Protein Anatomy: Spontaneous Formation of Filamentous Helical Structures from the N-Terminal Module of Barnase

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ABSTRACT: This paper reports the conformation of the N-terminal module (24 amino acid residues) of barnase in aqueous solution. This module contains the first of three helices in the intact protein. Circular dichroism spectra showed the peptide fragment to have a predominantly random coil structure immediately following dissolution in aqueous solution and to be gradually converted to a helical structure at 5 °C. This was mediated by aggregation, and an electron micrograph indicated the aggregate to be comprised of filamentous helical structures. Scanning tunneling microscopy showed the filamentous structures to be made up of protofilamentous structures containing many disks apparently stacked on top of each other. A monomer of the peptide predominantly took on a random coil conformation in aqueous solution and the multimer, a stable helical structure. A local amino acid sequence would thus appear to determine the secondary structure corresponding to that in a native protein but stability to be governed by other factors such as tertiary interactions. Helical wheel representation indicated the peptide fragment to have the features of an amphiphilic helix. Hydrophobic burial may provide the driving force for producing a stable helical structure in aqueous solution.

Two models have been proposed for the folding pathway of proteins; they differ in regard to whether the formation of secondary structures precedes that of a compact globular structure. The one supporting such a view is a framework model (Kim & Baldwin, 1990). Peptides with sequences derived from proteins have been shown to form cognate secondary structures corresponding to the parent protein. Some peptides encompassing helical regions in native proteins appear to have mainly random coil structures and subtle populations each with a helical structure having marginal stability (Dyson et al., 1992; Waltho et al., 1989). One exception is the C-peptide of RNase A, possessing a stable helical structure in aqueous solution (Brown & Klee, 1971). The mechanism by which the α-helix is stabilized has been extensively studied, and salt-bridge formation and charge–helix dipole interaction have been shown factors of stability [see a recent review of Scholtz and Baldwin (1992)]. Local amino acid sequence thus certainly has preference for secondary structures although stability is marginal. Secondary structures of peptide fragments would thus not be highly populated, since small proteins each consisting of a single structural domain unfold/refolded cooperatively, indicating the absence of a stable intermediate (Jaenicke, 1987).

A "module" is a compact structural unit in a globular protein or domain (Gō, 1983; Gō & Nosaka, 1987). Module boundaries are closely correlated with intron positions of genes that encode proteins (Gō, 1981; Gilbert et al., 1986). Modules and/or their assembly may have functioned as primitive proteins in prebiological evolution. For confirmation of this "module hypothesis", examination was made of the assembly of disconnected modules in aqueous solution. The conformation of the N-terminal module fragment (M1, 24 residues) in barnase was studied to clarify the initiation of protein folding and stability of secondary structures.

Barnase, a ribonuclease from *Bacillus amyloliquefaciens*, is a small monomeric protein whose X-ray structure is known (Mauguen et al., 1982; Baudet & Janin, 1991). The tertiary structure of barnase was decomposed into six modules (M1–M6) by compactness criterion using centripetal and extension profiles (Gō & Nosaka, 1987). The N-terminal fragment consisting of 24 residues is a compact segment containing the first α-helix in the native protein (Noguti et al., unpublished data). Sancho et al. (1992) determined the structure of an N-terminal peptide fragment (36 residues) of barnase using circular dichroism (CD)[1] and 2D-NMR spectroscopy. They observed the formation of a helical structure with marginal stability (the nascent helix) at cognate regions in the intact protein (Sancho et al., 1992; Wright et al., 1988). The conformation of M1 as determined by CD spectroscopy is presented in this paper. The peptide was shown to take on a predominantly random coil structure in a monomer state and a helical structure to be stabilized in a multimer state.

EXPERIMENTAL PROCEDURES

Peptide Synthesis and Purification. The peptide M1, AQVINTFDGVADYLQTYHKLPDNY, was synthesized by solid-phase methodology of *tert*-butoxycarbonyl chemistry on a Biosearch 9500 peptide synthesizer. After hydrogen fluoride cleavage from the resin, the crude product was purified by gel filtration chromatography on a Sephadex G-50 column, ion-exchange chromatography on a DEAE-52 (Whatman) column, and reverse-phase HPLC on an ODS column with a gradient of acetonitrile in 0.1% trifluoroacetic acid. The structure and purity of the peptide were confirmed by analytical HPLC, amino acid analysis, amino acid sequence analysis, and FAB-MS measurement.

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[1] Abbreviations: CD, circular dichroism; 2D-NMR, two-dimensional nuclear magnetic resonance; UV, ultraviolet; STM, scanning tunneling microscopy; FAB-MS, fast atom bombardment mass spectrometry.

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Peptide Concentration. Peptide concentrations were determined from UV absorbance at 276 nm. The extinction coefficient of the peptide, $\epsilon_{276} = 4350\ M^{-1}\ cm^{-1}$, was calculated by the method of Gill and von Hippel (1989).

CD Measurement. CD spectra were measured on a Jasco J-600 spectropolarimeter equipped with a personal computer at 5 °C, using a quartz cell with a light path length of 1 mm. The results were expressed as mean residue ellipticity, $[\theta]$. Temperature was controlled using a thermostatically controlled cell holder. The peptide was dissolved in 50 mM Tris-HCl (pH 7.5) in an Eppendorf tube and incubated at 5 °C with shaking. Just before measurement, the peptide solution was diluted to 20 μM in 5 mM Tris-HCl (pH 7.5) to enhance the signal-to-noise ratio. After dilution, no change in CD spectra of the peptide could be detected for at least a few days.

Analytical Ultracentrifugation. Molecular weights were determined at 5 °C by sedimentation equilibrium (for low molecular weight) or sedimentation velocity measurement (for high molecular weight) using a Hitachi Model 282 analytical ultracentrifuge equipped with an UV absorption system. The partial specific volume of the peptide was estimated from its amino acid composition as 0.72 (Cohn & Edsall, 1943). For the equilibrium run, the peptide solution was centrifuged at 32 000 rpm for 18 h at peptide concentrations of 0.2, 0.3, and 0.4 mg/mL, respectively. For velocity measurement, centrifugation was performed at 8000 rpm.

Electron Microscopy. A drop of the peptide solution was placed on a carbon-coated collodion grid, followed by removal of any excess with filter paper. The sample stained by 4% uranyl acetate was examined under a JEOL 1200 EX electron microscope at 100 kV.

Scanning Tunneling Microscopy (STM). STM was performed using a Nanoscope II (Digital Instruments Inc.). The sample (5 μL) was placed on the surface of a highly oriented pyrolytic graphite (6 mm × 6 mm) for 30 min, and excess was removed with filter paper. It was washed with distilled water and immediately examined after being dried. All image processings were conducted using Nanoscope II software. Mechanically cut Pt/Ir (80/20) probes with a D head (10-μm maximum scan size) and an A head (1-μm maximum scan size) were used. Tunneling bias voltage was between 80 and 800 mV, and current set points were between 0.2 and 0.5 nA.

RESULTS

An aqueous solution of M1 showed characteristic CD spectra following incubation at 5 °C (Figure 1A). The CD spectrum measured immediately after dissolution had a negative maximum below 200 nm, indicating the peptide predominantly to take on a random coil structure. The aqueous solution showed gradual change in its CD spectrum after incubation at 5 °C. A striking increase was noted in the negative Cotton effect with two peaks at 208 and 222 nm, indicating the formation of an α-helical structure. The magnitude of $[\theta]_{222}$ after incubation for 37 days was about 12 times that immediately following dissolution. The CD spectra at different incubation times showed an isodichroic point at 201 nm, indicating a two-state transition.

Short linear peptides may be considered flexible and should take on various conformations on a nanosecond time scale. Examination was made to determine whether a much slower conformational change would be due to intermolecular association. Sedimentation velocity measurement showed the peptide after incubation for over 2 months, which had a CD spectrum indicative of α-helix, to sediment as 70 S (data not shown). For a globular molecule, this value corresponds to a molecular weight of about 3 000 000. Assuming there to be a globule, the aggregate was considered to be comprised of more than 1000 molecules. A sedimentation equilibrium experiment showed the peptide immediately following dissolution, which had a CD spectrum indicative of a random coil, to have a molecular weight of about 4000, this being close to the monomeric molecular weight, 2786 (data not shown).

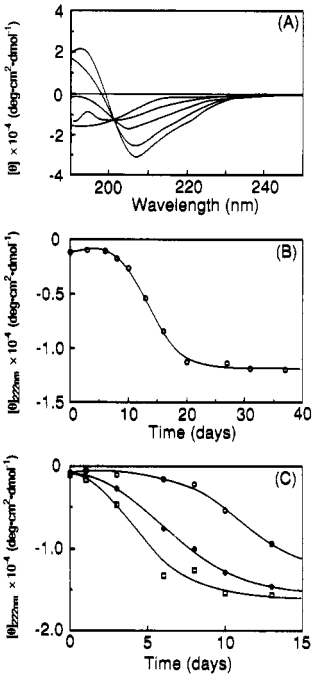

FIGURE 1: (A) CD spectra of M1. The CD spectrum of a 0.2 mM aqueous solution of M1 incubated for various periods, reading downward at 222 nm, of 0, 10, 13, 16, and 37 days at 5 °C. (B) Time course of mean residue ellipticity at 222 nm of M1. (C) Rate enhancement for the formation of the helical structure by the preformed aggregate. Helical formation was monitored as the increase in negative ellipticity at 222 nm. An aqueous solution (1 mM) of M1 in 50 mM Tris-HCl (pH 7.5) was incubated at 5 °C with the preformed aggregate at a concentration of 0 μM (circle), 5 μM (diamond), and 10 μM (square), respectively.

Figure 1B shows the time course of ellipticity at 222 nm. The magnitude did not change for a week but thereafter gradually increased, attaining a maximum in 20 days. Helical structure formation thus has a lag time of about a week, increases logarithmically, and finally reaches a plateau in 20 days. That the aggregate formed could subsequently accelerate the formation of an α-helical structure appears to reflect this sequence of events.

Figure 1C shows the effect of the preformed aggregate on the rate of structural change from a random coil to an α-helix. Increase in negative ellipticity at 222 nm was significantly accelerated by the presence of a small amount of preformed aggregate. The change was proportional to the amount of added aggregate. It thus follows that structural change is mediated by a mechanism such that the preformed aggregate autocatalytically accelerates the formation of the aggregate. That is, the preformed aggregate acts as a template for subsequent formation of the aggregate. The rate-determining step for the formation of helical structures should thus be the initial formation of a small amount of the aggregate.

Figure 2 shows an electron micrograph of the negatively stained aggregate. The aggregate formed filamentous helical structures of 10–20 nm in diameter. The geometry of the helical structures was right-handed. Differences in diameter

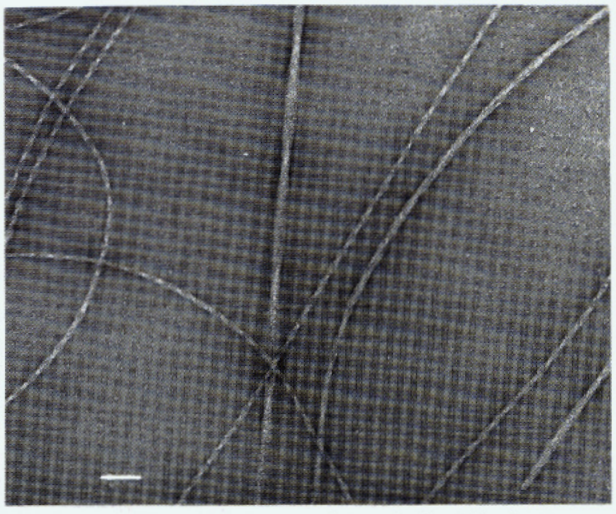

FIGURE 2: Electron micrograph of negatively stained aggregates from M1. The scale bar represents 50 nm.

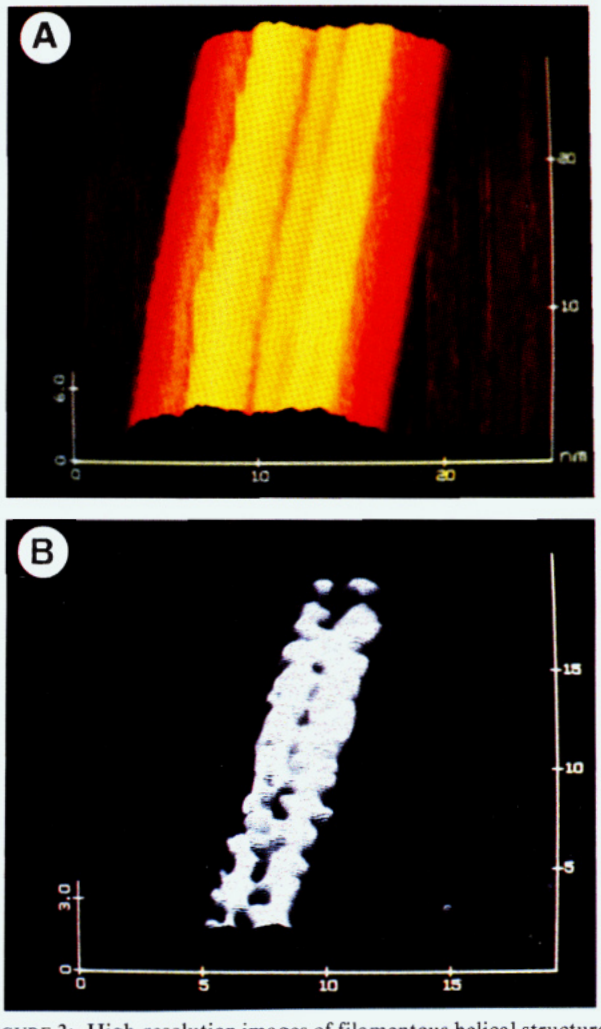

FIGURE 3: High-resolution images of filamentous helical structures taken by STM. Perspective views of a single filamentous helical structure (A) and protofilamentous helical structures aligned in parallel (B). Scales in panel B are represented by a nanometer unit.

and helical pitch, i.e., twisted or extended ribbon, were noted. STM showed each filament to be comprised of a bundle of thinner protofilaments of 1.5–2.0 nm in diameter (Figure 3A). Therefore, STM indicates the twisted and extended ribbon structures in Figure 2 to consist of a twisted bundle of protofilaments and protofilaments aligned in parallel, respectively. These protofilaments were apparently comprised of several stacked disk like structures of ≈2 nm in diameter and ≈1.5 nm in thickness (Figure 3B).

At 30 °C, M1 formed filamentous helical structures quite similar to those formed at 5 °C (electron microscopic data not shown), but it is unclear whether both fine filamentous structures are identical.

DISCUSSION

The above data show M1 to be capable of taking on a stable α-helix in an aggregated state but not in a monomer state. The local sequence of M1 thus has the same preference for a secondary structure observed in the native protein. Formation of the native-like secondary structure through aggregation does not always occur since intermolecular interactions in an aggregated state must differ from the tertiary interaction in the native protein. The α-helix in the native barnase may thus be an intrinsic secondary structure preferred by the amino acid sequence of M1. Peptides derived from protein sequences have been clearly shown to take on native-like secondary structures possessing low stability, thus supporting the above view (Brown & Klee, 1971; Dyson et al., 1985, 1990, 1992; Waltho et al., 1989; Williamson et al., 1986; Sancho et al., 1992). These examples indicate that peptide fragments of proteins have propensity for forming their native-like secondary structures. It should be noted that native structures of proteins would not be determined by a rule to maximize energy gain of secondary structure formation. If larger conformation stability is obtained by formation of a particular tertiary structure, the tertiary structure would not stabilize the locally preferred secondary structure. This is one of the reasons for a limited success rate in prediction of the secondary structure by only a short-range interaction between amino acids. It would thus be important to search for independent folding units in protein.

Hydrogen bonds of barnase were found mainly within each of the modules, indicating that their locations are so designed as to stabilize the primarily individual module (Noguti et al., unpublished data). This implies that the native conformation of modules is specified predominantly by interactions within the modules themselves. M1 may thus take on a native-like conformation even in filamentous helical structures in which M1 interacts with each other.

Helical wheel representation of residues from the 7th to 24th M1 indicated hydrophobic residues such as leucine, phenylalanine, tyrosine, and valine to be concentrated on one side of the α-helix, this being a feature of an amphiphilic helix (data not shown). Such a feature should contribute to the formation of a stable helical structure through aggregation. The UV absorption maximum of M1 shifted to a longer wavelength from 276 to 278 nm with formation of the helical structure (data not shown). This shift in UV absorption maximum was reflected by changes in the environment of the aromatic rings, indicating partition of tyrosine residues with aggregate formation. Hydrophobic interactions may thus be the predominant force for stabilizing the structure.

Among filamentous helical structures, a protofilamentous structure is the most fundamental structural unit. The STM image shown in Figure 3B indicated this structure to consist of stacked disks of ≈2 nm in diameter and ≈1.5 nm in thickness. The compact conformation of M1 and the architecture of barnase are shown in Figure 4. The front, back side, and side views of M1 indicated M1 to consist of disks of ≈2 nm in diameter and ≈1.5 nm in thickness, with hydrophobic amino acid residues between the 7th and 24th residues (drawn in

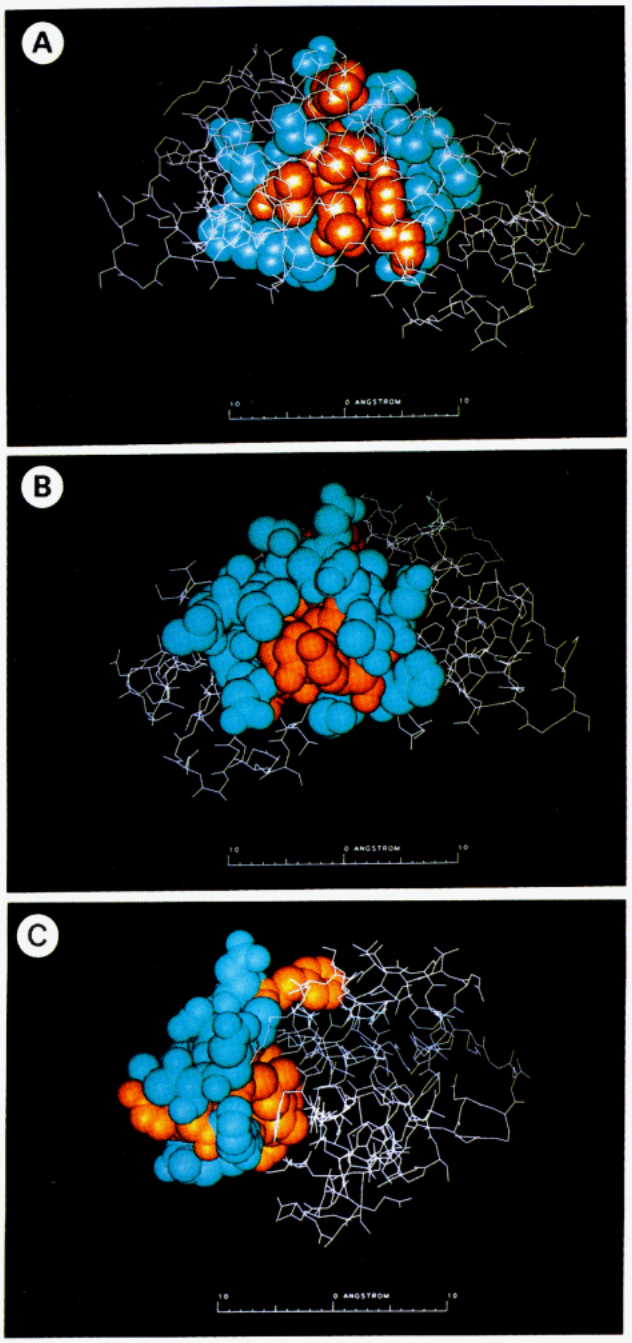

FIGURE 4: Three-dimensional structures of M1 and barnase. The M1 moiety of barnase is shown in a space-filling model, and other moieties are shown by a wire frame representing the main and side chains. In M1, hydrophobic amino acid residues between the 7th and 24th residues (Phe-7, Val-10, Tyr-13, Leu-14, Tyr-17, Leu-20, Pro-21, and Tyr-24) appear in gold and other amino acid residues in blue. M1 is shown from the direction of the catalytic sites (A), back side (B), and side (C), respectively.

gold) present in the center and others (drawn in blue) situated along the margin. Disk diameter and thickness of the structures were essentially in agreement with those measured by STM. The protofilamentous structure would thus appear to consist of disks stacked on top of each other through interactions between hydrophobic amino acid residues in aqueous solution. These interactions may thus be the driving force to form helical protofilamentous structure formation. A possible schematic model for helical protofilamentous structures consisting of stacked disks is given in Figure 5.

Sancho et al. (1992) examined the structure of a predicted nucleus site of protein folding in barnase using an N-terminal peptide fragment of 36 residues. The CD spectra of an

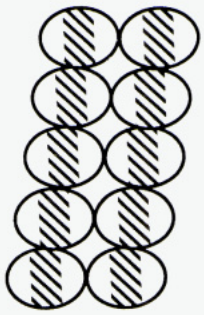

FIGURE 5: A possible schematic model for protofilamentous structures. The hydrophobic cores are hatched.

N-terminal peptide fragment of 36 residues in aqueous solution showed the peptide to be predominantly populated as a random coil as also observed for M1 in a monomer state. However, the 2D-NMR spectrum showed a fraction of the population to possess a helical structure near the predicted region as a nucleus site. The predicted nucleus site was comprised of amino acids from the 10th to the 21st, fully included in M1. The helical structure in an N-terminal peptide fragment of 36 residues was shown to be highly populated by the addition of trifluoroethanol capable of stabilizing helical structures by which it acquired marginal stability (nascent helix) (Sancho et al., 1992; Dyson et al., 1992). Helical formation by the addition of trifluoroethanol was also observed for M1 largely populated as a random coil structure immediately following dissolution (data not shown). M1 in a monomer state may also take on a helical structure in aqueous solution, but this could not be shown by CD spectroscopy owing to the smaller population or lower stability. The helical structure with marginal stability in a monomer state should be stabilizable by aggregation accompanying hydrophobic burial, this indicating structural change from a random coil to α-helix to possibly occur cooperatively. It is of interest that M1, even though a short peptide, could form filamentous helical strands. The mechanism for this remains to be understood.

M1 lacks interactions to stabilize the tertiary structure in native barnase, such as the formation of a triad salt bridge among Asp-8, Asp-12, and Arg-110, and interaction of His-18 with Trp-94 (Horovitz et al., 1990; Loewenthal et al., 1992). M1 also loses a complementary hydrophobic surface. M1 would thus not be capable of taking on a stable secondary structure in a monomer state. The elimination of tertiary interactions made the peptide populate as a random coil structure. The secondary structure of the N-terminal region of barnase thus largely has stability through tertiary interactions with the remaining C-terminal region. There may be alternative interactions in the aggregate for stabilizing the helical structure.

The tertiary structure of barnase consists of six modules (M1–M6). M2, M3, and M6, all of which form a shallow but wide cavity for RNA binding in native barnase, bind to RNA and possess RNase activity (Yanagawa et al., 1993). However, modules M1 and M5, which support other modules from the back side, and M4 have been shown not to bind to RNA and to possess no RNase activity. The present results strongly support the possibility that M1 is capable of spontaneously assembling with other modules to form a more active catalytic protein than modules alone.

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