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Hypothesis

Mechanochemical mechanism for peptidyl free radical generation by amyloid fibrils

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Abstract β-Amyloid peptides (Aβ) form the core of Alzheimer's disease (AD) senile plaques, and are implicated in AD neurotoxicity. Aβ and some derivatives generate free radicals upon fibrilogenesis. A mechanism for free radical generation is proposed, based upon fibril cross β-sheet structure: (1) During fibrilogenesis there is a small probability of mispacking of Aβ monomers, resulting in abnormal fibril packing. (2) Continued fibrilogenesis traps a packing defect within the β-sheet. Surrounding β-sheet resists distortion, and the abnormally packed polypeptide(s) is strained. (3) Thermal processes cause homolytic bond scission and radical production from strained polypeptide through mechanically activated thermal decomposition. (4) Reaction with oxygen produces peroxy radicals, prevents unproductive radical recombination, and promotes observed cross-linking, production of reactive oxygen species and peptide fragmentation. Adiabatic mapping suggested significant strain would be generated by β-sheet misalignment. The mechanism relates the common structure of fibrils to radical production, and may be relevant to cytotoxicity in prion and other amyloidoses.

Key words: Amyloid beta-protein; Alzheimer's disease; Prion disease; Free radical

1. Introduction

Alzheimer's disease (AD) is characterized by the presence of insoluble, extracellular, fibrous deposits comprised principally of β-amyloid protein (Aβ). Studies of the biological activity of Aβ suggest it may potentiate neurotoxicity, or be directly neurotoxic [1]. Recent evidence has shown that synthetic Aβ and certain truncated Aβ peptides, particularly Aβ(25–35)[1], generate peptidyl free radicals and reactive oxygen species (ROS) in the presence of oxygen and apparent absence of metal ions or other free radical initiators [2,3]. A close correlation has been demonstrated between free radical production by these peptides and the ability to promote oxidative reactions associated with AD etiology, including cultured neuronal cell lipid peroxidation [4], inhibition of glutamine synthetase activity [5], inhibition of creatine kinase activity [5], and inhibition of ion-motive ATPases [6]. The demonstration of Aβ neurotoxicity [7–9] led to the recent proposal that neurotoxicity in AD was caused by Aβ-generated free radicals [2].

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[1]β-Amyloid-derived polypeptides are abbreviated βA with the terminal amino acid numbers given in parentheses.

Aβ toxicity is closely correlated with the aggregation state of the polypeptide. Freshly solubilized Aβ may only exhibit neurotoxicity after several days of preincubation [9,10], while shorter peptides such as Aβ(25–35) aggregate rapidly with concomitant free radical production and neurotoxicity [5,7,9]. Agents such as Congo red [11] and related sulfonated dyes [12] attenuate neurotoxicity and may inhibit fibrilogenesis. A key observation is that fibril-associated neurotoxicity is only observed during the process of fibril assembly. Moderately aggregated Aβ is neurotoxic while soluble and extensively aggregated forms are not [2,13,14].

Amyloidogenic fibrils are rigid [15] or semi-rigid [16], strongly resisting chemical and thermal denaturation. X-ray analysis of oriented Aβ fibrils revealed antiparallel cross β-sheet in which the polypeptide backbones and interchain hydrogen bonds are oriented perpendicular and parallel to the fiber axis, respectively [17–20]. This arrangement results in extensive regions of β-pleated sheet, presumably extending along the length of the fiber. Recently, Lansbury et al. [21] derived a structural model for Aβ(34–42) fibril β-sheet by ^{13}C-NMR and FTIR which demonstrated that the pleated antiparallel β-sheet was characterized by specific intermolecular alignment of Aβ(34–42).

Because other amyloidogenic peptides, such as human amylin and β_2-microglobulin, also appear to produce cell-toxic free radicals [22], and because amyloidogenic peptides and prion rods [23] appear to possess common general structure (antiparallel cross β-sheet), yet are diverse in terms of composition and sequence [15], this study examined whether the common general structure could account for spontaneous peptidyl free radical generation during fibril growth. Mechanical strain can generate free radicals within polymers by mechanically activated thermal decomposition in which stress lowers the thermal activation energy for homolytic bond cleavage [24]. In crystals, stress occurs at dislocations and other imperfections [25]. The unusual stability and crystallite packing of neurotoxic fibrils suggested that imperfections in their assembly could likewise lead to stress and subsequent radicalization. A general model for free radical generation in fibrils is presented, based upon heterogeneity in β-strand alignment during fibril assembly, accumulation of strain, and radicalization through mechanically activated thermal decomposition.

2. Methods

Modelling was performed using a molecular model of silk fibroin type II from *Bombyx mori* [26] (Protein Databank file 2SLK). The coordinates of the central chains (B, C, D, G, H,

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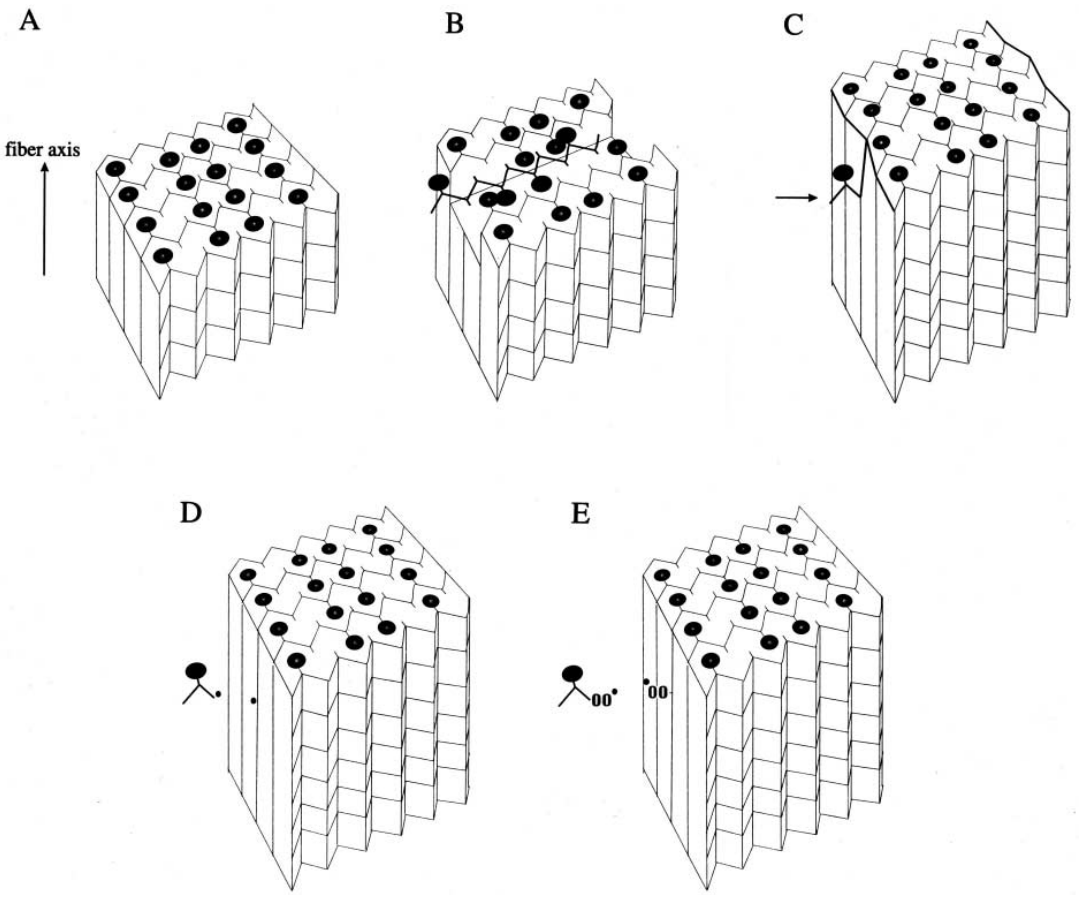

Fig. 1. Mechanochemical mechanism for peptidyl free radical generation. (A) Cartoon of normal fibrilogenesis. Polypeptide chains and interchain hydrogen bonds are aligned perpendicular and parallel to the fiber axis, respectively, as indicated by X-ray diffraction studies. Bulky side-chains are depicted as spheres. (B) Addition of a polypeptide with abnormal intrachain hydrogen bond alignment. (C) Continued fibril growth induces strain in the fibril. The arrow indicates the location of the trapped incorrectly aligned polypeptide. Thicker lines indicate distortion of the growing fibril. (D) Mechanically activated thermal decomposition of the strained polypeptide produces peptidyl radicals. (E) Reaction with oxygen produces peroxy radicals, preventing radical recombination and, through further reactions, permitting resumption of unstrained fibrilogenesis.

I, L, M and N) were used to construct an extended structure consisting of 35 hexapeptides (alternating chains of Ac-(Gly-Ala)-NHMe and Ac-(Ala-Gly)-NHMe) organized into 5 β-sheets of 7 β-strands each. Minimization in vacuo using the cvff91 class II forcefield to a rms derivative of less than 0.002 kcal mol^{-1} $Å^{-1}$ (Discover, Biosym, Inc., San Diego) resulted in a structure essentially identical in conformational parameters to that of Fossey et al. [26], but larger.

Adiabatic mapping was performed beginning with the minimized extended silk fibroin structure, by successively displacing the entire central β-sheet in 0.25 Å increments along the axis corresponding to the fiber axis depicted in Fig. 1A. To preserve the displacement during energy minimization, the terminal 2 carbon atoms of each β-strand were fixed during minimization. Each structure was extensively energy minimized as described above by a conjugate gradient algorithm in vacuo to a rms derivative of less than 0.02 kcal mol^{-1} $Å^{-1}$.

3. A mechanochemical model for free radical production

Step 1. Fibrilogenesis proceeds by the binding of a soluble Aβ monomer onto the growing fibril end (Fig. 1A), using the existing fibril as a template [27]. In the present model, template-directed fibrilogenesis does not ensure complete fidelity, and there is a small probability that a newly added monomer binds to the template in an unusual conformation (termed 'abnormal'), different from that of the template itself (termed 'normal'). For example, interchain hydrogen bonds between the newly incorporated Aβ monomer and the template may differ from the norm, resulting in abnormal fibril packing (Fig. 1B). The abnormality may consist of one or several monomers.

Step 2. Normal fibril growth may continue, templated by adjacent normal β-sheet, trapping the abnormal polypeptide(s) and preventing dissociation or facile rearrangement to the normal conformation (Fig. 1C). To the extent that surrounding normal fibril structure is perturbed by the abnormal region (thick lines, Fig. 1C), the abnormal region introduces stress within the fibril. The rigidity of the normal fibril structure attempts to deform the abnormal region towards the conformation of normal fibril. As fibrilogenesis continues, stresses accumulate at the interface of normal and abnormal regions.

Step 3. Thermal processes act upon strained polypeptides to cause homolytic bond scission and radical production through mechanically activated thermal decomposition (Fig. 1D). Mechanically activated thermal decomposition is well documented for strained polymers as described below. Molecular

strain reduces the activation energy for homolytic bond cleavage, resulting in an exponential increase in the rate of bond cleavage with increasing strain (Eq. 1).

Step 4. In the presence of dissolved molecular oxygen, peroxy free radicals are produced. (Fig. 1E). Reaction with oxygen prevents non-productive radical recombination. Further radical reactions result in cross-linking, isomerization, fragmentation, and production of reactive oxygen species. In the process, strain is dissipated, permitting resumption of unstrained fibrilogenesis.

4. Relation of model to existing studies

4.1. Fibrilogenesis, free radical production and neurotoxicity

Free radical production, detected by spin-trapping and EPR, has been reported for Aβ(1–40), Aβ(25–35), Aβ(35–25) (reverse sequence), and Aβ(25–35) (scrambled sequence) [2,3,28]. In addition, human amylin and β_2-microglobulin and other amyloidogenic proteins also damage cells by a free radical-dependent, fibril-dependent mechanism [22]. Peptidyl free radicals appear to be produced as a consequence of fibrilogenesis. In the present model, it is significant that HPLC-purified Aβ(25–35) polypeptide fibrils produce free radicals. Many opportunities for the introduction of packing defects into a nascent fibril can be envisaged in vivo, but there are fewer possible mechanisms in fibrilogenesis of an homogenous polypeptide. The maximum extent of spin trapped radical from Aβ(25–35) was reported to be one radical per 25 polypeptides [5], which suggested that the mechanism of radical production was not coupled to fibrilogenesis in a strictly stoichiometric mechanism. Similarly, the extent of peroxide-equivalent production by Aβ(25–35) was recently reported to be 10 μM per mg/ml peptide incubate [3], or roughly one per 70 Aβ(25–35) monomers. Given the low stoichiometry, it is here proposed that abnormal association during fibrilogenesis better accounts for the observed level of free radical and peroxide production than does a model based upon the chemistry of an homogenous fibril structure.

The lack of consensus sequence among amyloidogenic polypeptides [15], but the common cross-β conformation [17–20], are explained in the present model by basing a mechanochemical mechanism upon the general properties of extended β-sheet rather than on the juxtaposition of specific amino acid residues within the fibril structures, as required in a specific chemical mechanism. Table 1 illustrates the sequence diversity exhibited by neurotoxic Aβ(25–35) variant peptides previously reported, which indicates that neurotoxicity cannot be attributed to specific side-chains within the fibril.

Table 1
Neurotoxic variants of Aβ(25–35)

G S N K G A I I G L M	Neurotoxicity	Reference
A	+,++	[35,40]
A	+,++	[35,40]
S	+	[35]
A	+	[40]
N	+	[35]
A	++	[40]
A	++	[35,40]
V	+	[35]
A	++	[40]
L	+	[35]
A	+	[35,40]
I	+	[35]
D	++	[35]
S	++	[35]
C	+++	[35]
A	+++	[40]

The wild-type sequence of Aβ(25–35) is shown (top) together with substitutions reported to yield significantly neurotoxic effects. +++, neurotoxicity greater than wild-type; ++, 50–100%; +, less than 50% the activity of wild-type peptide. In all cases neurotoxicity was associated with aggregation of the peptide.

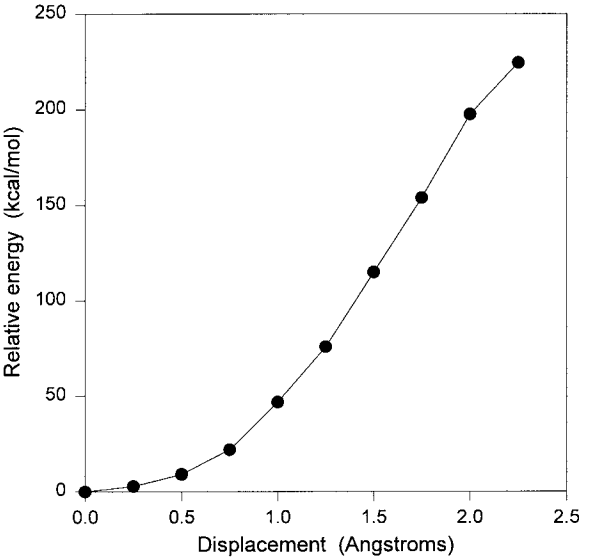

Fig. 2. Adiabatic mapping of sheet motion in an extended silk fibroin model. Energetic cost of motion of the central β-sheet of a silk fibroin type II model along an axis equivalent to a fibril fiber axis (see Fig. 1A). The energies of minimized structures are plotted as a function of central β-sheet displacement.

Fibrilogenesis is coupled to neurotoxicity and free radical production [13,28]. The amyloid fibril-binding dye Congo red inhibits both fibrilogenesis and neurotoxicity in β-amyloid plaques [11]. Similarly, rifampicin prevents Aβ(1–40) fibril formation and neurotoxicity [29]. Diffuse plaques appear to lack the crystallite fibrillar order present in compact plaques, and are not neurotoxic [11]. The association of neurotoxicity with the process of fibrilogenesis is also indicated by observations that unaggregated Aβ polypeptide and aged fibrils lack neurotoxic potential, which is only exhibited in moderately aggregated fibrils [2,14]. The instability of neurotoxic polypeptide in aqueous solution is lost in solvents such as trifluoroethanol that prevent aggregation [30]. Partial reversal of Aβ(1–42) aggregation also reversed neurotoxicity [9]. The model presented in this paper accounts for these observations by requiring strain, arising during fibrilogenesis, as a necessary prerequisite for mechanically induced thermal decomposition to produce free radicals. Aged fibrils would not be predicted to be neurotoxic because the strain would have already dissipated through previous radical production and rearrangement.

Hensley et al. [2] proposed that free radical production by β-amyloid is responsible for the neurotoxic properties of these fibrils. A correlation has been established between the extent of free radical spin trapping, neurotoxicity and other oxidative damage [5,13,28,31]. Molecular oxygen is required for free radical trapping [5] and to induce oxidative damage. In the present model, oxygen would be required in order to react

with peptidyl free radicals to produce reactive peroxy free radicals and ROS. In the absence of oxygen the majority of peptidyl free radicals would be expected to undergo rapid recombination but conversion to peroxy free radical would prevent this [32]. The lack of spin trapping reported under anaerobic conditions may reflect the absence of peroxy radical derivatives: Hensley et al. [31] presented EPR evidence that the spin trapped species were derived from peroxy free radicals. The model therefore explains the observed oxygen dependence of free radical trapping, and may also explain the observation of Harris et al. [28] that CD spectra of Aβ(25–35) peptide indicated more β-sheet under aerobic compared to anaerobic condition: continued fibrilogenesis may be impeded by incorrectly associated polypeptides if peptidyl free radical recombination is not prevented by reaction with oxygen. In accord with this model, antioxidants impeded fibril formation [33].

Free radical production may be necessary but not sufficient for neurotoxicity [4]. This is suggested from the observation that Aβ(35–25), the reverse sequence of the potent neurotoxic agent Aβ(35–25), produced spin trap-detectable free radical, but was not significantly neurotoxic. As observed by Mattson [34], conversion of free radical production to neurotoxic effects may depend on key residues, such as Met-35, which was oxidized to methionine sulfoxide during free radical production in Aβ(25–35) [5]. However, Met-35 is not absolutely essential for neurotoxicity: substitution of Met-35 with Asp, Ser, Ala or Cys also yielded strong neurotoxicity and aggregation (Table 1), while substitutions that destroyed neurotoxicity also diminished aggregation [9,35]. Recent studies indicate that Aβ peptides can interact with cell surfaces and induce lipid peroxidation [4,36]. Although free-radical production by Aβ peptides has been demonstrated in the absence of cell membranes or lipids [2,3], close proximity of the fibril to the membrane would be expected to enhance propagation of oxidative damage from the fibril to the cell surface. The present proposal does not address possible further chemical steps which may be required to convert free radical production into neurotoxic effects, but only the initial process of peptidyl free radical generation.

Lot-to-lot variability of Aβ peptide neurotoxicity has been reported that was not due to impurities, and correlated with the EPR species spin trapped. Inactive preparations often acquired neurotoxicity after freezing of the lyophilized powder at −80°C and 5 h aqueous incubation at room temperature [5]. These results may relate to the present model in that seeding of fibril crystallite growth is required [27]. The initial formation of β-sheet was shown by Yang and Honig [37] to be exergonic, and so initiation of fibril growth may be slow in the initial absence of suitable nuclei. Jarrett and Lansbury [27] have shown that small amounts of Aβ(1–42) can seed growth of fibrils of Aβ(1–40), which has a lower propensity for fibrilogenesis.

4.2. *Implications of fibril cross-β structure*

A general model of fibril structure, from fiber X-ray studies [15,17–20], is shown schematically in Fig. 1A. The planes of the extended antiparallel β-sheets lie along the fiber axis. Lansbury et al. [21] have demonstrated specific intermolecular alignment within the β-sheet in the case of β(34–42). Birefringence and ultrastructural studies also indicate a high degree of order within the fibril. The stacked β-sheets confer the observed rigidity upon the fibril and are very stable to chemical and thermal denaturation [16].

Fibrilogenesis has been viewed as a 'one-dimensional crystallization' [27] in which nucleation is followed by ordered addition of polypeptide chains to the fibril end. Sequential assembly permits, in the present model (Fig. 1), trapping of abnormally associated polypeptides within the growing fibril. Some abnormal association of polypeptides with the fibril tip would seem unavoidable on statistical-mechanical grounds. The crystallite packing of fibrils would impede subsequent structural transitions of a trapped, abnormally associated, polypeptide.

Misalignment of the main-chain hydrogen bonds of a polypeptide during incorporation into a growing β-sheet would perturb fibril structure both within and between β-sheets because of side-chain packing constraints and main chain conformational preferences [21]. As fibrilogenesis continues, a large stabilization (the sum total of all fibril destabilization subsequent to the misalignment) may be realized by a small concerted motion to correct the originating defect. Stress would be focused upon the abnormality in a manner analogous to that of a crystal dislocation [25], resulting in strain within the incorrect polypeptide. The stability and rigidity of the native fibril dictate that the incorrect polypeptide, rather than the surrounding fibril, would experience the deformation toward the native conformation necessary for continued fibril growth. This would be enhanced by the parallel stacking of surrounding β-sheets.

Fibril stability correlates with β-sheet forming propensity [38]. The stability of β-sheets derives mainly from inter- and intra-sheet non-polar side-chain-side-chain interactions [36], therefore these packing constraints would be significant during fibrilogenesis. In the model of Aβ(34–42) presented by Lansbury et al. [21], two prominent ridges of non-polar side-chain-side-chain interactions run the length of the fibril, separated by two central glycine residues.

Of particular relevance to the present model is the observation that the β-sheets are organized parallel to the fiber axis so that fibrilogenesis depends upon the correct stacking of new polypeptides onto the extant fibril. The resulting periodic inter-sheet interactions would strongly resist displacement of one β-sheet relative to another with a force approximately proportional to the area displaced. Conversely, if one sheet is distorted by an incorrect polypeptide alignment event, such that further sheet extension is displaced relative to adjacent sheets, then this force will be exerted upon the defect, and the force will increase for as long as new sheet formation is distorted. From ultrastructural analysis it can be inferred that approx. 4 β-sheets must be present to account for observed fibril dimensions [18], and that the fibrils, although straight, contain a periodic twist (periodicity 80 Å [15]) suggesting twisted β-pleated sheet. Thus, both inter- and intra-sheet interactions may guide correct fibrilogenesis, and intersheet interactions in particular may guide correct fibrilogenesis following an incorrect polypeptide alignment.

4.3. *Mechanical stress and free radical production in polymers*

Mechanical deformation has long been known to produce homolytic bond scission and free radical production in artificial polymers through mechanically activated thermal decomposition ([24,32] and references therein). Mechanically activated thermal decomposition is a two step process compris-

ing (1) mechanical stress upon polymer bonds, and (2) subsequent homolytic scission of bonds through thermal processes. Mechanical stress lowers the activation energy for homolytic bond scission according to a modified Arrhenius equation [24]:

$$k = k_o \exp\{-(E_A - \alpha\sigma)/RT\} \quad (1)$$

where k is the observed rate constant for bond scission, E_A is the activation energy in the unstrained state, σ is tensile stress, and α is an activation volume. The pre-exponential term k_o represents atomic vibration. Rates of bond cleavage are observed to increase exponentially with tensile stress. Bond cleavage and free radical production have been directly observed by EPR in a number of polymers, including silk, aramid yarns, polyethylenes and polyamides [24]. In the present proposal, stresses of internal, rather than external, origin are responsible for reducing the effective activation energy for free radical production (Eq. 1), although the underlying mechanism is identical. At stresses close to the failure point of artificial polymers, radical densities of the order of 10^{17} cm^{-3} have been observed [39]. An important observation for the present mechanism is that free radical production begins at stresses well below that required for failure. Zhurkov and Korsukov [24] detected by IR spectroscopy the appearance of aldehyde end groups, indicative of free radical production and subsequent reaction with oxygen, in polyethylene under very small stress, long before the specimen broke. Reactive aldehyde groups and other oxidation products are also produced on fibrils [2,3]. Radical production at low stress leads to the observed fatigue properties of many polymers. The common phenomenon of polymer relaxation under stress, in which strain is slowly relieved at constant polymer extension, is relevant to the present model, in that free radical induced rearrangement toward a more stable state may parallel the oxygen-dependent, antioxidant inhibited, enhancement of β-sheet structure reported in fibril aging. In the present model, much lower stresses than are required to cause failure of artificial polymers, and correspondingly smaller radical densities, are required to account for observed free radical and peroxide-equivalent production by Aβ(25–35) described above [3,5].

Peroxides can initiate free radical processes through thermal dissociation. The activation energies for thermolysis for a range of peroxides range from 76 to 156 kJ mol^{-1} [32]. An activation energy of approx. 100 kJ mol^{-1} yields a $t_{1/2}$ for decay of 10 h at ambient temperature, and the present mechanism would require strain to lower the activation energy for polypeptide bond scission to this magnitude.

Adiabatic mapping of silk fibroin type II [26] was performed to estimate the magnitude of forces generated upon distortion of an extended β-sheet structure. Silk fibroin was chosen because at present no experimental molecular model, including intersheet packing, exists for an Aβ fibril. Small-scale translation of a central sheet comprised of 7 hexapeptides (see Section 2), along the axis corresponding to a fibril fiber axis (Fig. 2, see also Fig. 1A) was strongly resisted with a maximum force of approx. 150 kcal mol^{-1} $Å^{-1}$ (Fig. 2), resulting mainly from non-bonded repulsion between Ala sidechains. Similar forces were observed along other trajectories within the plane of the β-sheet, and suggested that large forces could be generated in a mechanism that attempts to disrupt β-sheet packing. The adiabatic mapping method can overestimate forces by trapping the trajectory in local minima rather than locating the lowest energy trajectory. In the present calculations, the incremental displacements (0.25 Å) were small to minimize this risk. Even so, these data represent an upper limit to the forces expected. Molecular dynamics calculations (results not shown) indicated that bulky mutations (Trp, Met and Tyr) introduced singly within the extended β-sheet, remained trapped in strained conformations that were not relieved by simulated annealing.

5. Conclusions

A general model is presented to account for peptidyl free radical generation during assembly of β-amyloid fibrils. The model is mechanochemical, rather than chemical, in nature, and is based upon the high stability and rigidity of the common cross β-sheet structure of amyloidogenic polypeptides, and upon the sequential mechanism of fibrilogenesis. The model makes specific predictions, which are currently being tested.

Because of the general structural nature of the proposed mechanism, other amyloidogenic peptides exhibiting high fibril stability would be predicted to also generate free radicals upon fibrilogenesis. This would be of considerable interest [34] because it may provide a general mechanism for cell damage in other amyloidogenic diseases in addition to Alzheimer's disease, including prion-associated diseases [23] and type II diabetes [15].

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