FOR THE RECORD

Characterization of peptide folding nuclei by hydrogen/deuterium exchange-mass spectrometry

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Abstract

Covalently linked pairs of well-chosen peptides can be good model systems for protein folding studies because they can adopt stable secondary, side-chain, and tertiary structure under certain conditions. We demonstrate a method for characterizing the structure in such peptide pairs by hydrogen/deuterium exchange of individual amide groups analyzed by collision-induced dissociation tandem mass spectrometry, in concert with circular dichroism spectroscopy. We apply the method to two peptides (and their three possible pairs) from bovine pancreatic trypsin inhibitor to address specific hypotheses regarding the stabilization of local secondary structure by long-range interactions.

Keywords: secondary structure; hydrogen/deuterium exchange; mass spectrometry; collision-induced dissociation; autonomous folding unit; structured peptides; bovine pancreatic trypsin inhibitor (BPTI)

Supplemental material: see www.proteinscience.org

Protein folding is a central problem of molecular biophysics. An early model of folding hypothesized that proteins adopt “flickering” native structure in high-propensity segments and that protein folding proceeds by the gradual association and mutual stabilization of such marginally stable segments (Lewis et al. 1970; Scheraga 1971). Consistent with this hypothesis, mixing peptide fragments of a protein in sufficiently

high concentrations can produce folded species analogous to those in the corresponding segments of the intact protein (Taniuchi and Anfinsen 1971). On the other hand, numerous experiments show that most peptides corresponding to secondary-structure elements (e.g., α -helices, β -hairpins, β -meanders, etc.) (definitions of the three types of structure discussed in this work—secondary structure, tertiary structure, and side-chain structure—are provided in the Supplemental Material) from naturally occurring proteins exhibit little secondary structure in aqueous solution (Montelione and Scheraga 1989), probably owing to competition between water–amide and amide–amide hydrogen bonds (H-bonds¹) (Harrington and Schellman 1956; Klotz and Franzen 1962). Therefore, long-range interactions are usually invoked to account for the stabilization of short-range structure in protein folding. However, the mechanisms by which interactions *between* peptides stabilize the structure *within* peptides have not been studied systematically. It would be useful, for example, to assess

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Abbreviations: H-bond, hydrogen bond; HDX, hydrogen/deuterium exchange; NMR, nuclear magnetic resonance; MS, mass spectrometry; MALDI-TOF, matrix-assisted laser-desorption/ionization time-of-flight; CID-MS/MS, collision-induced dissociation tandem mass spectrometry; BPTI, bovine pancreatic trypsin inhibitor; TCEP, tris-(2-carboxyethyl)phosphine hydrochloride; TFA, trifluoroacetic acid; GdnHCl, guanidinium hydrochloride; HPLC, high-performance liquid chromatography; CD, circular dichroism; FA, formic acid.

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the correlations between secondary, side-chain, and tertiary structure in pairs of covalently linked peptides and to characterize quantitatively the stabilization of local structure conferred by various factors, e.g., amino acid sequence, specific side-chain packing, steric, and/or hydrophobic exclusion of competing water molecules, etc.

Hydrogen/deuterium exchange (HDX), as monitored by mass spectrometry (MS) and nuclear magnetic resonance (NMR) spectroscopy, has been used widely to study conformations and conformational dynamics of proteins in various conditions ranging from isolated states to noncovalent interactions (Englander 2000; Kaltashov and Eyles 2002). However, HDX has been rarely applied to study oligopeptide systems (Carulla et al. 2000). We chose to measure HDX using MS because it requires significantly less material than NMR and tolerates poorly soluble peptides. Collision-induced dissociation tandem mass spectrometry (CID-MS/MS) can assess the deuterium incorporation of individual amides from the *b*-series of CID fragment ions (Deng et al. 1999; Kim et al. 2001; Hoerner et al. 2004). Recently, HDX-CID-MS/MS results were reported in a study of two oligopeptides and their homodimers formed by non-covalent interactions using electrospray ionization with an ion-trap mass spectrometer (Cai and Dass 2005).

The current understanding of protein folding suggests three connected hypotheses that can be tested by HDX on pairs of covalently linked peptides:

- H1. The association of any two peptides should lower the rate of HDX (even in the absence of significantly increased secondary structure) because steric hindrance alone will inhibit water from encountering the amide groups.
- H2. The association of any two peptides should increase the secondary structure of both peptides because amide–amide H-bonds will have less competition from amide–water H-bonds.
- H3. The association of any two sufficiently nonpolar peptides may provoke stable secondary, side-chain, and/or tertiary structure.

These hypotheses will be tested by comparing the structure in monomeric and dimeric peptides with HDX and circular dichroism (CD). To enforce the association of designated peptides, we restrict our study here to (1) individual monocysteinyll peptides that are linked by a disulfide bond in the native structure of a folded protein and (2) all possible covalent dimers of these peptides.

We illustrate application of our method using two cysteinyll peptides and their three possible disulfide-linked pairs, denoted P_α , P_β , $P_{\alpha\alpha}$, $P_{\beta\beta}$, and $P_{\alpha\beta}$. The model peptides mimic selected segments of the sequence of bovine pancreatic trypsin inhibitor (BPTI);

specifically, the monomers, P_α and P_β , correspond to residues 43–58 (NNFKSAEDCMRTAGGA) and 20–33 (RYFYNAKAGLCQTF), respectively, which form the disulfide-linked C-terminal α -helix and central β -hairpin, respectively, in native BPTI (Fig. 1A, Supplemental Material). Because P_α and P_β each have a cysteine, they can be disulfide-bonded to form the $P_{\alpha\alpha}$ and $P_{\beta\beta}$ homodimers and the $P_{\alpha\beta}$ heterodimer. The $P_{\alpha\beta}$ heterodimer corresponds to the (Cys30–Cys51) disulfide species of intact BPTI, which accumulates in the one-disulfide ensemble and seems to be partially folded (Weissman and Kim 1991; Staley and Kim 1994). Oas and Kim have shown that the $P_{\alpha\beta}$ heterodimer has stable secondary structure by CD and 1D NMR, whereas the P_α and P_β monomers do not (Oas and Kim 1988). The $P_{\alpha\alpha}$ and $P_{\beta\beta}$ dimers have not been studied structurally. However, it was reported recently that the homodimer of another peptide model for the BPTI β -sheet folds into a four-stranded anti-parallel β -sheet when the two monomers are connected by an “optimized” synthetic linker, although homodimers formed using “unoptimized” linkers and the monomer peptide do not form stable structure (Carulla et al. 2002). Throughout this paper, the residues are numbered according to their position in native BPTI (e.g., Cys30, Ser47, etc.).

Results and Discussion

Results from HDX and matrix-assisted laser-desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) of the five peptides are reported as total deuterium incorporation determined by the observed mass shift (Table 1). The monomeric P_α and P_β peptides showed average mass shifts of 10.2 and 8.6 Da, respectively, from their 15 and 13 exchangeable hydrogens. Hence, P_α and P_β show some protection against HDX under our experimental conditions (see Materials and Methods). This protection may reflect “flickering” local structure, as has been observed in other experiments (Goodman and Kim 1989; Carulla et al. 2000) and in our CD and CID-MS/MS experiments reported below. Much smaller mass shifts of 6.8 and 3.5 Da were measured for the subunits of the $P_{\alpha\alpha}$ and $P_{\beta\beta}$ dimers, respectively; the native-like $P_{\alpha\beta}$ dimer showed slightly smaller mass shifts of 5.5 Da (α subunit) and 2.9 Da (β subunit). Hence, all three dimers showed significantly more protection than their component monomers, consistent with hypothesis H1.

To better understand the enhanced protection against HDX in the peptide dimers relative to the monomers, we used CD spectroscopy to probe the secondary structure. The CD spectra of P_α and $P_{\alpha\alpha}$ (Fig. 1A), P_β and $P_{\beta\beta}$ (Fig. 1B), and $P_{\alpha\beta}$ (Fig. 1C) suggest that the

Table 1. HDX results for α and β subunits as assessed by MALDI-TOF-MS

Peptide model	P_{α}	P_{β}	$P_{\alpha\beta}$	$P_{\alpha\alpha}$	$P_{\beta\beta}$
Mass shift ^a	10.2	8.6	5.5 (α -chain) 2.9 (β -chain)	6.8 (each α -chain)	3.5 (each β -chain)

^a Mass shift (adjusted for back-exchange) is the average of triplicate runs. The uncertainty of the reported values is ± 0.1 Da expressed as standard deviation.

presence of the disulfide bond increases the secondary structure content of all three dimers ($P_{\alpha\alpha}$, $P_{\beta\beta}$, and $P_{\alpha\beta}$) relative to that in their component monomers (P_{α} and P_{β}). The clearest evidence of secondary structure is visible in $P_{\alpha\beta}$ (Fig. 1C), which has been shown previously to contain stable tertiary structure (Oas and Kim 1988). At 0°C, the CD spectrum of $P_{\alpha\beta}$ exhibits two minima near those expected for helical proteins (208 nm and 222 nm) (Woody et al. 1996); at 60°C, these wells are replaced by one well at 204 nm characteristic of random-coil structure (Woody et al. 1996). The summed CD spectrum of the P_{α} and P_{β} peptides resembles the spectrum of $P_{\alpha\beta}$ at 60°C, but shifted to lower wavelengths; this contrasts with results from the earlier study (Oas and Kim 1988), which found little deviation. Comparison of P_{α} and $P_{\alpha\alpha}$ shows that $P_{\alpha\alpha}$ at 0°C has more α -helical structure than P_{α} at 60°C, which has more α -helical structure than P_{α} at 0°C (judging by ellipticity at 222 nm). Similarly, $P_{\beta\beta}$ at 0°C has significantly more secondary structure than its corresponding monomer P_{β} at 0°C (judging by ellipticity at 217 nm) (Woody et al. 1996). These results are consistent with hypothesis H2 that local secondary structure content is stabilized upon the association of two peptides, presumably by the exclusion of water from attacking the amide–amide H-bonds. However, the increase in secondary structure in $P_{\alpha\alpha}$ seems small and local, not global; the $P_{\alpha\alpha}$ CD spectrum is not fully α -helical even at 0°C, and the difference spectrum of $P_{\alpha\alpha}$ and P_{α} does not exhibit the double minima at 208 nm and 222 nm expected for α -helical structure (Fig. 1A). Hence, the enhanced protection of $P_{\alpha\alpha}$ against HDX (which rivals that of the native-like $P_{\alpha\beta}$) likely stems from the association of the two P_{α} monomers connected by the disulfide bond, rather than from enhanced secondary structure within each, which supports hypothesis H1. By contrast, the CD spectrum of the $P_{\beta\beta}$ peptide at 0°C (but not at 60°C) suggests the presence of side-chain structure; specifically, the positive peak at 230 nm likely corresponds to the CD signal of one or more ordered tyrosine side chains (Sreerama et al. 1999). This suggests that $P_{\beta\beta}$ may also have stable tertiary structure, similar to that in $P_{\alpha\beta}$; further evidence is cited below.

To identify the position(s) of the enhanced HDX protection induced by the dimerization in the β -unit, we used CID-MS/MS to probe the HDX of individual

amide groups in P_{β} , $P_{\beta\beta}$, and $P_{\alpha\beta}$. These measurements were done using direct infusion electrospray ionization on an LCQ^{Deca} ion trap mass spectrometer. HDX-CID of b ions has been reported to be reliable for assessing deuterium content at the single amino acid level, whereas that of y ions is unreliable (Deng et al. 1999; Kim et al. 2001; Hoerner et al. 2004). A series of b ions (b_3 – b_{10}) was formed during the CID process for the P_{β} subunit; however, only y ions were produced for the P_{α} subunit. The masses of the b_3 – b_{10} ions were used to assess deuterium incorporation in residues Tyr23–Leu29 of the P_{β} subunit in P_{β} , $P_{\beta\beta}$, and $P_{\alpha\beta}$ (Table 2). The results indicate that the amide hydrogens of Tyr23, Asn24, Lys26, and Ala27 exchange readily in P_{β} (0.6–1.1 Da), but are protected in $P_{\beta\beta}$ (0.1–0.3 Da) and even more so in $P_{\alpha\beta}$ (0.0 Da for Lys26, –0.1 Da for Ala27). By contrast, the Ala25 amide hydrogen exchanges equally well and moderately in P_{β} , $P_{\beta\beta}$, and $P_{\alpha\beta}$ (0.4 Da). Finally, the Gly28 and Leu29 amide hydrogens exchange weakly in P_{β} (0.2 Da) and less so in $P_{\beta\beta}$ and $P_{\alpha\beta}$ (0.1 and 0.0 Da, respectively). Due to the missing fragment of the b_1 , b_2 , and b_{11} – b_{13} ions, deuterium incorporations for Tyr21, Phe22, and Cys30–Phe33 are not available. However, the 2.0-Da mass shift of the b_3 ion of P_{β} indicates that Tyr21 and Phe22 amide hydrogens exchange readily in the monomer. In addition, the 1.0-Da decrease in the mass shift of the b_3 ion in $P_{\beta\beta}$ and $P_{\alpha\beta}$ suggests that one of these two amide hydrogens is protected in the dimers. Similarly, segment Cys30–Phe33 of the β sequence shows less protection in P_{β} (2.3 Da) than in $P_{\beta\beta}$ and $P_{\alpha\beta}$ (0.9 Da and 1.0 Da, respectively).

These site-specific HDX results for the P_{β} subunit are generally consistent with the solvent exposure and H-bonding of the native structure (see Fig. 1B, Supplemental Material). If the β -chain adopts its native structure, the amide hydrogens of Phe22, Asn24, Gly28, Gln31, and Phe33 should be well protected because they are all involved in strong backbone (amide–amide) H-bonds; however, the Phe33–Arg20 H-bond is unlikely to be maintained in the peptide model, owing to the well-known flexibility of chain termini. Consistent with these predictions, significant protection for Asn24 and Gly28 is observed in $P_{\beta\beta}$ and $P_{\alpha\beta}$ (Table 2). Similarly, the mass shifts of the b_3 and b_{14} ions of $P_{\beta\beta}$ and $P_{\alpha\beta}$ are consistent with significant protection of Phe22 and Gln31 against HDX, which are mutually H-bonded

Figure 1. (A) CD spectra of P_α at 0°C, $P_{\alpha\alpha}$ at 0°C, $P_{\alpha\alpha}$ at 60°C, and the difference spectrum of $P_{\alpha\alpha}$ and P_α at 0°C. (B) CD spectra of P_β at 0°C, $P_{\beta\beta}$ at 0°C, $P_{\beta\beta}$ at 60°C, and the difference spectrum of $P_{\beta\beta}$ and P_β at 0°C. (C) CD spectra of $P_{\alpha\beta}$ at 0°C, $P_{\alpha\beta}$ at 60°C, and the summed spectrum of P_α and P_β at 0°C.

in the native structure. The amide hydrogen of Leu29 is well protected in $P_{\beta\beta}$ and $P_{\alpha\beta}$ (Table 2); in the native structure, the amide hydrogen of Leu29 is protected from exchange by the surrounding residues and is

weakly H-bonded to the backbone oxygen of Asn24 (although it competes with the amide hydrogen of Gly28). Interestingly, the amide hydrogens of Gly28 and Leu29 exchange relatively slowly in monomeric P_β , suggesting that P_β adopts local structure in its β -hairpin (residues 5–10). Finally, the Lys26 and Ala27 amide hydrogens form a bifurcated H-bond with the side-chain oxygen of Asn24 in the native structure of BPTI. These hydrogens are well protected in $P_{\beta\beta}$ and even more so in $P_{\alpha\beta}$, consistent with a structured Asn24 side chain. These two hydrogens are chiefly responsible for the difference between the protection of the P_β subunit against HDX in $P_{\alpha\beta}$ and $P_{\beta\beta}$. Combined with the CD results, these data suggest significant native secondary and side-chain structure in the $P_{\beta\beta}$ homodimer, as well as the $P_{\alpha\beta}$ heterodimer (Oas and Kim 1988).

The *absence* of protection against HDX also can be a cross-check of native structure; the amide hydrogens of Ala25, Cys30, and Thr32 should exchange readily because they are solvent-exposed and not H-bonded in the native structure. The increased structure in $P_{\beta\beta}$ and $P_{\alpha\beta}$ does not provide enhanced protection to Ala25 (Table 2). Notably, Ala25 does not fully exchange even in P_β ; this effect remains to be explained, although slow exchange at this position has been noted in BPTI variants (Schulman and Kim 1994), and may reflect a stable β -turn at residues Asn24–Leu29. The mass shifts of b_{14} and b_{10} from $P_{\beta\beta}$ and $P_{\alpha\beta}$ suggests that at least one residue in the segment Cys30–Phe33 is unprotected. Finally, the amide hydrogens of Tyr21 and Tyr23 should be protected in the $P_{\alpha\beta}$ dimer owing to *intermolecular* H-bonds with Phe22 and Asn20 from the P_α subunit. Consistent with this prediction, no protection is observed for Tyr21 and Tyr23 in P_β , but modest and roughly equal protection of Tyr23 is observed in *both* $P_{\beta\beta}$ and $P_{\alpha\beta}$ (Table 2). This protection of Tyr23 in the $P_{\beta\beta}$ dimer suggests stable intermolecular H-bonding (and, thus, tertiary structure) in $P_{\beta\beta}$, in addition to the secondary and side-chain structure cited above.

Conclusions

We have demonstrated the usefulness of HDX-MS, when combined with CD, for studying conformational features of model peptide folding systems. Our protocol uses MALDI-MS to assess overall deuterium incorporation and CID-MS/MS to obtain residue-level HDX data. These data coupled with CD spectra (with thermal transitions) allow us to assay secondary and other structural changes that occur upon dimer formation in model peptides. Our method is general, and should be useful in studying the physics of protein folding and disorder.

Applied to peptide models from BPTI, our results provide evidence for the three hypotheses outlined in

Table 2. HDX results for the P_β subunit as assessed by CID-MS/MS

Ion	P_β		$P_{\beta\beta}$		$P_{\alpha\beta}$	
	Mass shift ^a	Mass shift of residue	Mass shift ^a	Mass shift of residue	Mass shift ^a	Mass shift of residue
b_3	2.0	2.0 (Tyr ₂₁ -Phe ₂₂)	1.0	1.0 (Tyr ₂₁ -Phe ₂₂)	1.0	1.0 (Tyr ₂₁ -Phe ₂₂)
b_4	3.1	1.1 (Tyr ₂₃)	1.3	0.3 (Tyr ₂₃)	1.2	0.2 (Tyr ₂₃)
b_5	4.0	0.9 (Asn ₂₄)	1.6	0.3 (Asn ₂₄)	1.6	0.4 (Asn ₂₄)
b_6	4.4	0.4 (Ala ₂₅)	1.9	0.4 (Ala ₂₅)	2.0	0.4 (Ala ₂₅)
b_7	5.3	0.9 (Lys ₂₆)	2.3	0.3 (Lys ₂₆)	2.0	0.0 (Lys ₂₆)
b_8	5.9	0.6 (Ala ₂₇)	2.4	0.1 (Ala ₂₇)	1.9	-0.1 (Ala ₂₇)
b_9	N/A	0.2 (Gly ₂₈)	N/A	0.1 (Gly ₂₈)	N/A	0.0 (Gly ₂₈)
b_{10}	6.3	0.2 (Leu ₂₉)	2.6	0.1 (Leu ₂₉)	1.9	0.0 (Leu ₂₉)
b_{14}	8.6	2.3 (Cys ₃₀ -Phe ₃₃)	3.5	0.9 (Cys ₃₀ -Phe ₃₃)	2.9	1.0 (Cys ₃₀ -Phe ₃₃)

^a Mass shift (adjusted for back-exchange) is the average of triplicate runs. The uncertainty of the reported values is ± 0.1 Da expressed as standard deviation.

the introduction. First, P_α shows some protection against HDX, but the $P_{\alpha\alpha}$ dimer has almost as much protection as the native-like $P_{\alpha\beta}$ heterodimer, despite having significantly less secondary structure than $P_{\alpha\beta}$ (as assessed by CD). This supports hypothesis H1 that the association of two peptides can inhibit HDX by lowering the local activity of water independently of secondary structure. Second, the $P_{\alpha\alpha}$ dimer exhibits more secondary structure than the P_α monomer, even at 60°C; analogous results pertain to P_β and $P_{\beta\beta}$. This supports hypothesis H2 that the decreased local activity of water stabilizes local secondary structure. Finally, the $P_{\alpha\alpha}$ dimer does not appear to be fully structured, judging from its nonhelical CD spectrum; by contrast, the data suggest that the $P_{\alpha\beta}$ and $P_{\beta\beta}$ dimers have stable secondary, side-chain, and even tertiary structure. Thus, our results are consistent with hypothesis H3 that stable secondary, side-chain, and even nonnative tertiary structure may develop when two sufficiently nonpolar peptides are linked covalently. The difference between the $P_{\alpha\alpha}$ and $P_{\beta\beta}$ dimers may result from the hydrophobicity of the two peptides; P_α has only two large hydrophobic residues (Phe45 and Met52) of 16 residues, whereas P_β has four (Tyr21, Phe22, Tyr23, and Leu29) of 14 residues. Therefore, the $P_{\beta\beta}$ dimer is better able to form a hydrophobic core around its disulfide bond, further shielding its amide-amide H-bonds from attack by water molecules. This is consistent with the global HDX results; $P_{\beta\beta}$ exchanges 27% (3.5/13) of its amide hydrogens, whereas $P_{\alpha\alpha}$ exchanges 45% (6.8/15).

Materials and methods

Materials

The P_α and P_β peptides were synthesized by the Genomics Technology Support Facility (GTSF) at Michigan State University and purified by reverse-phase high-performance

liquid chromatography (HPLC) using a Vydac C18 analytical column (catalog no. 218TP54). All other reagents were of the highest purity commercially available and used without further purification. Dibasic sodium phosphate (Na_2HPO_4) and sodium sulfate (Na_2SO_4) were purchased from Spectrum Chemical Mfg. Corp. and Columbus Chemical Industries, Inc., respectively. Guanidine hydrochloride (GdnHCl) was purchased from Invitrogen. Acetonitrile UV and acetonitrile (HPLC grade) were purchased from Honeywell International, Inc. and EMD Chemicals, respectively. The tris-(2-carboxyethyl) phosphine (TCEP) hydrochloride solutions were freshly prepared before use. All other chemicals were purchased from Sigma.

All pH/pD values were measured using a Beckman $\phi 40$ pH meter or ColorpHast pH indicator strips (pH 0–14; EM Science). pD values were computed by adding 0.4 to the corresponding pH readings. The standard buffer in this study contains 200 mM Na_2SO_4 , 10 mM NaH_2PO_4 , adjusted to pH 6.0. For the monomeric P_α and P_β peptides, the buffer also contained 1 mM reduced dithiothreitol to prevent dimerization of the monomers. No noticeable oxidation was detected after the experiment, as checked by reverse-phase HPLC.

Preparation of the peptide dimers

The $P_{\alpha\alpha}$ and $P_{\beta\beta}$ homodimers and the $P_{\alpha\beta}$ heterodimer were prepared as described elsewhere (Oas and Kim 1988). Briefly, roughly equal amounts of the P_α and P_β monomers were mixed in a solution containing 5 M GdnHCl and 200 mM Tris-HCl at pH 8.0 and were air-oxidized for 48 h at 25°C. The dimers were separated and purified on a Vydac C18 analytical column using a water/acetonitrile gradient in the presence of 0.1% trifluoroacetic acid (TFA). Each dimer was collected manually and dried under reduced pressure for further use.

Circular dichroism

CD spectra were obtained at 0°C on a Jasco J-810 CD spectropolarimeter (Jasco, Inc.) using a thermostatted 1-mm path-length cell. The samples were dissolved in the standard buffer. The concentrations were determined by UV absorbance at 205 nm (Scopes 1974) and were typically 0.15 mg/mL.

Hydrogen/deuterium exchange (HDX)

The HDX experiments were carried out in a cold room, typically at 4°C. Each peptide sample was equilibrated with the standard buffer (1 nmol/μL) for about half an hour before each experiment. HDX was initiated by diluting 1 μL of aqueous protein solution with 19 μL of standard buffer made with D₂O at pD 6.4. After 15 sec, 18 μL of aqueous TCEP solution (500 mM in 2M GdnHCl [pH 2.5], 4°C) were added to quench the exchange by decreasing the pH to 2.5 and to reduce the disulfide bond. The pH was maintained at 2.5 in all subsequent steps. Incubation of the sample with D₂O for 15 sec should be long enough to exchange exposed amide hydrogens with deuteriums (Bai et al. 1993), but short enough to avoid significant exchange with H-bonded/protected amide hydrogens.

Analysis by MALDI-TOF mass spectrometry

Mass spectra were acquired on a PerSpective Biosystems Voyager DE STR instrument (PE Biosystems) in the positive ion reflectron mode of operation. The accelerating voltage was 20 kV; the grid voltage and guide wire voltage were 76% and 0.04% of the accelerating voltage, respectively. The mirror voltage ratio was 1.12, whereas the extraction delay time was 150 nsec. The matrix was α-cyano-4-hydroxycinnamic acid in a 5 mg/mL solution containing acetonitrile, ethanol, and H₂O (1:1:1, v:v:v); the final pH was adjusted to 2.5 with TFA. The measurement was done using next-spot external calibration with the P_α and P_β monomers as the calibrants, for which the calculated monoisotopic [M + H]⁺ masses are 1671.7219 Da and 1681.8160 Da, respectively. The average mass of each peptide was measured experimentally by determining the centroid of the distribution of its isotope peak intensities using Data Explorer TM 4.0. Ion current from 256 laser shots was accumulated for each spectrum.

Immediately after quenching the HDX process, the sample was desalted with cold water containing 0.1% TFA (pH 2.5) using a ZipTip (Millipore), eluted with matrix solution, and spotted on a chilled MALDI target. The target was immediately placed in a desiccator under moderate vacuum to dry the spot within 1–2 min. The target was immediately transferred to the MALDI instrument. A highly deuterated standard was prepared by dissolving the sample in D₂O at pH 7.0 with 8 M D₄-urea and incubating at room temperature for over 48 h. Treating the highly deuterated standard by the same protocol indicated that the extent of back-exchange during the experimental procedures was 35%. The uncertainty of the mass shift values is ±0.1 Da, expressed as the standard deviation of results from triplicate runs.

Analysis by CID tandem mass spectrometry

Mass spectra were acquired on a Finnigan LCQ^{Deca} ion-trap mass spectrometer (ThermoQuest) with an ion-spray voltage of 3 kV, a capillary voltage of 24 V, a tube lens offset of 55 V, and a capillary temperature of 150°C. Data-dependent MS/MS conditions were set with a default collision energy of 35%, a default charge state of 2, and an isolation width of 5 (*m/z*). Scans were taken over the range *m/z* 235–1500. Typically, 100 scans were accumulated per spectrum.

Immediately after quenching the HDX process, the sample was desalted with cold water containing 0.1% formic acid

(FA) (pH 2.5) using a ZipTip and eluted with 100 μL of a water/acetonitrile solution (1:1, v:v) preadjusted to pH 2.5 with FA. The processed sample was infused into the LCQ^{Deca} immediately with a prechilled (ice) 100-μL syringe at a flow rate of 20 μL/min; the syringe was kept buried in ice throughout the experiment. The extent of back-exchange during the infusion-CID-MS/MS procedures was 25%, as determined from analysis of the highly deuterated standard prepared as described above. Mass spectra were obtained with the operating software Xcalibur, and the centroid values were calculated with the Magtran software (Zhang and Marshall 1998).

Electronic supplemental material

Three items were provided as supplementary material: definitions of structural terms; Figure 1A, ribbon diagram of bovine pancreatic trypsin inhibitor (BPTI), with peptides P_α and P_β highlighted in red and blue, respectively; and Figure 1B, ribbon diagram of the natively structured P_β peptide.

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