of two-state fits of cytochrome c melting⁴⁷. That suggests that thermodynamic fitting of NHX data (EX2 regime) for proteins with PUFs may estimate global stability more accurately than two-state fitting of denaturant melts. Possible population of PUFs during the denaturant melting transition of KI-FHA might also systematically decrease estimates of ΔG and m -value from such melts.

There is a second major consideration regarding the appearance of discrepancies in KI-FHA stability. Comparing $m_{g,NHX} = -5.5 \pm 0.8$ kcal/mol/M with $m_{NI,GdmCl} = -3.5$ kcal/mol/M (Table 2) suggests the $N \rightarrow I$ transition of chemical denaturation fails to unfold as much surface area as the rare global unfolding events monitored by NHX; two-state fitted $\Delta G_{NI,GdmCl}$ does not suffice to account for the unfolding behavior of KI-FHA. The history of PUFs being reported only in proteins with stabilities of about 8 kcal/mol or more^{14; 25} is consistent with $\Delta G_{NI,GdmCl}$ (Table 3) being an underestimate of the global stability of KI-FHA. For barnase which folds through an intermediate, it was concluded that the open state for rapid hydrogen exchange is the unfolded state U for all variants, based upon correlation of $\Delta \Delta G_{g,NHX}$ with $\Delta \Delta G_{NU}$ between each mutant and wild-type⁴⁸. Provided the open state of KI-FHA for hydrogen exchange is also U (see below), it is more appropriate to compare $\Delta G_{g,NHX}$ with $\Delta G_{NI,GdmCl} + \Delta G_{IU,GdmCl} = \Delta G_{NU,GdmCl} \approx 6.5 \pm 1.1$ kcal/mol, since $K_{NU} = K_{NI} \cdot K_{IU}$ (equation 9). $\Delta G_{g,NHX}$ needs a correction for the five *trans* prolines of folded KI-FHA (cf. 1mzk.pdb) undergoing *cis-trans* isomerization in the unfolded ensemble, a process too slow to occur during the transient openings monitored by NHX^{33; 34}. Applying the proline correction³⁴, $\Delta G_{g,NHX}$ is corrected downward by 0.38 kcal/mol to 7.15 ± 0.44 kcal/mol. This agrees, within the uncertainties, with $\Delta G_{NU,GdmCl}$ of 6.5 ± 1.1 kcal/mol. This suggests that super-protection need not be invoked to account for the global stability of the folding core of KI-FHA.

However, Glu242 and Leu243 of β -strand 6 average a more stable 8.7 kcal/mol (proline corrected). This suggests about 1.5 kcal/mol of super-protection of $\beta 6$. Residual structure or hydrogen bonding that impedes hydrogen exchange of these $\beta 6$ residues in the open state U to inflate their estimate $\Delta G_{g,NHX}$ is possible. Such super-protection and proposed residual structure has been reported for cytochrome *c*³³. Residual β -barrel structure in denatured staphylococcal nuclease was reported⁴⁹.

At higher [GdmCl] above the main unfolding transition in denaturant melts, further loss of ~10% of the UV signal across the $I \leftrightarrow U$ transition suggests melting of residual structure. The NMR peaks of I being at random coil peak positions implies that I is mostly unfolded (Figure 6A,B). This suggests I could be open for hydrogen exchange. However, since $\Delta G_{g,NHX}$ clearly exceeds $\Delta G_{NI,optical}$, the I state may be unlikely to be the open state accessed in the infrequent, transient global opening events detected by NHX. The open state reported by $\Delta G_{g,NHX}$ may instead be the higher energy unfolded state U .

Why global stability is markedly higher at pH 7.3 relative to 6.3 is unresolved, but it might depend on histidine protonation. Possibilities are that histidine deprotonation might either stabilize the folded structure or destabilize residual structure. The N ϵ of a conserved histidine near the binding site was proposed to accept a hydrogen bond from the amide of the i-3 conserved serine¹. However, this Ser226 in KI-FHA is not protected from HX at pH 7.3, dismissing a role in global stability.

Cooperative folding-unfolding units and hierarchy of states of KI-FHA

Thermodynamic and spatial clustering suggests a stable, central core and two flanking foldons in KI-FHA. Large $\Delta G_{g,NHX}$ and m_g identify the central core of $\beta 1$, 1/2 loop, $\beta 5$, $\beta 6$, $\beta 9$, 9/10 loop, $\beta 10$ and $\beta 11$ as the main foldon with global stability (blue, Figures 3 and 4). The m_g -values of the principal foldon (blue) vary from -3.4 to -6.7 kcal $\cdot$ mol $^{-1}\cdot$ M $^{-1}$ when excluding the two super-protected residues (Table 1). Raising [GdmCl] promotes the rare opening reactions that expose large surface area of the blue foldon. Two less stable green foldons flank the stable core. β -strands 2 and 3 form one of the green foldons. This is supported by both thermodynamics and structure. The m_{sg} -values are similar for His196 from $\beta 2$ and Val208 from $\beta 3$, namely -1.9 and -1.5 kcal $\cdot$ mol $^{-1}\cdot$ M $^{-1}$ (Table 1), respectively. $\beta 2$ and $\beta 3$ pack together from opposite faces of the β -sandwich (see Figure 2 in ref³). The 6/7 and 10/11 phosphopeptide recognition loops appear to form the other green foldon. The m_{sg} -values of these two adjacent

loops range from -1.2 to -2.1 kcal·mol⁻¹·M⁻¹ (Table 1). Compared to the blue core foldon, the modest m_{sg} -values of the green foldons (Tables 1 and 2) suggest that they under subglobal opening events that expose perhaps roughly 35% or less of the surface exposed by global unfolding events⁴⁵.

Figure 7 presents a model of the states of KI-FHA accessed by infrequent to rare unfolding fluctuations from the native state N at equilibrium. Red-colored sites in the 4/5 and 8/9 recognition loops and one end of β -strand 7 (Figures 4 and 7) seem to undergo the smallest scale subglobal openings of the most accessible PUF1 state. The 8/9 loop affected has a paucity of NOEs and large RMSD in its solution structure³ and is enriched in nanosec-scale motions⁴. PUF1 has a similarly low stability as the least stable PUF reported in apo cyt b₅₆₂²⁰. Next most accessible PUF2 has the two green foldons melted. This includes (i) β -strands 2 and 3 on an edge of the β -sandwich and (ii) neighboring 6/7 and 10/11 recognition loops (Figure 7). The structural separation of these two foldons suggests their independence. Yet prior unfolding of unstable β 4 and the 4/5 loop may facilitate unfolding of both green foldons. The largest and rare global fluctuations open a large surface area of the core of H-bonded β -strands 1, 10, 11 and 9/10 loop sandwiched against the hairpin of β -strands 5, 6 and 9 (blue in Figure 7). Super-protection of Glu242 and Leu243 suggests a residual structure of β 6 (dark gray in Figure 7). Subtle, residual structure that melts with greater stability is consistent with spectroscopic changes of KI-FHA observed over higher [GdmCl] (Figures 5 and 6).

Implications of decreased NHX stability towards edges of β -sandwich

The stabilities of β -strands drop at three of the four edges of the β -sandwich of KI-FHA. The stability progressions of these edges are $\beta 1 > \beta 2$, $\beta 5 > \beta 3 > \beta 4$, and $\beta 10 > \beta 7 > \beta 8$ (Table 2 and Figure 4). (H/D exchange protection is absent for $\beta 4$ and $\beta 8$; see Figures 2 and 4). The drop at the edges raises two major issues. First, it accounts for structural separation of subglobal motifs of similar (green) stability (Figure 4B). The spatial separation of subglobal motifs at each level of stability (Figure 4B and 7) may suggest a succession of parallel subglobal opening events, a possibility that has been anticipated¹⁴. Some parallelism in openings with shared stabilities contrasts the more strictly linear progressions demonstrated in the well-characterized folding pathways of all-helical cytochrome c and apo cytochrome b₅₆₂^{12; 14}. (Typical foldons of cyt c and cyt b₅₆₂ are helices that H-bond internally).

The second major issue raised by decreased stabilities of β -strands towards the edges of the β -sandwich is a paradox that can complicate categorizing the strands into foldons. That is, the strands on the edge could reasonably be predicted to share the same stability and foldon category with the more interior strands that they H-bond with, due to their shared network of H-bonds. However, this is observed at only one of four edges of the β -sandwich of KI-FHA ($\beta 6$ - $\beta 9$; Figure 4B). The lower stabilities and m -values at the other three edges of the sandwich clearly suggest them to be enriched in subglobal and local openings compared to their H-bonding partners in the interior. Why might this be? The edges (β -strands 2, 4, 8 and 9) of the β -sandwich possess clearly greater surface accessibility than their H-bonding partners towards the interior ($\beta 1$, $\beta 3$, $\beta 7$ and $\beta 6$, respectively). Perhaps the greater surface exposure of the edges accounts for their greater frequency of smaller scale openings than their more buried partners. All four strands on the edges of the β -sandwich appear to be more mobile on the psec scale in that their S^2 order parameters are lower than S^2 of the other β -strands⁴. The first example of the paradox in the KI-FHA sequence is β -strand 1 of the blue foldon H-bonded to $\beta 2$ on the edge. The H-bonding network could suggest that $\beta 2$ should join $\beta 1$ in the blue foldon. Yet, $\beta 2$ has clearly less denaturant dependence and less stability (Table 1 and green in Figure 4). (His196 is the sole residue of $\beta 2$ with HX protection observed). The thermodynamics distinguish $\beta 2$ from $\beta 1$ in our opinion. In the structure, $\beta 2$ can instead be grouped with $\beta 3$ to form a green foldon since their side chains pack together in the interior. $\beta 2$'s lower sensitivity

to denaturant than that of $\beta 1$ seems to correlate with (i) the greater surface accessibility of 24% for $\beta 2$ than the 11% of $\beta 1$ and (ii) slightly greater flexibility in $\beta 2$ ($S^2 = 0.846$ for His196) than in $\beta 1$ (S^2 of 0.914) (Ding et al., 2005). The NHX results on OspA show that some of the strands on the edges of its β -sheets (β -strands 1, 4 and 21 for example) also have lower m -values and stability than their neighbors to the interior of the sheet²¹. This suggests that the apparent paradox of lower stability on exposed edges of some β -sheets could turn out to be commonplace.

The minimal stability of β -strand 8, lacking H/D exchange protection, is relevant to FHA domain structure. The presumed local fluctuations of $\beta 8$ might allow for its rearrangement to form the segment-swapped dimer observed in crystals of the FHA domain of human Chfr⁵⁰.

Reversible, rapid folding of transiently visited states back to N (Figure 7) is implicit in the equilibrium NHX measurements. This suggests the possibility of these PUFs being visited in a non-equilibrium kinetic folding progression from U to N . PUFs were found to correspond to the kinetic progression of near-native folding intermediates in cyt c^{16; 25}, RNase H^{17; 26}, CD2¹⁹, OspA^{21; 51}, barnase²² and Rd-apocyt b₅₆₂⁵², but not T4 lysozyme¹⁸.

Consequently, it is reasonable to hypothesize that PUF1 or PUF2 (Figure 7) is related to near-native folding intermediate C of KI-FHA. The blue core of six stable β -strands would be formed early in the folding process (PUF2). β -hairpins have been observed to zip up as rapidly as the microsec-millisecond scale^{46; 53}, apparently due to low contact order⁵⁴. In the blue stable core, the hairpin of β -strands 5 and 6 packs against the hairpin of β -strands 10 and 11 (Figure 4B and 7). Early zipping up and stacking of these two β -hairpins of the blue foldon is likely to nucleate the formation of the β -sandwich. N-terminal β -strand 1 hydrogen bonds to C-terminal β -strand 11 in the blue foldon proposed to form early. This is consistent with the high prevalence of the contact of N- and C-termini, proposed to promote initial folding and subsequent stability of proteins⁹. The stable blue foldon (PUF2, Figure 7) includes the 1/2 loop that could poise $\beta 2$ and its $\beta 3$ neighbor for formation (green in PUF1, Figure 7). Perhaps the foldon of the 6/7 and 10/11 recognition loops (green in PUF1, Figure 7) form in parallel. The last elements to form in reaching the native state are hypothesized to be the 4/5 and 8/9 recognition loops, part of $\beta 7$. Lastly, unstable $\beta 4$ and $\beta 8$ and the other loops are hypothesized to become ordered in reaching the native state.

Summary

The folding of the FHA domain from Arabidopsis KAPP is clearly more complex than two-states. Both rollover in folding arm kinetics and subglobal motifs of NHX protection of the EX2 regime suggest near-native, compact intermediate(s) or transiently sampled PUFs. Kinks in unfolding arm kinetics, a minor component at high denaturant in equilibrium melts and its NMR spectra suggest a merely partly folded state I of KI-FHA. Superprotection of $\beta 6$ is consistent with subtle residual structure in the denaturant-unfolded ensemble. KI-FHA is more than 3 kcal/mol more stable at pH 7.3.

The NHX approach suggests two partially unfolded forms of KI-FHA. The stable core foldon (blue) comprises β -strands 1, 5, 6, 9, 10 and 11, as well as 1/2 and 9/10 loops. Two foldons of intermediate stability (green) appear to be (i) β -strands 2 and 3 and (ii) the 6/7 and 10/11 recognition loops. The structural separation and shared stability of these two green foldons suggest possible parallel (un)folding events. Subglobal motifs of modest stability and localized opening are found at the 4/5 and 8/9 recognition loops and $\beta 7$ (red). The NHX results suggest the hypothesis of a folding process in which the central nascent core of the β -sandwich (blue) may accrue flanking β -strands and loops progressively outward (Figure 7).

Materials and Methods

Folding kinetics by stopped-flow fluorescence

The kinetics of unfolding and refolding of KI-FHA, as a function of final [GdmCl], were monitored using a KinTek Model SF-2001 stopped-flow spectrofluorimeter. Intrinsic tryptophan fluorescence emission was collected at 20 °C using a 340 nm cutoff filter with excitation at 280 nm. In both unfolding and refolding experiments, 6-fold changes in [GdmCl] were obtained from the 1:5 ratio of volumes mixed. After rapid dilution, the final concentration of KI-FHA was 2.5 μM in 20 mM sodium phosphate at pH 6.3 and 120 mM NaCl in 99.9% D₂O. The KI-FHA solutions containing GdmCl were pre-incubated from two hours to a maximum of six hours in fresh GdmCl prior to experiments. The kinetic traces, an average of 30 transients each, were fitted with single exponentials using KinTek's software version 6.0. Residuals indicate that single exponential terms sufficient to fit the traces well. Segments of the chevron plot were fitted with exponential terms using Origin 7.5 (Microcal).

NMR spectroscopy of KI-FHA for NHX

KI-FHA was expressed and purified as described previously⁵⁵, using M9 minimal medium augmented with Celtone N (Spectral Stable Isotopes, Inc.). Pure KI-FHA was dialyzed against the NMR buffer of 20 mM sodium phosphate (pH 6.3) and 120 mM NaCl, concentrated by ultrafiltration and lyophilized. The GdmCl used was deuterated by dissolving it in D₂O, adjusting its pH to 6.3 using 0.1 M NaOH, lyophilizing, dissolving the powder in D₂O and lyophilized again. No attempt was made to correct pH correction for the isotope effect. Deuterium oxide (D₂O; D, 99.96%) was purchased from Cambridge Isotope Laboratories, Inc. and guanidinium hydrochloride (Ultrapure Bioreagent) from Mallinckrodt Baker, Inc.

NMR spectra were collected on a Varian INOVA 600 with a cryogenically cooled probe and samples set to 20 °C. The NMR peak assignments of KI-FHA were reported⁵⁵. Prior to initiating H/D exchange, shims were first adjusted on a similar sample. H/D exchange was initiated by dissolving lyophilized KI-FHA in D₂O containing appropriate concentrations of the deuterated GdmCl and centrifuging briefly to remove undissolved protein.¹⁵N TROSY spectra were recorded as an average of 8 transients over 80 *t*₁ increments. The spectra were processed using NMRPipe⁵⁶ and analyzed with Sparky⁵⁷.

Thermodynamic analysis of hydrogen exchange rates

The exchange rates *k*_{ex} were obtained by fitting a decaying exponential of TROSY NMR peak heights *A*(*t*) vs. duration *t* of hydrogen exchange: *A*(*t*) = *A*₀ *exp*(−*k*_{ex}*t*). The intrinsic rate of random coil HX, *k*_{rc}, was calculated by an established method^{32; 58}. Manual and automated estimations of *k*_{rc} using the program SPHERE from the Roder laboratory at the Fox Chase Cancer Center, Philadelphia at www.fccc.edu/research/labs/roder/agreed. The protection factor *P* for each amide proton in the protein is defined as *k*_{rc}/*k*_{ex}. The free energy of hydrogen exchange was calculated from equation 4³³:

Δ*G*_{HX} is influenced by opening transitions resulting from global unfolding, subglobal fluctuations, and local fluctuations^{16; 17; 59}. Δ*G*_{HX} can thus be expressed⁵⁹:

$$\Delta G_{HX} = -RT \ln \left[e^{-\frac{(\Delta G_g + m_g D)}{RT}} + e^{-\frac{(\Delta G_{sg} + m_{sg} D)}{RT}} + e^{-\frac{\Delta G_{loc}}{RT}} \right] \quad (6A)$$

where *D* refers to denaturant concentration, the *m*-value is the slope of the dependence on *D*, and subscripts *g*, *sg* and *loc* refer to global, subglobal and local fluctuations, respectively. Each Δ*G* is the free energy in absence of denaturant. Slope *m* reports on the surface area exposed in the unfolding reaction⁴⁵. Fitting of all five parameters Δ*G*_{*g*}, *m*_{*g*}, Δ*G*_{*sg*}, *m*_{*sg*}, Δ*G*_{*loc*} of equation

6A was not warranted for any residue of KI-FHA. For a majority of residues of KI-FHA, non-linear fits of the global and local terms proved sufficient (see Table 1):

$$\Delta G_{HX} = -RT \ln \left[e^{-\frac{(\Delta G_g + m_g D)}{RT}} + e^{-\frac{\Delta G_{loc}}{RT}} \right] \quad (6B)$$

For nine residues with subglobal behavior, another simplification of equation 6A was used in which only the subglobal term was fitted and the values of ΔG_g and m_g were fixed to the average among residues with global behavior (see Table 1):

$$\Delta G_{HX} = -RT \ln \left[e^{-\frac{(\Delta G_{g,avg} + m_{g,avg} D)}{RT}} + e^{-\frac{(\Delta G_{sg} + m_{sg} D)}{RT}} \right] \quad (6C)$$

For six residues with a linear, subglobal dependence on D , a simpler linear fit of only the subglobal term was sufficient:

$$\Delta G_{HX} = \Delta G_{sg} + m_{sg} D \quad (6D)$$

For two residues lacking any dependence on D , only the local term was fitted: $\Delta G_{HX} = \Delta G_{loc}$. The most appropriate and parsimonious fitting equation was selected by optimizing correlation coefficient R , minimizing χ^2/dof where dof represents degrees of freedom, and visually identifying the fit with smallest residuals. All curve-fitting was done using Origin 7.5 (Microcal).

Circular dichroism (CD) spectroscopy of denaturant-dependent unfolding and folding

CD measurements were carried out using an AVIV Model 62DS circular dichroism spectrometer. Denaturant melts were plotted using the ellipticity at 216 nm, taken from spectra scanned from 200 to 250 nm. All CD spectra were recorded at 20 °C using a 0.1 cm path-length quartz cuvette. Each protein sample contained 450 µg/ml KI-FHA in 20 mM sodium phosphate at pH 6.3 and 120 mM NaCl, usually with a solvent of 99.9% D₂O. No isotope correction was made in reading pH of solutions containing D₂O. In both denaturation and renaturation experiments, KI-FHA equilibrated for two hours in fresh, high GdmCl was mixed with KI-FHA in low or zero denaturant to reach the desired [GdmCl] and maintain [KI-FHA] constant. At each [GdmCl], mixing lasted two minutes, followed by 30 sec. without mixing, and then five CD spectral scans of 1.5 min. each. Each of the five CD scans at each concentration were essentially identical, indicating that the solutions had reached equilibrium within the 2.5 minutes of mixing and resting. Unfolding transitions were non-linear least squares fitted in Origin 7.5 (Microcal). Individual transitions were fitted initially to a two-state expression⁶⁰ that agrees well with the linear extrapolation method. The CD-detected unfolding curves in GdmCl were then fitted to a three-state expression, one transition at a time. We obtained the expression by simplifying a reported four-state expression⁶¹. The three state expression is:

$$F_{\text{fold,app}} = 1 - \frac{K_{NI}(Z_I + K_{IU})}{(1 + K_{NI} + K_{NI} \cdot K_{IU})} \quad (7)$$

where $F_{\text{fold,app}}$ is the apparent fraction of folded protein, K_{NI} and K_{IU} are the equilibrium constants for the native to intermediate and intermediate to unfolded transitions. Z_I is the apparent unfolded fraction, approaching a value of 1 at U or 0 at N. The equilibrium constants are defined as:

$$K_{NI} = e^{-\frac{(\Delta G_{NI} + m_{NI} D)}{RT}} \quad (8A)$$

$$K_{IU} = e^{\frac{-(\Delta G_{IU} + m_{IU}D)}{RT}} \quad (8B)$$

$$K_{NU} = K_{NI} \cdot K_{IU} \quad (9)$$

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

We thank Professors C. R. Matthews, S. Marqusee and T. Sosnick for insightful comments at early stages of the work. We thank Prof. P. Tipton for orienting us to the stopped-flow fluorimeter. This work was supported in part by NSF grant MCB 0111589 to S.R.V. NSF grant DBI0070359 and University of Missouri funds purchased the 600 MHz NMR system. NIH grant R01GM57289 contributed to purchase of the cryogenic NMR probe.

References

1. Durocher D, Taylor IA, Sarbassova D, Haire LF, Westcott SL, Jackson SP, Smerdon SJ, Yaffe MB. The molecular basis of FHA domain:phosphopeptide binding specificity and implications for phospho-dependent signaling mechanisms. *Mol Cell* 2000;6:1169–82. [PubMed: 11106755]
2. Qin BY, Liu C, Lam SS, Srinath H, Delston R, Correia JJ, Derynck R, Lin K. Crystal structure of IRF-3 reveals mechanism of autoinhibition and virus-induced phosphoactivation. *Nat Struct Biol* 2003;10:913–21. [PubMed: 14555996]
3. Lee GI, Ding Z, Walker JC, Van Doren SR. NMR structure of the forkhead-associated domain from the Arabidopsis receptor kinase-associated protein phosphatase. *Proc Natl Acad Sci U S A* 2003;100:11261–6. [PubMed: 14500786]
4. Ding Z, Lee GI, Liang X, Gallazzi F, Arunima A, Van Doren SR. PhosphoThr Peptide Binding Globally Rigidifies much of the FHA Domain from Arabidopsis Receptor Kinase-Associated Protein Phosphatase. *Biochemistry* 2005;44:10119–34. [PubMed: 16042389]
5. Westerholm-Parvinen A, Vernos I, Serrano L. Kinesin subfamily UNC104 contains a FHA domain: boundaries and physicochemical characterization. *FEBS Letters* 2000;486:285–90. [PubMed: 11119720]
6. Stone JM, Colinge MA, Smith RD, Horn MA, Walker JC. Interaction of a protein phosphatase with an *Arabidopsis* Serine-Threonine Receptor Kinase. *Science* 1994;266:793–795. [PubMed: 7973632]
7. Li J, Smith GP, Walker JC. Kinase interaction domain of kinase-associated protein phosphatase, a phosphoprotein-binding domain. *Proc Natl Acad Sci USA* 1999;96:7821–7826. [PubMed: 10393905]
8. Williams RW, Wilson JM, Meyerowitz EM. A possible role for kinase-associated protein phosphatase in the *Arabidopsis* CLAVATA1 signaling pathway. *Proc Natl Acad Sci USA* 1997;94:10467–10472. [PubMed: 9294234]
9. Krishna MM, Englander SW. The N-terminal to C-terminal motif in protein folding and function. *Proc Natl Acad Sci U S A* 2005;102:1053–8. [PubMed: 15657118]
10. Ptitsyn OB. Structures of folding intermediates. *Curr Opin Struct Biol* 1995;5:74–8. [PubMed: 7773749]
11. Pandya MJ, Williams PB, Dempsey CE, Shewry PR, Clarke AR. Direct kinetic evidence for folding via a highly compact, misfolded state. *J Biol Chem* 1999;274:26828–37. [PubMed: 10480890]
12. Feng H, Zhou Z, Bai Y. A protein folding pathway with multiple folding intermediates at atomic resolution. *Proc Natl Acad Sci U S A* 2005;102:5026–31. [PubMed: 15793003]
13. Feng H, Vu ND, Bai Y. Detection and structure determination of an equilibrium unfolding intermediate of Rd-apocytochrome b562: native fold with non-native hydrophobic interactions. *J Mol Biol* 2004;343:1477–85. [PubMed: 15491625]
14. Maity H, Maity M, Krishna MM, Mayne L, Englander SW. Protein folding: the stepwise assembly of foldon units. *Proc Natl Acad Sci U S A* 2005;102:4741–6. [PubMed: 15774579]

15. Bai Y, Englander SW. Future directions in folding: the multi-state nature of protein structure. *Proteins* 1996;24:145–51. [PubMed: 8820481]
16. Bai Y, Sosnick TR, Mayne L, Englander SW. Protein folding intermediates: native-state hydrogen exchange. *Science* 1995;269:192–7. [PubMed: 7618079]
17. Chamberlain AK, Handel TM, Marqusee S. Detection of rare partially folded molecules in equilibrium with the native conformation of RNaseH. *Nat Struct Biol* 1996;3:782–7. [PubMed: 8784352]
18. Llinas M, Gillespie B, Dahlquist FW, Marqusee S. The energetics of T4 lysozyme reveal a hierarchy of conformations. *Nat Struct Biol* 1999;6:1072–8. [PubMed: 10542101]
19. Parker MJ, Marqusee S. A kinetic folding intermediate probed by native state hydrogen exchange. *J Mol Biol* 2001;305:593–602. [PubMed: 11152615]
20. Chu R, Pei W, Takei J, Bai Y. Relationship between the native-state hydrogen exchange and folding pathways of a four-helix bundle protein. *Biochemistry* 2002;41:7998–8003. [PubMed: 12069590]
21. Yan S, Kennedy SD, Koide S. Thermodynamic and kinetic exploration of the energy landscape of *Borrelia burgdorferi* OspA by native-state hydrogen exchange. *J Mol Biol* 2002;323:363–75. [PubMed: 12381326]
22. Vu ND, Feng H, Bai Y. The folding pathway of barnase: the rate-limiting transition state and a hidden intermediate under native conditions. *Biochemistry* 2004;43:3346–56. [PubMed: 15035606]
23. Feng H, Vu ND, Bai Y. Detection of a hidden folding intermediate of the third domain of PDZ. *J Mol Biol* 2005;346:345–53. [PubMed: 15663949]
24. Clarke J, Itzhaki LS, Fersht AR. Hydrogen exchange at equilibrium: a short cut for analysing protein-folding pathways? *Trends Biochem Sci* 1997;22:284–7. [PubMed: 9270297]
25. Rumbley J, Hoang L, Mayne L, Englander SW. An amino acid code for protein folding. *Proc Natl Acad Sci U S A* 2001;98:105–12. [PubMed: 11136249]
26. Raschke TM, Kho J, Marqusee S. Confirmation of the hierarchical folding of RNase H: a protein engineering study. *Nat Struct Biol* 1999;6:825–31. [PubMed: 10467093]
27. Linderstrøm-Lang, K. *Chem Soc (London) Spec Publ.* 2. 1955. Deuterium exchange between peptides and water; p. 1-20.
28. Hvidt A, Nielsen SO. Hydrogen exchange in proteins. *Adv Protein Chem* 1966;21:287–386. [PubMed: 5333290]
29. Li R, Woodward C. The hydrogen exchange core and protein folding. *Protein Science* 1999;8:1571–1590. [PubMed: 10452602]
30. Clarke J, Itzhaki LS. Hydrogen exchange and protein folding. *Curr Opin Struct Biol* 1998;8:112–8. [PubMed: 9519304]
31. Bai Y, Milne JS, Mayne L, Englander SW. Primary Structure Effects on Peptide Group Hydrogen Exchange. *Proteins* 1993;17:75–86. [PubMed: 8234246]
32. Zhang, Y-Z. Protein and peptide structure and interactions studied by hydrogen exchange and NMR. Ph.D., University of Pennsylvania
33. Bai Y, Englander JJ, Mayne L, Milne JS, Englander SW. Thermodynamic parameters from hydrogen exchange measurements. *Methods Enzymol* 1995;259:344–56. [PubMed: 8538461]
34. Huyghues-Despointes BM, Scholtz JM, Pace CN. Protein conformational stabilities can be determined from hydrogen exchange rates. *Nature Structural Biology* 1999;6:910–2.
35. Woodward C, Carulla N, Barany G. Native state hydrogen-exchange analysis of protein folding and protein motional domains. *Methods Enzymol* 2004;380:379–400. [PubMed: 15051346]
36. Woodward CK, Hilton BD. Hydrogen isotope exchange kinetics of single protons in bovine pancreatic trypsin inhibitor. *Biophys J* 1980;32:561–75. [PubMed: 7248461]
37. Bai Y, Milne JS, Mayne L, Englander SW. Protein stability parameters measured by hydrogen exchange. *Proteins* 1994;20:4–14. [PubMed: 7824522]
38. Huyghues-Despointes BM, Pace CN, Englander SW, Scholtz JM. Measuring the conformational stability of a protein by hydrogen exchange. *Methods in Molecular Biology* 2001;168:69–92. [PubMed: 11357629]
39. Baldwin RL. On-pathway versus off-pathway folding intermediates. *Fold Des* 1996;1:R1–8. [PubMed: 9079355]

40. Oliveberg M. Alternative Explanations for "Multistate" Kinetics in Protein Folding: Transient Aggregation and Changing Transition-State Ensembles. *Acc Chem Res* 1998;31:765–772.
41. Silow M, Oliveberg M. Transient aggregates in protein folding are easily mistaken for folding intermediates. *Proc Natl Acad Sci U S A* 1997;94:6084–6. [PubMed: 9177173]
42. Otzen DE, Kristensen O, Proctor M, Oliveberg M. Structural changes in the transition state of protein folding: alternative interpretations of curved chevron plots. *Biochemistry* 1999;38:6499–511. [PubMed: 10350468]
43. Jonsson T, Waldburger CD, Sauer RT. Nonlinear free energy relationships in Arc repressor unfolding imply the existence of unstable, native-like folding intermediates. *Biochemistry* 1996;35:4795–802. [PubMed: 8664269]
44. Sauder JM, MacKenzie NE, Roder H. Kinetic mechanism of folding and unfolding of *Rhodobacter capsulatus* cytochrome c2. *Biochemistry* 1996;35:16852–62. [PubMed: 8988024]
45. Myers JK, Pace CN, Scholtz JM. Denaturant *m* values and heat capacity changes: relation to changes in accessible surface areas of protein unfolding. *Protein Sci* 1995;4:2138–48. [PubMed: 8535251]
46. McCallister EL, Alm E, Baker D. Critical role of beta-hairpin formation in protein G folding. *Nature Structural Biology* 2000;7:669–73.
47. Mayne L, Englander SW. Two-state vs. multistate protein unfolding studied by optical melting and hydrogen exchange. *Protein Sci* 2000;9:1873–7. [PubMed: 11106159]
48. Perrett S, Clarke J, Hounslow AM, Fersht AR. Relationship between equilibrium amide proton exchange behavior and the folding pathway of barnase. *Biochemistry* 1995;34:9288–98. [PubMed: 7626599]
49. Wrabl J, Shortle D. A model of the changes in denatured state structure underlying *m* value effects in staphylococcal nuclease. *Nat Struct Biol* 1999;6:876–83. [PubMed: 10467101]
50. Stavridi ES, Huyen Y, Loreto IR, Scolnick DM, Halazonetis TD, Pavletich NP, Jeffrey PD. Crystal Structure of the FHA Domain of the Chfr Mitotic Checkpoint Protein and Its Complex with Tungstate. *Structure* 2002;10:891–899. [PubMed: 12121644]
51. Yan S, Gawlak G, Smith J, Silver L, Koide A, Koide S. Conformational heterogeneity of an equilibrium folding intermediate quantified and mapped by scanning mutagenesis. *J Mol Biol* 2004;338:811–25. [PubMed: 15099747]
52. Zhou Z, Huang Y, Bai Y. An on-pathway hidden intermediate and the early rate-limiting transition state of Rd-apocytochrome b562 characterized by protein engineering. *J Mol Biol* 2005;352:757–64. [PubMed: 16125200]
53. Varley P, Gronenborn AM, Christensen H, Wingfield PT, Pain RH, Clore GM. Kinetics of folding of the all-beta sheet protein interleukin-1 beta. *Science* 1993;260:1110–3. [PubMed: 8493553]
54. Baker D. A surprising simplicity to protein folding. *Nature* 2000;405:39–42. [PubMed: 10811210]
55. Lee, G.; Li, J.; Walker, JC.; Van Doren, SR. *J Biomol NMR*. 25. 2003. ¹H, ¹³C and ¹⁵N resonance assignments of kinase-interacting FHA domain from *Arabidopsis* phosphatase KAPP; p. 253-254.
56. Delaglio F, Grzesiek S, Vuister GW, Zhu G, Pfeifer J, Bax A. NMRPipe: a multidimensional spectral processing system based on UNIX pipes. *J Biomol NMR* 1995;6:277–93. [PubMed: 8520220]
57. Goddard, TD.; Kneller, DG. SPARKY 3 edit. University of California, San Francisco; San Francisco: 2000.
58. Bai Y, Milne JS, Mayne L, Englander SW. Primary structure effects on peptide group hydrogen exchange. *Proteins* 1993;17:75–86. [PubMed: 8234246]
59. Wildes D, Marqusee S. Hydrogen-exchange strategies applied to energetics of intermediate processes in protein folding. *Methods Enzymol* 2004;380:328–49. [PubMed: 15051344]
60. Santoro MM, Bolen DW. Unfolding free energy changes determined by the linear extrapolation method. 1. Unfolding of phenylmethanesulfonyl alpha-chymotrypsin using different denaturants. *Biochemistry* 1988;27:8063–8. [PubMed: 3233195]
61. Gualfetti PJ, Bilsel O, Matthews CR. The progressive development of structure and stability during the equilibrium folding of the alpha subunit of tryptophan synthase from *Escherichia coli*. *Protein Sci* 1999;8:1623–35. [PubMed: 10452606]

Abbreviations

HX	hydrogen exchange
NHX	native-state HX
FHA	forkhead-associated
KAPP	kinase-associated protein phosphatase
KI-FHA	kinase-interacting FHA domain
EX1	monomolecular exchange
EX2	bimolecular exchange
PUF	partially unfolded form
Gdm	guanidinium
N	native state
U	unfolded state
foldon	cooperative folding–unfolding unit

Figure 1.

Chevron plot of [GdmCl]-dependence of folding and unfolding kinetics of KI-FHA suggests non-two-state behavior. The inset is an expansion of the low denaturant region. Standard deviations of triplicate measurements are plotted as error bars. Single exponential fits to the folding arm and sections of the unfolding arm are marked with broken lines. The fit to the first and steep phase of the unfolding arm is an estimate of $k_u(0\text{M})$ of $7.7 \cdot 10^{-4} \text{ s}^{-1}$ derived from supposing two states, $k_f(0\text{M})=318 \text{ s}^{-1}$ and $\Delta G = \Delta G_{\text{g,NHX}} = 7.53 \text{ kcal/mol}$.

Figure 2.

Residue-specific stability estimates of KI-FHA by hydrogen exchange indicate (i) five segments are most stable, (ii) stability is greater at pH 7.3 than at pH 6.3, and (iii) the sensitivity to low [GdmCl] additions below the unfolding transition. ΔG_{HX} was estimated from k_{ex} values, observed at 20 °C using ^{15}N TROSY spectra after plunging lyophilized KI-FHA into D_2O , coupled with k_{rc} estimates (equation 4)^{31; 33}. Open bars represent pH 7.3 and all other bars pH 6.3. Black designates 0 M GdmCl, medium gray for 0.15 M GdmCl, light gray for 0.275 M, and hatched bars for 0.425 M. Residue numbering reflects the location of KI-FHA in the larger Kinase-Associated Protein Phosphatase sequence.

Figure 3.

[GdmCl]-dependence of ΔG_{HX} at pH 6.3 and 20 °C fitted with variations of equation 6. The color code designates four categories of stability and m -value: blue for global stability; black for higher-than-global stability, suggestive of superprotection; green for intermediate, subglobal stability; and red for the low subglobal stability. Dashed lines mark the average most stable term for each color category. Panel A plots isotherms from β -strands 5 and 6 of the 5-stranded sheet, plus the 9/10 loop, fits to equation 6B. Panel B plots isotherms, fitted to equation 6B, from β -strands 1, 9, 10, and 11 plus the 1/2 loop, all from the 6-stranded sheet. Panel C plots isotherms of residues with subglobal stability fitted to equation 6C or 6D (Table 1). The symbol legend refers to the residue number. Panel D plots fitted ΔG_{sg} (if needed for fits) or fitted ΔG_g versus fitted absolute value of m_{sg} or m_g , respectively. The subscript “sg” refers to subglobal and “g” to global openings.

Figure 4.

Mapping of NHX stability properties onto sequence and structure of KI-FHA. The color code of Figure 3 is used in both panels A and B here, plus pink for local stability. (A) The most stable fitted NHX free energy variable, ΔG_g or ΔG_{sg} , is plotted against sequence position using the numbering within Arabidopsis KAPP. (B) The backbone ribbon of the KI-FHA NMR structure³ is colored by NHX-derived stability. Gold side chains were proposed to be critical to the folding core throughout the superfamily of FHA domains and SMAD MH2 domains³. “L” in the labels refers to a loop. Stereo viewing of the stereo pair clarifies the labeling.

Figure 5.

Chemical denaturation suggests pH-dependence and a partly folded intermediate of KI-FHA. The [GdmCl]-dependent changes in far UV CD spectra were monitored at 216 nm at 20°C with the same buffer used for NHX, i.e. 99.9% D₂O, 120 mM NaCl and 20 mM sodium phosphate. Red triangles and blue circles represent denaturing and refolding data, respectively, at pH 6.3. Black squares represent denaturing data at pH 7.3. The fitted curves use the three-state equilibrium expression of equation 7 with fitted parameters listed in Table 3.

Figure 6.

^{15}N TROSY indicates random-coil peak positions that shift and broaden subtly from 1.5 to 5 M GdmCl. The GdmCl concentrations shown are (A) 1.5 M, (B) 2.5 M, (C) 3.5 M, and (D) 5.0 M. The standard buffer was used, but using H_2O as solvent. Two of the regions with shifting patterns of peaks are boxed.

Figure 7.

Proposed model for the energy landscape of KI-FHA folding and structure of its PUFs minutely populated at equilibrium. NHX suggests two near-native, partially unfolded states (PUF1 and PUF2), an unfolded state *U* open to HX and a higher energy unfolded state *U** with residual structure melted. The structural states use the same color code of Figure 3 and 4 with red and green for progressively higher subglobal stabilities, blue for global stability and black for superprotection.

Table 1

Fitted global, subglobal and local terms describing the HX behavior.

Residue	Global ^A		Subglobal		Local	
	ΔG , kcal/mol	m_g kcal mol ⁻¹ M ⁻¹	ΔG_{sg} kcal/mol	m_{sg} kcal mol ⁻¹ M ⁻¹	ΔG , kcal/mol	
E242	9.24	-8.73			6.94	
L243	8.92	-8.64			6.98	
K240	8.43	-6.28			7.67	
L184	8.23	-6.43			7.04	
F183	8.20	-6.61			7.04	
I281	7.88	-6.73			6.52	
A188	7.75	-5.58			7.05	
K288	7.72	-5.42			7.70	
T233	7.72	-6.20			7.07	
Q231	7.71	-5.60			7.64	
R292	7.63	-5.18			7.53	
V291	7.62	-6.33			6.99	
V289	7.57	-5.66			6.95	
Y290	7.53	-5.44			7.34	
L182	7.53	-5.94			6.75	
D279	7.51	-5.55			7.07	
L275	7.50	-6.23			6.74	
D278	7.49	-5.11			7.17	
T282	7.39	-5.53			7.01	
I232	7.35	-5.26			6.89	
I280	7.30	-5.09			7.18	
A230	7.25	-4.00			7.16	
A276	7.16	-4.57			7.03	
V186	7.03	-4.70			6.64	
L194	6.74	-4.47			5.49	
V273	6.46	-4.12			6.20	
G193	5.98	-3.44	6.01	-1.77	5.34	
S248 ^C			6.00	-1.94		
H196 ^C			5.93	-1.98		
T287 ^C			5.81	-1.17		
G284 ^B			5.78	-4.36		
W241 ^B			5.71	-1.47		
V208 ^B			5.63	-1.32		
G251 ^C			5.42	-1.53		
L253 ^B			5.38	-1.25		
D245 ^B			5.23	-2.01		
F239 ^C			5.12	-1.85		
K209 ^B			5.05	-2.09		
T252 ^C			4.27	-0.92		
L264 ^B			4.17	-0.92		
V254 ^B			4.05	-1.17		
V225 ^B					4.01	
I192					3.79	
L206						

^A Fitted to equation 6B.

B Fitted to equation 6C, using an estimated average ΔG_{G} and m_{G} of 7.58 kcal/mol and $-5.60 \text{ kcal mol}^{-1} \text{ M}^{-1}$, respectively, for residues with global stability (blue in Figure 3 and 4).

C Fitted to equation 6D.

Table 2
Average stability of foldons and structural segments of KI-FHA at pH 6.3, 20°C.

Foldon	Global ^A		Subglobal ^B	
	ΔG kcal/mol ^C	m kcal mol ⁻¹ M ⁻¹	ΔG kcal/mol	m kcal mol ⁻¹ M ⁻¹
Black / Super-protected	9.08 ± 0.23	-8.69 ± 0.06		
Blue / Global	7.53 ± 0.44	-5.50 ± 0.76		
Green / PUF2			5.62 ± 0.34	-2.01 ± 0.91
Red / PUF1			4.16 ± 0.11	-1.00 ± 0.14
Structural element				
β-strand 1	7.75 ± 0.58	-5.92 ± 0.86		
β-strand 2 ^D			6.00	-1.94
β-strand 3			5.42 ± 0.42	-1.66 ± 0.27
β-strand 5	7.50 ± 0.24	-5.27 ± 0.93		
β-strand 6	7.55 ± 1.82	-5.85 ± 3.15		
β-strand 7			4.80 ± 0.88	-1.23 ± 0.43
β-strand 9 ^D	6.98	-5.18		
β-strand 10	7.52 ± 0.25	-5.73 ± 0.70		
β-strand 11	7.61 ± 0.07	-5.61 ± 0.44		
1/2 loop			6.82 ± 0.89	-4.49 ± 1.07
6/7 loop			5.56 ± 0.49	-1.73 ± 0.38
9/10 loop	7.38 ± 0.20	-5.30 ± 0.85		
10/11 loop			5.87 ± 0.09	-1.58 ± 0.57

^A From fits using equation 6B.

^B From fits using equation 6C or 6D.

^C Without subtracting the 0.38 kcal/mol proline correction described in the text.

^D Standard deviation is not reported here as only residue is protected in this strand.

Table 3
Stability of KI-FHA against denaturation by GdmCl at 20 °C, detected optically.

pH	Solvent	Detection	Transition ^C	<i>m</i> -value ^D kcal/(mol.M)	Δ <i>G</i> kcal/mol ^D
6.3	D ₂ O	CD ^A	Δ <i>G</i> _{Ni} , 90%	-3.37 ± 0.06	2.79 ± 0.05
6.3	D ₂ O	CD ^A	Δ <i>G</i> _{TU} , 10%	-1.15 ± 0.31	3.71 ± 1.1
6.3	D ₂ O	Trp fluorescence ^B	Δ <i>G</i> _{Ni}	-3.51 ± 0.25	3.08 ± 0.22
6.3	H ₂ O	CD ^A	Δ <i>G</i> _{Ni} , 94%	-3.31 ± 0.07	3.11 ± 0.07
6.3	H ₂ O	CD ^A	Δ <i>G</i> _{TU} , 6%	-1.12 ± 0.35	3.22 ± 1.25
7.3	D ₂ O	CD ^A	Δ <i>G</i> _{Ni} , 90%	-3.91 ± 0.08	6.17 ± 0.13
7.3	D ₂ O	CD ^A	Δ <i>G</i> _{TU} , 10%	-1.26 ± 0.19	5.0 ± 0.7

^A Monitored at 216 nm.
^B Monitored as a difference between emission peak at 354 nm and baseline at 295 nm. Signal-to-noise was insufficient to characterize a minor transition at high [GdmCl].
^C Proportion of total optical change measured is given as a percentage where determined.
^D The uncertainties are the fitting uncertainties reported by Origin 7.5.