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Engineering of betabellin-15D: A 64 residue beta sheet protein that forms long narrow multimeric fibrils

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Abstract

The betabellin target structure is a β-sandwich protein consisting of two 32 residue β-sheets packed against one another by interaction of their hydrophobic faces. The 32 residue chain of betabellin-15S (HSLTAKI**pk**LTFSIA**ph**TYTCAV **pk**YTAKVSH, where **p** = DPro, **k** = DLys, and **h** = DHis) did not fold in water at pH 6.5. Air oxidation of betabellin-15S provided betabellin-15D, the 64 residue disulfide bridged two-chain molecule, which also remained unfolded in water at pH 6.5. By circular dichroic spectropolarimetry, the extent of β structure observed for betabellin-15D increased with the pH and ionic strength of the solution and the betabellin-15D concentration. By electron microscopy, in 5.0 mM MOPS and 0.25 M NaCl at pH 6.9, betabellin-15D formed long narrow multimeric fibrils. A molecular model was constructed to show that the dimensions of these betabellin-15D fibrils are consistent with a single row of β-sandwich molecules joined by multiple intersheet H-bonds.

Keywords: betabellin; β-sandwich; β-sheet; circular dichroism; de novo design; fibril formation; protein engineering

One way to study protein folding is to start with an amino acid sequence and attempt to predict its folded protein structure. Another way is to start with a folded protein structure and attempt to predict an amino acid sequence that will fold into this structure. Most natural protein structures are too complicated to be easy folding targets. De novo protein engineering involves designing a simple folded protein structure from scratch, synthesizing a peptide chain having an amino acid sequence that may fold into the designed structure, and studying how it folds (DeGrado, 1997).

This practical approach has provided many designed α-helical proteins (Hodges et al., 1988; Hecht et al., 1990; Engel et al., 1991; Handel et al., 1993; Bryson et al., 1995; Betz & DeGrado, 1996; Betz et al., 1996, 1997; Hodges, 1996; Lombardi et al., 1996; Dahiyat & Mayo, 1997; Kohn et al., 1997). Fewer proteins have been designed with the β-sheet as the main element of secondary structure (Kullmann, 1984; Moser et al., 1985; Osterman & Kaiser, 1985; Richardson et al., 1992; Pessi et al., 1993; Quinn et al., 1994; Wagner et al., 1994; Yan & Erickson, 1994; Krause et al., 1996; Mayo et al., 1996; Nesloney & Kelly, 1996; Smith & Regan, 1997; Zappacosta et al., 1997). Because β-sheets are less modular than α-helices and tend to aggregate in solution, it is more challenging to design a native-like β-sheet protein (Hecht, 1994). Designing a water-soluble β-sheet protein involves understanding of the noncovalent interactions between β-strands and between β-sheets. Therefore, engineering of a β-sheet protein should increase our understanding of the interactions of β-turns, β-strands, and β-sheets. This paper describes the de novo design, solid phase synthesis, and biophysical characterization of betabellin-15D, a 64 residue disulfide bridged two-chain protein that forms long narrow multimeric fibrils.

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Results and discussion

Design of betabellin-15

Betabellin target structure

The word "betabellin" denotes a β-sandwich bell-shaped protein. The betabellin target structure is a de novo design for a folded protein structure consisting of two 32 residue β-sheets that are packed against each other by hydrophobic interactions to form a β-sandwich (Erickson et al., 1986, 1988; Richardson & Richardson, 1987, 1990; Daniels et al., 1988; McClain et al., 1990, 1992; Richardson et al., 1992). The amino acid sequence of each 32 residue betabellin chain is designed to have a palindromic pattern of polar (p), nonpolar (n), end (e), and β-turn (**t**, **r**) residues

```
chirality LLLLLLLDDLLLLLLDDLLLLLLDDLLLLLLL  chirality
position  1   5   9  13  17  21  25  29 32  position

   12  HTLTASIpdLTYSINpdTATCKVpdFTLSIGA   12
 12=14 | |||||  ||       ||||  |   |  |   Δ = 17
   14  HSLTASIkaLTIHVQakTATCQVkaYTVHISE   14
 14=15 |||||  |  ||       | ||  |  ||  |   Δ = 17
   15  HSLTAKIpkLTFSIAphTYTCAVpkYTAKVSH   15
 12=15 | ||| || || || |  | || ||   |       Δ = 15
   12  HTLTASIpdLTYSINpdTATCKVpdFTLSIGA   12
pattern epnpnpnttnpnpnprrpnpnpnttnpnpnpe  pattern

12=14=15 H_LTA_I  LT____  T_TC_V  T____   12=14=15
```

Fig. 1. Comparison of the amino acid sequences of the betabellin-12, betabellin-14, and betabellin-15 peptide chains. The 32 residue pattern of polar (p), nonpolar (n), end (e), and β-turn (**t**, **r**) residues is indicated. Each half chain contains the same 14 residue palindromic pattern (underlined). The chiral pattern of L amino acid (L) and D amino acid (D) residues, the residues in common (|) between pairs of betabellin chains, the number (Δ) of different residues between pairs of chains, the 12 residues in common to all three chains (12 = 14 = 15), and the positions of the four 6 residue strands (double underlined) are also shown.

(Fig. 1). Each half of this large palindrome contains a smaller 14 residue palindromic pattern (pnpnpn**tt**npnpnp). If the residue pairs **tt** and **rr** are the L1–L2 loop residues of inverse common (type I′) β-turns, the peptide chain might fold into a four strand antiparallel β-sheet that is stabilized by the presence of 18 intrachain interstrand H-bonds (Fig. 2B), which places 12 polar residues on the polar face of the β-sheet and 12 nonpolar residues on its nonpolar face. Hydrophobic interactions between the nonpolar faces of two such β-sheet might form a β-sandwich.

The shape of the β-sandwich is roughly a rectangular parallelepiped with width a, length b, and height c (Fig. 2C). The ab surfaces are the top and bottom faces, the ac surfaces are the upper and lower sides, and the bc surfaces are the **rr** and **tt** ends. If the two peptide chains are identical, a C_2 symmetry axis passes through the center of each end, so that the β-sandwich has two identical faces and two identical sides but two different ends.

The two betabellin chains might fold into a β-sandwich without the presence of a covalent bond between the two β-sheets (Wagner et al., 1994). Alternatively, this folding might require the presence of a covalent cross link, such as an interchain disulfide bridge, between the two β-sheets (Erickson et al., 1986; Yan & Erickson, 1994). The 32 residue chain of betabellin-15 has a Cys residue at position 21 (Fig. 1) (Kroll et al., 1996). When this residue is in the reduced, sulfhydryl (S-H) form (cysteine), the resulting single-chain covalent molecule is designated by the letter "S" (betabellin-15S). Alternatively, when it is in the oxidized, disulfide (S-S) form (half-cystine), the resulting double-chain covalent molecule is designated by the letter "D" (betabellin-15D). Thus, the betabellin target structure might be achieved by the folding of two molecules of betabellin-15S or of one molecule of betabellin-15D.

D Amino acids

The β-sandwich geometry of the betabellin target structure is most compatible with having each **tt** and **rr** segment as the L1–L2 loop of an inverse common (type I′) β-turn (Richardson & Richardson, 1987). Through molecular dynamics simulations of model dipeptides (Yan et al., 1993, 1995), the prototypic D,D-dipeptide CH_3CO-DAla-DAla-NH_2 was found to be thermodynamically more stable when folded into an inverse common (type I′) β-turn than into a common (type I), glycine (type II), or inverse glycine (type II′) β-turn. Betabellin-12 (Fig. 1) had DPro-DAsp (**pd**) at the **tt** and **rr** segments (McClain et al., 1992; Richardson et al., 1992), and betabellin-14 had DLys-DAla (**ka**) at both **tt** segments and DAla-DLys (**ak**) at the **rr** segment (Yan & Erickson, 1994). Betabellin-12S folded into a β-structure in water, but betabellin-14S did not (Wagner et al., 1994; Yan & Erickson, 1994). In order to test if having DPro at the L1 loop residue of each β-turn would induce folding into a β-structure in water, betabellin-15 was designed to have DPro-DLys (**pk**) at both **tt** segments and DPro-DHis (**ph**) at the **rr** segment.

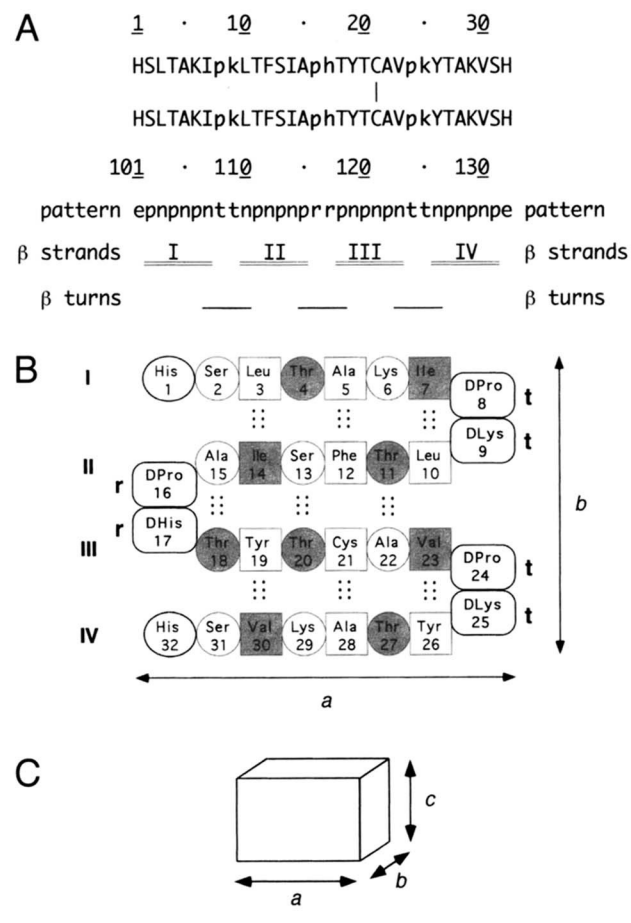

Fig. 2. Structure of betabellin-15D. **A:** Covalent structure of the 64 residue disulfide bridged two-chain molecule. The 12 D amino acids (**h**, **k**, **p**), the six His residues (H, **h**), the single interchain disulfide bond (|), the eight predicted β-strands (═══), and six predicted inverse common (Type I′) β-turns (____) are indicated. **B:** Predicted β-sheet structure of each betabellin-15 chain looking down on the nonpolar face. The 18 β-sheet H-bonds ($\cdots$) between three pairs of polar residues (circled, side chains in back), and six pairs of nonpolar residues (boxed, side chains in front) are indicated. The nine residues in grey are β-branched. **C:** A parallelepiped with dimensions $a = 3$ and $b = c = 2$.

Net charge

Betabellin-12D was more soluble in water than previous betabellins but formed insoluble aggregates at higher concentrations, which precluded an NMR study of its structure in water (McClain et al., 1992). So betabellin-14D was designed to contain fewer

residues with negatively charged side chains (Asp, Glu) and more residues with positively charged side chains (His, Lys) than the betabellin-12D chain (Yan & Erickson, 1994). The nominal net charge at pH 5 increased from −2 for betabellin-12D to +10 for betabellin-14D, a desirable property for NMR studies in an acidic solution. As a result, betabellin-14D was soluble in water and showed well-dispersed NMR resonances at pH 6.5 (Yan & Erickson, 1994). Betabellin-15D was designed to contain no residues with negatively charged side chains and 14 residues with positively charged side chains at pH 5 (4 Lys and 3 His per chain), so it has a nominal net charge of +14 at pH 5.

Positively charged faces

In order to minimize aggregation, betabellin-15D was designed to have positively charged faces. Eight Lys residues were strategically placed at the **tt** end and in the β-strands of the β-sandwich. Lys6 was placed in β-strand I, DLys9 in the first β-turn, DLys25 in the third β-turn, and Lys29 in β-strand IV (Fig. 2B). The two chains of the betabellin target structure are distinguished by numbering the positions of the bottom chain from 1 through 32 and of the top chain from 101 through 132 (Fig. 2A). When betabellin-15D is folded into a β-sandwich, it has two Lys residues (Lys6, Lys129) on the upper side, two (Lys106, Lys29) on the lower side, and four (DLys9, DLys25, DLys109, DLys125) at the **tt** end. In addition, at pH ≤ 5 it also has six positively charged His residues at the **rr** end.

Cu(II) binding sites

Folding of the betabellin-14 chain (Fig. 1) into the predicted β-sheet places His13 and His29 in the middle of the polar face (Yan & Erickson, 1994). These His residues were moved to positions 17 and 32 in the betabellin-15 chain so that all three His residues (His1, DHis17, and His32) are at the **rr** end (Fig. 2B). If Cys21 of the first sheet were covalently linked to Cys121 of another sheet through a disulfide bond, the resulting molecule of betabellin-15D might have all six His residues at the **rr** end of the β-sandwich. The two β-sheets might be oriented such that two clusters of three His residues are formed. If His1 of the first sheet were close to DHis117 and His132 of the second sheet, then by symmetry His101 of the second sheet should be close to DHis17 and His32 of the first sheet. Each of the three His clusters (His1/DHis117/His132, His101/DHis17/His32) might correspond to one Cu(II) site (Lim et al., 1998).

Interior aromatic residues

The aromatic residues at positions 12, 19, and 26 of betabellin-15 were derived from the corresponding residues of betabellin-12 by reversing the positions of Tyr12 and Phe26 and by adding a phenol ring to Ala19. As a result, betabellin-15 has Phe12 in β-strand II, Tyr19 in strand III, and Tyr26 in strand IV.

Comparison of betabellin-15D with betabellins-12D and -14D

The remaining residues of betabellin-15 were selected for a variety of reasons. To mimic the distribution of β-branched residues in natural proteins (Richardson et al., 1992), nine β-branched residues (*Ile*, *Thr*, *Val*) are juxtaposed to β-unbranched residues on the adjacent β-strands, such as Leu3//*Ile*14//Tyr19//*Val*30 and Lys6//*Thr*11//Ala22//*Thr*27 (Fig. 2B). The net result is that the residues at 12 positions of the betabellin-15 chain are also present in betabellin-12 and betabellin-14, the residues at five positions (DPro8, Ser13, Ile14, DPro16, DPro24) are just in betabellin-12, the residues at three positions (Ser2, Tyr26, Ser31) are just in betabellin-14, and the residues at 12 positions are not present in either of the other two betabellin chains (Fig. 1).

Synthesis of betabellins-15S and -15D

The 32 residue peptide chain of betabellin-15S was assembled by solid phase synthesis using tert-butyloxycarbonyl chemistry, desalted by gel filtration, and purified by preparative reversed phase high performance liquid chromatography (RP-HPLC) on an octadecyl-silica (Vydac C-18) column. By analytical RP-HPLC, the 32 residue peptide thiol was eluted from the column at 32.4% acetonitrile (Fig. 3). Amino acid analysis was consistent with the expected residue composition: found (calculated),

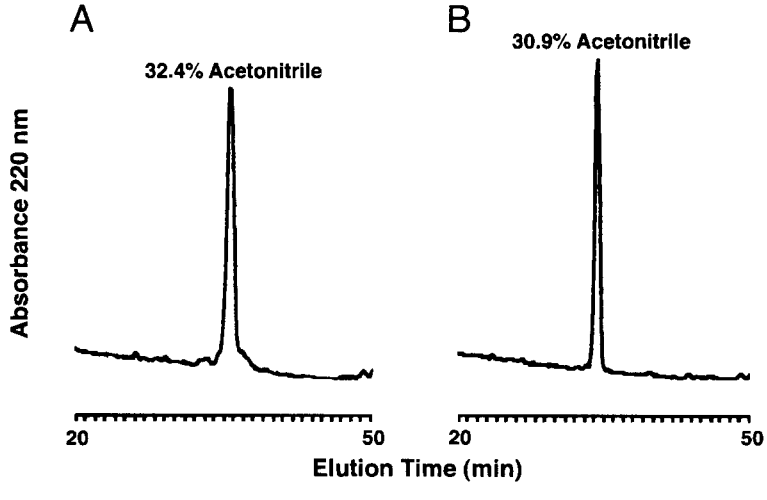

Fig. 3. Analytical RP-HPLC chromatograms of betabellins-15S (**A**) and 15D (**B**). A linear gradient of 0–70% acetonitrile over 60 min was used to elute each betabellin from a Vydac C-18 column. The elution time of betabellin-15S was 35.8 min (32.4% acetonitrile) and that of betabellin-15D was 34.5 min (30.9% acetonitrile).

Ala 4.2 (4), His 2.9 (3), Ile 2.0 (2), Leu 2.1 (2), Lys 4.1 (4), Phe 1.0 (1), Pro 3.2 (3), Ser 2.8 (3), Thr 4.7 (5), Tyr 1.9 (2), and Val 2.0 (2). The average molecular mass of betabellin-15S as determined by electrospray ionization mass spectrometry (ESI-MS) was that expected for the 504 atoms of a single betabellin-15 chain (calculated for $C_{162}H_{256}N_{42}O_{43}S$, 3,512.1 Da; found, 3,511.8 ± 0.1 Da).

The 64 residue two-chain molecule of betabellin-15D was formed by air oxidation (Tam et al., 1991) of betabellin-15S in 20% dimethyl sulfoxide at 37 °C. By analytical RP-HPLC, betabellin-15D was eluted from a Vydac C-18 column at 30.9% acetonitrile (Fig. 3). Evidently, on formation of the disulfide bond of betabellin-15D, some amino acid residues are not as accessible to interact with the hydrophobic stationary phase as they are in betabellin-15S. Thus, the disulfide bond decreases the overall hydrophobicity of betabellin-15D (Lee et al., 1991). Amino acid analysis was also consistent with the expected residue composition: found (calculated), Ala 8.0 (8), His 6.0 (6), Ile 4.1 (4), Leu 4.3 (4), Lys 7.3 (8), Phe 2.2 (2), Pro 6.6 (6), Ser 5.7 (6), Thr 9.3 (10), Tyr 4.3 (4), and Val 4.2 (4). The average molecular mass of betabellin-15D as determined by ESI-MS was that expected for the 1,006 atoms of two betabellin-15S chains linked by a single disulfide bond (calculated for $C_{324}H_{510}N_{84}O_{86}S_2$, 7,022.2 Da; found, 7,022.0 ± 0.6 Da).

Biophysical characterization of betabellin-15D

pH dependence of the folding of betabellin-15D

The circular dichroic (CD) spectra of 40 mM betabellin-15S and 28 mM betabellin-15D in water at pH 6.5 were featureless (Fig. 4A), so both peptides were unfolded under these conditions. Dithiothreitol (10 mM) was present in the solution of betabellin-15S to prevent its oxidation to betabellin-15D. But the CD spectra of 50 mM betabellin-15D in 50 mM sodium chloride and 50 mM potassium phosphate at pH 6.0–7.5 (Fig. 4B) showed a negative band at 218 nm, which is characteristic of β-structure (Woody, 1985, 1995). At the ionic strength of this solution (0.13 M at pH 7.0), betabellin-15D was mostly folded into a β-structure at pH 7.0, mainly folded at pH 6.5, and still substantially folded at pH 6.0 (Fig. 4B,C). Since betabellin-15D precipitated at pH 8.0 (Yan, 1994), the amplitude of the band seen at pH 7.5 probably reflects its limited solubility at this pH. The imidazole rings on the side chains of the six His residues of betabellin-15D are expected to have pK_a values near pH 6.5. Protonation of one or more of these imidazole rings is evidently sufficient to disrupt the β-structure of betabellin-15D.

Ionic strength dependence of the folding of betabellin-15D

At pH 6.5 in the absence of added salts, betabellin-15D was not folded (Fig. 4A). At pH 6.5 and 0.11 M ionic strength (Fig. 4B), however, betabellin-15D folded into a β-structure, so the dependence of the folding of betabellin-15D on the ionic strength was examined. The CD spectrum of a solution of 50 mM betabellin-15D in 5.0 mM 3-(*N*-morpholino)propanesulfonic acid (MOPS) at pH 6.9 and ionic strength of 0.0025 M was featureless (Fig. 5), so betabellin-15D did not fold at low ionic strength. When NaCl was added to this solution, the negative band at 218 nm due to β-structure appeared (Fig. 5A). At pH 6.9, betabellin-15D was about half folded at an ionic strength of 0.062 M, mostly folded at an ionic strength of 0.12 M, and fully folded at ionic strengths of 0.19–0.50 M. At pH 6.9, the side chains of all eight Lys residues of betabellin-15D bear positively charged ammonium groups and the side chains of some of the six His residues bear positively charged imidazolium groups. Increasing the concentration of chloride ions evidently diminishes the repulsion of these positively charged groups, which induces the protein to fold.

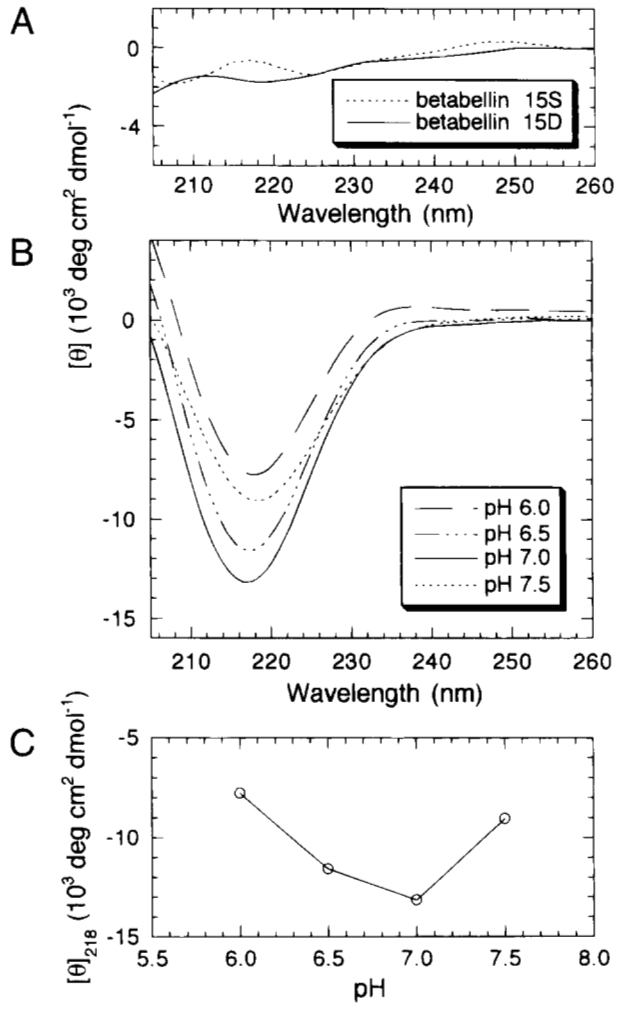

Fig. 4. Dependence of the β-structure of betabellin-15D on the pH. **A:** CD spectra of 40 μM betabellin-15S and 10 mM dithiothreitol and of 28 μM betabellin-15D, both in water at pH 6.5. **B:** CD spectra of 50 μM betabellin-15D in 50 mM NaCl/50 mM potassium phosphate buffer at pH 6.0–7.5. **C:** Amplitude of $[\theta]_{218}$ from the spectra in panel B as a function of pH.

Concentration dependence of the folding of betabellin-15D

Next, the dependence of the folding of betabellin-15D on its concentration was examined under folding conditions (5.0 mM MOPS, 0.25 M NaCl, pH 6.9). As the concentration of betabellin-15D was increased from 4.0 to 60 μM, the amplitude of the negative band at 218 nm due to β-structure increased threefold (Fig. 6). For example, the mean residue ellipticity (in deg cm^2 dmol^{-1}) at 218 nm of betabellin-15D was −5,300 at 4.0 μM, −12,000 at 15 μM, and −15,400 at 60 μM. These results are consistent with the formation of a noncovalent betabellin-15D multimer. In contrast, under conditions adequate for folding of betabellin-

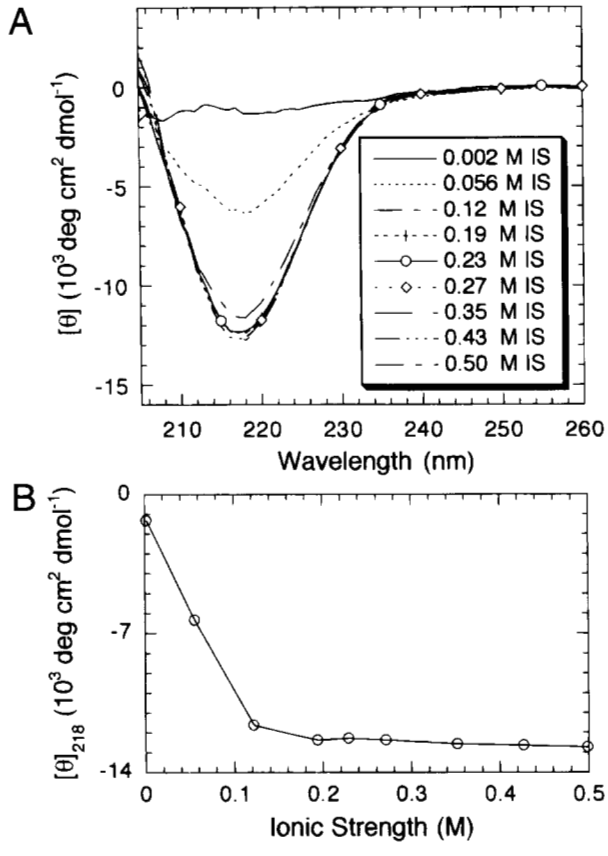

Fig. 5. Dependence of the β-structure of betabellin-15D on the ionic strength (IS). **A:** CD spectra of 50 μM betabellin-15D in 5.0 mM MOPS at pH 6.9. **B:** Amplitude of $[\theta]_{218}$ from the spectra in panel A as a function of ionic strength.

15D (5.0 mM MOPS, 0.25 M NaCl, pH 6.9), betabellin-15S remained unfolded at a concentration of 30 or 60 μM in the presence of 1 mM dithiothreitol (data not shown).

Analytical ultracentrifugation

The average molecular mass of betabellin-15D in solution was determined by sedimentation velocity experiments in an analytical ultracentrifuge (Fig. 7). Under folding conditions (5.0 mM MOPS, 0.25 M NaCl, pH 6.9) at 20°C, 58 μM betabellin-15D monomer (the 64 residue two-chain molecule) formed multimers having an average molecular mass of 1,140,000 Da, which corresponds to about 160 β-sandwiches of mass 7,022 Da. From these sedimentation velocity data, the betabellin-15D multimer was estimated to have an average length of 240 nm and an average diameter of 3.8 nm.

Electron microscopy

The appearance of the betabellin-15D multimers was examined by electron microscopy. Figure 8 shows electron micrographs of a solution of 30 μM betabellin-15D or 62 μM betabellin-15D under folding conditions (5.0 mM MOPS, 0.25 M NaCl, pH 6.9) that had been stored at 4°C for six months. Under these conditions, 30 μM betabellin-15D formed long narrow fibrils (Fig. 8A) with an average length of (100 ± 56) nm (mean ± SD) (n = 206, number of fibrils measured) (Fig. 9A) and an average diameter of (3.5 ± 0.5) nm (n = 20). For example, the lower half of Figure 8A shows a single straight fibril 400 nm in length. These dimensions suggested that each fibril consists of a single row containing a large number of β-sandwiches joined lengthwise by multiple intersheet H-bonds.

Under folding conditions at the higher concentration of 62 μM, betabellin-15D formed longer fibrils that associated to form multifibril bundles (Fig. 8B). When 10 s was allowed to elapse between the addition of the betabellin-15D solution to the carbon foil and addition of the 2% uranyl acetate stain, the fibrils deposited on the carbon foil had an average length of (350 ± 200) nm (n = 50). After a deposition time of 30 s, the average fibril length was (400 ± 220) nm (n = 138) (Fig. 9B). Since these two samples of the populations of fibril lengths are statistically indistinguishable, the deposited fibrils were already formed before deposition rather than induced by the carbon foil during deposition. These results show that the average length of the deposited fibril increased about fourfold as the concentration of betabellin-15D increased twofold.

Although the two betabellin-15D solutions described above were examined by electron microscopy after standing for six months, fibril formation was rapid. For example, a stock solution of 258 μM betabellin-15D in water (which does not promote folding)

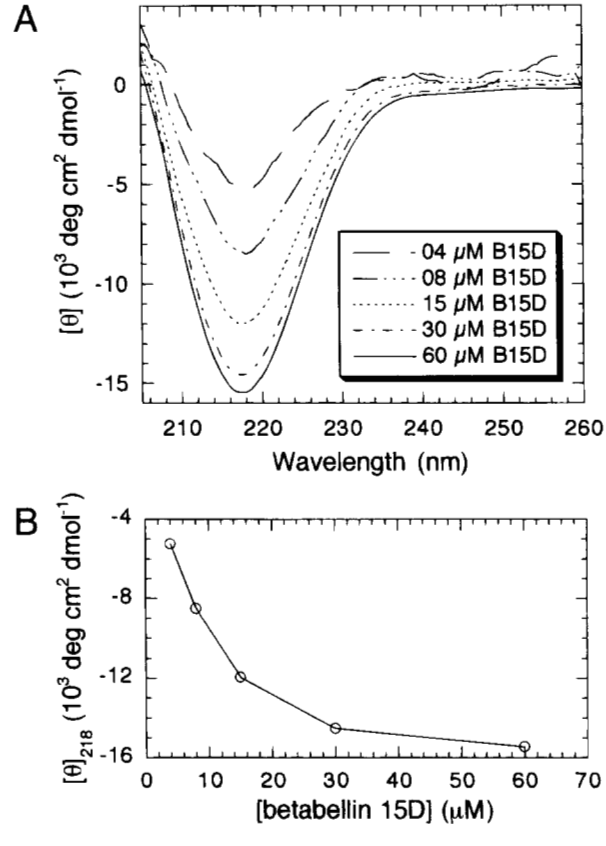

Fig. 6. Dependence of the β-structure of betabellin-15D on its concentration. **A:** CD spectra of 4.0–60 μM betabellin-15D (B15D) in 5.0 mM MOPS and 0.25 M NaCl at pH 6.9. **B:** Amplitude of $[\theta]_{218}$ from the spectra in panel A as a function of the concentration of betabellin-15D.

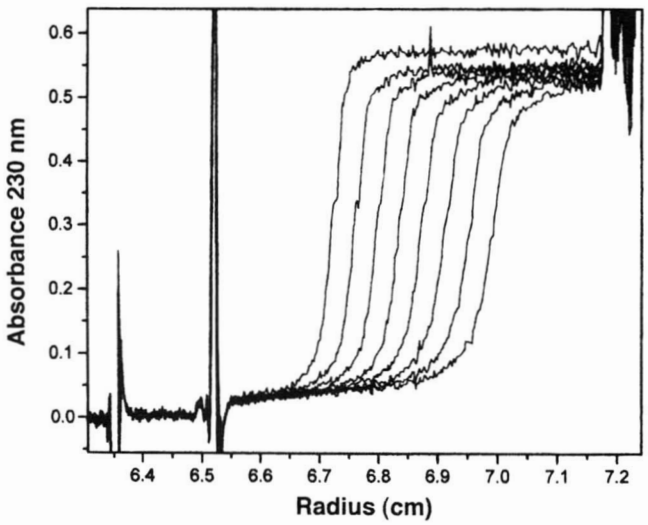

Fig. 7. Sedimentation velocity scans of 58 μM betabellin-15D in 5.0 mM MOPS and 0.25 M NaCl at pH 6.9 and 20 °C. Absorbance at 230 nm of scans 6–13 with 6 min between scans as a function of the radial distance from the rotor axis. The rotor was spun at 40,000 rpm.

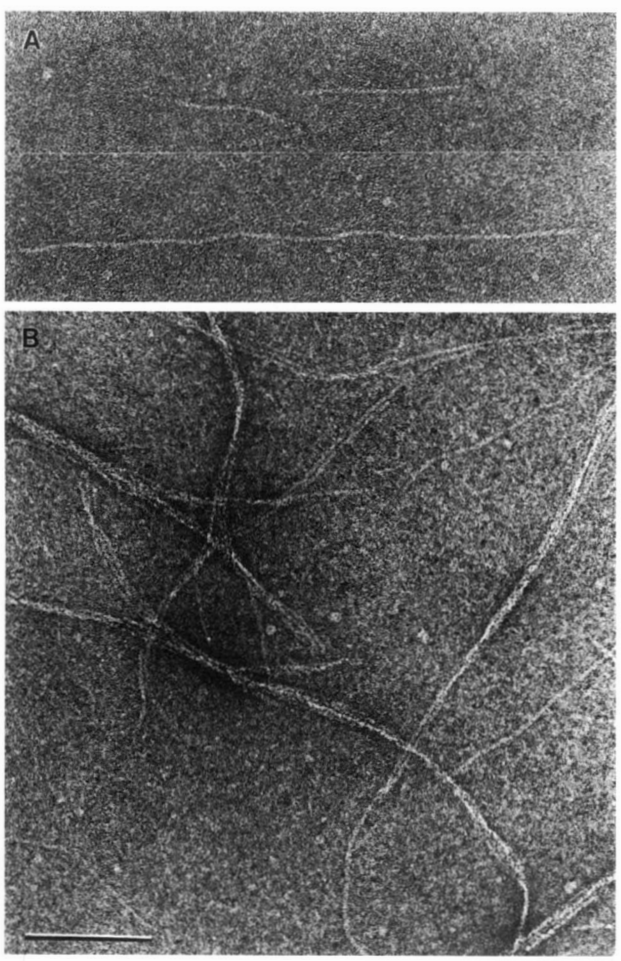

Fig. 8. Transmission electron micrographs of a solution of 30 μM (**A**) or 62 μM (**B**) betabellin-15D in 5.0 mM MOPS and 0.25 M NaCl at pH 6.9 (stored at 4 °C for six months) deposited on carbon foil and negatively stained with 2% uranyl acetate. The scale bar at the lower left represents 100 nm.

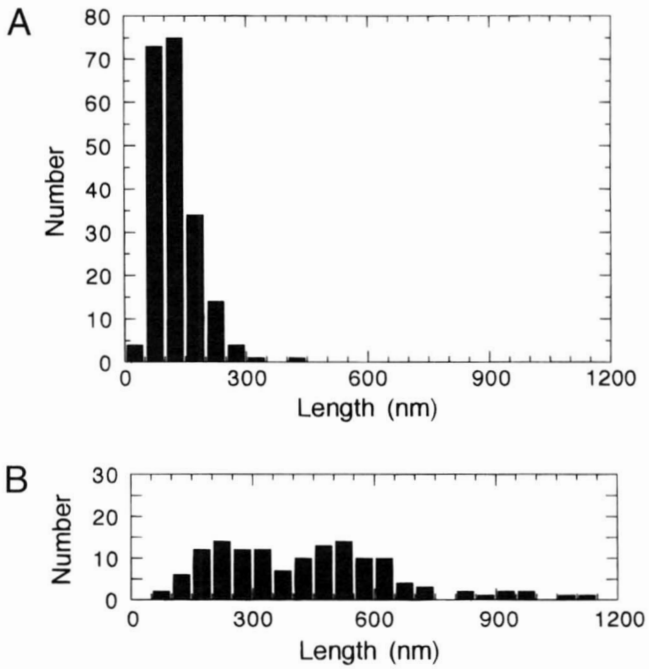

Fig. 9. Length distribution of betabelin-15D fibrils. The data in **A** and **B** were obtained under the conditions described in Figure 8.

was mixed with a solution of folding buffer (5.0 mM MOPS, 0.25 M NaCl, pH 6.9). After 5 min, an aliquot of the resulting solution of 50 μM betabellin-15D under folding conditions was applied to the carbon foil. After 30 s, the foil was stained with 2% uranyl acetate and examined by electron microscopy. The fibrils seen after 5 min in this 50 μM betabellin-15D solution looked very similar to those of the 30 μM betabellin-15D solution that had been stored at 4 °C for six months.

Model of the betabellin-15D fibril as a linear multimer of β-sandwiches

A single molecule of betabellin-15D is predicted to fold into a disulfide bridged β-sandwich in which each β-sheet is stabilized by the formation of 18 intrachain H-bonds between strands I–IV (Fig. 2B). For example, bottom strand II should form 12 intrachain H-bonds (six to strand I, six to strand III). Likewise, bottom strand III should also form 12 intrachain H-bonds (six to strand II, six to strand IV). In contrast, bottom strand I should form only six intrachain H-bonds (to strand II), and bottom strand IV should form only six intrachain H-bonds (to strand III). These interactions leave six residues in the bottom β-sheet (Ser2, Thr4, and Lys6 of strand I; Thr27, Lys29, and Ser31 of strand IV) and six residues in the top β-sheet (Ser102, Thr104, and Lys106 of strand I; Thr127, Lys129, and Ser131 of strand IV) unable to form intrachain H-bonds.

Four pairs of these residues can form eight intersheet H-bonds by joining the lower side of sandwich-S to the upper side of sandwich-T (Fig. 10B). We considered two ways to form these H-bonds, as illustrated by the patterns of H-bonds shown between bottom strand IV of sandwich-S and bottom strand I of sandwich-T in Figure 11. Pattern A has two H-bonds between Ser31S and Thr4T and two between Lys29S and Lys6T (Fig. 11A). Pattern B,

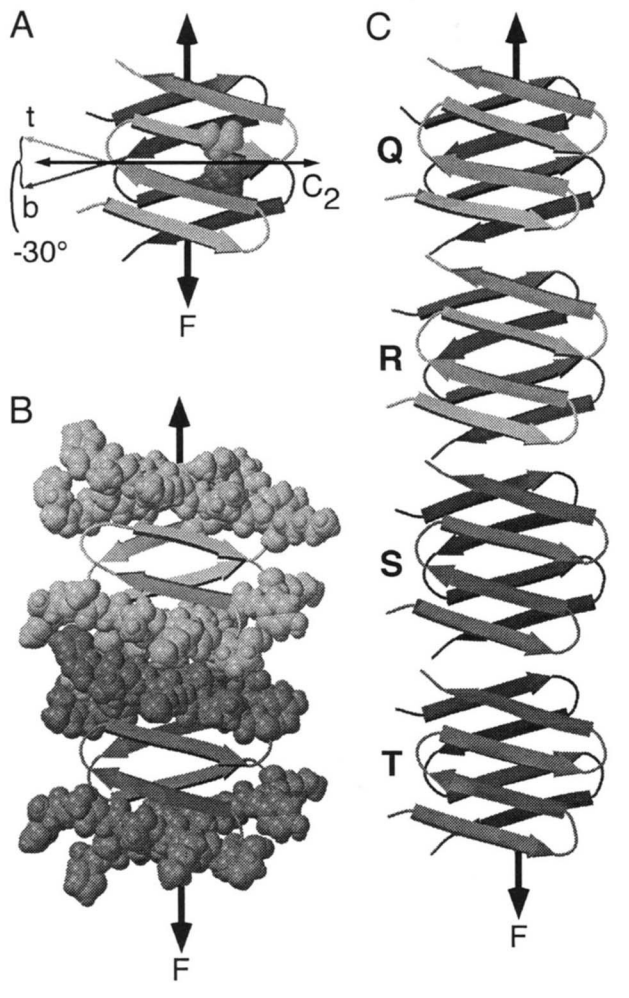

Fig. 10. A molecular model of the betabelin-15D fibril. The vertical closed arrows (F) represent the fibril axis. **A:** Ribbon model of a betabelin-15D β-sandwich showing the central disulfide-bridged residues Cys21 and Cys121 as space filling atoms. The horizontal double-headed arrow represents the C_2 axis of rotation through the β-sandwich. The dihedral angle between the bottom strand axis b and the top strand axis t is $-30°$. **B:** Ribbon model of two betabellin-15D sandwiches with outer strands I and IV of each β-sheet shown as space filling atoms. **C:** Ribbon model of four β-sandwiches (Q, R, S, T) of the betabelin-15D fibril.

however, has two H-bonds between Lys29S and Ser2T and two between Thr27S and Thr4T (Fig. 11B).

Pattern A is favored for two reasons. First, the β-sheets of natural β-sandwiches stack with an intersheet dihedral angle of about $-30°$ (Chothia & Janin, 1981; Cohen et al., 1981). The intersheet Cys21/Cys121 disulfide bond of the betabellin-15D β-sandwich is compatible with this value for the dihedral angle between the strands of the bottom (b) sheet and the top (t) sheet (Fig. 10A). In contrast, the intersheet dihedral angle is about $+30°$ for Pattern B. Second, it is unusual for an adjacent pair of interstrand residues of an antiparallel β-sheet to both be β-branched residues (Val, Ile, Thr) (Richardson & Richardson, 1989). In the betabellin-15D β-sheet, for example, none of the 18 adjacent pairs of interstrand residues (Ser2/Ala15, Leu3/Ile14, ... , Tyr19/Val30, Thr18/Ser31) are both β-branched (Fig. 11A). In the interface between strand IV of sandwich-S and strand I of sandwich-T, Pattern A does not contain an unfavorable pair of adjacent interstrand β-branched residues. In contrast, Pattern B contains the unfavorable pair Thr27S/Thr4T.

The molecular model in Figure 10B illustrates the close packing between strands I and IV by using Pattern A to specify the eight intersheet H-bonds between sandwiches-S and -T (four between the top sheets and four between the bottom sheets). These interactions should decrease the hydrophobic surface area of two otherwise partially exposed hydrophobic residues (Ile7, Val30) of each internal sandwich. Adding other β-sandwiches in this manner would form a long fibril measuring sandwich width a wide, sandwich height c tall, and sandwich length b long (Fig. 2B,C). Electron microscopic measurements revealed that the average width of an individual fibril was 3.5 ± 0.5 nm. This value is consistent with the value of approximately 3.7 nm for sandwich width a, which was measured from a space filling version of the four sandwich model shown in Figure 10C. As shown in Figure 12, this model was stable during molecular dynamics simulations and reached equilibrium on the basis of the leveling with time of the root-mean-square deviation (RMSD) of the positions of the backbone atoms. Three-fourths of the H-bonds shown in Figure 11A were maintained during the simulation. The sandwich repeat length b from this four sandwich model was 2.5 ± 0.1 nm. Thus, the 400 nm fibril shown in the lower half of Figure 8A should contain about 160 betabellin-15D β-sandwiches.

Conclusion

Betabellin-15D forms long narrow multimeric fibrils but betabellin-15S remains unfolded in 5.0 mM MOPS and 0.25 M NaCl at pH 6.9. This designed synthetic protein may provide a useful model for studying the mechanism of fibril formation and for designing potential inhibitors of fibril formation.

Materials and methods

Synthesis of betabellin-15S

The 32 residue peptide chain of betabellin-15S was assembled by solid phase synthesis using tert-butyloxycarbonyl chemistry (0.5 mmol scale) on an Applied Biosystems Model 430A peptide synthesizer. Cleavage of the peptide from the peptide resin was achieved by treatment with 7:6:3:1:37 (v/v) trimethylsilyl bromide/thioanisole/ethanedithiol/*m*-cresol/trifluoroacetic acid for 1 h at 0 °C and for 2 h at room temperature. The peptide and resin were washed with ice cold diethyl ether and dried under nitrogen. The peptide was extracted into water, acidified in 1% acetic acid, and lyophilized. The residue was redissolved in water, neutralized with ammonium carbonate (1 g), and lyophilized. The residue, plus excess dithiothreitol, was dissolved in 6.0 M guanidine hydrochloride/0.25 M Tris HCl at pH 7.5 and desalted by gel filtration through a Bio-Gel P-4 column (40×2.6 cm, 90–180 μm hydrated beads, Bio-Rad) with 1% acetic acid as the mobile phase at 2.0 mL/min. A solution of peptide (58 mg) and excess dithiothreitol (58 mg) in 6.0 M guanidine hydrochloride/0.25 M Tris HCl (7 mL) at pH 7.5 was applied to a preparative Vydac C-18 column (25×1.0 cm, 10 μm particle size) and eluted at 5.0 mL/min over 60 min with a gradient of 15–29% acetonitrile in 0.1% trifluoroacetic acid. By RP-HPLC

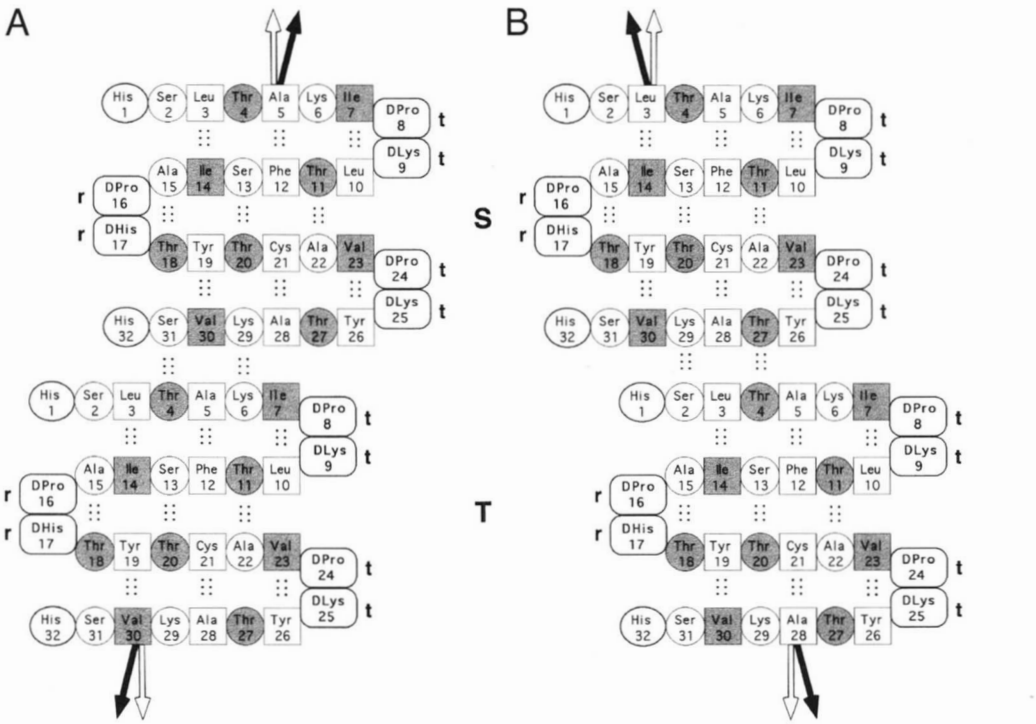

Fig. 11. Schematic diagrams of (**A**) favorable Pattern A or (**B**) unfavorable Pattern B for the intersheet H-bonds (···) between the bottom β-sheets of two β-sandwiches (S, T) of a betabellin-15D fibril. The angle between the fibril axis (closed arrows) and the H-bonding axis (open arrows) is 15°. The β-branched residues are shaded in grey.

on an analytical Vydac C-18 column (25 × 0.46 cm, 5 μm particle size), the solution containing betabellin-15S was eluted at 1.0 mL/min over 60 min with a linear gradient of 0–70% acetonitrile in 0.1% trifluoroacetic acid. Betabellin-15S had an elution time of 35.8 min (32.4% acetonitrile). By this criterion, the preparative HPLC fractions containing 25–27% acetonitrile were combined and lyophilized to give pure betabellin-15S (40 mg).

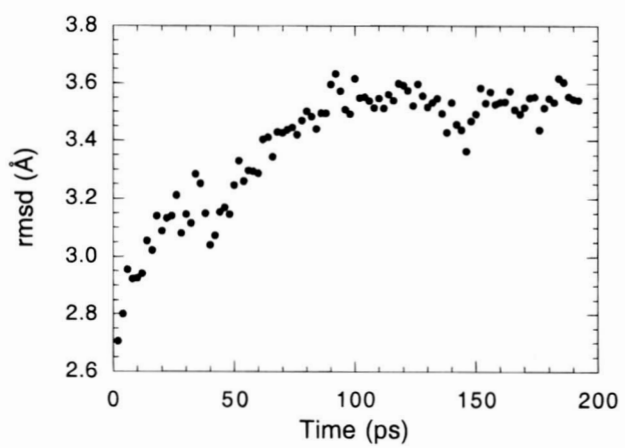

Fig. 12. RMSD of the positions of the backbone atoms (relative to the configuration at t = 0 = ps) versus time for a molecular dynamics simulation of the four sandwich model of part of a betabellin-15D fibril (Fig. 10C).

Synthesis of betabellin-15D

Pure betabellin-15S (28 mg) in 20% dimethyl sulfoxide (4.0 mL) was air oxidized at 37 °C (Tam et al., 1991) to provide betabellin-15D. After 24 h, the reaction was complete as monitored by analytical RP-HPLC and ESI-MS. The reaction mixture was diluted with water (6.0 mL) and applied to a preparative Vydac C-18 column (25 × 1.0 cm, 10 μm particle size) and eluted at 5.0 mL/min over 60 min with a gradient of 14–28% acetonitrile in 0.1% trifluoroacetic acid. By RP-HPLC on an analytical Vydac C-18 column (25 × 0.46 cm, 5 μm particle size), the solution containing betabellin-15D was eluted at 1.0 mL/min over 60 min with a linear gradient of 0–70% acetonitrile in 0.1% trifluoroacetic acid. Betabellin-15D had an elution time of 34.5 min (30.9% acetonitrile). By this criterion, the preparative HPLC fractions containing 22–24% acetonitrile were combined and lyophilized to give pure betabellin-15D (17 mg).

Electrospray ionization mass spectrometry

ESI-MS was carried out in the positive ion mode by using a Perkin-Elmer Sciex API-I mass spectrometer equipped with a nebulizer assisted electrospray (ion spray) and calibrated with poly(propylene glycol) ions. A solution of 2 μM protein in 10:10:0.4 (v/v) methanol/water/acetic acid at pH 3.2 was infused at 5 μL/min

with a Harvard Apparatus Model 11 syringe pump through the ion spray needle, which was kept at 5,000 V relative to the orifice potential at 60 V.

Circular dichroic spectropolarimetry

CD spectra were recorded in quartz cuvettes (1 mm path length) with an Aviv Model 62DS CD spectropolarimeter equipped with a thermoelectric temperature controller. Each data point was corrected by baseline subtraction and converted into mean residue ellipticity, $[\theta]$, as calculated by the equation $[\theta] = 100 * \theta_{obs}/(l * C_r)$ where θ_{obs} is the measured ellipticity (deg), l is the path length of the cuvette (cm), and C_r is the molar protein concentration as determined by amino acid analysis times the number of amino acid residues of the protein. Data for the pH and ionic strength studies were collected every 1.0 nm from 190 to 280 nm by using a bandwidth of 1.0 nm and an averaging time of 5.0 s. Data for the protein concentration studies were collected every 0.5 nm from 200 to 260 nm by using a bandwidth of 1.0 nm and an averaging time of 4.0 s. For all CD experiments, the temperature of the thermostatted cell holder was kept at 25 °C.

Analytical ultracentrifugation

Sedimentation velocity data were obtained with a Beckman Optima Model XL-A analytical ultracentrifuge equipped with absorption optics set at 230 nm. A solution of 58 μM betabellin-15D, 0.25 M NaCl, and 5.0 mM MOPS at pH 6.9 and 20 °C was spun at 40,000 rpm. The sample cell was scanned 18 times with 6 min between scans. Sedimentation and diffusion coefficients were determined from these data by using the Beckman XLA-VELOC computer program. The partial specific volume of betabellin-15D was calculated by using the VBAR computer program developed by Dr. Les Holladay. The molecular mass was estimated from the Svedberg equation, $M_r\ (RTs)/[D(1 - \nu\rho)]$, where M_r is the relative molecular mass (Da), R the gas constant [8.314×10^7 g cm^2/(s^2 mol K)], T the absolute temperature (293 K), s the solute sedimentation coefficient (9.07×10^{13} s), D the solute diffusion coefficient (8.07×10^{-8} cm^2/s), ν the solute partial specific volume (0.752 mL/g), and ρ the solvent density (1.0091 g/mL) as determined at 20 °C using a Mettler Toledo Model DA-110M density/specific gravity meter. The dimensions of the betabellin-15D multimer were estimated (Tinoco et al., 1985) from the values shown above for s, D, ν, T, and ρ, the calculated solvent viscosity (1.027 centipoise), and the amount of hydration of betabellin-15D (0.20 g water/g betabellin-15D) by using the AXIAL computer program, which models the protein as a prolate ellipsoid of revolution, provided by Dr. Les Holladay.

Electron microscopy

A solution of betabellin-15D was applied to a glow charged carbon foil supported on a 400 mesh copper grid. After 10–30 s, the foil was negatively stained with 2% (w/v) uranyl acetate/water and examined in a Philips Model CM12 electron microscope equipped with a Gatan video system and image intensifier. The diameter of an individual protein fibril was measured by digital image analysis with the NIH IMAGE computer program. The contour length of an individual fibril was measured by projecting its micrograph image onto a Summagraphics digitized tablet coupled to a Macintosh computer and analyzing the digital image with software developed by Dr. Jack Griffith.

Molecular modeling

To construct a model of the betabellin-15D fibril (Fig. 10C), a flat sheet was first constructed from Ala residues by using standard β-sheet dihedral angles ($\phi = -147°$, $\psi = +145°$) for sheet residues (Salemme, 1983) and standard type I′ dihedral angles for the β-turns (Yan et al., 1993, 1995). The Ala residues were then replaced with the residues of betabellin-15D using a rotamer library (Ponder & Richards, 1987) to maintain proper side-chain orientations. After this sheet was energy minimized using the INSIGHT II/DISCOVER modeling software (Molecular Simulations, Inc.), it was duplicated and translated to form the desired H-bonds. This process was repeated twice to give a continuous β-sheet consisting of four betabellin-15S β-sheets. This sheet was duplicated, rotated 180°, and translated to form a segment containing four β-sandwiches of a betabellin-15D fibril. Its four chain sheets were slowly brought within van der Waals contact by repetitively moving them closer together and minimizing the energy of the interior side chains. Finally, the four disulfide bridges were formed, and the charged side chains on the surface were neutralized by placing chloride counterions near all of the Lys ϵ-ammonium groups. This neutralized structure was energy minimized by using a Silicon Graphics Origin R10000 6-processor workstation, the AMBER-4.1 molecular modeling program (Pearlman et al., 1995), the second generation AMBER force field (Cornell et al., 1995), and a distant dependent dielectric constant to simulate a solvent. Initially only the side chains were allowed to move (100 steps by steepest descent and then 5,000 steps by conjugate gradient), then all atoms in the outer two sandwiches (100 steps by steepest descent and then 10,000 steps by conjugate gradient), and finally, all atoms (100 steps by steepest descent and then 10,000 steps by conjugate gradient). The structure (Fig. 10C) did not change appreciably after these 25,000 steps of minimization.

This minimized structure was solvated in a TIP3P water box with each side at least 12.5 Å from the nearest protein atom (box size: 122.2 Å × 53.4 Å × 47.7 Å). The solvent was energy minimized (10,000 steps by conjugate gradient) at constant volume with the protein fixed followed by 20 ps of belly dynamics. The waters were then reminimized by using 10,000 steps of conjugate gradient, and then the whole system was minimized using another 10,000 steps of conjugate gradient. After 10 ps of heating in 50 K intervals, the solvated fibril underwent 192 ps of molecular dynamics at 300 K. The simulation used the second-generation AMBER force field and a step size of 1 fs. Neighbor lists were updated every five steps. An 8 Å nonbonded atom cutoff was used to account for short range interactions, and the particle mesh Ewald method was used to accommodate the long range electrostatic forces (Essmann et al., 1995).

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