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Conformational Indeterminism in Protein Misfolding: Chiral Amplification on Amyloidogenic Pathway of Insulin

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Abstract: Unlike folding, protein aggregation is a multipathway, kinetically controlled process yielding different conformations of fibrils. The dynamics and determinism/indeterminism boundaries of misfolded conformations remain obscure. Here we show that, upon vortexing, insulin forms two distinct types of fibrils with opposite local chiral preferences, which manifest in the opposite twists of bound dye, thioflavin T. Occurrence of either type of fibrils in a test tube is only stochastically determined. By acting through an autocatalytic, "chiral amplification"-like mechanism, a random conformational fluctuation triggers conversion of the macroscopic amount of insulin into aggregates with uniformly biased chiral moieties, which bind and twist likewise the achiral dye. Although a convection-driven chiral amplification in achiral systems, which results in randomly distributed excesses of optically active forms, is known, observation of such a phenomenon in misfolded protein built of L-amino acids is unprecedented. The two optical variants of insulin fibrils show distinct morphologies and can propagate their chiral biases upon seeding to nonagitated insulin solutions. Our findings point to a new aspect of topological complexity of protein fibrils: a chiral feature of hierarchically assembled polypeptides, which is partly emancipated from the innate left-handedness of amino acids. Because altering chirality of a molecule changes dramatically its biological activity, the finding may have important ramifications in the context of the structural basis of "amyloid strains".

Introduction

While studies on protein misfolding and fibrillation are medically motivated,[1,2] from a physicochemical standpoint, the phenomenon seems to reflect a basic, generic feature of proteins as polyamides.[3] One of the consequences of fibrillation is the loss of structural unambiguity of folding, resulting in polymorphism of misfolded conformations, especially in the form of amyloid "strains" (i.e., multiple, self-propagating conformational templates that can form out of a single, unmodified protein sequence). This scenario seems to be a generic property among amyloids, and its biological as well as physicochemical consequences have been demonstrated for a number of proteins, including yeast prions[4] insulin fibrils,[5] Alzheimer β-amyloid,[6] or fibrils from the K3 fragment of β_2-microglobulin.[7] Whenever optical isomers of misfolded, aggregated polypeptides are studied, the focus is placed on L−D chain packing effects[8] and possible therapy-oriented applications as inhibitors of amyloid growth.[9] However, thus far the "strain"-like conformational variability in amyloid fibrils has not been thought of in the context of possible optical or chiral isomerization. On the contrary, the chiral aspects of amyloid fibrils, such as their ubiquitous single-handedness, are rationalized in terms of hierarchical consequences of the fundamental left-handedness of the amino acid building blocks,[10] highlighting the notion of determinism in amyloidogenic self-assembly. This stance has also been taken in our recent communication showing that, upon binding to fibrils obtained through vortexing of insulin at 60 °C, a strong negative Cotton effect (induced circular dichroism, ICD) is enforced in the dye, thioflavin T (ThT), known also as "Basic Yellow 1", which is an optically inactive molecule consisting of a pair of benzothiazole and benzaminic

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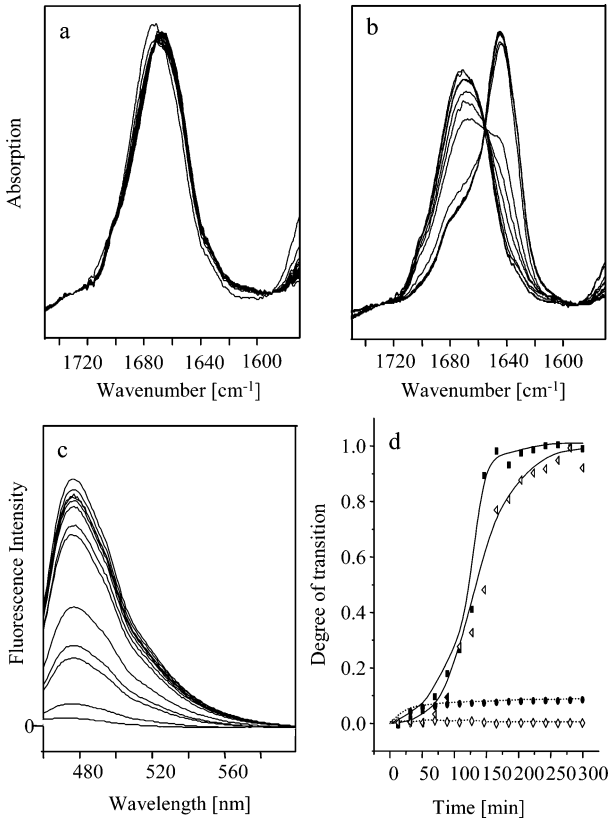

Figure 1. Effect of vortexing on kinetics of insulin fibrillation at 45 °C. The infrared (a and b) spectra reflect the progress of fibrillation of insulin at ambient conditions (a) and vortexing (b). (c) Corresponding time-lapsed fluorescence spectra of ThT bound to the fibrillating insulin (black: vortexing, gray: ambient). (d) Normalized intensities of ThT fluorescence (empty symbols) and β-sheet infrared component at 1628 cm^{-1} (filled symbols) calculated from the spectra in panels a−c for vortexing-assisted (solid lines) and ambient conditions (dotted lines) of fibrillation.

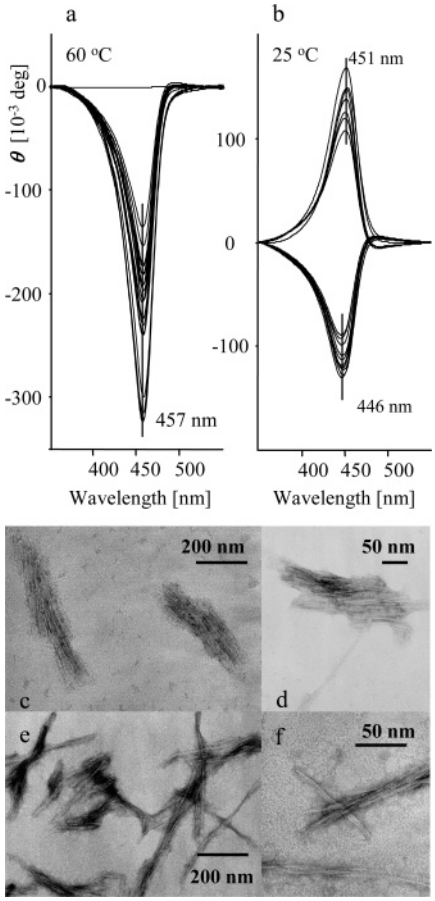

Figure 2. Conformational variants of insulin fibrils with opposite ICDs of bound ThT molecules. (a) A pronounced and uniformly negative Cotton effect hallmarks CD spectra of ThT bound to insulin fibrils formed through vortexing insulin at 1400 rpm and 60 °C (20 spectra correspond to 20 separate samples). The CD data was collected at 25 °C. The horizontal flat line is CD spectrum of ThT bound to "ambient" insulin fibrils formed at 60 °C without vortexing. (b) A similar vortexing routine carried out at 25 °C yields two populations of amyloid samples inducing strong positive or negative CD in ThT. (c−f) Negatively stained TEM images of fibrils with positive (c, d) and negative (e, f) CD signals reflect distinct morphologies.

rings.[11] According to density functional theory (DFT) calculations, the two rotational energy minima of ThT (dihedral angles ψ of ±34.6°) are separated by a barrier of only 13.2 kJ/mol, which accounts for the rapid interconversion between the two enantiomers rendering the molecule with an on the average C_s symmetry and therefore optically inactive.[11] When the low barrier to rotation is hindered (e.g., through entrapment in chirally biased left-handed amyloid fibrils), one sterically favored chiral conformer prevails. Because ThT molecules with positive dihedral angles ψ exhibit strong negative circular dichroism for the HOMO−LUMO transition, we concluded that such conformers are apparently trapped by chiral binding sites on the surface of insulin fibrils.[11]

Experimental Section

Preparation of Amyloid Samples. Fibrils were prepared through 72-h-long vortexing of 1.7 mM bovine insulin solution (Sigma, St. Louis, MO) in 0.1 M NaCl, in D_2O, pD 1.9. Because samples of grown amyloid were subjected to FT-IR measurements, H_2O was replaced with D_2O, and the pD (pH*) was adjusted with diluted DCl according to uncorrected readout from a pH meter.[5] For vortexing-assisted fibrillation of insulin, Eppendorf thermomixer comfort was used. The device enables simultaneous vortexing of 24 × 1.5 mL Eppendorf probes in a temperature-programmable manner. The appliance permits only an anticlockwise mode of spinning. Each amyloid preparation routine began with distributing a fresh solution of native insulin into 20 identical pristine 1.5-mL disposable Eppendorf probes placed in the thermomixer accessory (1 mL per probe). After 72 h of vortexing, CD, fluorescence, FT-IR, linear dichroism, and TEM measurements followed. On the basis of the analysis of amide I and II infrared bands, it can be estimated that, within the accuracy of the FT-IR method and under the conditions of this study, all insulin molecules are converted into solvent-protected β-sheet-rich aggregates. Once the fibrillation process was completed, amyloid samples were equilibrated for 30 min at room temperature prior to staining with ThT.

Effect of Vortexing on Kinetics of Insulin Fibrillation. A 1.7 mM insulin solution in 0.1 M NaCl, D_2O, pD 1.9, was incubated at 45 °C for the period of 5 h with or without intensive vortexing at 1400 rpm in the thermomixer accessory. Every 20 min, a small (30 μL) portion was collected and immediately subjected to FT-IR and fluorescence measurements. While samples for FT-IR were undiluted, for a ThT-fluorescence measurement a 2 μL protein sample was diluted with 100 μL of 0.1 M NaCl, pD 1.8, containing 2×10^{-5} M ThT.

Circular Dichroism. CD spectra were acquired at 25 °C with a Jasco 715 CD System equipped with 1-, 2-, or 10-mm path length cuvettes. Far-UV CD spectra were obtained for original 1.7 mM insulin amyloid samples diluted 20 times with 0.1 M NaCl, pD 1.9. For near-UV CD spectra, the original amyloid samples were diluted 1:1 with the buffer. For measurements of ICD in thioflavin T, stock 0.3 wt %

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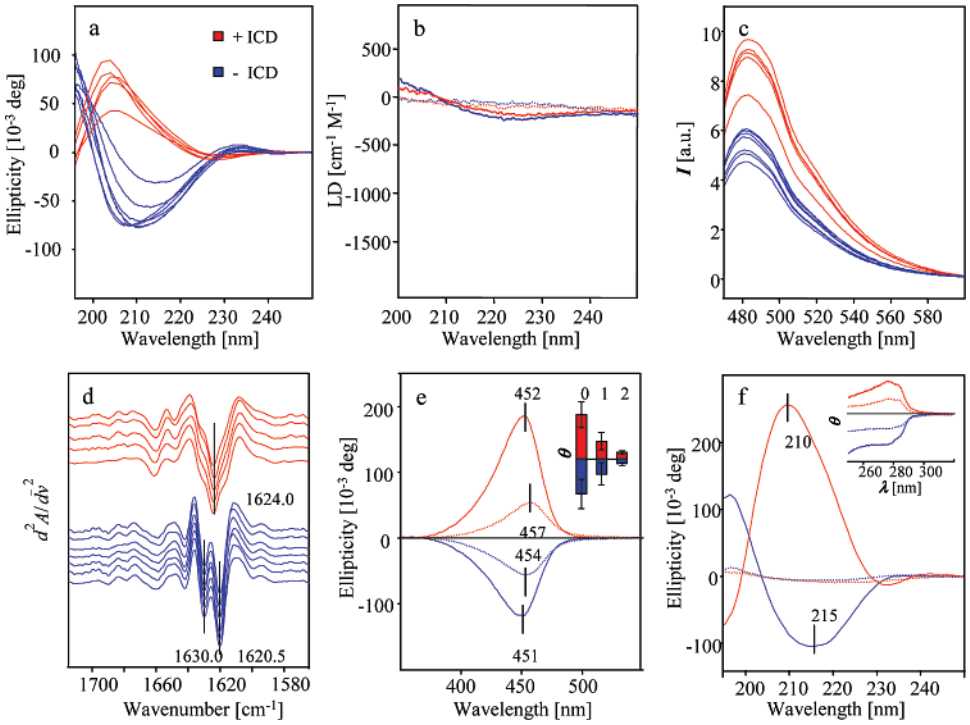

Figure 3. Spectral characteristics of two optical variants of insulin fibrils. (a) Far-UV CD spectra of insulin fibrils acquired in the absence of ThT. The spectra corresponding to the samples with positive ICD of ThT give also positive far-UV CD signals (red), and vice versa. (b) Control data showed no evidence of static linear dichroism in either mother or daughter samples. (c, d) Intensity of fluorescence of ThT−amyloid complexes, $\lambda_{ex} = 450$ nm (c), and second derivative FT-IR spectra (amide I band, d) of samples formed at 25 °C, 1400 rpm, coincide with the sign of ICD of bound ThT molecules (red: positive; blue: negative). Higher quantum yields of ThT fluorescence (c) and weaker interstrand hydrogen bonding in the corresponding infrared absorption spectra (d) of the +ICD samples are observed. (e) ThT ICD spectra of mother amyloid samples grown from insulin at 30 °C, 1400 rpm (solid lines), and their daughter generations obtained through seeding of unstirred insulin solutions at 30 °C at 100:1 native protein-to-amyloid weight ratio (dotted lines). Intensity of ThT ICD signal decreases by 50−80% per generation (e, inset). (f) In corresponding far-UV CD spectra of mothers and daughters, even more dramatic decrease of ellipticity is observed. Inset spectra show simultaneous decay of ellipticity in the near-UV.

ThT solution in 0.1 M NaCl, pD 1.9, was prepared. A quantity of 100 μL of original, fresh, but equilibrated at room temperature amyloid sample was mixed with 200 μL of 0.1 M NaCl, pD 1.9, buffer and 10 μL of the stock ThT solution. After brief incubation at room temperature, ICD was measured in the 350−550 nm range.

Fluorescence. For fluorescence experiments, we used samples prepared for the ThT ICD; however, the samples were further diluted 15 times with 0.1 M NaCl, pD 1.8, prior to measurement. Spectra were collected at room temperature on an AMINCO Bowman Series 2 luminescence spectrometer ($\lambda_{ex} = 450$ nm, five repetitions, 10-mm cuvette).

Fourier Transform Infrared Spectroscopy. FT-IR measurements at 25 °C with a CaF_2 transmission cell and 0.05-mm Teflon spacers were carried out only on deuterated amyloid samples in the absence of ThT. FT-IR spectra were collected on a Nicolet NEXUS FT-IR spectrometer equipped with a liquid nitrogen-cooled MCT detector. Typically, for a single spectrum 256 interferograms of 2 cm^{-1} resolution were co-added. Second-derivative Savitzky−Golay spectra were computed with GRAMS software (ThermoNicolet, Waltham, MA).

Linear Dichroism. The measurement were performed on a Jasco J-720 circular dichroism spectropolarimater equipped with a dedicated linear dichroism accessory, using a 1-mm path length quartz cell at room temperature. Insulin fibrils concentrations were 1.7×10^{-2} mM.

Transmission Electron Microscopy. Samples of insulin amyloid were diluted 10-fold prior to being applied onto formvar carbon-coated 400 mesh per inch copper grids. The samples were left to adsorb for 30 s. Excess material was removed through a triplicate washoff with the buffer. In the following step, the remaining specimens were negatively stained with 1 wt % of uranyl acetate solution. After 1 min, the excess stain was removed and the grid was left to dry off. The fibrils were examined on a Joel JEM 1200 EX transmission electron microscope at an accelerating voltage of 80 kV and at a nominal magnifications of 40 000× and 100 000×.

Results and Discussion

The rapid agitation appears to have two distinct effects on insulin fibrillation. One is rather trivial and consists in the pronounced acceleration of the process, which is illustrated in Figure 1, wherein the kinetics of insulin fibrillation in agitated and nonagitated solutions are compared. No evidence of fibrils was found in nonagitated samples after 5 h of incubation. The minor spectral shift of the amide I band in Figure 1a should be attributed to ongoing H/D exchange of the amide backbone.

Another and more interesting consequence of vortexing of fibrillating insulin is that fibrils formed under such conditions twist bound ThT molecules uniformly.[11] ICD spectra of an amyloid−ThT complex obtained from such fibrils formed at 60 °C are shown in Figure 2a, whereas Figure 2b presents an analogous set of ICD data obtained for fibrils formed at room temperature. Surprisingly, almost half of the 20 samples produced positive ICDs, which must reflect negative dihedral angles in twisted ThT molecules,[11] and therefore the opposite local chirality of moieties capturing the dye. The positive ICD spectra appear to be mirror images of the negative ICDs, although the two states must be related diastereomerically because of the identity of their amino acid chiral constituents. Despite the dramatic differences in ICD, the corresponding UV absorption spectra of ThT are affected by interactions with amyloid fibrils of either type to a much lesser degree (Supporting Information). Corresponding TEM images (Figure 2c−f) show

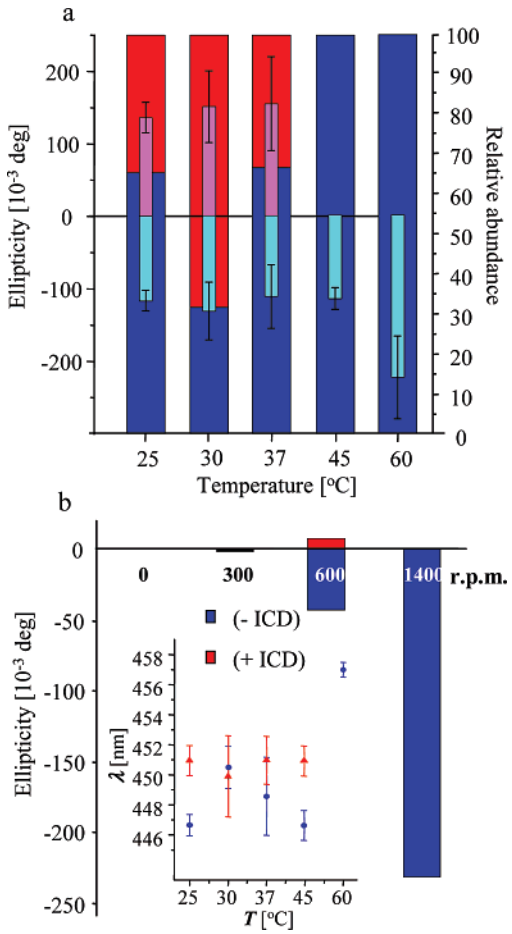

Figure 4. Temperature- and convection-rate dependencies of ICD of ThT bound to insulin fibrils. (a) Relative abundances (wide bars) and corresponding mean ICD intensities (thin bars) of ICD of ThT bound to amyloid samples formed by vortexing insulin at 1400 rpm at different temperatures (statistics based on $n = 20$ independently vortexed samples, error bars mark standard deviations). Red and blue colors reflect positive and negative ICD of ThT, respectively. Room temperature is permissive to the formation of both the variants. At 45 and 60 °C, only the −ICD form is observed. (b, inset) Spectral shifts of the ICD peaks. (b) Dependence of vortexing rate on mean ThT ICD in insulin amyloid samples obtained at 60 °C. Intensity of the ICD signal increases with the convection rate. In unstirred samples, the phenomenon is not observed.

that both types of fibrils have different morphologies revealing clearly their diastereoisomeric relationships. The +ICD fibrils are shorter and laterally aligned, while the −ICD fibrils are longer, thicker, and resemble "wild-type" insulin fibrils. While it is interesting to compare handedness of both types of fibrils in light of the observed opposite ICDs, this task was hampered by persistently insufficient quality of negatively stained TEM images of +ICD fibrils, suggesting a degree of surface amorphization.

Figure 3a reveals another interesting feature of the samples. While there is a broader distribution of signal intensities in far-UV CD data, there is agreement between the signs of far-UV CD and ICD in the corresponding sample with ThT.

That radical perturbation of the far-UV CD spectra in +ICD samples resembling the CD of a D-protein concerned us as to whether optical artifacts stemming from linear dichroism could be overlapping the benign ellipticity of the protein.[12−14] Those were ruled out through control measurements (Figure 3b) showing lack of appreciable static linear dichroism signal. In fact, neither CD nor ICD was affected by stirring, realignment of the optical cell, or brief sonication, all of which would highlight the presence of linear dichroism or birefringence related artifacts. Moreover, the observation of the positive ICDs cannot be rationalized in terms of an L/D-isomerization of the protein. Although such an event seems quite improbable, this can be independently proved by disassembling the +ICD fibrils at pD 12 and then refolding the native protein upon pD readjustment to 7, after which the samples yielded the typical negative Cotton effect of the native predominantly α-helical conformation (data not shown).

For amyloid samples formed at 25 °C and 1400 rpm, the signs of ICD (Figure 2b) coincide with the corresponding morphologies (Figure 2c−f), higher quantum yield of +ICD-amyloid-bound ThT (Figure 3c), and subtle spectral patterns visible in the corresponding infrared absorption spectra (IR amide I band in Figure 3d). In +ICD/−ICD samples formed at higher temperatures, these correlations were absent, however, implying that the various spectral characteristics of both the conformers may not have a common molecular origin, and therefore may be "inherited" independently. Although one may make some links between pieces of the data (e.g., considering earlier studies pointing to lateral aggregation of insulin fibrils, such as in the +ICD samples (Figure 2c,d) as weakening interchain hydrogen bonding,[15] which is in accordance with the corresponding infrared spectra in Figure 3d), these certainly do not allow for a comprehensive structural model of the two optical conformers.

Although ethanol-induced conformational variants of insulin fibrils cross-seed quite effectively,[5] according to Figure 3e,f seeding of either optical conformer to nonagitated insulin solution produces aggregates with ICD decreasing by 50−80% intensity per generation, while the decay of far-UV CD is even more pronounced. These seeding experiments were carried out at 30 °C, which turned out to be the optimal temperature because, on the one hand, it prevents competitive "de novo" fibrillation of insulin and, on the other hand, it permits using relatively low concentration of the seeds. The observed gradual decay of the ICD signals upon seeding is puzzling. It can be hypothesized at this point that a 3D particle of the amyloid seed of either type (±ICD) has only one of its surface "walls" capable of imprinting its "chiral bias", while other walls promote ambient and optically inactive fibrils, which could explain the increasing ratio of the latter and the simultaneously decaying ICD in the following generations (inset in Figure 3e). In other words, vortexing of insulin solution appears to be indispensable for a sustained "transgenerational" proliferation of the ICD signal. This is reminiscent of the situation of aggregating porphyrins, wherein bifurcated pathways of chiral stacking can be controlled by vortexing.[16,17] It was shown recently that chirality of macroscopic spinning can be "memorized" also in spin-coated films formed by aggregated derivatives of porphyrins.[18]

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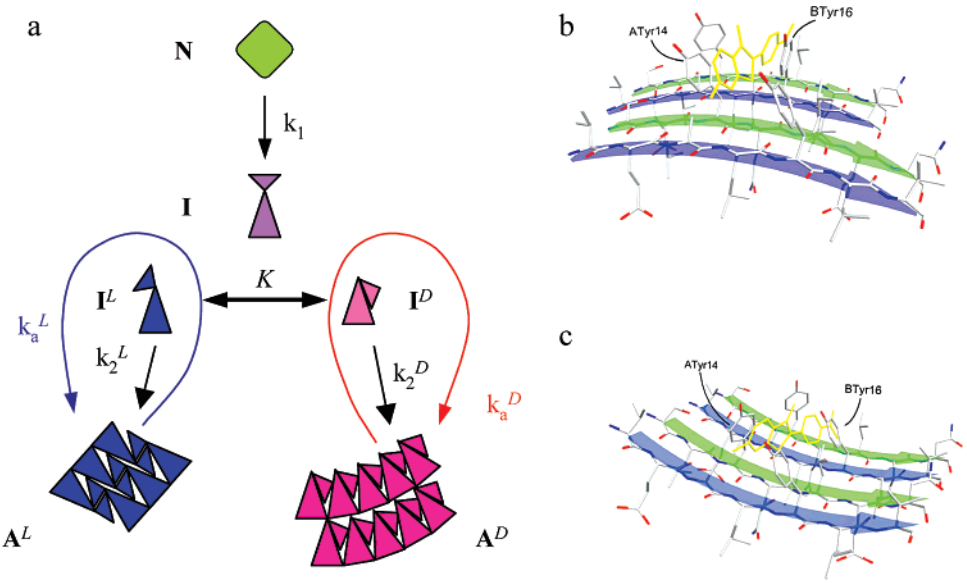

Figure 5. Hypothetical mechanisms underlying the optical polymorphism induced in insulin fibrils. (a) Conformational transition from native state (*N*) to intermediate (*I*) consisting of two interconverting populations of conformational substates (I^{L} and I^{D}) with opposite chiral biases. The chiral bias of each substate appears to be determined at a higher hierarchical level of topological complexity of aggregated polypeptide conformations and is partly emancipated from the fundamental bias of left-handed amino acids residues. I^{L} and I^{D} are not energetically equivalent, and at higher temperature the equilibrium K is shifted toward one form favored by the innate chiral bias of the ingredient L-amino acids. The formation of stacked aggregates of either type from the chirally biased precursor follows the "nucleation and growth scenario". The autocatalytic character of fibrillation and the specific docking of monomeric intermediates to existing aggregates (A^{L} or A^{D}) underlie the observed chiral amplification effect ($k_a^{L} \gg k_2^{L}$; $k_a^{D} \gg k_2^{D}$). (b, c) Tilting of the β-sheet structure and following perturbation of $\pi-\pi$ interactions between ThT molecule and insulin's aromatic side chains could underlie the observed inversion of signs of the ICD of ThT bound to amyloid fibrils (b, +ICD, c, −ICD).

Figure 4 presents effects of temperature (a, at 1400 rpm vortexing rate) and vortexing rate (b, at 60 °C) of fibrillating insulin samples on intensities of ±ICDs of bound ThT. In a "conventional" case of a convection-assisted chiral-amplification process, when there is no initial chiral bias in the system, one would expect that (i) there would be an equal probability of formation of either enantiomer and (ii) changing achiral parameters, such as temperature, should not affect the ratio of the enantiomers.[19−24] However, given the fact that aggregating insulin is chirally biased from the very beginning, the temperature dependence of the prevailing chiral sense of the aggregates becomes less surprising. In fact, a temperature-induced reversal of helical senses in certain polyisocyanates containing chiral centers has been predicted theoretically and verified experimentally.[25,26] It is quite likely that there are more very close analogies in the domain of polymer sciences that would parallel the chiral conflict between deterministic, "left-handed" bias of amino acid building blocks and the chirally unbiased indeterminism of molecular fluctuations reported in this study. It is remarkable that at 45 and 60 °C only −ICD conformer is observed, suggesting that a profound unfolding of the native structure and formation of high entropy disordered conformations renders the system more deterministically controlled. Namely, any residual native structure in the aggregation-prone intermediates might act as a kinetic trap along the fibrillation process, affecting its kinetics and conformation of fibrils.

Figure 5a is a hypothetical kinetic mechanism of induction of the two distinct conformers of insulin fibrils. The key element of this model is the presence of an intermediate state (*I*) consisting of two interconverting populations of substates (I^{L} and I^{D}) with opposite local chiral preferences. The nature of this chiral bias of each substate is, at least at a moderate temperature (vide Figure 4a), not determined by the principal bias of left-handed amino acid residues. This could be achieved on a higher hierarchy level of topologies of aggregated polypeptide conformations, for example, through tilting planes of aggregated β-sheets (Figure 5b,c). At this point, we may only speculate that tilted, strained geometries of β-sheets underlie conformational variations leading to the observed dramatic changes in optical activity. Such conformational events, along with possible realignment of β-strands, could help rationalize the opposite ICD of docking ThT molecules (Figure 5c). The I^{L} and I^{D} intermediates are neither energetically nor entropically equivalent. At higher temperatures and with increasing structural disorder, the equilibrium $I^{L} \leftrightarrow I^{D}$ is shifted toward the form favored by the innate chiral preference of the ingredient L-amino acids.

While some of the observations reported in this work may appear paradoxical, in fact there are theoretical and experimental studies on synthetic polymers that provide very close and interesting analogies. For example, both left- and right-handed helical crystals were obtained from polymers having identical right-handed chiral centers.[27,28] Obviously, the relative confor-

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mational simplicity makes it easier to propose a comprehensive structural model explaining such behavior for synthetic sequenceless polymers than for a protein. Another very recent and very relevant observation in the context of our findings concerns a number of filamentous phages, which form nematic liquid crystals "oblivious" to the chirality of their molecular components.[29]

A number of studies indicate that the helical sense of polymers, or tubular aggregates, may be controlled by means of interactions with a chiral solvent.[30,31] We applied a similar approach, looking at whether the presence of pure enantiomers of lactic acid, or 2-butanol (at 20 v/v %), would affect the *K* constant; however, within the examined range of temperature, pH, and convection rate, such a "chiral solvation" effect was not observed (data not shown). In the end, from the kinetic standpoint, the observed phenomenon can be described on the basis of the "nucleation and growth scenario" coupled to the chiral amplification-like effect.[32]

Conclusions

Our findings unravel not only a thus-far unknown aspect of protein misfolding, but also the topological organization and symmetry of an amyloid fibril itself. Apart from the aforementioned possible clinical implications for studies on "prion strains", optical isomers of a misfolded protein described in this work provide a new tool to study the determinism/indeterminism boundaries in protein folding. According to the recent study by Corrigan et al.,[33] aggregating lysozyme may form nematic liquid crystal phases. Such optical properties, when coupled to the chiral complexity of protein aggregates described in this work, constitute a new nanotechnological rationale for undertaking studies on the polymorphism of protein non-native β-pleated aggregates.

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Supporting Information Available: UV spectra of insulin amyloid, thioflavin T, and the insulin amyloid−thioflavin T complex. This material is available free of charge via the Internet at http://pubs.acs.org.

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