

Freezing of a Fish Antifreeze Protein Results in Amyloid Fibril Formation

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ABSTRACT Amyloid is associated with a number of diseases including Alzheimer's, Huntington's, Parkinson's, and the spongiform encephalopathies. Amyloid fibrils have been formed *in vitro* from both disease and nondisease related proteins, but the latter requires extremes of pH, heat, or the presence of a chaotropic agent. We show, using fluorescence spectroscopy, electron microscopy, and solid-state NMR spectroscopy, that the α -helical type I antifreeze protein from the winter flounder forms amyloid fibrils at pH 4 and 7 upon freezing and thawing. Our results demonstrate that the freezing of some proteins may accelerate the formation of amyloid fibrils.

INTRODUCTION

Amyloidosis describes the presence of proteinaceous deposits with specific morphological, staining, and structural characteristics. The existence of these insoluble and proteolytically resistant deposits can seriously interfere with normal organ function (Pepys, 2001). Amyloid deposits have been associated with a number of diseases, including Alzheimer's, Huntington's, Parkinson's, AA amyloidosis, type II diabetes, dialysis-related amyloidosis, and scrapie. When stained with traditional dyes, amyloid deposits appear amorphous. However, when stained with Congo Red and viewed through cross-polarizing lenses, amyloid deposits show a characteristic green birefringence, which suggests an underlying structure. Under an electron microscope, the fibrils appear unbranched and have a diameter of 50–130 Å (Serpell et al., 1997). X-ray diffraction studies reveal that these fibrils have a "cross- β " structure, wherein the protein aggregates as β -strands forming a sheet that winds around the fibril axis in a helical manner (Serpell et al., 1997). Solid-state NMR work has refined the fibril model by showing that the β -amyloid peptides form parallel β -sheets (Balbach et al., 2002; Benzinger et al., 2000), although an antiparallel arrangement has also been detected for a shorter β -amyloid peptide (Balbach et al., 2000). Other NMR experiments have demonstrated that the backbone conformation differs from the classical β -sheet (Costa et al., 1997; Spencer et al., 1991). Despite these similar morphological features, no consistent similarity in sequence, structure, or function has been found for proteins that are able to form amyloid fibrils (Kisilevsky, 2000; Chiti et al., 2001; Dobson, 1999).

One of the first clues that the formation of amyloid fibrils may not be limited to the disease state was the study on the SH3 domain of the phosphatidylinositol kinase (Guijarro et al., 1998). Exposure of this protein to low pH resulted in

the formation of a gel with morphological properties similar to amyloid fibrils. This has also been repeated with a number of other, nonpathological proteins including fibronectin (Litvinovich et al., 1998), cold-shock protein B (Gross et al., 1999), and myoglobin (Fandrich et al., 2001).

Type I antifreeze protein (AFP, also known as thermal hysteresis protein and as ice-structuring protein) is found at relatively high concentrations in the circulatory system (10–15 mg/mL) and in the skin of fish living in subzero seawater, and protects the organism from macromolecular ice growth by adsorption inhibition (Fletcher et al., 2001). Structural studies have revealed that the 37-residue protein forms an α -helical structure (Sicheri and Yang, 1995; see Fig. 1). The role of hydrogen bonds and van-der-Waals forces in ice binding has been extensively studied (Jia and Davies, 2002); however, their relative contribution to the interaction remains unclear.

During our studies of the AFP mechanism, we found that the winter flounder type I AFP in solution formed a translucent gel upon freezing and thawing at physiological pH and below. It has been established that gel formation of a protein at or above the critical concentration of polymerization (Harper and Lansbury, 1997) may indicate the formation of amyloid fibrils. To determine if the gel was amyloidotic in nature, we examined three properties specific to amyloid: morphology by electron microscopy, fluorescence staining by the amyloid-specific dye Thioflavin T (ThT), and structural characteristics by solid-state ^{13}C -NMR spectroscopy. The data presented here demonstrate that type I AFP can be converted into amyloid fibrils upon freezing and thawing, conditions appropriate to its physiological function.

MATERIALS AND METHODS

Preparation of AFP fibril

The wild-type winter flounder AFP HPLC-6 was synthesized according to standard solid-phase peptide synthesis methods as described previously (Hodges et al., 1988). For ^{13}C -labeled samples, a $^{13}\text{C}\alpha$ -alanine residue (2- ^{13}C -alanine, Cambridge Isotopes Laboratories, Andover, MA) was incorporated at position 17 (Fig. 1).

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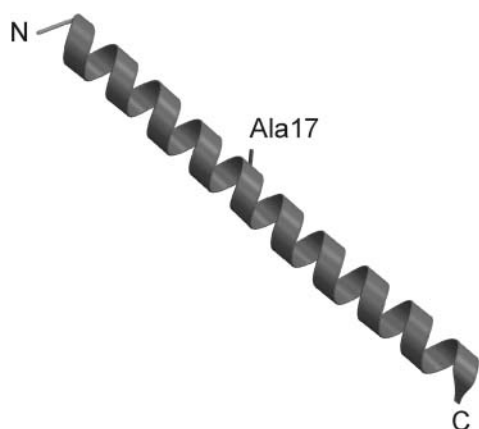


FIGURE 1 Structure of type I AFP. A ribbon diagram of the type I AFP crystal structure (PDB code 1wfb, chain B; see Sicheri and Yang, 1995). The protein is 37 residues long, with 23 residues consisting of alanine. The location of the single $^{13}\text{C}\alpha$ -alanine is labeled.

For the preparation of unlabeled antifreeze protein for use with the fluorescence experiments, 3 mg of protein was dissolved in 31 μL of H_2O . The pH was adjusted to ~ 4 by the addition of 9 μL of 100 mM NaOH, resulting in a final protein concentration of 75 mg/mL (23 mM). An aliquot of the sample was stored at 4°C as a negative control. The remaining aliquots were frozen and thawed between one and three times.

For the preparation of labeled protein for NMR experiments, 9 mg of $^{13}\text{C}\alpha$ -Ala17 type I AFP was dissolved in 90 μL of H_2O . To adjust the pH to ~ 4 , 30 μL of 100 mM NaOH was added, giving a final concentration of 75 mg/mL (23 mM). The sample was frozen and thawed as indicated in Fig. 4.

Electron microscopy

Electron micrographs were acquired on a Hitachi Transmission Electron Microscope H-7000 (Tokyo) operating at a 75-kV excitation voltage. The sample was prepared by applying 3 μL of ~ 50 mg/mL protein gel on a Formvar grid and subsequently dried. The gel was negatively stained with 2% uranyl acetate.

ThT fluorescence

ThT fluorescence was performed as described by LeVine (1999). Briefly, 1 μg of protein was mixed with 1 mL of 5 μM ThT in 50 mM glycine-NaOH, pH 8.5. The fluorescence of the sample was measured on a Shimadzu RF5301-PC fluorescence spectrophotometer (Kyoto, Japan) with the emission intensity traced from 460 to 520 nm with a 10-nm slit width using an excitation wavelength of 450 nm with a 5-nm slit width. Relative fluorescence values are reported at 482 nm. The three negative controls consisted of ThT alone, AFP fibril alone, or AFP in solution with 5 μM ThT.

NMR spectroscopy

The NMR spectra were collected at 7.04 Tesla corresponding to a ^{13}C Larmor frequency of 75.416 MHz on a Varian Unity Spectrometer (Palo Alto, CA) equipped with a Varian/Chemagnetics double-resonance MAS probe with a 5.0-mm PENCIL-I MAS rotor. For the ^{13}C -CP/MAS experiments, the cross-polarization contact time was 3.0 ms during which the ^1H decoupling field strength was 78.1 kHz. During acquisition, the decoupling field strength was reduced to 55.3 kHz. 13,312 transients were collected with a recycle delay of 5 s. The proton 90° pulsewidth was

calibrated to be 3.6 μs . For the ^{13}C -MAS experiments, a ^1H decoupler field strength of 15.6 kHz was applied to 144 or 512 transients with a recycle delay of 3 s. All MAS experiments were collected with 2972 complex data points, a sweepwidth of 37,140 Hz, and performed with the sample spinning at 3.333 kHz. Chemical shifts were referenced relative to the carbon chemical shift of 2,2'-dimethyl-2-silapentane-5-sulfonate (0 ppm; see Wishart et al., 1995).

RESULTS

Electron microscopy reveals unbranched fibrils

Electron microscopy was used to examine uranyl acetate stained gel preparations of type I AFP. Fig. 2 shows unbranched fibrils that are ~ 50 Å in diameter, which is in agreement with previous studies on fibrils in amyloid deposits (Serpell et al., 1997). In addition, studies have shown that these fibrils associate into larger, mature fibrils, where several protofilaments coil around one another (Chiti et al., 1999). This is illustrated for type I AFP in Fig. 2 B.

Type I AFP gel binds the fluorescent dye ThT

To provide further evidence that the type I AFP fibrils were amyloidotic in nature, we examined the AFP gel in the

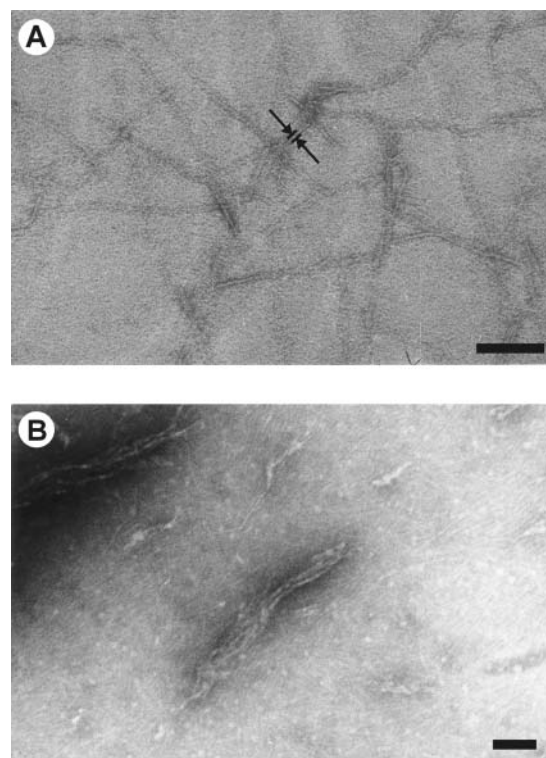


FIGURE 2 Transmission electron micrographs of AFP gel. Uranyl acetate stained preparations of the AFP gel were examined under an electron microscope to determine its nature. (A) Fibrils formed after freezing and thawing a 75 mg/mL, pH 4 sample three times. The arrows show an individual fibril with a diameter of ~ 50 Å. (B) Protofilaments. Scale bars, 100 nm.

presence of the fluorescent dye ThT. ThT undergoes a specific excitation and emission redshift when bound to amyloid fibrils (LeVine, 1999). Congo Red (Puchtler et al., 1962), a stain commonly used for detecting amyloid fibrils, was not employed inasmuch as it has been shown to bind soluble proteins including β -helical proteins (Khurana et al., 2001). Fig. 3 shows a bar graph demonstrating the increase in fluorescence intensity over time with successive freeze-and-thaw cycles. Immediately after one freeze/thaw cycle, there is little change in the fluorescence intensity. Subsequent freeze/thaw cycles increased the fluorescence signal, suggesting that more of the protein was being converted into amyloid fibril. Similar results were obtained with type I AFP at pH 7, 100 mM NaCl (data not shown). In addition, the warming and cooling of a type I AFP solution between 5°C and 25°C did not cause a gel to form. These two results demonstrate that it is the freeze/thaw procedure and not the low pH or temperature cycling that promote amyloid formation. The control sample of type I AFP that was not frozen had approximately the same fluorescence as the ThT dye alone. Interestingly this sample, upon storage at 4°C for several days before the fluorescence experiments, had also become gel-like, but was transparent and not firm like the translucent, amyloidotic gel.

Solid-state ^{13}C -NMR spectroscopy shows a β -sheet structure

The third assessment for amyloid formation is the presence of β -sheet structure. To follow changes in protein conformation during freezing and thawing, ^{13}C -NMR was used as $^{13}\text{C}\alpha$ chemical shifts are especially sensitive to secondary structure; a positive deviation from random coil chemical

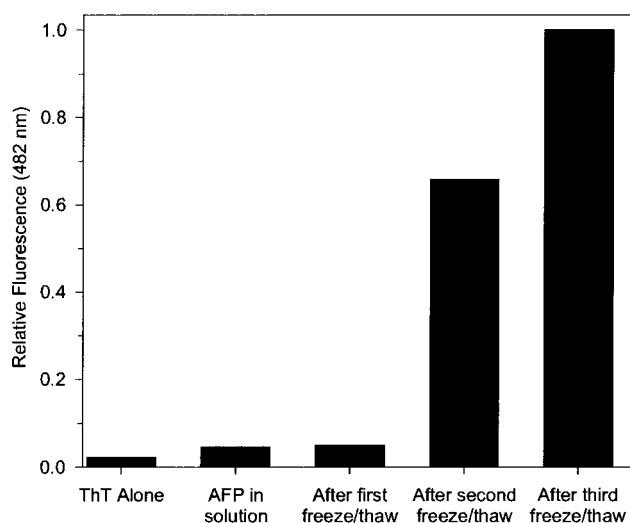


FIGURE 3 ThT fluorescence of fibrillar AFP and controls. Samples of the AFP gel after various stages of freezing and thawing were treated with the amyloid-specific dye ThT. A bar graph shows the normalized fluorescence emission maximum at 482 nm after excitation at 450 nm.

shifts (51.7 ppm for alanine) is indicative of an α -helical conformation whereas a negative deviation is indicative of β -sheet conformation (Wishart et al., 1994). Because 23 of the 37 residues in type I AFP are alanine, the protein was synthesized with a single $^{13}\text{C}\alpha$ label at position Ala17 to simplify analysis (Fig. 1). Solid-state ^{13}C -1D magic-angle spinning direct polarization (^{13}C -MAS) experiments revealed that before freezing, the protein was α -helical (54.1 ppm chemical shift at 20°C) (Fig. 4 A). This value is similar to the previously measured chemical shift of 54.5 ppm at 21°C (Graether et al., 2001). Upon freezing, the signal was lost (Fig. 4 A). A cross-polarization/magic-angle spinning (^{13}C -CP/MAS) spectrum subsequently taken at -20°C (Fig. 4 B) indicated that the peptide remained α -helical in ice with a chemical shift of 55.1 ppm. This also agrees with experiments demonstrating that the protein remains α -helical when supercooled (Graether et al., 2001) and rules out denaturation during the freezing process as the cause of fibril formation.

Upon thawing the sample at 20°C, the ^{13}C -MAS spectrum revealed a lower intensity than before freezing. This intensity decreased over 20 h (at 20°C) during which a ^{13}C -CP/MAS spectrum was taken. Three peaks were found centered at 54.0, 51.5, and 50.9 ppm. The peak at 54.0 ppm is consistent with an α -helical structure, whereas the peaks at 51.5 and 50.9 are consistent with a β -sheet structure. The heterogeneity in β -sheet chemical shifts may indicate that the fibril is dynamic. Refreezing of the sample produced a spectrum with chemical shifts of 55.0 and 51.4 ppm. The peak at 51.4 ppm is consistent with the chemical shift observed for an alanine residue in a parallel β -sheet structure found in amyloidotic prion protein fragments (Laws et al., 2001). Further thawing and freezing of the protein results in a total loss of intensity in the ^{13}C -MAS experiment indicating a loss of free protein. Concomitantly, the ^{13}C -CP/MAS experiments revealed an increase in the amount of protein in the parallel β -sheet conformation. Interestingly, at a certain point the peak at 55 ppm appears to change little upon freezing and thawing. This was also observed for the prion protein and may be due to the trapping of the α -helical form of AFP in the gel matrix.

DISCUSSION

Conformational stability and amyloidogenesis

It has been shown that destabilization of the native conformation of several proteins render it prone to amyloid formation (Kelly, 1998; Dobson, 2001; Chiti et al., 1999). To induce amyloid formation, these proteins require unusual conditions such as extremes of pH, high concentrations of chaotropic agents, such as TFE, or high temperatures (Guijarro et al., 1998; Chiti et al., 2000; Fandrich et al., 2001; Chiti et al., 2001; Bucciantini et al., 2002; Litvinovich

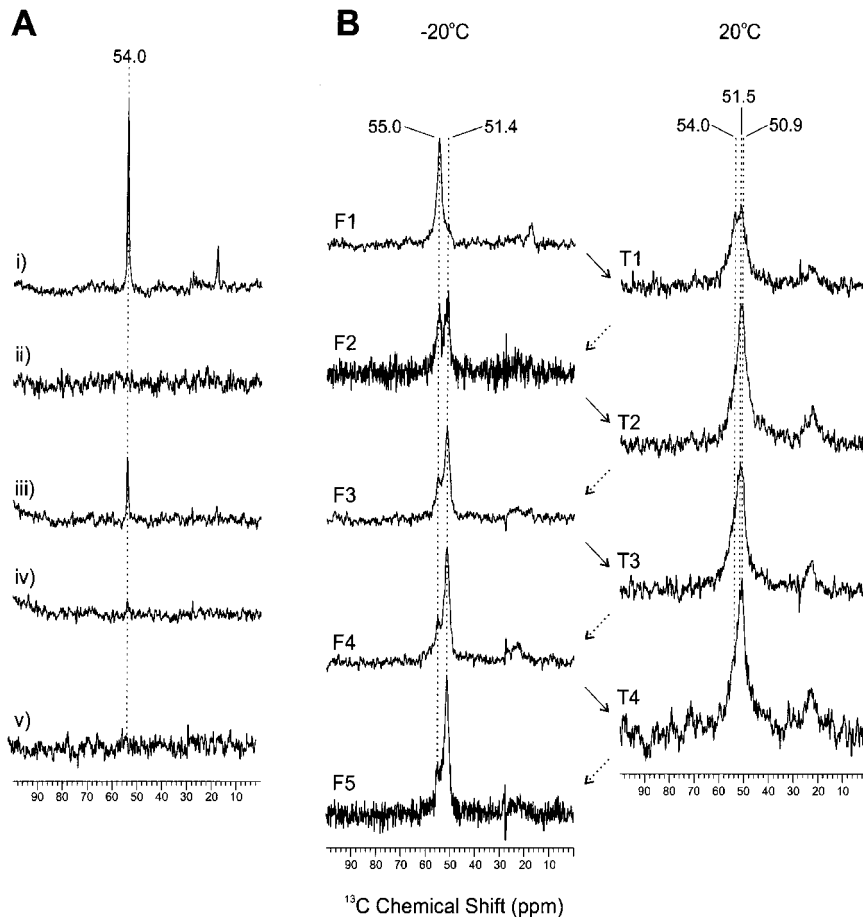


FIGURE 4 ^{13}C -NMR spectra of type I AFP. The conformation of the type I AFP was examined after sequential freeze/thaw cycles by following the $^{13}\text{C}\alpha\text{-Ala17}$ chemical shift. Values greater than 51.7 ppm indicate that the residue is in an α -helical conformation, whereas lesser values indicate β -strand conformation. Numbers indicate the chemical shift of the $^{13}\text{C}\alpha\text{-Ala17}$ peak(s). (A) ^{13}C -MAS direct polarization: (i) Before first freeze, 20°C; (ii) Frozen, -20°C; (iii) 30 min after first thaw, 20°C; (iv) 20 h after first thaw, 20°C; and (v) After second thaw, 20°C. (B) ^{13}C -CP/MAS spectra of AFP at -20°C (left) and 20°C (right). The arrows indicate the spectra after sequential freeze (dashed line) and thaw (solid line) cycles. The number indicates the cycle of freezing (F1-5) or thawing (T1-5). Chemical shifts are referenced to 2,2'-dimethyl-2-silapentane-5-sulfonate at 0 ppm (Wishart et al., 1995).

et al., 1998). For type I AFP, amyloid formation can occur at neutral pH and physiological salt concentrations suggesting that the chemical environment is not responsible for amyloid formation for this protein.

Antifreeze proteins have a number of unusual properties. Analysis of the type I AFP amino-acid sequence using the program SEQSEE (Wishart et al., 1994) predicts that due to its high hydrophobicity (68% of the residues are hydrophobic), the protein forms multimers and is therefore not expected to be soluble. Nonetheless, type I AFP can dissolve to a high concentration (>50 mg/mL) and remain monomeric and α -helical, presumably because of its high alanine content (23 of 37 residues). Interestingly, a de novo designed helical peptide was shown to form fibrils at neutral pH (Fezoui et al., 2000). However, this peptide appeared to be only partially folded at pH 7.4 whereas at pH 3.6, where the peptide was most stable, fibril formation was not favored. This is in direct contrast to type I AFP, which was able to form fibrils at neutral and acidic pHs, and appears to have an increase in stability as the temperature is decreased (Graether et al., 2001).

For the most part, amyloid fibril formation is associated with disease states. However, there are examples where

amyloid formation can be beneficial to an organism. In *Escherichia coli* and *Salmonella* spp., a class of proteins known as curli are responsible for forming extracellular amyloid-like fibers which are responsible for colonization of inert surfaces, biofilm formation, and mediation of binding to host proteins (Chapman et al., 2002). Fish embryos of *Austrofundulus limnaeus* are surrounded by an egg envelope composed of two proteins that together form a structure similar to amyloid fibrils. This amyloid chorion protects the fish embryo from mechanical disruption, serves as a barrier to polyspermy, microbes, and low molecular weight solutes, and is able to help prevent dehydration of the embryo by preventing water loss (Podrabsky et al., 2001). It is possible that a conformational change of type I AFP at physiological concentrations may play a role in ice inhibition. Indeed, the insect antifreeze proteins have a parallel β -helix structure (Graether et al., 2000; Liou et al., 2000), a motif that has been shown to bind Congo Red (Khurana et al., 2001). Possibly the remarkable properties of AFP (such as alignment of proteins on the ice surface) promote the ordered aggregation of neighboring molecules into the parallel β -sheet structure, that, under high concentrations, can form amyloid. We are currently investigating

how this may be involved in AFP function and fibril formation.

Amyloidogenesis and applications of AFPs

The type I AFP is found in the circulatory system of fish at a concentration of 10–15 mg/mL (Fletcher et al., 2001). The relatively low physiological concentration of the protein and its ability to bind to membranes (Tomczak et al., 2002) may prevent it from self-association. However, there is a question concerning the freezing of fish for long-term storage, and whether other potentially fibril-forming proteins could similarly form amyloid-like fibrils upon freezing and thawing. Inasmuch as type I AFP can form amyloid, its use in applications (Fletcher et al., 1999) such as cryopreservation, cryosurgery, as a food additive, and in expression in transgenic organisms needs to be addressed (Crevel et al., 2002).

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REFERENCES

- Balbach, J. J., Y. Ishii, O. N. Antzutkin, R. D. Leapman, N. W. Rizzo, F. Dyda, J. Reed, and R. Tycko. 2000. Amyloid fibril formation by A beta(16–22), a seven-residue fragment of the Alzheimer's beta-amyloid peptide, and structural characterization by solid-state NMR. *Biochemistry*. 39:13748–13759.
- Balbach, J. J., A. T. Petkova, N. A. Oyler, O. N. Antzutkin, D. J. Gordon, S. C. Meredith, and R. Tycko. 2002. Supramolecular structure in full-length Alzheimer's beta-amyloid fibrils: evidence for a parallel beta-sheet organization from solid-state nuclear magnetic resonance. *Biophys. J.* 83:1205–1216.
- Benzinger, T. L., D. M. Gregory, T. S. Burkoth, H. Miller-Auer, D. G. Lynn, R. E. Botto, and S. C. Meredith. 2000. Two-dimensional structure of beta-amyloid(10–35) fibrils. *Biochemistry*. 39:3491–3499.
- Bucciantini, M., E. Glannoni, F. Chiti, F. Baroni, L. Formigli, J. Zurdo, N. Tadel, G. Ramponi, C. M. Dobson, and M. Stefani. 2002. Inherent toxicity of aggregates implies a common mechanism for protein misfolding diseases. *Nature*. 416:507–511.
- Chapman, M. R., L. S. Robinson, J. S. Pinkner, R. Roth, J. Heuser, M. Hammar, S. Normark, and S. J. Hultgren. 2002. Role of *Escherichia coli* Curli operons in directing amyloid fiber formation. *Science*. 295:851–855.
- Chiti, F., M. Bucciantini, C. Capanni, N. Taddei, C. M. Dobson, and M. Stefani. 2001. Solution conditions can promote formation of either amyloid protofilaments or mature fibrils from the HypF N-terminal domain. *Prot. Sci.* 10:2541–2547.
- Chiti, F., N. Taddei, M. Bucciantini, P. White, G. Ramponi, and C. M. Dobson. 2000. Mutational analysis of the propensity for amyloid formation by a globular protein. *EMBO J.* 19:1441–1449.
- Chiti, F., P. Webster, N. Taddei, A. Clark, M. Stefani, G. Ramponi, and C. M. Dobson. 1999. Designing conditions for in vitro formation of amyloid protofilaments and fibrils. *Proc. Natl. Acad. Sci. USA*. 96:3590–3594.
- Costa, P. R., D. A. Kocisko, B. Q. Sun, P. T. Lansbury, and R. G. Griffin. 1997. Determination of peptide amide configuration in a model amyloid fibril by solid-state NMR. *J. Am. Chem. Soc.* 119:10487–10493.
- Crevel, R. W., J. K. Fedyk, and M. J. Spurgeon. 2002. Antifreeze proteins: characteristics, occurrence and human exposure. *Food Chem. Toxicol.* 40:899–903.
- Dobson, C. M. 1999. Protein misfolding, evolution and disease. *Trends Biochem. Sci.* 24:329–332.
- Dobson, C. M. 2001. The structural basis of protein folding and its links with human disease. *Phil. Trans. R. Soc. Lond. B.* 356:133–145.
- Fandrich, M., M. A. Fletcher, and C. M. Dobson. 2001. Amyloid fibrils from muscle myoglobin. *Nature*. 410:165–166.
- Fezoui, Y., D. M. Hartley, D. M. Walsh, D. J. Selkoe, J. J. Osterhout, and D. B. Teplow. 2000. A de novo designed helix-turn-helix peptide forms nontoxic amyloid fibrils. *Nat. Struct. Biol.* 7:1095–1099.
- Fletcher, G. L., S. V. Goddard, and Y. Wu. 1999. Antifreeze proteins and their genes: From basic research to business opportunities. *Chemtech*. 30:17–28.
- Fletcher, G. L., C. L. Hew, and P. L. Davies. 2001. Antifreeze proteins of Teleost fishes. *Annu. Rev. Physiol.* 63:359–390.
- Graether, S. P., M. J. Kuiper, S. M. Gagné, V. K. Walker, Z. Jia, B. D. Sykes, and P. L. Davies. 2000. β -helix structure and ice-binding properties of a hyperactive antifreeze protein from an insect. *Nature*. 406:325–328.
- Graether, S. P., C. M. Slupsky, P. L. Davies, and B. D. Sykes. 2001. Structure of type I antifreeze protein and mutants in supercooled water. *Biophys. J.* 81:1677–1683.
- Gross, M., D. K. Wilkins, M. C. Pitkeathly, E. W. Chung, C. Higham, A. Clark, and C. M. Dobson. 1999. Formation of amyloid fibrils by peptides derived from the bacterial cold shock protein CspB. *Protein Sci.* 8:1350–1357.
- Guijarro, J. I., M. Sunde, J. A. Jones, I. D. Campbell, and C. M. Dobson. 1998. Amyloid fibril formation by an SH3 domain. *Proc. Natl. Acad. Sci. USA*. 95:4224–4228.
- Harper, J. D., and P. T. Lansbury. 1997. Models of amyloid seeding in Alzheimer's disease and scrapie: mechanistic truths and physiological consequences of the time-dependent solubility of amyloid proteins. *Annu. Rev. Biochem.* 66:385–407.
- Hodges, R. S., P. D. Semchuk, A. K. Taneja, C. M. Kay, J. M. Parker, and C. T. Mant. 1988. Protein design using model synthetic peptides. *J. Pept. Res.* 1:19–30.
- Jia, Z., and P. L. Davies. 2002. Antifreeze proteins: an unusual receptor-ligand interaction. *Trends Biochem. Sci.* 27:101–106.
- Kelly, J. W. 1998. The alternative conformations of amyloidogenic proteins and their multi-step assembly pathways. *Curr. Opin. Struct. Biol.* 8:101–106.
- Khurana, R., V. N. Uversky, L. Nielsen, and A. L. Fink. 2001. Is Congo Red an amyloid-specific dye? *J. Biol. Chem.* 276:22715–22721.
- Kisilevsky, R. 2000. Review: Amyloidogenesis—unquestioned answers and unanswered questions. *J. Struct. Biol.* 130:99–108.
- Laws, D. D., H. M. L. Bitter, K. Liu, H. L. Ball, K. Kaneko, H. Wille, F. E. Cohen, S. B. Prusiner, A. Pines, and D. E. Wemmer. 2001. Solid-state NMR studies of the secondary structure of a mutant prion protein fragment of 55 residues that induces neurodegeneration. *Proc. Natl. Acad. Sci. USA*. 98:11686–11690.
- LeVine, H. 1999. Quantification of β -sheet amyloid fibril structures with Thioflavin T. *Meth. Enzymol.* 309:274–284.
- Liou, Y., A. Tocilj, P. L. Davies, and Z. Jia. 2000. Mimicry of ice structure by surface hydroxyls and water of a β -helix antifreeze protein. *Nature*. 406:322–324.
- Litvinovich, S. V., S. A. Brew, S. Aota, S. K. Akiyama, C. Haudenschild, and K. C. Ingham. 1998. Formation of amyloid-like fibrils by self-association of a partially unfolded fibronectin type III module. *J. Mol. Biol.* 280:245–258.
- Pepys, M. B. 2001. Pathogenesis, diagnosis and treatment of systemic amyloidosis. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* 356:203–210.

- Podrabsky, J. E., J. F. Carpenter, and S. C. Hand. 2001. Survival of water stress in annual fish embryos: Dehydration avoidance and egg envelope amyloid fibers. *Am. J. Physiol. Regulatory Integrative Comp. Physiol.* 280:R123–R131.
- Puchtler, H., F. Sweat, and M. Levine. 1962. On the binding of Congo Red by amyloid. *J. Histochem. Cytochem.* 10:355–364.
- Serpell, L. C., M. Sunde, and C. C. F. Blake. 1997. The molecular basis of amyloidosis. *Cell. Mol. Life Sci.* 53:871–887.
- Sicheri, F., and D. S. Yang. 1995. Ice-binding structure and mechanism of an antifreeze protein from winter flounder. *Nature.* 375:427–431.
- Spencer, R. G. S., K. J. Halverson, M. Auger, A. E. McDermott, R. G. Griffin, and P. T. Lansbury. 1991. An unusual peptide conformation may precipitate amyloid formation in Alzheimers disease: application of solid-state NMR to the determination of protein secondary structure. *Biochemistry.* 30:10382–10387.
- Tomczak, M. M., D. K. Hinch, S. D. Estrada, W. F. Wolkers, L. M. Crowe, R. E. Feeney, F. Tablin, and J. H. Crowe. 2002. A mechanism for stabilization of membranes at low temperatures by an antifreeze protein. *Biophys. J.* 82:874–881.
- Wishart, D. S., C. G. Bigam, J. Yao, F. Abildgaard, H. J. Dyson, E. Oldfield, J. L. Markley, and B. D. Sykes. 1995. ^1H , ^{13}C and ^{15}N chemical shift referencing in biomolecular NMR. *J. Biomol. NMR.* 6:135–140.
- Wishart, D. S., R. F. Boyko, L. Willard, F. M. Richards, and B. D. Sykes. 1994. SEQSEE: a comprehensive program suite for protein sequence analysis. *Comp. Appl. Biosci.* 10:121–132.