Amyloid fibril formation by bovine cytochrome c

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Abstract. Bovine heart cytochrome c is an all-α globular protein containing a covalently bound heme group. Prolonged incubation at 75°C in mild alkaline solution damages the prosthetic group and results in permanent unfolding of the polypeptide chain. Under this conditions, cytochrome c aggregates into fibrillar structures. Characterization by transmission electron microscopy and thioflavin-T binding assays shows that these species posses the characteristics of fibrils associated with the family of amyloid diseases. Our findings indicate that destabilization of the native fold of this highly α-helical protein can lead to its polymerization into β-sheet rich structures and suggest that this process does not depend on the population of partially folded monomeric states with extensive β-sheet structure.

Keywords: Amyloid formation, cytochrome c, protein misfolding, protein denaturation, helical proteins

Abbreviations: CD = circular dichroism; FTIR = Fourier-transform infrared.

1. Introduction

The deposition of amyloid fibrils has been linked to a variety of slow-onset degenerative diseases, such as Alzheimer's disease, senile systemic amyloidosis, Parkinson's disease, dyalisis-related amyloidosis, and transmissible spongiform encephalopathies [1–4]. The proteins responsible for these diseases do not share structural or sequential identities [5]. In spite of this diversity, all amyloid fibrils display similar structural features, exhibiting a cross-β structure. In the last few years, proteins unrelated to any known human disease have been found to convert *in vitro* into higher order structures that also present a cross-β structure and fulfill all characteristics of amyloid fibrils [6–12]. This has suggested that amyloid represents a generic form of polypeptide conformation, and most peptides/proteins have the potential to form amyloid-like structures under appropriate conditions [13].

The mechanism of fibril assembly is still controversial. For long time it has been accepted that amyloid fibril formation involved the docking of monomeric partially folded states, which display at least partial β-sheet structure [8,14–19]. Nevertheless, recent studies suggest that whereas this can occur in specific cases it may not be the general rule. This way, it has been shown that in the case of myoglobin, an ordinary all-α protein, amyloid fibril formation correlates whit environments in which the protein backbone is unfolded, rather than with conditions that may allow population of partially structured states [11,20]. In this context, it is likely that the study of different protein models with predominant helical secondary structure should provide new insights into the onset of the aggregation process.

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Bovine heart cytochrome c is a small heme protein with 104 amino acid residues, and is an important electron-transfer protein in the respiratory chain. The three-dimensional native structure of this protein fold has been well characterized [21,22], and consists of four α-helices forming a compact core around the covalently attached heme moiety without any β-sheet segment. In this study we show that destabilization of the native fold of this helical protein promotes the formation of amyloid fibrils from an essentially unfolded state.

2. Material and methods

Heart bovine cytochrome c, thioflavin T (ThT), and Trizma base were purchased from Sigma. Unless otherwise mentioned, all solutions were made in 50 mM Tris-HCl buffer (pH 9.0). Controlled heating of protein samples were obtained by using an Erycom PCR system for the desired incubation time.

Circular dichroism. Circular dichroism (CD) spectra in the far- and near-UV region were obtained by using a Jasco 710 spectropolarimeter at 25°C. Protein was assayed at 5–100 μM. Ten accumulations were averaged to obtain each spectrum.

Dye binding assays. Thioflavin-T binding assays were carried out using aliquots of 50 μl drawn from 90 μM protein samples incubated as indicated above. These aliquots were stained with 0.5% Thioflavin-T, washed twice with H_2O and air-dyed. Samples were viewed under UV light using a Leica fluorescence microscope.

Transmission electron microscopy. Samples containing 90 μM protein were incubated as indicated above. A 5 μl aliquot was then placed on carbon-coated copper grids, and allow them to stand for 2 min. The grids were then washed with distilled water and stained with 2% uranyl acetate for another 2 min prior to analysis using a Hitachi H-7000 transmission electron microscope operating at accelerating voltages of 75 kV.

Fourier-transform infrared (FTIR) spectroscopy analysis. Aggregates were dried for 1 h in a speed-vac system prior to analysis to reduce H_2O interference in the infrared spectra. The structure of the dry aggregates was directly analysed in a Bruker Tensor FT-IR spectrometer. FT-IR spectrum of the native protein was acquired after air-drying the protein solution. For each spectrum, 20 interferograms were collected and averaged. All processing procedures were carried out so as to optimize the quality of the spectrum in the amide I region, between 1550 and 1700 cm^{-1}. Second derivatives of the amide I band spectra were used to determine the frequencies at which the different spectral components were located.

3. Results and discussion

Bovine heart cytochrome c posses a predominant helical secondary structure (48.2%) under mild alkaline conditions (pH 9.0) at room temperature (calculated using the Contin method with CDPro suite[1]). This is illustrated by the far-UV CD spectrum shown in Fig. 1, which displays the typical 210 and 222 nm minima. Heating of cytochrome c to 75°C results in partial unfolding of the protein and reduced helical content (38.8%), as denoted by the decrease in the signal strength of the minima at 222 nm and a shift of the band at 210 nm toward lower values (Fig. 1). When the protein is heated just for 5 min
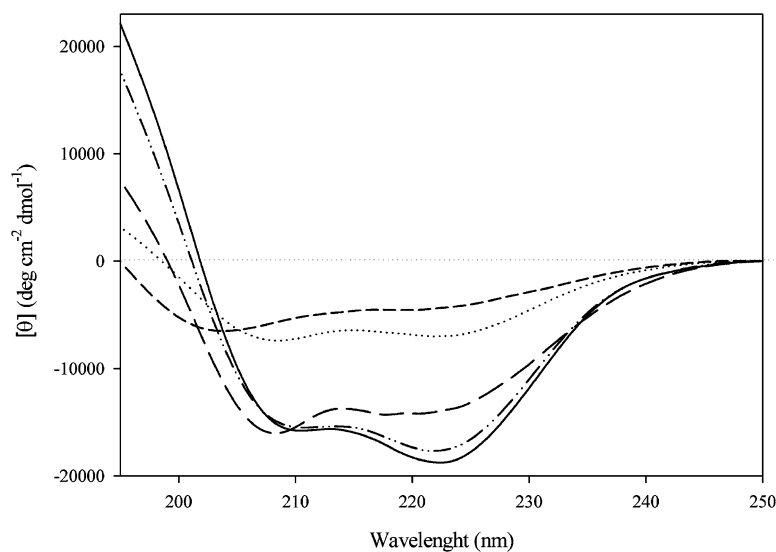
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[1]CDPro suite at http://lamar.colostate.edu/~sreeram/CDPro/main.html.

Fig. 1. Far UV-CD spectra of bovine heart cytochrome c samples at 25°C (—), at 75°C (– –), incubated at 75°C for 5 min and cooled down to 25°C (–··–), incubated at 75°C for 4 h and cooled down to 25°C (· · · ·) and incubated at 75°C for 12 h and cooled down to 25°C (- - - -). Buffer was Tris-HCl 50 mM pH 9.0.

these structural changes are almost fully reversible and the original CD spectra shape and helical content (46.5%) are recovered upon cooling (Fig. 1). Incubation of the protein at 75°C for 4 h results in permanent conformational changes with a significant decrease in helical structure (21.4%) and large increase in random coil conformation (Fig. 1). Further heating of the sample up to 12 h promotes permanent protein unfolding and almost complete loss of the helical content (0.1%). Under these conditions the polypeptide chain is found mainly in random coil conformation (Fig. 1).

Prolonged heating of the protein at 75°C in 50 mM Tris-HCl pH 9.0 also causes a significant loss of the typical red colour present in cytochrome c solutions. No protein or heme group aggregation was detected after 12 h incubation at 75°C and 5 μM protein concentration. Furthermore, no soluble dissociated prosthetic group could be found upon gel filtration of the protein solution, being all colour associated to the protein fraction (data not shown). The visible absorption spectra of a bovine cytochrome c preparation incubated at 25°C for 12 h shows the typical Soret-band maximum at 408 nm due to the heme iron in its oxidized form (Fig. 2A). In addition, bands at 219 and 550 nm attributable to the presence of some reduced cytochrome c species, are also detected (Fig. 2B). The same cytochrome c solution heated at 75°C for 12 h lacks any reduced-state associated band (Fig. 2B) and exhibits a 6 fold decrease in absorbance at 408 nm (Fig. 2A). These results suggest irreversible structural changes in the covalently bound heme group of cytochrome c upon prolonged incubation at 75°C. These changes in the prosthetic group appear to promote unfolding of the polypeptide chain, as assessed by CD. The extent of protein conformational change depends on the time of incubation and presumably on the degree of heme group alteration. Our data are consistent with the notion that the heme group in cytochrome c is not only the redox center of the protein, but is also critical for maintaining the native structure: its removal produces apocytochrome c, and has been shown to cause disruption of the native fold and loss of most of the secondary structure under physiological conditions in different cytochromes [23–28].

Recently it has been reported that prolonged incubation at room temperature of an apo form of cytochrome c_{552} from *Hydogenobacter thermophilus* resulted in the formation of protein aggregates with
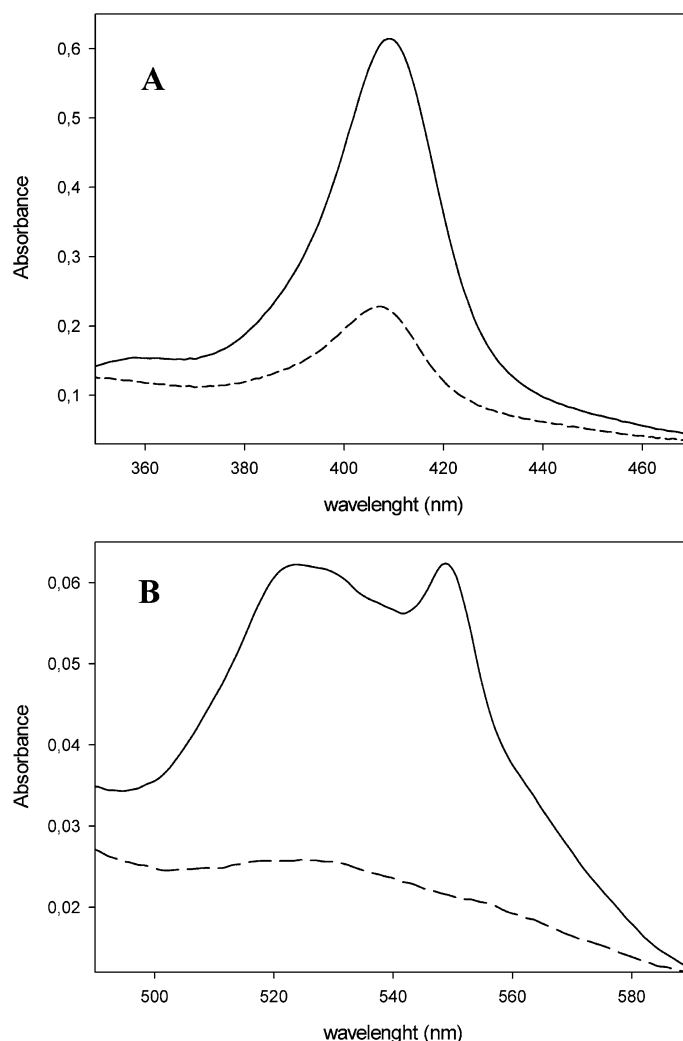
Fig. 2. Absorption spectra of 5 μM bovine heart cytochrome c solutions after incubation for 12 h at 25°C (—), or 75°C (– –). The band at 408 nm correspond to oxidized heme forms (A) and the bands at 519 and 550 nnm to reduced species (B).

amyloid-like properties [28]. In addition, it has been shown that incubation of the apo form of muscle myoglobin, another helical heme protein, at pH 9.0 and 65°C causes the formation of large quantities of fibrillar structures [20]. Under these conditions the native fold of apomyoglobin is, as it happens with apocytochrome c_{552}, substantially destabilized [20,28]. The conformational properties of bovine heart cytochrome c when incubated at 75°C in a pH 9.0 solution for 12 h resemble very much those exhibited by the apo forms of the above mentioned and related heme proteins. Hence, we focused on the possibility that it may also aggregate into amyloid-like structures. We screened for conditions that might promote protein aggregation of bovine cytochrome c and found that aggregation was strongly dependent on protein concentration (data not shown). Slight precipitation of cytochrome c was detected at 90 μM protein concentration at the end of the 12 h incubation period at 75°C. Further characterization of the protein aggregates by electron microscopy revealed the presence of fibrillar structures, which resemble those formed by disease-related proteins (Fig. 3A). Thioflavin-T (Th-T) is an amyloid azo-free diag-
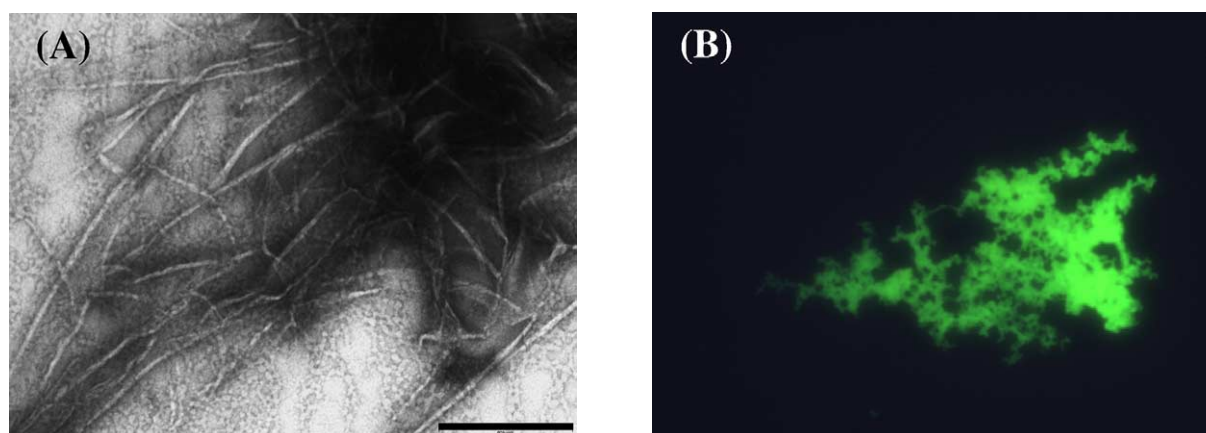
Fig. 3. Features of the fibrils formed by bovine heart cytochrome c after 12 h incubation at 75°C and pH 9.0 at 90 μM protein concentration. (A) Transmission electron micrograph of negatively stained aggregated protein (bar = 500 nm). (B) Thioflavin-T fluorescence of stained amyloid-like material.

nostic dye that by a so far unknown mechanism specifically interacts with the crossed-β-pleated sheet structure common to a variety of amyloid fibrils. Binding of this dye to cytochrome c fibrils was probed by fluorescence microscopy. The areas rich in protein fibrous material appeared stained with Th-T, giving a bright green–yellow fluorescence against a dark background (Fig. 3B), reinforcing the amyloid-like nature of the aggregated protein.

To further characterize the nature of the cytochrome c aggregates we used FTIR spectroscopy. In myoglobin the formation of amyloid-like aggregates resulted in a significant reduction of the α-helix content and new formation of β-sheet structure [20]. To see whether this was also the case for cytochrome c, we recorded the FTIR spectrum of the native and aggregated states in the amide I region. The difference spectrum between both states shows a strong formation of new β-sheet structure with a concomitant loss of α-helix content upon aggregation (Fig. 4). Because native cytochrome c posses only α-helical secondary structure and because amyloid fibrils are always associated with β-sheet structure, the aggregated β-sheets are constructed from residues that form α-helices in the folded protein. Thus, it is clear that to enable fibril formation, these structural elements need to be previously unfolded.

Taken together our data argue that long-lasting incubation of bovine cytochrome c at 75°C in a pH 9.0 solution somehow damages/denatures the heme group in the native protein, resulting in a loss of the cooperative native structure in these conditions. This leads to unfolding of the protein, which backbone adopts chiefly a random coil conformation. Consequently, the polypeptide chain becomes exposed to solvent allowing the establishment of intermolecular interactions, resulting in concentration dependent protein aggregation. At low protein concentration this aggregation occurs in the form of ordered amyloid fibrils of the type formed by disease related proteins. The behaviour of bovine heart cytochrome c is especially interesting because, as it happens with cytochrome c_{552} and muscle myoglobin [28,29], is a helical protein devoid of β-sheet elements in the native state, whereas amyloid fibrils posses mainly β-sheet structure. Besides, predictions of the secondary structure content of bovine cytochrome c clearly show the absence of stretches with β-sheet propensity for this protein sequence (43.5% α-helix and 0% β-sheet are predicted with the PSIPRED algorithm[2]). Thus, bovine cytochrome c constitutes yet another example in which secondary structure propensity and amyloid fibril formation are not related.

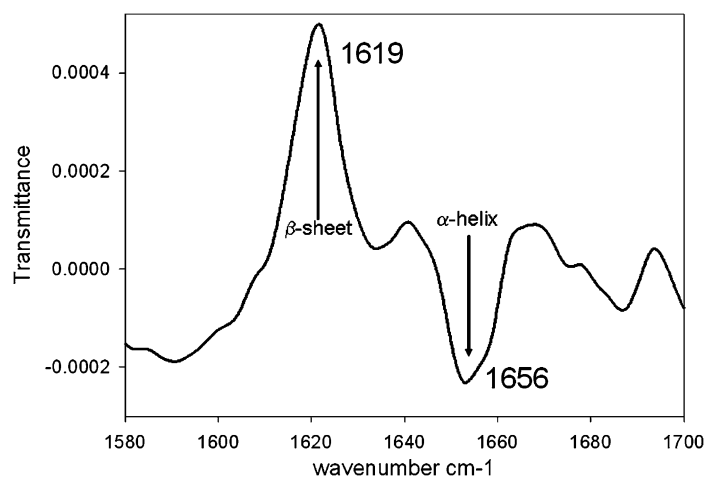[2]PSIPRED at http://www.psipred.net.

Fig. 4. Difference FTIR spectrum in the amide I region between the aggregated and native states of bovine heart cytochrome *c*. Arrows indicate the increase of β-sheet and decrease of α-helix structures upon aggregation.

The present results support the idea that amyloid fibril formation is an intrinsic property of many polypeptide chains with independence of the conformation of their native state [4]. Although the structural, thermodynamic and kinetic factors determining the polymerisation of helical cytochrome *c* into β-sheet rich fibrils should be studied in much more detail before we can understand the rules underlying this self-assembling process, our data suggest that, resembling what happens to myoglobin, amyloid fibril formation occurs for this protein under conditions in which the polypeptide backbone is predominantly unfolded. Thus, it appears that amyloid fibril formation by bovine cytochrome *c* does not require significant population of partially folded intermediates with β-sheet conformation as those reported for other protein models. This behaviour is not exclusive of highly helical proteins, since it is now clear that amyloid fibrils can be formed by very short peptides [29,30] or polyaminoacids [31,32], which neither fold nor populate partially structured states. Hence, it is likely that the presence of unfolded protein regions may be a general requirement for the formation of amyloid fibrils.

The data reported herein show that an intact prosthetic group permits the recovery of the native cytochrome *c* structure after a moderate conformational stress situation, avoiding prolonged exposition of unfolded protein regions to solvent and thus reducing aggregation propensity. This observation provides a possible explanation for the role of covalently linked heme groups in this protein family and supports the suggestion that natural protein sequences have evolved in part to code for structural characteristics other than those included in the native fold, such us avoidance of aggregation.
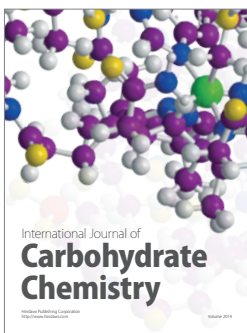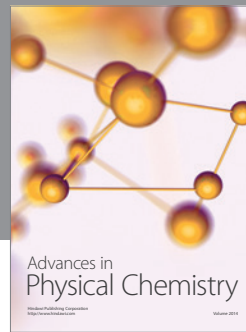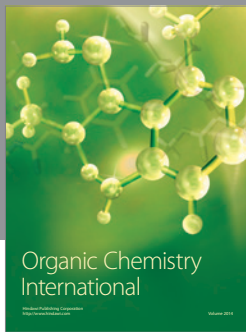
Acknowledgements

References

[1] S.Y. Tan and M.B. Pepys, *Histopathology* **25** (1994), 403–414.
[2] J.W. Kelly, *Curr. Opin. Struct. Biol.* **8** (1998), 101–106.

[3] J.C. Rochet and P.T. Lansbury, Jr., *Curr. Opin. Struct. Biol.* **10** (2000), 60–68.
[4] C.M. Dobson, *Phil. Trans. R. Soc. Lond.* **356** (2001), 133–146.
[5] M. Sunde and C.C. Blake, *Quart. Rev. Biophys.* **31** (1998), 1–39.
[6] J.I. Guijarro, M. Sunde, J.A. Jones, I.D. Campbell and C.M. Dobson, *Proc. Natl. Acad. Sci. USA* **95** (1998), 4224–4228.
[7] S.V. Litvinovich, S.A. Brew, S. Aota, S.K. Akiyama, C. Haudenschild and K.C. Ingham, *J. Mol. Biol.* **280** (1998), 245–258.
[8] F. Chiti, P. Webster, N. Taddei, A. Clark, M. Stefani, G. Ramponi and C.M. Dobson, *Proc. Natl. Acad. Sci. USA* **96** (1999), 3590–3594.
[9] M. Ramirez-Alvarado, J.S. Merkel and L. Regan, *Proc. Natl. Acad. Sci. USA* **97** (2000), 8979–8984.
[10] V. Villegas, J. Zurdo, V.V. Filimonov, F.X. Aviles, C.M. Dobson and L. Serrano, *Protein Sci.* **9** (2000), 1700–1708.
[11] M. Fandrich, M.A. Fletcher and C. Dobson, *Nature* **410** (2001), 165–166.
[12] R.J. Ellis and T.J. Pinheiro, *Nature* **416** (2002), 483–484.
[13] C.M. Dobson, *Trends in Biochemical Sciences* **24** (1999), 329–332.
[14] J.W. Kelly, *Proc. Natl. Acad. Sci. USA* **95** (1998), 930–932.
[15] Z. Lai, W. Colon and J.W. Kelly, *Biochemistry* **35** (1996), 6470–6482.
[16] D.R. Booth, M. Sunde, V. Belloti, C.V. Robinson, W.L. Hutchinson, P.E. Fraser, P.N. Hawkins, C.M. Dobson, S.E. Radford, C.C. Blake and M.B. Pepys, *Nature* **385** (1997), 787–793.
[17] V.N. Uversky, J. Li and A.L. Fink, *J. Biol. Chem.* **276** (2001), 10737–10744.
[18] V.J. McParland, N.M. Kad, A.P. Kalverda, A. Brown, P. Kirwin Jones, M.G. Hunter, M. Sunde and S.E. Radford, *Biochemistry* **39** (2000), 8735–8746.
[19] R. Khurana, J.R. Gilleppie, A. Talapatra, L.J. Minert, C. Jonesiu-Zanetti, I. Millett and A.L. Fink, *Biochemistry* **40** (2001), 3525–3535.
[20] M. Fandrich, V. Forge, K. Buder, M. Kittler, C.M. Dobson and S. Diekmann, *Proc. Natl. Acad. Sci. USA* **100** (2003), 15463–15468.
[21] P.X. Qi, D.L. Di Stefano and A.J. Wand, *Biochemistry* **33** (1994), 64082–6417.
[22] G.W. Bushnell, G.V. Louie and G.D. Brayer, *J. Mol. Biol.* **214** (1990), 585–595.
[23] C.D. Moore and J.T.J. Lecomte, *Biochemistry* **29** (1990), 1984–1989.
[24] Y. Feng and S.G. Sligar, *Biochemistry* **30** (1991), 10150–10155.
[25] M.F. Jeng, S.W. Englander, G.A. Elove, A.J. Wand and H. Roder, *Biochemistry* **29** (1990), 10433–10437.
[26] D. Hamada, M. Hoshino, M. Kataoka, A.L. Fink and Y. Goto, *Biochemistry* **32** (1993), 10351–10358.
[27] Q. Feng, S.G. Sligar and A.J. Wand, *Nat. Struct. Biol.* **1** (1994), 30–35.
[28] T.A. Pertinhez, M. Bouchard, E.J. Tomlinson, R. Wain, S.J. Ferguson, C.M. Dobson and L.J. Smith, *FEBS Let.* **495** (2001), 184–186.
[29] E. Gazit, *Curr. Med. Chem.* **9** (2002), 1725–1735.
[30] M. Lopez De La Paz, K. Goldie, J. Zurdo, E. Lacroix, C.M. Dobson, A. Hoenger and L. Serrano, *Proc. Natl. Acad. Sci. USA* **99** (2002), 16052–16057.
[31] M. Fandrich and C.M. Dobson, *EMBO J.* **21** (2002), 5682–5690.
[32] M.F. Perutz, B.J. Pope, D. Owen, E.E. Wanker and E. Scherzinger, *Proc. Natl. Acad. Sci. USA* **99** (2002), 5596–5600.