Molecular chaperone properties of serum amyloid P component

Alun R. Coker[a], Alan Purvis[a], Douglas Baker[a], Mark B. Pepys[b], Steve P. Wood[a,*]

[a] *Division of Biochemistry and Molecular Biology, School of Biological Sciences, University of Southampton, Bassett Crescent East, Southampton SO16 7PX, UK*

[b] *Centre for Amyloidosis and Acute Phase Proteins, Department of Medicine, Royal Free and University College Medical School, Rowland Hill Street, London NW3 2PF, UK*

Received 11 April 2000

Edited by Jesus Avila

Abstract The selective binding of serum amyloid P component (SAP) to proteins in the pathological amyloid cross-β fold suggests a possible chaperone role. Here we show that human SAP enhances the refolding yield of denatured lactate dehydrogenase and protects against enzyme inactivation during agitation of dilute solutions. These effects are independent of calcium ions and are not inhibited by compounds that block the amyloid recognition site on the B face of SAP, implicating the A face and/or the edges of the SAP pentamer. We discuss the possibility that the chaperone property of SAP, or its failure, may contribute to the pathogenesis of amyloidosis.

Key words: Serum amyloid P component; Amyloidosis; Protein folding; Chaperone

1. Introduction

The mechanisms by which protein molecules attain their biologically active conformations are of great fundamental interest, and recognition of the role of molecular chaperones in these processes within the cell has been a critical contribution to cell biology [1,2]. The recent appreciation that aberrations of protein folding are responsible for a number of human diseases, has focused additional attention in this area [3]. In particular, the propensity for a group of otherwise unrelated, soluble, globular proteins to aggregate and deposit extracellularly as amyloid fibrils is important because amyloidosis is a feature of Alzheimer's disease, type 2 diabetes and the prion diseases, as well as the fatal, acquired and hereditary, systemic amyloidoses [4].

Serum amyloid P component (SAP) is a non-fibrillar plasma glycoprotein that undergoes specific calcium dependent binding to all types of amyloid fibrils, even though it shows no affinity for the same proteins in their native state [5]. Binding of SAP stabilizes amyloid fibrils in vitro [6] and amyloid deposition is reduced and delayed in SAP knockout mice [7]. This capacity of SAP to recognize misfolded proteins of the fibrils resembles the behavior of molecular chaperones and suggested to us that SAP may affect protein folding.

The SAP molecule is an oligomer of five identical subunits non-covalently associated in a disc-like particle with cyclic pentameric symmetry, diameter 100 Å, and depth 35 Å. The atomic resolution three-dimensional (3D) structure of human SAP reveals a β-sheet sandwich fold for the protomer, with flattened jelly roll topology, and with the two calcium ions, required for ligand binding, bound 4 Å apart by loop side chains that congregate on one face of the sheet [8]. One surface of the pentamer carries the five double calcium sites on the binding, B, face, while the other, A, face is characterized by five α-helices. The calcium dependence of SAP binding to amyloid fibrils [9] and the fact that the cyclic pyruvate acetal of galactose, 4,6-*O*-(1-carboxyethylidene)-β-D-galactopyranoside (MOβDG), that binds into the double calcium site displaces SAP from amyloid [10], indicate that this is the amyloid recognition site.

2. Materials and methods

SAP and the closely related pentraxin, C-reactive protein (CRP), were purified as described previously [11,12]. SAP was stored at 56 mg/ml in 10 mM Tris/HCl buffer pH 8.0 containing 140 mM sodium chloride and 10 mM EDTA. CRP was stored at 4.4 mg/ml in 10 mM Tris/HCl buffer pH 8.0 containing 140 mM sodium chloride and 2 mM calcium chloride. Lactate dehydrogenase (LDH) from *Bacillus stearothermophilus* was purchased from Porton Products, or Sigma, UK. SAP was immobilized on cyanogen bromide activated Sepharose as described previously [13] to provide ~1 mg SAP/ml of settled matrix. LDH was denatured by incubation for 2 h at room temperature in 4.2 M guanidine hydrochloride in 50 mM MOPS/KOH, 150 mM NaCl, 10 mM β-mercaptoethanol, pH 7.0. Refolding was initiated at 25°C by a 200-fold dilution of the denatured LDH to a final protomer concentration of between 40 and 70 nM in the same buffer but without guanidine and β-mercaptoethanol. The effects were tested of the presence during refolding of SAP, or CRP, with or without calcium ions, MOβDG, or dAMP. In pilot experiments the refolding cocktail included the LDH assay substrates and activity was monitored continuously. In follow-up experiments LDH activity was assayed at discrete time intervals by diluting 900 μl aliquots of the refolding mixture with 100 μl of MOPS buffer including 100 mM pyruvate and 2 mM β-NADH, and monitoring absorption at 340 nm for 60 s at 25°C. Isolated human SAP rapidly autoaggregates in the presence of calcium ions and this was monitored by light scattering at 500 nm excitation and emission. The concentration of residual soluble SAP was determined by absorbance at 280 nm (E(1%, 1 cm) = 17.1).

To induce inactivation by agitation, 500 μl of 50 nM LDH in MOPS/NaCl buffer pH 7.0, in a 1.5 ml plastic microfuge tube, were rotated on a blood tube mixer at 20 rpm. Aliquots (10 μl) were removed at intervals for estimation of remaining LDH activity as described above. SAP, or CRP, with or without calcium ions and MOβDG, were included in the sample at the start of mixing.

3. Results and discussion

LDH from *B. stearothermophilus* is denatured by 4 M gua-

*Corresponding author. Fax: (44)-1703-594459.
E-mail: steve@soton.ac.uk

Abbreviations: CRP, C-reactive protein; LDH, lactate dehydrogenase; MOβDG, 4,6-*O*-(1-carboxyethylidene)-β-D-galactopyranoside; SAP, serum amyloid P component

PII: S0014-5793(00)01530-1

nidine hydrochloride to a state showing no secondary structure [14]. Only 3–6% of active dimeric enzyme was slowly regained by diluting out the denaturant as described. Recovery rose to about 8% if β-mercaptoethanol was included in the refolding step, but it was usually omitted to avoid reduction of the single intrachain disulphide bridge within each SAP protomer. Addition of SAP to the refolding solution markedly enhanced the yield of active LDH. A typical experiment, shown in Fig. 1, included 400 nM SAP (with respect to pentamer), 3 mM calcium ions and 40 nM LDH. In the same conditions, inclusion of 2 mM MOβDG, which blocks the calcium dependent ligand binding site of SAP and also the calcium dependent aggregation of SAP, did not reduce the SAP induced enhancement of LDH refolding and in fact increased the yield.

No change in the refolding yield was observed when CRP was included in place of SAP at 0.4 or 2.0 μM with respect to pentamers, representing a 10–40-fold excess over LDH subunits (Fig. 1). This powerful control demonstrates the highly specific nature of SAP enhanced refolding of LDH, since CRP

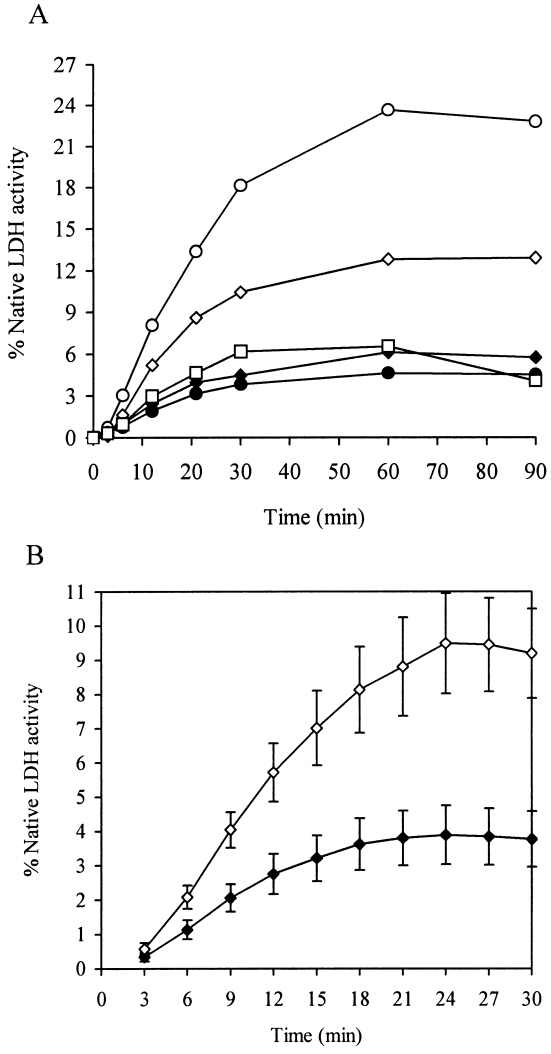

Fig. 1. Reactivation of chemically denatured LDH (40 nM) in the absence (♦) or presence (◇) of 400 nM SAP, 400 nM CRP (□), 2 mM MOβDG (●), or SAP and MOβDG (○) in 50 mM MOPS–KOH pH 7.0 buffer containing (A) 3 mM $CaCl_2$ or (B) 150 mM NaCl and 7 mM $CaCl_2$.

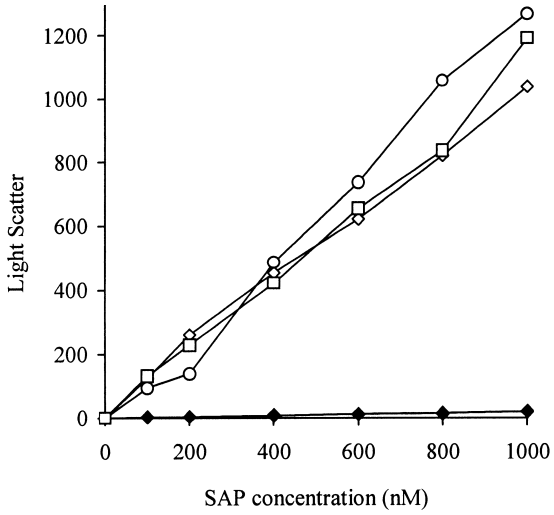

Fig. 2. Concentration dependence of SAP aggregation induced by calcium ions measured by light scattering at an excitation and emission wavelength of 500 nm, at room temperature in 50 mM MOPS–KOH pH 7.0 with 2 mM EDTA (♦), 3 mM $CaCl_2$ (◇), 7 mM $CaCl_2$ (□), or 3 mM $CaCl_2$ and 140 mM NaCl (○).

and SAP share 52% sequence identity, a common subunit fold and pentameric oligomeric structure and both bind calcium ions in a similar way, although in contrast to human SAP, CRP does not autoaggregate in the presence of calcium [15].

Increasing the concentration of SAP to 2 μM increased the refolding yield but this did not exceed the value of ∼25% shown with MOβDG in Fig. 1. The effective concentration and form of SAP in these experiments was difficult to quantify because of the severe calcium dependent aggregation of SAP. Light scattering measurements showed that even at SAP concentrations as low as 100 nM some aggregation occurred immediately after mixing with 3 mM calcium ions (Fig. 2). At 2 μM SAP we estimate that only 10% remained in solution. These results are likely to change with time after mixing and suggest that the protein does not exhibit a simple solubility limit.

The enhanced refolding of LDH was also observed in the absence of calcium ions (Fig. 3) where SAP is highly soluble, but even at SAP concentrations as high as 4 μM the maximal enhancement (∼25%) was never exceeded. SAP immobilized by covalent attachment to Sepharose beads, so that calcium induced aggregation was prevented, also increased the yield of refolding LDH in the presence of calcium ions.

The calcium induced aggregation of SAP is apparently due to binding of carboxylate ligands on the A face by the calcium dependent ligand binding sites on the B face, as we have previously reported [5,16], leading to A:B face stacking of pentamers. The MOβDG concentration used here completely inhibits aggregation (K_d ∼0.1 mM), probably by occupying these sites, and the SAP remains in solution as free individual pentamers [17]. The enhancement of SAP induced refolding yield produced in some experiments by addition of MOβDG (Fig. 1A) presumably resulted from increased solubility of SAP with more of it therefore readily available for interaction with LDH folding intermediates. Although SAP clearly promoted refolding in the absence of calcium, indicating that the B face calcium dependent ligand binding sites are apparently not involved, it is conceivable that an inhibitory action of

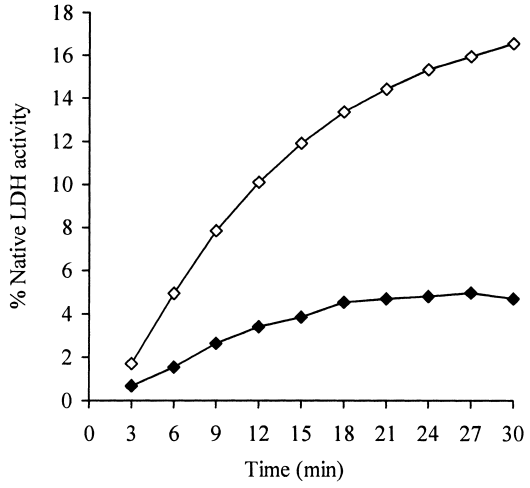

Fig. 3. Reactivation of chemically denatured LDH (40 nM) in the presence (◇) or absence (◆) of 400 nM SAP in 50 mM MOPS–KOH pH 7.0.

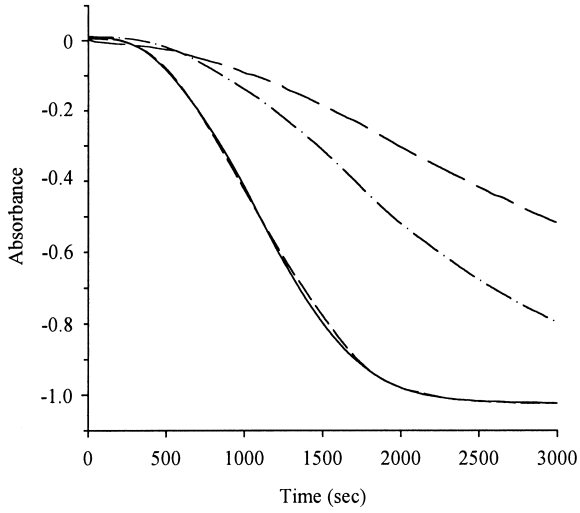

Fig. 4. Reactivation of chemically denatured LDH (70 nM) in the absence (dashed line, long dashes) or presence (solid line) of 4 μM SAP, 5 mM dAMP (dot dashed line), or SAP and dAMP (dashed line, short dashes) in 50 mM MOPS–KOH pH 7.0, 150 mM NaCl, 7 mM $CaCl_2$ buffer. Activity regain was monitored continuously by absorbance change at 340 nm at room temperature.

MOβDG could be masked by its capacity to increase the concentration of soluble SAP in the presence of calcium. However, if the calcium site in SAP did contribute to the refolding enhancement, and the K_d of the SAP–MOβDG interaction was in the region of 0.1 mM, then in the mixture of 400 nM SAP/3 mM calcium/2 mM MOβDG (Fig. 1) the effect of the SAP calcium site on LDH refolding would be attributable to just 5% of the SAP (20 nM). It is unlikely that the calcium site could have such potency as we have determined that the 200 nM soluble SAP that remains on mixing 2 μM SAP with 3 mM calcium is less effective in enhancing LDH refolding. Furthermore we found that SAP in the absence of calcium enhanced the refolding yield of LDH to a similar extent to calcified SAP at the same concentration in the presence of MOβDG. The effect of MOβDG on SAP solubility therefore seems to provide a good explanation for the apparent increase in activity, and the increased stability of SAP conferred by the bound ligand [6] could provide additional benefit to the complex.

Calcium dependent aggregation of SAP is also prevented by the ligand, dAMP, which, at appropriate molar excess is bound by SAP and then induces formation of the B:B face decamers that we have previously characterized [18]. Under these conditions, both with 4 μM SAP, 5 mM dAMP and 7 mM calcium in the refolding mixture together with LDH and substrates (Fig. 4), and when activity was assayed separately after refolding (Table 1), there was no difference in enhancement of refolding compared to control mixtures without dAMP.

Table 1
Effect of dAMP on reactivation of LDH after guanidine dilution in presence of SAP

Reagents present during refolding	% Initial LDH activity (mean (S.D.))
LDH alone	2.28 (0.78)
LDH+SAP	8.61 (0.45)
LDH+SAP+dAMP	9.33 (0.21)
LDH+dAMP	2.24 (0.56)

LDH (50 mM) was refolded from guanidine in the presence of 3 mM calcium, 500 mM SAP and 5 mM dAMP and restored activity determined. $n = 3$.

Gentle agitation of LDH at 50 nM in the presence of 3 mM calcium ions for 30 min eliminated about 75% of the enzyme activity (Table 2). Similar but less dramatic inactivation also took place in gently stirred solutions. It is likely that physical forces such as shear at the air/liquid or liquid/container interface, foaming or cavitation are responsible for this loss of activity. Inclusion of β-mercaptoethanol does not protect LDH activity in these conditions, suggesting that cysteine oxidation is not involved. However, in the presence of ~4 μM immobilized SAP or 500 nM free SAP (with respect to pentamer) only 25% of the LDH activity was lost irrespective of the presence or absence of 2 mM MOβDG.

Molecular chaperones function by binding to aggregation-prone folding intermediates and we presume that SAP enhances the efficiency of LDH refolding by similarly interacting with intermediates on the refolding landscape to increase the flux through productive routes [19]. The effect of SAP is saturable, with a maximum of 25% regain of activity in the present conditions. This might reflect differential rates of partitioning of intermediates between states that interact with SAP and others. A small proportion clearly proceed directly to the native state but the majority go to an inactive misfolded product. SAP might bind to a misfolded state and enhance LDH folding by removing species capable of forming an unproductive dimer or act in a more direct way by enabling the formation of productive species. The parallel we drew between

Table 2
Effect of immobilized SAP on loss of LDH activity during mixing

	Residual LDH activity
LDH without mixing	96.8
LDH alone	26.0
LDH+Sepharose-SAP	74.9
LDH+Sepharose-SAP+MOβDG	65.4
LDH+MOβDG	26.9

LDH (50 mM) was mixed for 30 min in the presence of 3 mM calcium, ~4 μM SAP immobilized covalently on Sepharose and 2 mM MOβDG, then assayed for residual activity.

recognition of the protein misfold of amyloid and protein folding intermediates would favor models in which the SAP interacts with misfolding species. However, we have shown that the site on SAP most likely to be involved in amyloid recognition, can be blocked by MOβDG without any inhibitory effect on the folding enhancement of LDH. The site can also be blocked by formation of B:B face decamers by interaction with dAMP without effect on the LDH refolding phenomenon. Furthermore, the refolding reaction can proceed in the absence of calcium, which is absolutely required for amyloid binding. We therefore conclude that this site is not involved in the folding enhancement process, and presume that the A face and/or 'edge' regions of the pentameric disc are involved.

It has lately become clear that even proteins unrelated to in vivo amyloidogenesis can be induced by suitable denaturation protocols in vitro to adopt the amyloid cross-β fold and to aggregate as typical amyloid fibrils [20–23]. This highlights the mysterious fact that only about 20 diverse and unrelated proteins are known to form amyloid fibrils in vivo. Here we demonstrate that for LDH only gentle agitation is sufficient to initiate unfolding reactions that must be a general feature in the production of amyloidogenic precursors. Although LDH is not itself an amyloid protein, our observations are reminiscent of results obtained with Aβ1–42, the major amyloid fibril protein in Alzheimer's disease. This spontaneously forms amyloid fibers in physiological, non-denaturing, solvent conditions in vitro. SAP binds the mature fibers in a calcium dependent reaction but SAP is also reported to retard fiber formation in a calcium independent reaction [24]. Since binding of SAP stabilizes amyloid fibrils in vitro [6] and participates in pathogenesis of amyloidosis in vivo [7], we wonder whether display by misfolding amyloidogenic precursor proteins of ligands recognized by the calcium dependent binding site of SAP may contribute to their clinical amyloidogenicity. In contrast, other proteins, not recognized by SAP in this way, may undergo productive folding interactions with SAP, or at least not be trapped or enabled by calcium dependent SAP binding to persist in the pathological conformation that causes amyloid disease.

An alternative scenario is suggested by the protection conferred by SAP against agitation induced denaturation of LDH. Here there is gradual production of misfolded species and mere binding of SAP to these would not be expected to prevent overall loss of enzyme activity unless such loss depended on a nucleated aggregation process, as occurs in experimental amyloid fibrillogenesis in vitro [21]. SAP may therefore have a surveillance role in vivo, binding to misfolded species and preventing the seeding of larger aggregates. The primary driving force towards clinical amyloidosis would then be the inherent instability that is now recognized as a common feature of amyloidogenic precursor proteins [25,26]. This may first overwhelm the normal productive folding role of SAP, and subsequent calcium dependent binding of SAP to mature fibrils could then stabilize and protect them from degradation [6]. Whatever the underlying mechanisms, the present observations support our novel approach to therapy of amyloidosis, which is about to be evaluated clinically, using a drug that inhibits SAP binding and dissociates it from the deposits [27].

Acknowledgements: This work was supported in part by MRC Programme Grant G7900510 to M.B.P.

References

[1] Ellis, R.J. (1994) Curr. Opin. Struct. Biol. 4, 117–122.
[2] Hartl, F.U. (1996) Nature 381, 571–596.
[3] Pepys, M.B. (2000) in: Proceedings of Protein Misfolding and Disease (Zeki, S., Ed.), Philos. Trans. R. Soc. Lond. B. Biol. Sci., The Royal Society, London, in press.
[4] Pepys, M.B. (1994) in: Samter's Immunologic Diseases, 5th Edn. (Frank, M.M., Austen, K.F., Claman, H.N. and Unanue, E.R., Eds.), pp. 637–655, Little, Brown and Company, Boston.
[5] Pepys, M.B., Booth, D.R., Hutchinson, W.L., Gallimore, J.R., Collins, P.M. and Hohenester, E. (1997) Int. J. Exp. Clin. Invest. 4, 274–295.
[6] Tennent, G.A., Lovat, L.B. and Pepys, M.B. (1995) Proc. Natl. Acad. Sci. USA 92, 4299–4303.
[7] Botto, M., Hawkins, P.N., Bickerstaff, M.C.M., Herbert, J., Bygrave, A.E., McBride, A., Hutchinson, W.L., Tennent, G.A., Walport, M.J. and Pepys, M.B. (1997) Nat. Med. 3, 855–859.
[8] Emsley, J., White, H.E., O'Hara, B.P., Oliva, G., Srinivasan, N., Tickle, I.J., Blundell, T.L., Pepys, M.B. and Wood, S.P. (1994) Nature 367, 338–345.
[9] Pepys, M.B., Dyck, R.F., de Beer, F.C., Skinner, M. and Cohen, A.S. (1979) Clin. Exp. Immunol. 38, 284–293.
[10] Hind, C.R.K., Collins, P.M., Caspi, D., Baltz, M.L. and Pepys, M.B. (1984) Lancet ii, 376–378.
[11] Hawkins, P.N., Tennent, G.A., Woo, P. and Pepys, M.B. (1991) Clin. Exp. Immunol. 84, 308–316.
[12] Vigushin, D.M., Pepys, M.B. and Hawkins, P.N. (1993) J. Clin. Invest. 91, 1351–1357.
[13] Pepys, M.B., de Beer, F.C., Dyck, R.F., Hind, C., Lantain, I., Fagan, E.A., Maton, P.N., Starke, I., Fox, K., Allan, R., Hodgson, H., Chadwick, V.S., Hughes, G.R.V., Goldinam, J., Catolvsky, D., Coalton, D., Krikler, D., Masen, A., Mallya, R.K., Demy, H., Hamilton, E.D.B. and Mace, D.E.N. (1982) Ann. N.Y. Acad. Sci. 389, 459–460.
[14] Smith, C.J., Clarke, A.R., Chia, W.N., Irons, L.I., Atkinson, T. and Holbrook, J.J. (1991) Biochemistry 30, 1028–1036.
[15] Baltz, M.L., de Beer, F.C., Feinstein, A. and Pepys, M.B. (1982) Biochim. Biophys. Acta 701, 229–236.
[16] Booth, D.R., Gallimore, J.R., Hutchinson, W.L., Hohenester, E., Thompson, D., Wood, S. and Pepys, M.B. (1999) in: Amyloid and Amyloidosis 1998 (Kyle, R.A. and Gertz, M.A., Eds.), pp. 23–25, Parthenon Publishing, Pearl River, NY.
[17] Hutchinson, W.L., Hohenester, E., Perkins, S.J., Herbert, J. and Pepys, M.B. (1999) in: Amyloid and Amyloidosis 1998 (Kyle, R.A. and Gertz, M.A., Eds.), pp. 32–34, Parthenon Publishing, Pearl River, NY.
[18] Hohenester, E., Hutchinson, W.L., Pepys, M.B. and Wood, S.P. (1997) J. Mol. Biol. 269, 570–578.
[19] Dill, K.A. and Chan, H.S. (1997) Nat. Struct. Biol. 4, 10–19.
[20] Litvinovich, S.V., Brew, S.A., Aota, S., Akiyama, S.K., Haudenschild, C. and Ingham, K.C. (1998) J. Mol. Biol. 280, 245–258.
[21] Canet, D., Sunde, M., Last, A.M., Miranker, A., Spencer, A., Robinson, C.V. and Dobson, D.M. (1999) Biochemistry 38, 6419–6427.
[22] Chiti, F., Webster, P., Taddei, N., Clark, A., Stepani, M., Ramponi, G. and Dobson, C.M. (1999) Proc. Natl. Acad. Sci. USA 96, 3590–3594.
[23] Jiménez, J.L., Guijarro, J.I., Orlova, E., Zurdo, J., Dobson, C.M., Sunde, M. and Saibil, H.R. (1999) EMBO J. 18, 815–821.
[24] Janciauskiene, S., García de Frutos, P., Carlemalm, E., Dahlbäck, B. and Eriksson, S. (1995) J. Biol. Chem. 270, 26041–26044.
[25] Booth, D.R., Sunde, M., Bellotti, V., Robinson, C.V., Hutchinson, W.L., Fraser, P.E., Hawkins, P.N., Dobson, C.M., Radford, S.E., Blake, C.C.F. and Pepys, M.B. (1997) Nature 385, 787–793.
[26] Kelly, J.W. (1996) Curr. Opin. Struct. Biol. 6, 11–17.
[27] Pepys, M.B. (1999) in: Horizons in Medicine (Williams, G., Eds.), Vol. 10, pp. 397–414, Royal College of Physicians, London.