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Engineering of betabellin 14D: Disulfide-induced folding of a β-sheet protein

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Abstract

The betabellin target structure consists of 2 32-residue β sheets packed against each other by hydrophobic interactions. We have designed, chemically synthesized, and biophysically characterized betabellin 14S, a single chain, and betabellin 14D, the disulfide-bridged double chain. The 32-residue nongenetic betabellin-14 chain (HSLTASI**ka**LTIHVQ**ak**TATCQV**ka**YTVHISE, **a** = D-Ala, **k** = D-Lys) has a palindromic pattern of polar (p), nonpolar (n), end (e), and β-turn (**t**, **r**) residues (epnpnpn**tt**npnpnp**rr**pnpnpn**tt**npnpnpe). Each half contains the same 14-residue palindromic pattern (underlined). Pairs of D-amino acid residues are used to favor formation of inverse-common (type-I′) β turns. In water at pH 6.5, the single chain of betabellin 14S is not folded, but the disulfide-linked betabellin 14D is folded into a stable β-sheet structure. Thus, folding of the covalent dimer betabellin 14D is induced by formation of the single interchain disulfide bond. The binary pattern of alternating polar and nonpolar residues of its β sheets is not sufficient to induce folding. Betabellin 14D is a very water-soluble (10 mg/mL), small (64 residues), nongenetic (12 D residues) β-sheet protein with properties (well-dispersed proton NMR resonances; T_m = 58 °C and ΔH_m = 106 kcal/mol at pH 5.5) like those of a native protein structure.

Keywords: circular dichroism; de novo design; NMR spectroscopy; protein synthesis; thermal denaturation

A major challenge in de novo protein design is the engineering of a protein having the folding stability of the native structure of a natural protein. Several proteins have been designed with the α helix as the major structural element (Ho & DeGrado, 1987; Hodges et al., 1988; Hahn et al., 1990; Hecht et al., 1990; Engel et al., 1991; Richardson et al., 1992; Handel et al., 1993; Kamtekar et al., 1993). Fewer proteins have been designed with the β sheet as the major structural element (Kullmann, 1984; Moser et al., 1985; Erickson et al., 1986; Richardson et al., 1992; Pessi et al., 1993). The β sheet is less modular than the α helix, requires more cooperativity to fold properly, and has a greater tendency to form aggregates in solution. A major difficulty in designing a structurally stable β protein is dealing with the interactions between 2 β strands or 2 β sheets. Study of these interactions has been hindered by the lack of a suitable model β protein. Engineering of a soluble native-like β-sheet protein would add to our knowledge of how β turns, β strands, and β sheets interact.

The betabellin target structure is a design for a simple β-sheet protein containing a variety of amino acids (Erickson et al., 1986, 1988; Richardson & Richardson, 1987, 1990; Daniels et al., 1988; McClain et al., 1990, 1992; McClain, 1991; Richardson et al., 1992; Yan, 1994; Yan & Erickson, 1994). It consists of two 32-residue β sheets packed against each other by interaction of the hydrophobic face of each sheet. By several biophysical criteria, betabellin 12S is a noncovalent β-sheet dimer, and 12D is a covalent disulfide-bridged β-sheet dimer (McClain, 1991; McClain et al., 1992; Richardson et al., 1992; Yan, 1994). But the aggregation of 12D in water precluded a detailed study of its NMR solution structure. A 2D-NMR study of betabellin 12D in $(CD_3)_2SO$ showed that the Asn 15–Pro 16 peptide bond is present in both the *trans* and *cis* conformations (Yan, 1994). This paper describes the design, synthesis, and characterization of betabellin 14D, a nongenetic protein designed to avoid the problems of aggregation and *cis/trans* peptide bond isomerization (Yan, 1994; Yan & Erickson, 1994). Synthetic betabellin 14D is a very water-soluble protein whose folding into a β-sheet structure is induced by formation of its single disulfide bridge and whose properties are similar to those of small native proteins.

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Results

Design of betabellin 14

The betabellin target structure consists of 2 β sheets held together by interaction of their hydrophobic faces. It is characterized by a 32-residue palindromic pattern of polar (p), nonpolar (n), end (e), and turn (**t**, **r**) residues (Fig. 1A). Each half of this larger palindrome contains the same smaller 14-residue palindromic

A

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position  1   5    10   15   20   25   32 position
   12  HTLTASIpdLTYSINpdTATCKVpdFTLSIGA  12
common  | |||||  ||     ||||  |   |  |  common
   14  HSLTASIkaLTIHVQakTATCQVkaYTVHISE  14
pattern epnpnpnttnpnpnprrpnpnpnttnpnpnpe pattern
chirality LLLLLLLDDLLLLLLDDLLLLLLDDLLLLLL chirality
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B

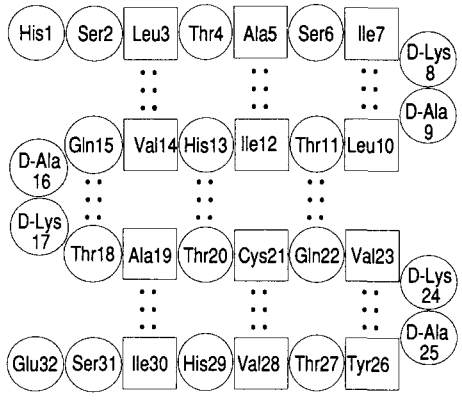

Fig. 1. Amino acid sequence of the betabellin-14 chain. **A:** Palindromic pattern of polar (p), nonpolar (n), end (e), and turn (**t**, **r**) residues. Each half contains the same 14-residue palindromic pattern (underlined). The chiral pattern of L-amino acid (L) and D-amino acid (D) residues and residues common (|) to the betabellin-12 chain are indicated. **B:** Betabellin target structure with 18 β-sheet hydrogen bonds ($\cdots$) between 3 pairs of polar residues (circled, side chains back) and 6 pairs of nonpolar residues (boxed, side chains forward).

pattern (pnpnpn**tt**npnpnp). When the adjacent pairs **tt** and **rr** are the loop residues L1 and L2 of inverse-common (type-I′) β turns, the peptide chain should fold in water into a 4-strand antiparallel β sheet that is stabilized by 18 peptide-peptide hydrogen bonds (Fig. 1B). The 12 polar side chains would constitute 1 face of this β sheet, and the 12 nonpolar residues would comprise the other face. This amphiphilic β sheet would probably not be stable by itself, but 2 such β sheets could mutually stabilize one another through hydrophobic interaction of their nonpolar faces to form an 8-strand β barrel. A disulfide bridge between the β sheets might be needed to stabilize the β barrel (Erickson et al., 1986).

Betabellin 12D is more water soluble than previous betabellins, but its aggregation in water precludes an NMR study of its solution structure (Yan, 1994). Betabellin 14D was designed by replacing more than half of the residues of betabellin 12D (Fig. 1). The expected net charge of the 2-chain dimer at pH 5 was changed from -2 to $+10$ by removing most of the Asp and Glu residues and by adding several Lys and His residues. The betabellin target structure is geometrically most compatible with type-I′ β turns at the **tt** and **rr** positions (Richardson & Richardson, 1987). Therefore, betabellin 12D was designed (McClain, 1991; McClain et al., 1992; Richardson et al., 1992) with D-Pro-D-Asp (**pd**) at the **tt** and **rr** positions (Fig. 1A). Through molecular-dynamics simulation of dipeptides models (Yan et al., 1993; Tropsha et al., 1994), we found that D-Ala-D-Ala segments are thermodynamically favored to fold into a type-I′ β turn rather than into a type-I, type-II, or type-II′ β turn. Thus, betabellin 14D was designed (Yan, 1994; Yan & Erickson, 1994) to test if it would fold into a stable β-sheet protein with D-Lys-D-Ala (**ka**) at the **tt** positions and D-Ala-D-Lys (**ak**) at the **rr** positions, which should also favor folding into type-I′ β turns.

Chemical synthesis

The 32-residue peptide chain of betabellin 14S was assembled on a solid-phase synthesizer using fluorenylmethoxycarbonyl (Fmoc) chemistry and was purified by reversed-phase HPLC on a butyl-silica column. By analytical HPLC, 14S was eluted from this column as a single symmetric peak at 34% acetonitrile/0.05% trifluoroacetic acid (TFA). Amino acid analysis of betabellin 14S was consistent with its residue composition: found (calculated), Asp + Glu 3.0 (3), Ser 2.5 (3), His 2.4 (3), Thr 3.0 uncorrected (5), Ala 5.0 (5), Tyr 1.0 (1), Val 2.7 (3), Cys 1.2 (1), Ile 2.7 (3), Leu 1.9 (2), Lys 3.2 (3). The average mass of betabellin 14S from electrospray ionization (ESI) mass spectrometry was that of a single betabellin-14 chain (calculated for $C_{152}H_{253}N_{43}O_{46}S$, 3,451.0 Da; found, 3,451 Da).

Air oxidation of betabellin 14S in 20% $(CH_3)_2SO$ at 37 °C for 24 h furnished betabellin 14D quantitatively. 14D was eluted from the butyl-silica HPLC column as a single symmetric peak at 29% acetonitrile/0.05% TFA. Therefore, 14D is more hydrophilic than 14S. The average covalent mass of betabellin 14D from ESI mass spectrometry was that expected for 2 betabellin-14 chains linked by a single disulfide bond (calculated for $C_{304}H_{504}N_{86}O_{92}S_2$, 6,900.0 Da; found, 6,900 Da).

β-Sheet structure

The circular dichroic spectra of betabellins 14S and 14D in water at pH 6.5 are strikingly different (Fig. 2). 14S has a negative band at 198 nm, which is characteristic of a nonfolded structure (Woody, 1985). In contrast, 14D has a negative band at 216 nm, a strong positive band at 197 nm, a crossover point at 190 nm, and a strong negative band at a shorter wavelength, all of which are characteristic of a β-sheet structure (Woody, 1985). The CD spectrum of betabellin 14D in water (pH 6.5) was unchanged over the concentration range of 5–247 μM. The amplitude of the mean residue ellipticity of the negative band at 216 nm, $[\theta]_{216}$, varied as the pH of a solution of 14D in 25 mM sodium acetate

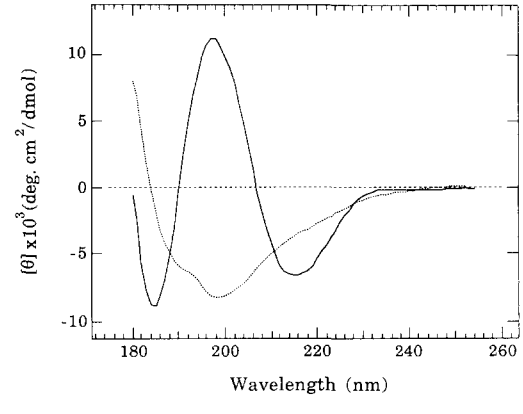

Fig. 2. CD spectra of betabellins 14S (------) and 14D (———) in water at pH 6.5 (Yan & Erickson, 1994).

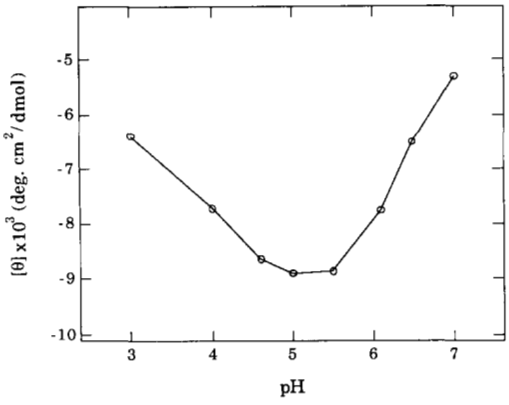

Fig. 3. Amplitude of $[\theta]_{216}$ for betabellin 14D in 25 mM sodium acetate buffer at pH 3.0–7.0.

buffer was changed from 3.0 to 7.0 (Fig. 3). By this criterion, the β-sheet structure of betabellin 14D is largest near pH 5.0–5.5 and decreases bimodally with pH. The decrease of $[\theta]_{216}$ above pH 5.5 might be due to change in the ionization of a His residue, and the decrease below pH 5.0 might be caused by change in the ionization of a Glu residue.

Proton environments

The 500-MHz proton NMR spectra of betabellins 14S and 14D in 10% D_2O at pH 6.5 are also quite different (Fig. 4). The chemical shifts for the side-chain resonances of 14S in the upfield, aliphatic region are approximately the same as those seen for denatured, "random coil" proteins (Wüthrich, 1986). The CH_3 resonances for the Val, Ile, and Leu groups cluster near 0.91 ppm with none upfield of 0.81 ppm. In contrast, the aliphatic resonances for 14D are well dispersed. The CH_3 resonances are spread over the range of 0.48–1.06 ppm with many upfield of 0.81 ppm. In addition, several αCH resonances appear farther downfield for 14D than for 14S. This dispersion of the aliphatic resonances for 14D suggests that the protons involved are located in well-folded environments. For example, the CH_3 resonances at 0.48–0.81 ppm may be due to nonpolar residues located in the interior between 2 β sheets. In contrast, the resonances assigned to the Thr γCH_3 (1.20 ppm), Lys γCH_2 (1.40 ppm), and Lys δCH_2 groups (1.67 ppm), which appear at the same chemical shifts for both 14S and 14D, may be due to solvent-exposed polar residues on the exterior.

The chemical shifts for the side-chain resonances of 14S in the downfield, aromatic/amide region are also approximately the same as those seen for denatured, "random coil" proteins (Wüthrich, 1986) except for a 0.05-ppm shift of the Tyr aromatic resonances. The His 2H resonances are clustered near 8.60 ppm. Except for the Gln δNH_2 resonances, few if any amide resonances for 14S are at 6.5–8.0 ppm, with none downfield of 8.70 ppm. In contrast, the amide and aromatic resonances for 14D are well dispersed and many amide resonances are downfield of 8.70 ppm. The downfield shift of the amide resonances and several αCH resonances for 14D compared to the nonfolded peptide 14S is consistent with betabellin 14D having a β-sheet structure in water (Wishart et al., 1992).

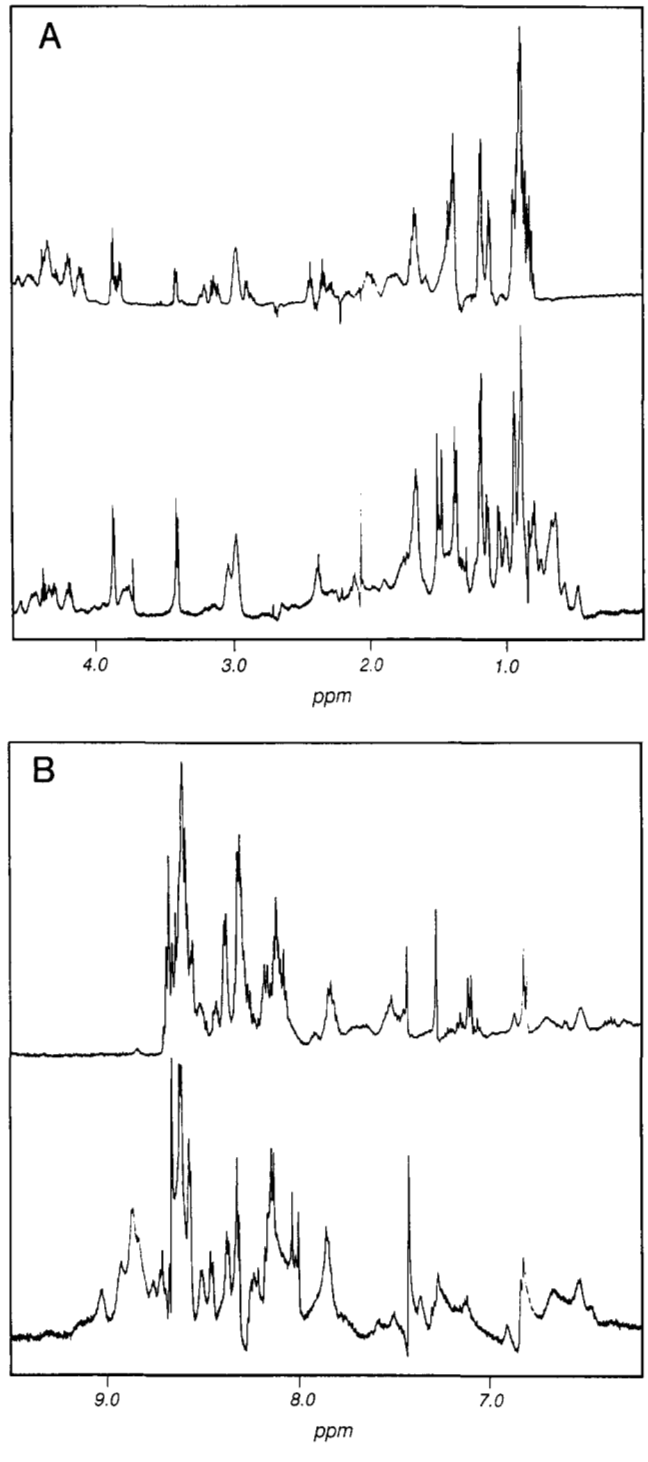

Fig. 4. Proton NMR spectra (at 500 MHz) of betabellins 14S (upper) and 14D (lower) in 10% D_2O at pH 6.5. **A:** Aliphatic region. **B:** Amide and aromatic region.

Thermal denaturation of betabellin 14D

The thermal denaturation of betabellin 14D in 25 mM sodium acetate buffer (pH 5.5) was monitored by measuring the amplitude of $[\theta]_{216}$ over the range of 5–95 °C (Fig. 5). Denaturation is reversible because the amplitude of $[\theta]_{216}$ for the sample af-

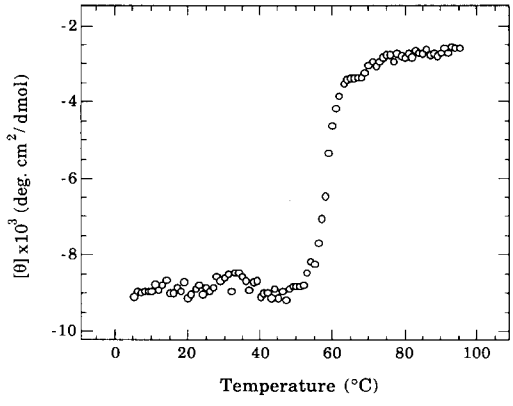

Fig. 5. Thermal denaturation of betabellin 14D in 25 mM sodium acetate buffer at pH 5.5 as monitored by the amplitude of $[\theta]_{216}$.

ter cooling back to 5 °C was within 5% of that for the sample at 5 °C before heating. This transition was modeled as a 2-state transition between a folded state and an unfolded state (Pace et al., 1989). A plot of ΔG against temperatures of 50–64 °C (Fig. 6) gave a linear correlation coefficient of 0.994. At m, the midpoint of denaturation, the temperature T_m was (58.4 ± 2.4) °C, the entropy change ΔS_m was (321 ± 9) cal/(mol·K), and the enthalpy change ΔH_m was (106 ± 5) kcal/mol.

Discussion

In water at pH 6.5, the single chain of betabellin 14S is not folded, but the disulfide-linked dimer betabellin 14D folds into a β-sheet structure. Thus, the single interchain disulfide bond of betabellin 14D is essential for its folding. Unlike the noncovalent dimer betabellin 12S (Yan, 1994), betabellin 14S is not able to fold into a stable noncovalent dimer. Once the disulfide bond is formed between the Cys 21 residues of 2 nonfolded betabellin-14 chains, they are induced to fold into the double-chain β-sheet structure of betabellin 14D.

Kamtekar et al. (1993) recently proposed that to induce the folding of a designed polypeptide chain into a compact native-like structure, the proper binary pattern of polar and nonpolar residues must be specified, but their identities do not. The binary pattern for a β sheet is the alternating polar and nonpolar residues of its β strands. The betabellin target structure contains 12 polar and 12 nonpolar β-strand residues per chain (Fig. 1A). The betabellin-12 and betabellin-14 chains have the same pattern of polar and nonpolar residues. Two betabellin-12 chains fold as a noncovalent β-sheet dimer, but the betabellin-14 chain remains nonfolded. A binary pattern of polar and nonpolar residues may be sufficient to induce the folding of an α-helical structure (Kamtekar et al., 1993), but it is not always sufficient to induce the folding of the β sheets of the betabellin target structure.

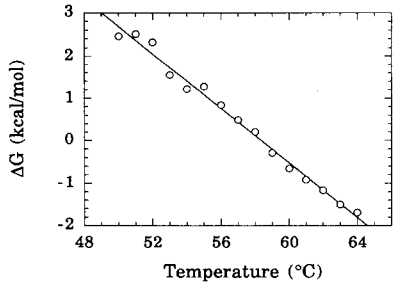

Fig. 6. Free-energy changes for the thermal denaturation of betabellin 14D in 25 mM sodium acetate buffer at pH 5.5.

Dyson et al. (1988) proposed that formation of β turns might induce folding of β sheets. The energetically most favorable chiral pattern (D, D) for type-I′ β-turn residues (Yan et al., 1993, 1994) is present at 3 turn sites of the betabellin-14 chain. But even this bias is not sufficient to induce its folding into a noncovalent β-sheet dimer. The disulfide bond of betabellin 14D, however, prevents its 2 β sheets from separating once they fold. The contrast between the similarities in folding of betabellins 12S and 12D and the differences in folding of betabellins 14S and 14D suggests that hydrophobic interaction of the nonpolar faces of 2 β sheets is necessary but not sufficient to stabilize the betabellin target structure.

In water at pH 6.5, the amplitude of $[\theta]_{216}$ for covalent dimers 12D and 14D is half that for the noncovalent dimer 12S. This difference may be due to distortion of the β strands imposed by the disulfide bridge. In 25 mM sodium acetate buffer at pH 5.5, betabellin 14D is folded at 40–45 °C but undergoes reversible thermal denaturation at 50–64 °C. The T_m value for betabellin 14D (58 °C) is slightly lower than those for betabellins 12S (66 °C) and 12D (77 °C) (Yan, 1994). But ΔH_m for 14D (106 kcal/mol at pH 5.5) is 3.5–5.5 times greater than for 12S or 12D. It is also greater than that for bovine pancreatic trypsin inhibitor, a native protein structure of similar size (58 residues), which has a T_m of 104 °C and a ΔH_m of 76 kcal/mol at pH 4.9 (Makhatadze et al., 1993). These results suggest that the thermal denaturation of betabellin 14D resembles the reversible unfolding of a native protein structure.

Betabellin 14D folds into a stable β-sheet protein with D-Lys-D-Ala at all 4 **tt** positions and D-Ala-D-Lys at both **rr** positions. Other pairs of D-amino acids might also favor type-I′ β turns at these 6 positions. Because it is very soluble in water (10 mg/mL) and is readily prepared by chemical synthesis, betabellin 14D should be useful as a model β-sheet protein for studying protein folding and as a molecular framework for engineering novel functional proteins.

Materials and methods

Synthesis of betabellins 14S and 14D

The 32-residue chain of betabellin 14S (Fig. 1) was assembled by solid-phase synthesis using Fmoc chemistry on an Applied Biosystems model 430 peptide synthesizer. The peptide was deprotected and cleaved from the resin by treatment for 2 h with 49:1 (v/v) TFA/ethanedithiol. The peptide was extracted from the resin, precipitated with ice-cold diethyl ether, centrifuged, dried under argon, dissolved in 0.05% TFA/water, and lyophilized. Crude peptide was stirred for 4 h at room temperature with a solution of 4 M guanidinium chloride/0.10 M Tris buffer (pH 8) containing 25 molar equivalents of dithiothreitol. The solution was chromatographed on a butyl-silica HPLC column (Vydac C-4, 250 × 10 mm).

Betabellin 14D was obtained by air oxidation of betabellin 14S (5 mg/mL) in 20% $(CH_3)_2SO$ (Tam et al., 1992) at 37 °C in a

capped reaction vial. Progress of the reaction was monitored by diluting a sample in 0.1% TFA/water and analyzing it by HPLC. After 24 h, the reaction mixture was diluted with 0.1% TFA/water and chromatographed on the butyl-silica HPLC column.

ESI mass spectrometry

Positive-ion ESI mass spectra were recorded with a Sciex model API-III mass spectrometer with the ion-spray needle at 5,300 V and the orifice potential at 80 V.

CD spectroscopy

CD spectra were recorded in quartz cells (path length of 10 or 1 mm) with an AVIV model 62DS CD spectrophotometer equipped with a thermoelectric temperature controller. Each data point was corrected by baseline subtraction and converted into mean residue ellipticity, $[\theta]$, based on the molar concentration of protein present as determined by quantitative amino acid analysis. Thermal denaturation of betabellin 14D in 25 mM sodium acetate buffer (pH 5.5) was monitored by measuring $[\theta]_{216}$ for each degree from 5 to 95 °C. Thermodynamic quantities for protein denaturation were determined as described by Pace et al. (1989).

NMR spectrometry

Betabellin 14S or 14D (5 mg) was dissolved in 10% D_2O (0.50 mL, pH 6.5). Proton NMR spectra were recorded at 25 °C with a Bruker model 500 AMX NMR spectrometer as an average of 64 transients centered at H_2O with a width of 6,020 Hz. The H_2O signal was suppressed by presaturation and chemical shifts were referenced to 2,2,3,3-tetradeuterio-3-(trimethylsilyl)propionic acid.

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