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A Molecular Model for Self-Assembly of Amyloid Fibrils: Immunoglobulin Light Chains†

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The formation and pathological deposition of amyloid fibrils are defining features of many acquired and inherited disorders, including primary or light-chain-associated amyloidosis, Alzheimer's disease, and adult-onset diabetes. No pharmacological methods exist to block this process or to effect the removal of fibrils from tissue, and thus, little can be done to prevent organ failure and ultimate death that result from deposition of amyloid. Knowledge of the pathogenesis, treatment, or prevention of these presently incurable diseases is limited due to the relative paucity of information regarding the biophysical basis of amyloid formation. Antibody light chains of different amino acid sequence show differential amyloid-forming tendencies and, as such, can provide insight into the structural organization of amyloid fibrils as well as into basic mechanisms of protein self-assembly. We have compared primary structures of 180 human monoclonal light chains and have identified particular residues and positions within the variable domain that differentiate amyloid- from nonamyloid-associated proteins. We propose a molecular model that accounts for amyloid formation by antibody light chains and might also have implications for other forms of amyloidosis.

The term amyloid refers to fibrillar tissue deposits formed by many types of native and/or mutated proteins or their proteolytically processed derivatives (Sipe, 1992; Kelly & Lansbury, 1994; Pascali, 1995). Despite the diverse nature of the precursor proteins involved in amyloid formation—e.g., immunoglobulin light chains, β-protein, islet amyloid polypeptide, β_2-microglobulin, transthyretin, etc.—all amyloid fibrils have characteristic physicochemical, tinctorial, and ultrastructural features. Regardless of the nature of the protein constituent, all forms of amyloid are stable assemblies mediated by noncovalent interactions between subunits forming nonbranching fibrils having diameters of 5−10 nm, are composed of a crossed β-pleated structure with alignment of the β-sheets parallel to the fibril axis, and exhibit green birefringence after Congo red staining. On the basis of these common features, definition of the molecular mechanisms that account for formation of amyloid, regardless of protein composition, will contribute to the general understanding of amyloid pathogenesis and treatment. Recent evidence suggests that prevention of fibril formation could significantly decrease cellular toxicity in both Alzheimer's disease and adult-onset diabetes (Lorenzo et al., 1994; Lorenzo & Yankner, 1994).

The primary structures of more than 50 κ and λ amyloid fibrils and amyloid-associated antibody light chains have been determined. Typically, each newly described sequence of the ~110 residue variable domain (V_L) reveals one or more amino acids previously unobserved at a particular

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position in other light chains, including other amyloidogenic light chains. However, the amino acid variations that distinguish nonamyloidogenic light chains from homologous polypeptides that are capable of forming pathogenic amyloid deposits have not been identified. This is in contrast to the obvious causative role played by the substitution of valine for glutamic acid at position 6 in the β-subunit of hemoglobin which imparts upon the deoxygenated tetramer the ability to form the extended polymers associated with sickle cell anemia. Light-chain amyloid is in this way distinct from the familial amyloids, which define a limited set of variant transthyretins, each of which differs from normal transthyretin at a single site. Unlike hemoglobin or transthyretin, thousands of immunoglobulin light chains of different amino acid sequence are produced by each individual. As a consequence, the extensive variability in light-chain primary structures among monoclonal light chains obtained from different patients obscures the identity of the residue(s) responsible for amyloid formation.

However, this same diversity of amyloidogenic light-chain sequences, in the context of a well-established three-dimensional structure, provides a valuable system in which to develop models to describe the architecture of fibril assembly. Because fibrils are not amenable for study by crystallography or high-resolution fiber diffraction, model building is the primary approach currently available by which to deduce the structural basis of fibril assembly and stability. A central issue is whether the precursor for amyloid formation is the native light-chain structure or a folding intermediate or an unfolded state of unknown conformation (Kelly & Lansbury, 1994; Hurle et al., 1994).

In addition to its disease-specific protein component, amyloid fibrils invariably contain other factors such as glycosaminoglycans and P component whose contributions to amyloid pathogenesis have not been established. In the case of light-chain amyloidosis, evidence that the immunoglobulin protein itself is responsible for the observed pathology was demonstrated in studies in which mice injected with monoclonal light chains, obtained from patients with amyloidosis, formed fibrillar deposits. In contrast, injection of nonamyloid-associated proteins failed to produce amyloid-related pathology (Solomon et al., 1991, 1992).

In an effort to determine the molecular basis of light-chain amyloidogenicity, we compared the V_L amino acid sequences of amyloid- and nonamyloid-associated proteins. We have identified residues located at specific positions within the V_L that differentiate amyloid from nonamyloid light chains. The locations and orientations of these sites would permit the noncovalent interactions necessary for polymerization of V_L domains into amyloid fibrils. These observations have allowed us to develop a three-step model for light-chain amyloid formation that could provide guidance for the identification of therapeutic agents that inhibit or prevent amyloidogenesis by light chains.

Primary Structural Correlates of Light-Chain Amyloid Formation. We have analyzed the amino acid sequences of 180 human monoclonal κ- and λ-type proteins, 52 of which were obtained from patients with known amyloidosis. The reference set consisted of all other human plasma cell dyscrasia related light chains, which may include a limited number of undocumented amyloidogenic proteins. Results of position-by-position comparison of the frequencies of particular V_L amino acid residues in amyloid-associated versus nonamyloid primary structures are provided in Table 1. Particular residues at a number of positions in both κ and λ light chains were identified as preferentially associated with amyloid formation. Potentially significant residues that have been observed fewer than three times were ignored. Using a standard difference of proportion test (Walpole & Meyers, 1978), the frequencies of the identified amino acid in the two populations were different at significance levels ranging from $P < 0.05$ to $P < 0.0005$. Because the likely presence of amyloidogenic sequences in the control population may inflate the apparent frequencies in the reference data, these significance levels are conservative estimates. Significant differences were found in both complementarity determining regions (CDRs) and the more conserved framework regions (FRs).

Table 1: Amyloid-Associated Light-Chain Residues

	κ			λ		
position[a]	res	freq[b]	$P <$[c]	res	freq	$P <$
13				Ala	0.178 (0.054)	0.040
20	Ile	0.167 (0.035)	0.025			
31	Asp	0.333 (0.035)	0.0005	Asn	0.571 (0.304)	0.020
32				Thr	0.208 (0.036)	0.020
40	pho[d]	0.167 (0.017)	0.010			
45	Asn	0.208 (0.053)	0.025			
49	Phe	0.167 (0.035)	0.025			
50	Asp	0.417 (0.176)	0.025			
51				Asn	0.321 (0.107)	0.010
52				Asp	0.214 (0.071)	0.040
55	Gln	0.375 (0.158)	0.025			
60				Asn	0.142 (0.035)	0.050
70	His	0.125 (0.017)	0.025	Ser	0.429 (0.232)	0.040
74				Gly	0.143 (0.018)	0.020
89				Gly	0.143 (0.018)	0.020
90				Thr	0.286 (0.107)	0.025
92				Thr	0.143 (0.036)	0.050
93	Asn	0.333 (0.158)	0.025			
	Gly	0.125 (0.000)	0.005			
96	Pro	0.125 (0.017)	0.025	Pro	0.214 (0.089)	0.070[e]
	Val	0.125 (0.000)	0.005			

[a] Position as designated by the Kabat–Wu numbering system (Johnson et al., 1995); CDR positions are in bold. [b] Frequency of appearance in 24 κ or 28 λ amyloid-associated proteins; frequency of appearance in 57 κ or 56 λ control proteins shown in parentheses. Residues were observed in at least three amyloidogenic light chains and have frequencies of appearance that differ from frequency in the control population at a significance level of at least $P < 0.05$. Light chains of the λVI subgroup are excluded due to invariant amyloid formation tendency. [c] Significance level as determined by the difference of proportion test. [d] pho: any hydrophobic residue. [e] Included on the basis of significance of Pro at position 96 in κ light chains.

The amyloid-associated residues were, in some cases, germline-encoded and, in other cases, resulted from somatic mutation. For instance, Asp50, located in CDR2, is encoded by the O18–O8 and L18 κI germline genes (Klein et al., 1993) and was found in 10 of 24 κ amyloid proteins, a frequency that differed from the control set ($P < 0.025$). In contrast, Asp31, an acidic CDR1 residue found in 7 out of 18 κI amyloid light chains resulted from somatic mutation and is highly associated with amyloidogenesis ($P < 0.0005$). Notably, two λ proteins that had an Asp residue at position 31 also formed amyloid. In FR2, the basic residue generally found at position 45 in control proteins was replaced with a neutral Asn; at position 20, the germline-encoded Thr or Ser was substituted by a hydrophobic Ile residue. Additionally, replacement of the highly conserved Pro at position 40 in both κ and λ light chains appeared significant. As previously

discussed, the presence of a non-proline hydrophobic residue at this position is invariantly associated with amyloid formation (Wilkins-Stevens et al., 1995).

For both κ and λ light chains, the majority of the amyloid-associated positions are distributed along the surface of the light chain involved in the binding of antigen. The side chains extend into the solvent and are accessible for interaction with other molecules in solution. Thus, the amino acids preferentially found among amyloid-associated proteins are not involved in the internal packing of the V_L domain and, nominally, should not dramatically affect molecular stability.

Molecular Mechanism for Amyloid-Related Light Chain Polymerization. On the basis of the identification of primary structural features that are shared among amyloid-associated light chains, we propose a three-step molecular mechanism by which these proteins form amyloid fibrils. These steps involve (1) association of two native V_L domains to form a noncovalent dimer, (2) stacking of dimers to form filamentous noncovalent polymers, and (3) packing of filaments to assemble stable fibrils.

Step 1: Dimer Formation. Light-chain dimers assemble as symmetric complexes that emulate the structure of the Fab of an antibody molecule (Epp et al., 1974). Dimerization equilibrium constants of closely homologous light chains have been found to range from $<10^4$ to $>10^6$ M^{-1} (Stevens et al., 1980; Kolmar et al., 1994; Wilkins-Stevens et al., 1995), with much of the variation apparently attributable to the chemical nature of various CDR residues.

Step 2: Dimer−Dimer Interaction. The basis of polymerization of certain light chains into tetramers or higher order complexes (Stevens et al., 1980; Myatt et al., 1994) has not been established. We propose that initially a proamyloid filament is formed by head-to-tail interactions between V_L dimers related by a rotation of ~90° around the 2-fold axis. Our finding that acidic residues in CDR1 and CDR2 (Asp31, Asp50) are found in amyloid-associated light chains is correlated with this interaction. Among κ proteins, FR segments located distally from the CDR loops tend to be basic with lysines typically found at positions 39, 42, 103, and 107 while arginines are found at positions 18 and 108. In the symmetric V_L dimer, the separations between the two residue 50 side chains and the two residue 42 side chains are approximately equal. When two V_L dimers are stacked on each other following an ~90° rotation about the local 2-fold axis, oppositely charged side chains at positions 42 and 50 would be able to form salt bridges. Due to the symmetry of the interacting dimers the resulting dual salt bridges would contribute significant free energy to V_L polymerization. In some amyloid-associated light chains, e.g., protein Col (Liepnieks et al., 1991), these interactions could be maintained in salt bridges of inverted polarity.

The $V_{\kappa I}$ subgroup encoded by the L12 germline gene (Klein et al., 1993) is characterized by lysyl residues at both position 42 and position 50. Due to the resulting juxtaposition of two basic residues, our model would predict light chains encoded by this gene would be underrepresented among κI amyloid proteins. In fact, of 18 amyloidogenic κI proteins, only one is apparently derived from the L12 gene as compared to 10 of the 37 nonamyloid κI proteins ($P < 0.03$).

Formation of hypothetical proamyloid filament assemblies as a result of salt bridges between Asp50 in one V_L dimer and Lys42 residues in a second is illustrated in Figure 1. Polymer formation can also result from other types of interactions; for example, the location of Asp31 may allow bonding with Lys42 or the highly conserved Lys103 residue. Although the V_L dimers of many κI proteins can self-associate through salt-bridge interactions, light chains of other κ (as well as λ proteins) can initiate this process *via* hydrogen bonds or van der Waals contacts. Such head-to-tail binding defines a polymerization mechanism that may generate filaments of indefinite length; this process is theoretically limited only by interaction affinity and dimer concentration. The free energy contribution of each amino acid side chain is functionally "doubled" due to the 2-fold symmetry of the interacting sites. The involvement of the structurally heterogeneous CDRs in light-chain aggregation provides a mechanism for idiosyncratic variations in polymerization as well as dimerization properties.

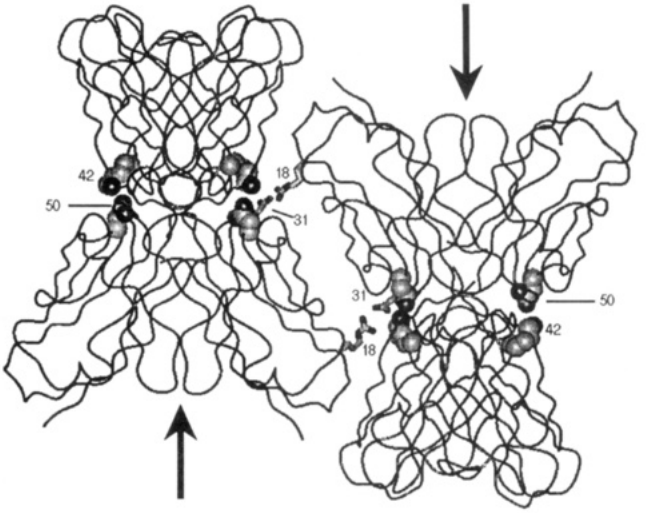

FIGURE 1: Representation of the longitudinal and lateral interactions between light-chain (variable domain) dimers in the proposed mechanism of light-chain fibril formation. Four V_L dimers are shown; the backbone structure is that of protein Rei (Brookhaven data base entry 1REI) in which Glu50 and Lys31 were each replaced by Asp. The two interacting dimers in the leftmost strand are arranged such that CDR loops are at the top of the dimer; the rightmost strand is oriented in an antiparallel manner. In this complex, Asp residues at position 50 are able to form intrafilament salt bridges with lysyl residues at position 42 (solid sphere representations); Asp residues at position 31 may participate in interfilament salt bridges with arginyl residues at position 18 (stick representations). Arrows represent filament orientations from the C-terminus to N-terminus.

Step 3: Fibril Formation. We propose that the assembly of the completed amyloid fibril is accomplished by lateral interactions of concurrently formed proamyloid filaments. As shown in Figure 1, antiparallel docking of two proamyloid filaments is facilitated by the convoluted surface of the filament resulting from the axial rotation of each successive dimer. The docking of two filaments creates an assembly of diameter ~10 nm. Fibril stability is enhanced through the multiplicity of intra- and interfilament interactions. For a monomeric constituent to be released from the assembled fibril, it must simultaneously dissociate at several interfaces, suggesting that fibril stability arises from functionally cooperative multiple interactions. Although we describe fibril formation as three stepwise processes, it is likely that all occur concurrently. The formation of dimers, as well as their linear and lateral aggregation, is likely to be a reversible process until a minimal closed assembly has formed that can

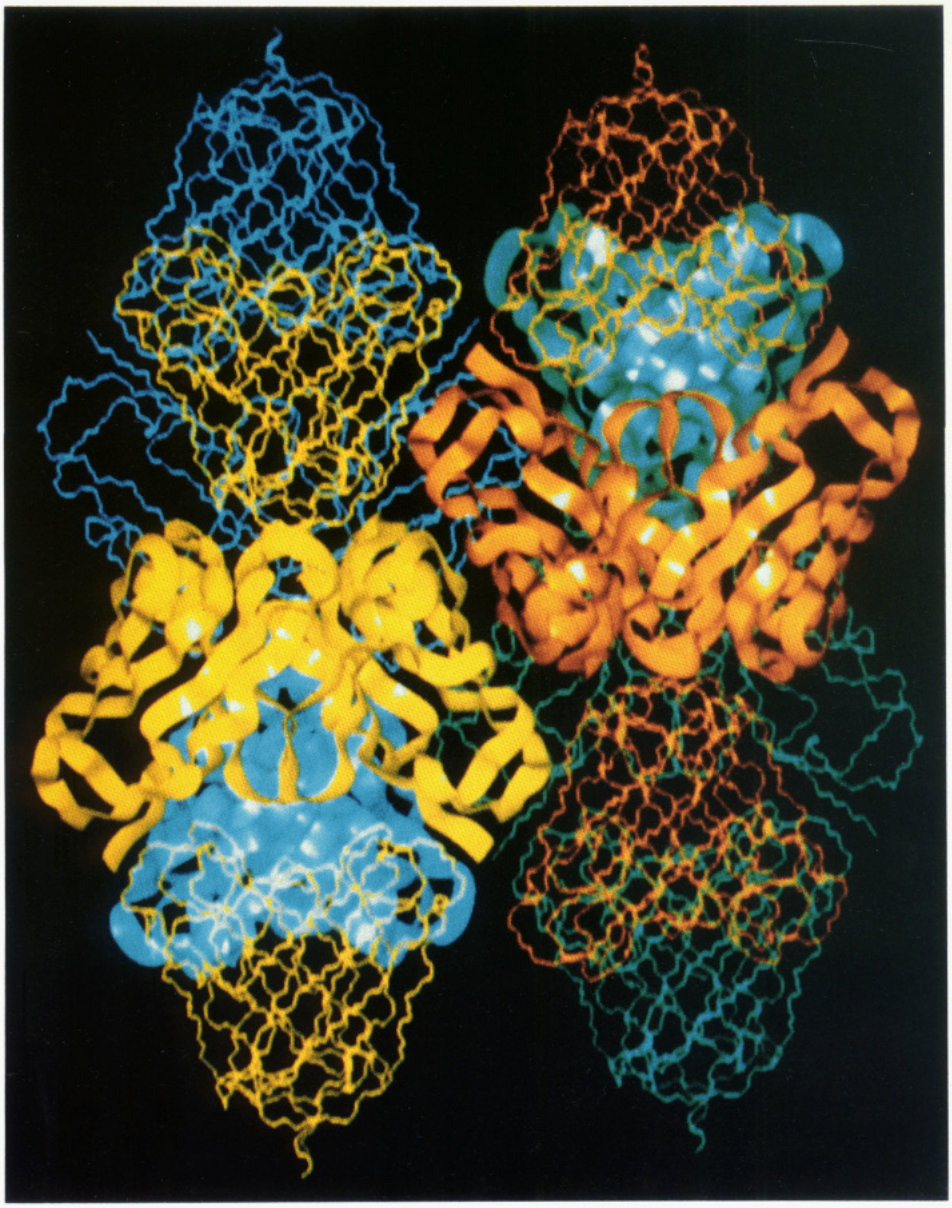

FIGURE 2: Formation of stable fibrils. Four antiparallel filaments are shown in which one dimer in each filament is depicted in a ribbon. Each filament interacts with two orthogonally arranged filaments in an antiparallel arrangement based on an assumption of a 90° rotation between successive dimers. Variation from this arrangement could be accommodated by this general packing scheme and would introduce a screw axis and helical aspect to the resulting assembly. Constant domains are not shown but could be readily accommodated on the outside of the assembly; removal of constant domains by proteolysis, as is typically found in light chains extracted from fibrils, would facilitate the lateral packing of filaments. Axial orientations of the forward filament pair are as in Figure 1.

serve as a nucleation center (Jarrett & Lansbury, 1993; Come et al., 1993).

Figure 2 depicts a possible "closed" model of an amyloid fibril consisting of four interdigitated filaments. This model of light-chain amyloid fibril assembly involves participation by most of the V_L-dimer surfaces, including its interface, the loops at both poles, and the exterior β-sheet surface. Consequently, most amino acid differences between two light chains either enhance or attenuate the ability of a light chain to form an amyloid fibril. For instance, the FR residues identified statistically, Ile20 and Asn45, could enhance the lateral interactions involved in fibril assembly. Favorable or unfavorable interactions may be accomplished by different amino acid pairs in different proteins. Therefore, locations of amino acids that distinguish amyloidogenic κ and λ light chains may differ due to extensive variation in the composition of their FR segments, despite the fact that their three-dimensional conformations are essentially the same.

The model shown in Figure 2 depicts only the variable domain component of the light chain. Typically, light-chain-derived material extracted from fibrils contains the V_L domains and a portion of the C_L domain. We would anticipate removal of all or part of the C_L domain would facilitate the fibril packing illustrated here. Potentially, the presence of C_L domains would affect optimal packing strategies. To our knowledge, no systematic study has correlated fibril morphology with the presence or absence of the C_L domain.

In our proposed model, the structural integrity of the V_L dimer is preserved, including its β-sheet architecture and the β interstrand and intersheet spacings of ~4 and ~10 Å, respectively. The features are compatible with X-ray fiber diffraction studies of fibrils (L. Makowski, personal communication) that have also shown the V_L-domain β-sheets

to be approximately parallel to the 2-fold axis of the dimer. Because we have postulated that the fibril axis is an extension of the V_L dimer 2-fold axis, and because the β-sheets of the dimer are parallel to the dimer axis, it follows that the β-sheets within the proposed fibril are aligned as required by diffraction data. Our model also provides a physicochemical basis for the characteristic tinctorial properties of Congo red-stained amyloid fibrils. The proposed lateral interactions of proamyloid filaments bring together β-sheet surfaces in a manner that is not found in the precursor protein or the proamyloid filament itself. This juxtaposition (that does not occur in the native molecule) creates a quasicrystalline array that provides sites for birefringent Congo red binding.

"Rules" of Amyloidogenesis. Identification of structural features responsible for light-chain amyloidogenicity is complicated by the fact that light chain proteins can also deposit in other pathological forms—e.g., as renal tubular casts, crystals, or basement membrane precipitates (Stevens et al., 1991). These three processes occur more rapidly than does amyloid formation in mice injected with pathological human light chains (Solomon et al., 1991). The rate of catabolism of radiolabeled light chains has been found to be similar in mice and humans (Solomon et al., 1964; Waldmann et al., 1972); thus, the slow appearance of light-chain amyloid deposits may reflect a molecular assembly process that is rate-limited by a nucleation event or by the induction of some form of "amyloid-enhancing factor" (Wood et al., 1988). Because cast and crystal formation as well as basement membrane deposition expresses rapid in vivo kinetics, an absence of amyloid in patients with these types of light-chain deposits does not necessarily imply that the primary structure of the protein is incompatible with amyloidogenesis. In essence, the lifetime of the protein in circulation may be too short to allow significant amyloid fibril formation to take place. We suggest that, in fact, most monoclonal light chains (if synthesized in sufficient quantity) are structurally capable of assembly into amyloid fibrils *unless* (1) cast or crystal formation, basement membrane deposition, or another preemptive process occurs first, (2) a single or limited number of fortuitous amino acids at any of several key locations within the V_L prevents dimer formation, filament formation, or filament docking, or (3) as yet undefined host factors prevent light-chain aggregation.

Conclusions. We have identified primary structural features of certain human antibody light chains that render these molecules amyloidogenic. This information has led to the development of a molecular mechanism for fibril formation that involves a progressive process of V_L self-association leading to dimer, filament, and, finally, fibril formation. Our model of light-chain amyloidogenesis is consistent with presently available biophysical data and accounts for the apparent idiosyncrasy by which only some light chains are amyloidogenic, the preferential presence in fibrils of V_L domains relative to intact light chains, and the physiological irreversibility of fibril formation. Although we suggest that the major determinant of light-chain amyloidogenesis is the primary structure of the light chain (because it determines the spatial distribution of atoms involved in the multiple interactions of fibril formation), the model excludes neither the importance of host factors involved in protein synthesis or catabolism nor the potential significance of other ancillary factors, such as light-chain concentration, protein glycosylation, or the presence of other constituents associated with amyloid deposition (Kisilevsky & Snow, 1988).

Our light-chain model evokes extensive interactions between β-domains, a structural motif common to other amyloid-associated proteins. Thus, detailed study of the molecular basis of light-chain amyloid formation, using both patient-derived specimens and recombinant protein variants that incorporate site-specific single amino acid replacements (Wilkins-Stevens et al., 1995), should provide further insight into the physicochemical foundation of amyloidogenesis. The lateral β-sheet interactions that result in light-chain fibril assembly and stability can also occur with other types of proteins that form amyloid deposits, such as in Alzheimer's disease and adult-onset diabetes. The three concurrent protein−protein interactions we propose to be the basis of light-chain amyloidogenesis are reversible and, thus, targets for agents that would affect V_L polymerization. The development of pharmacological compounds that inhibit or prevent this process would have obvious clinical importance for patients with amyloidosis. In addition to the broad clinical relevance of amyloidogenesis, it is clear that fibril formation is driven by the same factors involved in other protein self-assembly processes. Therefore, study of the underlying protein chemistry of amyloidogenesis may provide new insight into the structural basis of self-assembly.

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REFERENCES

Come, J. H., Fraser, P. E., & Lansbury, P. T. (1993) *Proc. Natl. Acad. Sci. U.S.A. 90*, 5959.

Epp, O., Colman, P. M., Fehlhammer, H., Bode, W., Schiffer, M., Huber, R., & Palm, W. (1974) *Eur. J. Biochem. 45*, 513.

Hurle, M. R., Helms, L. R., Li, L., Chan, W., & Wetzel, R. (1994) *Proc. Natl. Acad. Sci. U.S.A. 91*, 5446.

Jarrett, J. T., & Lansbury, P. T. (1993) *Cell 73*, 1055.

Johnson, G., Kabat, E. A., & Wu, T. T. (1995) *Kabat Database of Sequences of Proteins of Immunological Interest,* National Center for Biotechnology Information, National Library of Medicine, National Institutes of Health, Bethesda, MD.

Kelly, J. W., & Lansbury, P. T. (1994) *Amyloid: Int. J. Exp. Clin. Invest. 1*, 186.

Kisilevsky, R., & Snow, A. D. (1988) *Med. Hypotheses 26*, 231.

Klein, R., Jaenichen, R., & Zachau, H. G. (1993) *Eur. J. Immunol. 23*, 3248.

Kolmar, H., Frisch, C., Kleemann, G., Götze, K., Stevens, F. J., & Fritz, H.-J. (1994) *Biol. Chem. Hoppe-Seyler 375*, 61.

Liepnieks, J. J., Benson, M. D., & Dwulet, F. E. (1991) in *Amyloid and Amyloidosis, 1990* (Natvig, J. B., Forre, O., Husby, G., Husebekk, A., Skogen, B., Sletten, K., & Westermark, P., Eds.) pp 153−156, Kluwer Academic Publishers, Dordrecht.

Lorenzo, A., & Yankner, B. A. (1994) *Proc. Natl. Acad. Sci. U.S.A. 91*, 12243.

Lorenzo, A., Razzaboni, B., Weir, G. C., & Yankner, B. A. (1994) *Nature 368*, 756.

Myatt, E. A., Westholm, F. A., Weiss, D. T., Solomon, A., Schiffer, M., & Stevens, F. J. (1994) *Proc. Natl. Acad. Sci. U.S.A. 91*, 3034.

Pascali, E. (1995) *Crit. Rev. Oncol./Hematol. 19*, 149.

Sipe, J. D. (1992) *Annu. Rev. Biochem. 61*, 942.

Solomon, A., Waldmann, T. A., Fahey, J. L., & McFarlane, A. S. (1964) *J. Clin. Invest. 43*, 103.

Solomon, A., Weiss, D. T., & Kattine, A. A. (1991) *N. Engl. J. Med. 324*, 1845.

Solomon, A., Weiss, D. T., & Pepys, M. B. (1992) *Am. J. Pathol. 140*, 629.
Stevens, F. J., Westholm, F. A., Solomon, A., & Schiffer, M. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 1144.
Stevens, F. J., Solomon, A., & Schiffer, M. (1991) *Biochemistry 30*, 6803.
Waldmann, T. A., Strober, W., & Mogielnicki, R. P. (1972) *J. Clin. Invest. 51*, 2162.
Walpole, R. E., & Meyers, R. H. (1978) *Probability and Statistics for Engineers and Scientists,* 2nd ed., Macmillan, New York.
Wilkins-Stevens, P., Raffen, R., Hanson, D. K., Deng, Y.-L., Berrios-Hammond, M., Westholm, F. A., Murphy, C., Eulitz, M.,Wetzel, R., Solomon, A., Schiffer, M., & Stevens, F. J. (1995) *Protein Sci. 4*, 421.
Wood, S. P., Oliva, G., O'Hara, B. P., White, H. E., Blundell, T. L., Perkins, S. J., Sarharwall, I., & Pepys, M. B. (1988) *J. Mol. Biol. 282*, 169.

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