Protofibril Formation of Amyloid β-Protein at Low pH via a Non-cooperative Elongation Mechanism*

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Deposition of the amyloid β-protein (Aβ) in senile or diffuse plaques is a distinctive feature of Alzheimer's disease. The role of Aβ aggregates in the etiology of the disease is still controversial. The formation of linear aggregates, known as amyloid fibrils, has been proposed as the onset and the cause of pathological deposition. Yet, recent findings suggest that a more crucial role is played by prefibrillar oligomeric assemblies of Aβ that are highly toxic in the extracellular environment. In the present work, the mechanism of protofibril formation is studied at pH 3.1, starting from a solution of oligomeric precursors. By combining static light scattering and photon correlation spectroscopy, the growth of the mass and the size of aggregates are determined at different temperatures. Analysis and scaling of kinetic data reveal that under the studied conditions protofibrils are formed via a single non-cooperative elongation mechanism, not prompted by nucleation. This process is well described as a linear colloidal aggregation due to diffusion and coalescence of growing aggregates. The rate of elongation follows an Arrhenius law with an activation enthalpy of 15 kcal mol⁻¹. Such a value points to a conformational change of peptides or oligomers being involved in binding to protofibrils or in general to a local reorganization of each aggregate. These results contribute to establishing a clearer relation at the molecular level between the fibrillation mechanism and fibrillar precursors. The observation of a non-cooperative aggregation pathway supports the hypothesis that amyloid formation may represent an escape route from a dangerous condition, induced by the presence of toxic oligomeric species.

A clear hallmark of Alzheimer disease is the presence in the brain of extracellular amyloid plaques containing a certain amount of cleavage products of the transmembrane amyloid β-protein precursor (1). These are small peptides of 39–42 residues with a hydrophobic domain at the C terminus. They have been found in the core of senile plaques in typical linear aggregates, known as amyloid fibrils. Similar amyloid fibrils made from different proteins or peptides are associated with other neurodegenerative diseases, such as Parkinson and Huntington diseases, and transmissible spongiform encephalopa-

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1 The abbreviations used are: Aβ, amyloid β-protein; QLS, quasielastic light scattering; M_w, weight average molecular mass.

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simplified conditions to highlight a few basic features of the molecular interactions and mechanisms that drive amyloid fibrillogenesis.

At physiological pH Aβ(1–40) forms small oligomers (of a few units) (24–28), whereas, at acid pH (below 3.4), it forms large mass oligomers (with tens of peptides) that readily aggregate into beaded protofibrillar chains (28, 38). It has been proposed that at pH 2, peptides may coexist with spherocylindrical micelles that act as nuclei for fibrillogenesis (35–37, 39).

Our light scattering experiments have shown that the initial oligomers, with an average aggregation number of 75 Aβ(1–40), assemble into elongated linear structures, consistent with previous observations (28) but without any observed nucleation step. Scaling of kinetic data at different temperatures on a single master curve reveals that the same mechanism of aggregation operates between 27 and 67 °C: aggregation proceeds via diffusion and coalescence of clusters and not just by addition of a single monomer to fibrils, as has often been reported under other conditions (35, 36, 40). Also, a single activation free energy barrier has been found to be responsible for the coagulation rate. Interestingly, these experiments go beyond the classic scheme of protein polymerization (41, 42) or amyloid formation (43, 44), which prescribes an elongation process preceded by a rate-limiting nucleation (35, 36, 45–47). In the present work, amyloid formation occurs via a non-cooperative coagulation process, which closely resembles linear colloidal aggregation (33).

EXPERIMENTAL PROCEDURES

Preparation of Peptide Solutions—Samples of 1 mg of Aβ(1–40) powder (purchased from Anaspec, Inc.) were dissolved in 0.2 ml of trifluoroacetic acid and gently stirred at 5 °C for 3 h to completely dissolve associated peptides (46, 49). Upon addition of 1.8 ml of Millipore SupergQ water, solutions were fractionated into five equal parts and lyophilized overnight. This procedure provided stock powder aliquots of the same amount (200 μg). Each aliquot was dissolved in 250 μl of 0.1 M sodium citrate buffer at pH 3.1 and filtered (via a 0.2-μm Millipore filter) into 1-cm square quartz cuvettes or 1-mm cylindrical capillaries. Different peptide concentrations were obtained by diluting the stock powder aliquots by a different amount of buffer solution. All chemicals were reagent grade.

Verification of Seed-free Condition—Pretreatment with trifluoroacetic acid and filtering ensured that no large aggregates (seeds) were present in solution (49). This was readily verified at the beginning of each experiment by quasielastic scattering measurements, which are very sensitive to high molecular weight objects. The high reproducibility of our kinetics further confirmed the absence of a significant amount of spurious nucleation centers within our peptide solutions, before and after filtering.

Concentration Determination—Concentration was determined by measuring tyrosine absorbance at 276 nm (extinction coefficient: 1390 cm⁻¹ m⁻¹) with a Jasco J-530 spectrometer (50). Peptide concentrations were 185 μM for the main set of experiments. Considering that the molecular mass of Aβ(1–40) is 4.3 kDa, the final concentrations were consistent with those calculated assuming the initial nominal peptide amount of 1 mg, in purchased vials. This confirmed that no material was lost through filtering and that peptide was efficiently dissolved via the trifluoroacetic acid treatment.

Quasielastic Light Scattering—Time-resolved light scattering experiments were performed at different temperatures, immediately after sample dissolution in buffer at pH 3.1. Samples were placed in a thermostatted cell compartment of a Brookhaven Instruments BI200-5M goniometer, equipped with a 100-milliwatt argon laser tuned at λ₀ = 514.5 nm. The temperature was controlled within ±0.05 °C with a thermostatted recirculating bath. Scattered light intensity at 90° and its time autocorrelation function, g(τ), were measured simultaneously by using a Brookhaven BI-9000 correlator. Correlation functions g(τ) were analyzed using a constrained regularization method (51) to determine the distribution P(D) of the apparent diffusion coefficients D = g(0) = 1 + |P(D)| exp(−4π²c³/D²), where q is the scattering vector, defined just below. The z-average hydrodynamic radius R_z was calculated by taking the z-average diffusion coefficient D_z and assuming the Stokes-Einstein relation: R_z = D_z/6πηR_0, where R_0 is the Boltzmann constant, T is the temperature, and η is the solvent viscosity (52).

RESULTS

Onset of Kinetics: Mass and Size of Oligomers—Peptides were dissolved into pH 3.1 buffer to obtain the final concentration of 185 μM. After dissolution, QLS measurements reveal the presence of small size oligomers in the solution, along with a negligible amount of large size aggregates (more than hundreds of nanometers). Apart from this, the distribution of hydrodynamic radii is unimodal with a mean value of 330 ± 60 nm (Fig. 1A, B). Measurement of the intensity scattered at 90° (scattering vector q = 23 μm⁻¹) provides the Rayleigh ratio I(q) that is related to the weight average molecular mass (Mw) by the relation: I(q) = 4π²n²dn/dcλ₀⁴N_A/M_w²P(q), with c the mass concentration, N_A Avogadro’s number, and P(q) the z-averaged form factor (52). By taking dn/dc = 0.18 cm²g⁻¹ and P(q) = 1 (because the initial size of oligomers is much smaller than q⁻¹), we obtain a weight average molecular mass M_w = 330 ± 60 kDa. Considering that the molecular mass of a single Aβ(1–40) is 4.3 kDa, the soluble oligomers found at the onset of kinetics are made up on average
of 75 (± 15) peptides, and they have a mean hydrodynamic radius of 7 nm. The same results have been obtained at each temperature used: 27, 37, 47, 42, 52, 57, and 67 °C.

We remark that the weight average molecular mass measured by light scattering experiments is defined as $M_w = \sum M_i c_i / c$, where $M_i$ and $c_i$ are, respectively, the molecular mass and the mass concentration of oligomers composed of $i$ peptides. Therefore, a certain amount of single peptides could be present in the solution even if not clearly observable by light scattering measurements, which are more sensitive to large mass objects. The tolerance in the analysis of dynamic light scattering at the initial stage of kinetics allows us to put an upper limit of ~0.4% for the contribution of monomers to scattered intensity. Consequently, in this limit condition, the mass concentration of monomers would be ~30%, and the actual average aggregation number of the oligomeric species would rise up to 110 units.

Early Stages of Kinetics at Different Temperatures—QLS experiments were performed at different temperatures and at the concentration of 185 μM focusing on the early stage of the kinetics. The time dependence of the 90° scattered intensity $I_0(q)$ is shown in Fig. 2a for different temperatures. Data were normalized with respect to the initial intensity $I_0(q)$ due to scattering by the initial oligomer distribution. The distribution of hydrodynamic radii at $T = 37^\circ C$ is shown for selected different times in Fig. 1. For each temperature studied, the distribution remains unimodal over time while its standard deviation and the mean value increase, so that their ratio (which is a measure of the polydispersity) is essentially stationary. The kinetics of the mean hydrodynamic radius is reported in Fig. 2b for each temperature.

As shown in the inset of Fig. 2a, the intensity starts increasing with no lag time. Such growth continues with a temperature-dependent rate and eventually saturates, while the growth of the hydrodynamic radii exhibits no reduction. The saturation of the intensity signal is due to the fact that the scattered intensity is proportional to the average mass of aggregates times the form factor $P(q)$, thus,

$$\frac{I}{I_0} = \frac{M_w P(q)}{M_w P(q)}$$  \hspace{1cm} (Eq. 1)

where $M_w$ is the weight average molecular mass and the subscript '0' refers to the zero time quantities. During the aggregation process, the average molecular mass increases so that aggregate size becomes larger than the reciprocal scattering vector $q = 23 \mu m^{-1}$ and the form factor decreases: $P(q) < 1$.

Empirical Fit and Scaling of Kinetics—The intensity growth versus time data have a sigmoidal shape on a log-log scale. To describe this behavior, the data were fit to the following empirical expression,

$$\frac{I}{I_0} = 1 + \left(\frac{t}{\tau_p}\right)^{\beta}$$  \hspace{1cm} (Eq. 2)

where $\beta = 0.87, \Delta = 8.0$, and $\tau_p = 7.3, 15.5, 26.0, 50.6, 51.2, 129.4, 293.8$ min, respectively, for $T = 67, 57, 52, 47, 42, 37$, and 27 °C. The exponent $\beta$ is less than unity, as expected for kinetics with no lag time.

The only fitting parameter depending on temperature is the time constant $\tau_p$, related to an aggregation rate. By plotting the intensity kinetics versus the renormalized time $t/\tau_p$, the data collapse on the same master curve (Fig. 3a). Remarkably, hydrodynamic radii also scale on a master curve by using the same parameter $\tau_p$ (Fig. 3b). Therefore, the same aggregation mechanism occurs throughout the temperature range studied.

Suitable Model for the Form Factor—To obtain a basic explanation of this aggregation mechanism, we need to work out a model to fit the experimental kinetic data. An expression for both the growth of the weight average molecular mass $M_w$ and for the form factor $P(q)$ is required, as is clear from Equation 1. In the present case, we will assume that the shape of aggregates is not critically related to the molecular mass, which is a very reasonable assumption when dealing with polymeric or fibrillar aggregates. Hence, the radius of gyration of aggregates can be written in the following scaling form with respect to the average molecular mass: $R_g = R_{g0}(M_w/M_w)^{1/2}$, where $R_{g0}$ is the initial z-averaged radius of gyration of the oligomers, and $d$ is an effective fractal dimension. Approximating the form factor with the Fisher-Burford expression (53) $P(q) = [1 + 2/3d^{-1}q^2 R_g^2]^{-d/2}$, the time evolution of scattered intensity reads,

$$\frac{I}{I_0} = \frac{M_w}{M_w} \left[1 + \frac{2}{3d}q^2 R_{g0}^2 (M_w/M_w)^{1/2}\right]^{-d/2}$$  \hspace{1cm} (Eq. 3)

Our modeling effort is now devoted to finding an expression for the weight average molecular mass, $M_w$.

Coagulation Theory—Protein aggregation can be studied in the framework of classic coagulation theory (54), by following an approach that has been widely used for colloids or aerosols (55, 56). The time-dependent aggregation number distribution can be described by the following Smoluchowski equation,

$$\frac{d}{dt} n_i(t) = \frac{1}{2} \sum_{j \neq i} K_{i,j} n_i(t) n_j(t) - n_i(t) \sum_j K_{i,j} n_j(t)$$  \hspace{1cm} (Eq. 4)
According to Equation 3 and 8, Fujii (64); production and loss of terms in the right-hand side of Equation 4 are, respectively, the only addition (assuming a time independent form factor).

Smoluchowski equation (Equation 4) with a kernel allowing monomer sivity hydrodynamic radius versus normalized scattering intensity. The c

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where $n_s$ is the number concentration of a cluster of $s$ units, and $K_{ij}$ is the reaction rate of two clusters of $i$ and $j$ units. The two terms in the right-hand side of Equation 4 are, respectively, the production and loss of $s$-mers by coagulation of two clusters of appropriate size. Here, we are assuming that aggregation is irreversible, so that no fragmentation or evaporation processes occur, and that three-body effects can be neglected. Also, following our discussion above, we do not introduce any nucleation term.

By multiplying Equation 4 by $s^k$ and summing over all $s$ one obtains the time evolution of the moments of the distribution $N_k = \sum s^k n_s$ (57, 58),

$$\frac{d}{dt} N_k(t) = \frac{1}{2} \sum_{w} \left( K_{w} p(t) n_j(t) \right)[(i + j - i^k - j^k)] \quad \text{(Eq. 5)}$$

In particular, the zero and first moments are proportional to the total number and to the mass concentration of clusters, respectively. Note that the conservation of total mass concentration is correctly issued by Equation 5 for $k = 1, dN_0/dt = 0$.

For our purposes, the most important quantity is the second moment of the distribution $N_2$, because it is proportional to the weight average molecular mass $M_w/M_w^0 = N_2/N_1$ and hence to the forward scattered intensity.

**Suitable Model for the Intensity Growth**—A simple model of fibrillar elongation requires that only monomers can bind to larger clusters. With this prescription, the kernel of the Smoluchowski equation is $K_{ij} = \gamma (\delta_{i1} + \delta_{j1})$, where $\gamma$ is a temperature-dependent parameter, and $\delta_{ij} = 1$ for $i = j$, and zero otherwise. This kernel is strongly and selectively mass-dependent. The weight average molecular mass $M_w$ is given by $M_w/M_w^0 = 1 + 2 M_0/M_w \ln[w(x(t))],$ where $M_0$ is the mass of a monomer, and $w(x)$ is the solution of the differential equation, $w'' + w^{-1} = 0$. The time-dependent variable $x(t)$ is determined by the initial total number concentration: $x = N_0(0)/\gamma t$. In Fig. 3a, a numerical solution of this model is reported for the weight average molecular mass by considering the oligomers as the monomeric units, that is $M_m = M_w^0$. It increases up to a saturation value that is too low compared with our experimental data, because this model implies a rapid consumption of available monomers. Note, *a fortiori*, that if we were to consider the time dependence of the form factor the saturation value for $I(t)/I_0$ would be further lowered from the value shown in the figure. A still lower saturation value would be reached if one considers that the growth is due to addition of single peptides to fibrils starting from an initial distribution of monomer and oligomers, which is $M_w^0 = 75 M_w^0$. Therefore, the simpler model where linear aggregates grow by sequential addition of single oligomer units or single peptides can be ruled out by the present experiments.

A suitable model to fit our experimental data should include a more realistic dependence on the mass and the size of aggregates. A simple analytic solution has been worked out theoretically and experimentally for colloidal aggregation (59–62). It requires a kernel that is a homogeneous function of the clusters’ mass, or, equivalently, of aggregation numbers, $i$ and $j$, that is,

$$K_{w,j} = a^K_{ij} \quad \text{(Eq. 6)}$$

for any number $a$. This assumption yields, for $\lambda < 1$ (nongelling condition),

$$\frac{M_w}{M_w^0} = \left( 1 + \frac{t}{\tau_0} \right)^{-z}, \quad \text{where } z = \frac{1}{1 - \lambda} \quad \text{(Eq. 7)}$$

For $\lambda = 0$, the classic solution with a constant mass-independent kernel is recovered (54). Data shown in Fig. 3a are well fit by Equations 3 and 7, with the following fitting parameters: $R_{g0} = 18 \text{ nm}, d = 1.65, z = 0.66$ (corresponding to $\lambda = -0.5$), and $t_0 = 0.757 \tau_0$. The initial $R_i$ is larger than the initial hydrodynamic radius as expected, because it is strongly affected by polydispersity, contrary to the mean hydrodynamic radius that is obtained by averaging over reciprocal values. The value of the fractal dimension $d$ is close
to that found for self-avoiding random coils (63), but in the present case it may be considered as a result of linear elongated flexible aggregates.

**Suitable Model for the Hydrodynamic Radius Growth**—
Along with an increase of the mean weight cluster mass, the increase of aggregate size is mirrored by the growth of the mean hydrodynamic radius. We fit the master curve of hydrodynamic radii (Fig. 3b) by using Equation 7 and assuming the following scaling relation between the mean hydrodynamic radius $R_h$ and the weight average molecular mass,

$$R_h = R_{h0} (\frac{M_g}{M_{g0}})^{\lambda_h} \quad \text{(Eq. 8)}$$

where $R_{h0} = 6.9$ nm and $\lambda_h = -0.5$. Also, Equations 3 and 8 have been used to fit data for the hydrodynamic radius versus intensity (Fig. 3c), with the same parameters obtained in the two previous fitting procedures reported in Fig. 3 (a and b): $R_{h0} = 6.9$ nm, $\lambda_h = -0.5$, and $R_{g0} = 18$ nm, $d = 1.65$. Unlike the radius of gyration, the hydrodynamic radius is not determined solely by the shape and size of an object but also by its diffusion properties. In fact, the exponents used for the scaling relations of hydrodynamic and gyration radii are, respectively, $\lambda_h = 0.5$ and $1/d = 0.6$, analogously to what has been found in the case of self-avoiding random polymers (63). We also note that the growth of these polymeric aggregates occurs in a very dilute regime due to the small length of the chains and the low peptide concentration, ruling out the effect of interparticle interaction on the measured hydrodynamic properties. A power law, as in Equation 8, with exponent 0.5, is a very good approximation for the case of flexible rods, when the mean aggregation number $\bar{s}$ is relatively small, as in the present experiments. In fact, we are considering that the building unit of a fibril is not the single Aβ peptide, but the “average” oligomer observed at the onset of kinetics: $\bar{s} = \frac{M_g}{M_{g0}} < 30$. To easily visualize the goodness of this approximation, in the inset of Fig. 3c we plot indeed the expression derived by Yamakawa and Fuji (64) for flexible rods with a persistence length equivalent to the diameter of the rod $d_\text{p} = 14$ nm, together with a power law $R_h = d_\text{p}^2/\bar{s}^{0.5}$. From the estimated persistence length we could calculate the contour length of these linear flexible aggregates. For example, when the hydrodynamic radius is 35 nm, the contour length would be 350 nm.

**Structure of the Kernel**—A very striking point of the present results is that the exponent $\lambda_h$ is equivalent to the exponent $\lambda$ of the coagulation kernel in the Smoluchowski equation (Equation 4). From coagulation theory (54), one can write a quite general expression for the kernel $K_{ij}$, depending upon both the diffusion coefficients of clusters made of $i$ and $j$ units, $D_i$ and $D_j$, and the correspondent spheres of influence of such clusters, with radii $R_i$ and $R_j$, which are related to the size of their binding sites,

$$K_{ij} = 4 \pi D_i D_j \frac{1}{(R_i + R_j)} g_i g_j e^{-\Delta G_{ij}T} \quad \text{(Eq. 9)}$$

where $\Delta G$ is the size-independent free energy change associated with coagulation of two clusters and $g_{ij}$ is a geometric factor related to coagulation probability. Expressions for the hydrodynamic radius (65) and for the sticking probability (66) of rigid fibrils have been reported and used to numerically integrate the Smoluchowski equation for the case of amyloid formation (36, 67, 68). In the present experiments, the kernel homogeneity and the equivalence between the mentioned exponents obtained from data analysis ($\lambda = \lambda_h = -0.5$) allow us to assign all the mass dependence to the hydrodynamic radius. In fact, by using the Stokes-Einstein relation and an expression equivalent to Equation 8 for any aggregation number $i$, the Equation 9 fulfills Equation 6 as follows.

**Temperature Dependence of the Aggregation Rate**—The free energy barrier associated with the aggregation process can be estimated from the temperature dependence of the aggregation rate. In Fig. 4, the quantity $-N_\Lambda k_B \ln(\tau_{c})$ (where $N_\Lambda$ is Avogadro’s number) is plotted versus the reciprocal temperature (Arrhenius plot). Data are well fit by a straight line,

$$N_\Lambda k_B \ln(\tau_{c}) = \Delta S - \frac{\Delta H}{T} \quad \text{(Eq. 11)}$$

where $\Delta S$ and $\Delta H$ are, respectively, the entropy and enthalpy costs associated with coagulation. From the fit, we obtain $\Delta S = 12.2 \pm 2$ cal mol$^{-1}$K$^{-1}$ and $\Delta H = 14.8 \pm 0.3$ kcal mol$^{-1}$, with a corresponding average free energy of $\Delta G = 11 \pm 1$ kcal mol$^{-1}$ (value at 37 °C). The error associated with $\Delta H$ is mainly due to the experimental error on the characteristic times $\tau_0$, which is on the order of 2%. On the other hand, the error in the entropic term $\Delta S$, and consequently in the free energy, is affected also by the intrinsic indetermination in the quantity $g$ that appears in Equation 10. If we assume an error in $g$ of 100%, the error in $\Delta S$ is on the order of $N_\Lambda k_B g$.

**Early Stages of Kinetics at Different Concentrations**—To check the validity of the proposed kinetic model, we report QLS experiments on the early stage of the kinetics performed at 37 °C and at two additional concentrations: 92 and 277 µM. Kinetics of intensity and hydrodynamic radius are reported in Fig. 5, together with the already shown kinetics at 185 µM concentration. Times are scaled according to the parameter $\tau_{c}^p(c)$, which depends on both temperature and concentration as $\tau_{c}^p(c) = \tau_\text{c} c/c_{185}$, where $\tau_\text{c}$ is the parameter already reported for the concentration of the main set of experiments (185 µM), and $c/c_{185}$ is the ratio between the concentration of each sample and the concentration of 185 µM. At the higher concentration, data can be scaled in agreement with the proposed model, whereas at the lower concentration the model seems to fail.

**Aggregation Kinetics from Oligomers to Large Size Tangles**—The kinetics of aggregation has been monitored up to the for-
mation of large size clusters of fibrils, which are clearly observable after 2 months (Figs. 6 and 7). By measuring the scattered intensity at different angles, which is at different scattering vectors, we have obtained the $z$-averaged form factor $P_z(q)$, which can be used to estimate the $z$-averaged radius of gyration $R_g$. In the early stage (first day), when aggregate size is still smaller than 100 nanometers, only a rough estimate of $R_g$ was possible via the Guinier expression: $P_z(q) = [1 + \frac{1}{3}q^2 R_g^2]^{-1}$. During the next weeks, the clusters size slowly increased up to tens of microns. The form factor of such clusters can be modeled by using the mentioned Fisher-Burford expression (53), with an effective fractal dimension $d$ close to one. The form factor measured after two months of aging reveals a clear power law behavior ($P_z(q) \sim q^{-d}$) and a fractal dimension $d = 1.3$, which mirrors the structure of clusters of fibers, as clearly seen by optical microscopy measurements (see Fig. 7). However, the aggregates observed at these late stages are not directly related to the mechanism revealed by the early stage experiments, and their formation and morphological variety are not addressed by the present work.

**DISCUSSION**

Aggregation kinetics of $\alpha\beta(1–40)$ have been monitored by light scattering techniques at pH 3.1 and at different temperatures: 27, 37, 42, 47, 52, 57, and 67°C. At this pH, the kinetics of $\alpha\beta(1–40)$ self-assembly are highly reproducible, mainly due to the lack of a significant amount of spurious nucleation seeds.

**Onset of Kinetics: Large Oligomers**—After dissolution of peptides in aqueous solution, QLS measurements reveal the presence of oligomeric species with a unimodal distribution of hydrodynamic radii peaked at the value of 7 nm (Fig. 1a). The average molecular mass is $390 \pm 60$ kDa, corresponding to an assembly of $75 \pm 15 \alpha\beta(1–40)$. As remarked in the previous section, the presence of monomeric $\alpha\beta$ is not excluded by the presence of larger size oligomers and by the high average molecular mass. These findings are in reasonable agreement with the results of Huang et al. (28) who have estimated, under analogous conditions, a molecular mass of $\alpha\beta(1–40)$ oligomers in the range between 0.5 and 1.4 MDa and a mean diameter of 15 nm. At more acid conditions (pH 2), Lomakin et al. (35–37, 39) have found that $\alpha\beta(1–40)$ are assembled into spherocylindrical micelles of mean hydrodynamic diameter of 14 nm and made up of about 25 peptides. Such low-$q$ holograms are considerably larger than those involved in $\alpha\beta$ fibrillogenesis under physiological conditions (9, 25, 28), as well as from typical oligomeric intermediates found in other protein aggregation processes (18, 69–71).

**Early Stage of Kinetics: One Single, Non-cooperative Process**—To unravel the mechanism of protofibril formation starting from oligomers, we focused on the early stage of aggregation kinetics, by measuring essentially two quantities: light scattered intensity, which is closely related to the average mass of aggregates, and hydrodynamic radius, which is related to the size and the diffusion properties (and hence the shape) of aggregates (Fig. 2). Kinetics at different temperatures (in the range 27–67°C) can be scaled on a single master curve by renormalizing the timescale, that is, by using only one parameter $t_0$, related to the reciprocal aggregation rate (Fig. 3). Also, each set of kinetic data shows no lag time, because the growth of both intensities and hydrodynamic radii have a non-zero derivative at time $t = 0$. Thus, present experiments show that at pH 3.1, protofibril formation occurs via a single elongation process, which operates at different temperatures, with no observable nucleation step.

Of course, the time resolution of our experiments does not allow us to completely rule out the occurrence of nucleation
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within the first minute after sample preparation. However, measurements at the onset of the kinetics at each temperature show the same distribution of oligomers with large molecular mass and size. Thus, nuclei formation, if any, has gone to completion within tens or hundreds of seconds with a very high rate. We can state that, under the present conditions, nucleation is not a rate-limiting step for amyloid formation.

**Arrhenius Plot: One Single Barrier**—The time-scale parameter $t_0$ is linearly correlated with reciprocal temperature (on a semilogarithmic scale) (Fig. 4). This implies that aggregation is controlled by one single free energy barrier, associated with the activation of the intermediate state in the coagulation process. We measured the enthalpic and entropic costs associated with this process to be $\Delta H = 14.8 \pm 0.3 \text{ kcal mol}^{-1}$ and $\Delta S = 12 \pm 2 \text{ cal mol}^{-1}\text{K}^{-1}$, with a corresponding average free energy at $37 \degree C$ of $\Delta G = 11 \pm 1 \text{ kcal mol}^{-1}$.

These results are consistent with the work of Kusumoto et al. (37), which is the only comparable report in the literature. They have estimated the following values for the elongation process of $\beta\alpha(1–40)$ at pH 2: $\Delta H = 23 \text{ kcal mol}^{-1}$, $\Delta S = 53 \text{ cal mol}^{-1}\text{K}^{-1}$, and $\Delta G_{-300\degree K} = 7 \text{ kcal mol}^{-1}$. As remarked by these authors, such a value of activation free energy is reminiscent of those expected for conformational changes of a protein or a peptide. At physiological pH, low molecular weight oligomers of $\beta\alpha(1–40)$ have been found to undergo a conformational change, with a subtle balance of $\alpha$ and $\beta$ structures, to bind to amyloid fibrils (72, 73). The estimated activation free energies are a few kcal mol$^{-1}$. In the present case, we can argue that along with a conformational change related to the single peptide, a local reorganization of each aggregate may be involved to incorporate large molecular weight oligomers, analogously to what is observed in rod-like micelles (74, 75).

**Modeling Coagulation: Diffusion and Reaction of Protofibrils and Exclusion of Monomer-only Addition**—Scaled kinetic data have been analyzed in terms of linear colloidal aggregation (33, 76). We have found that the growth of cluster mass and size can be described by a Smoluchowski equation with a homogeneous kernel associated with the coagulation rate (Equations 4 and 9). These results have been achieved by simultaneous fitting of intensity and hydrodynamic radius growth with time at different temperatures (Fig. 3). In particular, it has been found that the exponent $\lambda_0$, used to model the dependence of the mean hydrodynamic radius $R_h$ on the average molecular mass (Equation 8) is equal to the exponent $\lambda$, which indicates the rank of homogeneity of the kernel (Equation 6). This implies that the coagulation rate has a mass dependence given entirely by the hydrodynamic radii of aggregates, i.e. by their diffusional properties, and that the free energy of activation related to cluster coalescence does not depend on their mass. Also, the typical model of fiber growth by monomer-only addition is straightforwardly ruled out. Thus, the elongation model seems to be different from that observed at pH 7.4, where elongation occurs by addition of single intermediate units (46, 47, 67). A reasonable explanation for this different behavior is that the distributions of oligomeric species initially present in solution are quite different in the two cases, because, as discussed above, they depend on pH and other thermodynamic quantities (28, 77).

**Model Validity and Generality: Dependence on the Initial Conditions**—In addition to the main set of kinetics at different temperatures and at the concentration of 185 µM, we performed two experiments at 37 °C at a lower (92 µM) and a higher concentration (277 µM) (Fig. 5). Although this is a very preliminary report of an ongoing work, it allows us to check the validity of our kinetic model. Kinetic data can be scaled according to the proposed model in the case of the higher concentration, whereas at the lower concentration the model fails. This is reasonably due to the different initial conditions that are observed at low concentration, where both the mean hydrodynamic radius and the average molecular mass are lower than the other two cases. Interestingly, this is in close agreement with the results of Lomakin et al. (35), who have found, for a solution of $\beta\alpha(1–40)$ at pH 2, a critical micellar concentration of about 100 µM. Thus, our model seems to be easily extendable to a high concentration range where the solution exhibits an analogous initial distribution of oligomers.

**Late Stage of Kinetics: Clusters of Fibrils**—The aggregation process has been monitored for several weeks, until amyloid fibrils assemble into large size clusters (hundreds of microns). Such clusters have an apparent fractal dimension of 1.3 on the submicron scale (Fig. 6), because they originate from the aggregation of already formed elongated fibrils (see Fig. 7). Their aggregation mechanism is of course not equivalent to that revealed by our early stage kinetic experiments.

**Concluding Remarks**—The kinetics of amyloid fibrillation are important to understand the mechanism of amyloid self-assembly and to eventually design molecular inhibitors. A complex panorama of different macromolecular structures (protoproteins, filaments, and fibers) is usually present both in the case of the Alzheimer amyloid $\beta$-protein and in other amyloidogenic proteins (78–80). Although our experiments do not directly investigate the physiology of the pathologic assembly, they address $\beta\alpha(1–40)$ self-organization at the molecular level and thus contribute to understand a few basic features of the molecular interactions and mechanisms that drive amyloid fibrillogenesis. Under the particular condition studied (pH 3.1 and high concentrations), flexible protofibrils are formed from a solution containing large molecular weight oligomers by diffusion and coagulation of aggregates of different size, through an activation free energy of about 11 kcal mol$^{-1}$. The specific structure of such oligomers and the nature of the activation barrier, which is still not established, is the subject of ongoing work. The existence of a non-cooperative elongation process casts amyloid formation into an anti-pathogenic process that sequesters toxic oligomeric products. Apart from this interesting suggestion, the present work puts the important mechanism of amyloid formation on firmer basic physical grounds.

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