Perturbation of the Stability of Amyloid Fibrils through Alteration of Electrostatic Interactions

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ABSTRACT The self-assembly of proteins and peptides into polymeric amyloid fibrils is a process that has important implications ranging from the understanding of protein misfolding disorders to the discovery of novel nanobiomaterials. In this study, we probe the stability of fibrils prepared at pH 2.0 and composed of the protein insulin by manipulating electrostatic interactions within the fibril architecture. We demonstrate that strong electrostatic repulsion is sufficient to disrupt the hydrogen-bonded, cross-β network that links insulin molecules and ultimately results in fibril dissociation. The extent of this dissociation correlates well with predictions for colloidal models considering the net global charge of the polypeptide chain, although the kinetics of the process is regulated by the charge state of a single amino acid. We found the fibrils to be maximally stable under their formation conditions. Partial disruption of the cross-β network under conditions where the fibrils remain intact leads to a reduction in their stability. Together, these results support the contention that a major determinant of amyloid stability stems from the interactions in the structured core, and show how the control of electrostatic interactions can be used to characterize the factors that modulate fibril stability.

INTRODUCTION

Amyloid fibrils are filamentous, β-sheet-rich protein superstructures with typical diameters of ~10 nm and lengths of the order of microns. In vivo, these structures can form as a result of aberrant misfolding and subsequent aggregation of normally soluble proteins, leading to their accumulation in various organs and tissues of the body, processes that are associated with more than 40 clinical disorders, including Alzheimer’s disease and type II diabetes (1). In other cases, however, controlled formation of amyloid structures is connected to important biological functionality such as the catalytic production of melanin (2) and the formation of functional coating materials (3). In addition, amyloid fibrils with structural characteristics in common with those formed in vivo can also be formed in vitro from many different unrelated peptides and proteins (4). This finding, in addition to the observations that amyloidogenic proteins can be remarkably variable in both sequence and native structure, has led to the suggestion that fibril formation represents a common or generic property of polypeptide chains (1) driven by common intermolecular interactions between the polypeptide main chains (5).

Structural studies on amyloid fibrils have revealed that they share a common cross-β structural motif, in which individual β-strands lie perpendicular to the fibril axis with the resultant β-sheets stacked in the parallel direction to produce protofibrils (6,7). In many cases, these protofibrils associate laterally to generate hierarchical structures. Amyloid fibrils typically demonstrate remarkable stability and their extreme resistance to high hydrostatic pressures and temperatures has been used to support the suggestion that the amyloid structure, under some conditions, can represent a lower free energy state than that of the native fold of proteins (8,9). The stability of these structures is of great importance from a biomedical perspective, as this characteristic is likely to impede their disaggregation and clearance in vivo. This property could, however, also underlie the benefits associated with exploitation of amyloid fibrils for functional purposes in nature (10) as well as providing inspiration for the design and implementation of novel nanomaterials based on protein nanostructures (5,11).

The molecular origin of fibril stability has recently been the focus of intense investigation, and the characteristic main-chain hydrogen bonding between the constituent polypeptides has been identified as an important contribution (5,12,13). Recent high-resolution structural studies of amyloid structures have also identified additional sources of fibril stability; these include intermolecular hydrogen bonding, ionic pairing, and aromatic π–π interactions between amino-acid side chains (7,12,14). In addition, the entropic energy resulting from the release of structured solvent molecules in the densely packed core (13,15) of amyloid fibrils is likely to be a significant factor in enhancing their stability.

In this article, we examine the contribution of electrostatic interactions to the stability of fibrils composed of the protein insulin, and use the active control of such interactions to probe the magnitude and nature of the stability of the amyloid structure. Insulin is a small, α-helical protein...
comprising two disulfide cross-linked polypeptide chains (A and B), which readily forms amyloid fibrils under acidic conditions (16). It has been established for many years that insulin fibrils produced in this way dissociate under alkaline conditions (17,18). Here, we examined the mechanism of this dissociation process by preparing fibrils at pH 2.0 and measuring their stability as a function of the pH of the solution in which the fibrils are subsequently immersed. The results show that the stability of insulin fibrils is sensitive to the net charge on the polypeptide chains, and the disruption of ion-pairings. Furthermore, by combining this approach with chemical denaturation, we observe a correlation between fibril stability and β-sheet content, suggesting that, at least in this case, the origin of fibril stability stems from the interactions within the hydrogen-bonded cross-β structural motif common to amyloid fibrils.

**MATERIALS AND METHODS**

**Preparation of insulin fibrils**

Insulin fibrils were prepared in seeded reactions to produce a homogeneous fibril suspension with minimal chemical degradation and very few higher order aggregates such as spherulites (as determined by cross-polarized light microscopy) (19,20). A single round of seeding using 2.5–5% w/w pre-formed fibrils was sufficient to produce a homogeneous fibril suspension. Seed fibrils were prepared by incubation of bovine insulin at 10 mg/ml in dilute HCl at pH 2.0 and 60 °C for 16 h. The seeded solutions were incubated at 60 °C until a thick gel formed (16).

**pH dependence of the extent of insulin fibril dissociation**

Aliquots of insulin fibrils in dilute HCl at pH 2.0 were added into buffered solutions as follows: glycine in the pH ranges 2.0–3.5 (I: ~10–30 mM) and 9.5–10.5 (I: ~10–30 mM); acetate, pH 4.0–5.5 (I: ~10–30 mM); citrate, pH 6.0–7.0 (I: ~10–30 mM); Tris-HCl, pH 7.5–9.0 (I: ~30–10 mM); and phosphate, pH 11.0–12.5 (I: ~60–150 mM) such that the final protein and buffer concentrations were 0.3 mg/ml and 30 mM, respectively. Over the range where significant dissociation was observed, the ionic strength varied from ~10 mM to 60 mM. However, similar results were obtained when the pH of a separate fibril stock solution was adjusted with NaOH, where the ionic strength varied from only 10 mM at pH 2.0 to 11 mM at pH 11 (data not shown).

The solutions were incubated at room temperature for 48 h, and the extent of fibril dissociation measured by sedimenting the fibrils in an Optima TLX ultracentrifuge (Beckman-Coulter, Fullerton, CA) at −288,000 × g for 40 min. The supernatants were subsequently neutralized via the addition of concentrated Tris-HCl (pH 7.5) and the absorbance measured at 280 nm to calculate the protein concentration using an extinction coefficient of 6335 M⁻¹ cm⁻¹ (www.expasy.org).

**Kinetics of insulin fibril dissociation**

Insulin fibrils were diluted to a final concentration of 0.2 mg/ml in dilute HCl at pH 2.0. Dissociation was initiated by the addition of small volumes of concentrated NaOH and monitored by changes in the absorbance at 280 nm using a Spectronic 100S spectrophotometer (Varian, Cary, NC) with a Peltier temperature controller. The pH of each solution was measured at the conclusion of each reaction.

**Transmission electron microscopy**

Samples were deposited onto Formvar- and carbon-coated 400-mesh copper grids and negatively stained with uranyl acetate. Electron micrographs were acquired on a Tecnai 20 transmission electron microscope (TEM; Philips, Amsterdam, The Netherlands) at a voltage of 120 kV.

**Fourier transform infrared spectroscopy and analysis**

Labile hydrogens in bovine insulin were exchanged for deuterons by dissolving the protein to a final concentration of 3 mg/ml in D₂O at pH 1.6 (the pH meter electrode reading in the deuterated solvent without correction for isotope effects). Solutions were incubated at 37 °C for 16 h and subsequently lyophilized. Seed fibrils were produced as outlined above, except that D₂O at pH 1.6 was used as the solvent and the protein was incubated at a concentration of 20 mg/ml. Fibril solutions were adjusted to the desired pH† by the addition of NaOD. Spectra were recorded with an Equinox 55 Fourier transform infrared (FTIR) spectrometer (Bruker Optics, Billerica, MA). Samples were held between CaF₂ windows using a 50-µm Teflon spacer. For each spectrum, 256 interferograms were coadded at room temperature and at 2 cm⁻¹ resolution, and the background absorption of D₂O was subtracted. Spectra between 1600 and 1700 cm⁻¹ were normalized to unity, and the fitting of component peaks to Gaussian functions (21) was performed using the OriginPro 7.5 software package (OriginLab, Northampton, MA).

**Guanidinium thiocyanate-induced fibril dissociation**

Insulin fibrils were added at a final concentration of 0.3 mg/ml to solutions containing various concentrations of guanidinium thiocyanate and either 30 mM glycine (pH 2.0), acetate (pH 4.0), citrate (pH 6.0), or Tris-HCl (pH 8.0). Solutions were incubated at room temperature for 48 h, before centrifugation in an Optima TLX ultracentrifuge (Beckman-Coulter) at −288,000 × g for 40 min. This incubation period was demonstrated to be sufficient for the samples to reach equilibrium (see Fig. S2 in the Supporting Material). The protein concentration of the supernatants was determined using the Bradford assay (22).

**RESULTS AND DISCUSSION**

**Fibril stability is susceptible to electrostatic repulsion between constituent polypeptide chains**

To determine the pH dependence of insulin fibril stability, solutions of fibrils produced at pH 2.0 were adjusted to a range of pH values between 2.0 and 12.5 and incubated at room temperature for 48 h. Determination of the soluble fraction of insulin in these solutions subsequent to ultracentrifugation showed no observable dissociation of the fibrils between pH 2.0 and 8.0 (Fig. 1, left panel). Between pH 8.0 and 11.0, however, increasing amounts of insulin were present in the supernatants indicating that dissociation had taken place in these solutions, consistent with recent findings of Malisauskas et al. (23) who demonstrated disaggregation of insulin amyloid fibrils (grown under similar conditions) in this pH range using Thioflavin T fluorescence as a probe. This conclusion was confirmed by examination of the fibril solutions by TEM before centrifugation (Fig. 1, right panel).
panels). In agreement with the sedimentation data described above, abundant fibrils were observed between pH 2.0 and pH 8.0; fewer fibrils were present at pH 10.0, however, and none were detected at pH 12.0, confirming their dissociation in this pH range. Although no significant dissociation into soluble species occurred below pH 8.0, fibrils at pH 6.0 and 8.0 are morphologically distinct from those at pH 2.0 and 4.0, appearing shorter with a higher degree of lateral association.

The fibril dissociation reaction was found to have reached completion and to be reversible (see the Supporting Material), so the observed dissociation reflects a change in stability between the soluble and pH 2-like fibrillar forms of insulin. Soluble insulin has been shown to have complex self-association behavior, particularly in the presence of zinc and at neutral pH (24). However, because insulin oligomers become less stable as the pH is increased above neutral (25), the decrease in fibrillar material must be caused by destabilization of fibrils, rather than stabilization of the soluble form. Furthermore, it is expected that the majority of soluble insulin is monomeric over the pertinent pH range. This is because of the high concentration dependence of the insulin oligomer population (26–28) and the relatively low soluble insulin concentrations utilized in these studies (≤0.3 mg/ml). In addition, insulin oligomers dissociate from pH 7 to pH 9 (25) even at 2.5 mg/ml, whereas the majority of fibril dissociation is observed from pH 9.0 to pH 10.5.

To gain insight into the driving forces behind the dissociation reaction, we estimated the net charge $Q$ on the insulin molecules as a function of pH based on the ionizable groups present in the insulin sequence (Fig. 2 A). An isoelectric focusing gel showed that native pH 10.0 bovine insulin incubated at room temperature for 48 h experienced no change in pI (data not shown), suggesting that in the soluble form at least, there is no significant deamidation of the asparagine or glutamine residues (to aspartic acid or glutamic acid, respectively) within the timescale of the experiment. The $pK_a$ values of the ionizable groups present in amyloid fibrils are likely to deviate from their intrinsic values due to the conditions of the experiment. The $pK_a$ values of the ionizable groups present in amyloid fibrils are likely to deviate from their intrinsic values due to the

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effects of the folded context of the fibril. Given the challenges associated with empirically determining these \( pK_a \) values for amyloid fibrils using conventional approaches such as solution-state NMR spectroscopy, we estimated \( Q \) by utilizing average \( pK_a \) values tabulated by Pace et al. (29) for some 541 ionizable moieties from 78 folded proteins (as described in the Supporting Material).

The fraction of soluble protein is shown as a function of the estimated polypeptide net charge in Fig. 2 B. Between pH 2.0 and 8.0, the range in which fibril dissociation is below detectable limits, the estimated net charge on the protein changes from 5.9 to -3.3, the isoelectric point being pH 5.5. Across the pH regime in which dissociation is readily detectable (pH 8.0–12.0), the estimated net charge increases from -3.3 to -9.2. We consider here that fibril stability is readily perturbed in this high pH regime, owing to the increasing repulsive forces between insulin molecules as the magnitude of the polypeptide net charge increases.

On this assumption, we can estimate the contribution of electrostatic effects to the dissociation of fibrils by considering a colloidal model where each monomer in the fibril experiences a repulsive electrostatic interaction with its nearest neighbors, modeled by a screened Coulomb potential as

\[
U_{ch} = \frac{Q^2}{4\pi\varepsilon_0\varepsilon r_0} e^{-r_0/r_D},
\]

where \( r_0 \) is the Debye length, \( r_D \) is the effective separation of the protein molecules in the fibril, \( \varepsilon_0 \) is the permittivity of a vacuum, and \( \varepsilon \) is the dielectric constant of water. In agreement with independent studies of other amyloid systems (30–33), the existence of a dynamic equilibrium between the fibrillar and soluble states of insulin was demonstrated in experiments exploring the reversibility of the dissociation reaction (see Fig. S3 and Fig. S4). We consider this equilibrium according to classical linear polymerization theory as: \( m + x_j \rightleftharpoons x_{j+1} \), where \( [m] = [x_1] \) is the monomer concentration, \( [x_j] \) the concentration of fibrils with polymerization number \( j \), and \( K = [x_{j+1}]/([x_j][m]) \) is the equilibrium constant, which we assume here to be constant for all polymerization numbers \( j \) (34). Similarly to an approach used by Narimoto et al. (30), we can then compute the fraction of soluble protein as

\[
f = \frac{[m]}{[m_{tot}]} = \frac{2[m_{tot}]e^{-[\Delta G_0 + U_{ch}(Q)]} + 1 - \sqrt{4[m_{tot}]e^{-2[\Delta G_0 + U_{ch}(Q)]} + 1}}{2m_{tot}^2 e^{-2[\Delta G_0 + U_{ch}(Q)]}},
\]

where \( [m_{tot}] = \sum_{j=1}^{\infty} [x_j] = [m]/(1 - K[m])^2 \) is the total concentration of protein in both fibrillar and soluble form, and \( \beta = (RT)^{-1} \) is the inverse temperature. We have expressed the equilibrium constant as \( K = e^{-[\Delta G_0 + U_{ch}(Q)]} \), where the free energy difference between the soluble and fibrillar state \( \Delta G_{tot} = \Delta G_0 + U_{ch}(Q) \) has been split into an electrostatic part \( U_{ch}(Q) \) given by Eq. 1, and the free energy difference between a soluble insulin monomer and a monomer within a fibril in the absence of charge, \( \Delta G_0 \).

A fit of Eq. 3 to the data in Fig. 2 B reveals that these simple considerations based on electrostatic effects, which are utilized extensively in colloidal science (35), can describe accurately the dissociation of highly charged fibrils at high pH values, where \( Q \leq 0 \). Furthermore, we can acquire an estimate for the fibril stability in the absence of charge, \( \Delta G_0 = -39.4 \pm 1.3 \text{ kJ/mol} \), as well as the only other free parameter, the average distance between charges in the fibril, calculated as \( r_0 = 1.48 \text{ nm} \) using a Debye length of 2 nm. Previously, charge per residue (rather than net charge) has been shown to be an accurate order parameter for predicting conformations of intrinsically disordered proteins (36).

Because this study involves only one protein, it has not been possible to distinguish between these two parameters. We note, however, that the obtained value of \( r_0 \) using \( Q \) is consistent with the expected distance between monomers in the fibril of \((2 \times 0.78 \text{ nm}) = 1.56 \text{ nm} \), based on each monomer occupying two \( \beta \)-strands in the fibril structure (37) and an intersheet distance of 0.78 nm (37). The stability of the fibrils determined here is of the same order of magnitude as that measured for amyloid fibrils composed of \( \beta \)-microglobulin and the \( \beta \)-amyloid peptide (30,31), indicative that the key interactions that stabilize amyloid fibrils in general are largely sequence-independent in nature. The ability to model accurately the characteristics of amyloid fibrils shown here using a coarse-grained approach is consistent with findings from previous studies that show that the rate of fibril elongation correlates inversely with polypeptide net charge (38). Taken together, these observations demonstrate that both fibril assembly and stability are highly sensitive to the global physicochemical character of the polypeptide chain.

**Thermodynamic stability of fibrils is reduced by partial disruption of \( \beta \)-sheet structure**

Although fibril dissociation was below detectable limits at pH values <pH 8.0, inspection of the fibrils by TEM showed substantial changes in their morphology between pH 4.0 and pH 8.0 (Fig. 1, right panels). To ascertain whether or not this phenomenon is related to changes in the intrinsic molecular structure of the fibrils under these conditions, we examined the fibrils by FTIR spectroscopy. The amide I’ region of the FTIR spectrum (1600–1700 cm\(^{-1}\)) arises predominantly from carbonyl-stretching vibrations within the peptide bonds, and as such is highly sensitive to the secondary structure present in the polypeptide chain (21). The amide I’ band in the FTIR spectrum of insulin fibrils at pH 8.1 is shown in Fig. 3 and demonstrates a dominant peak at 1627 cm\(^{-1}\)
attributable to the presence of intermolecular β-sheet structure, a second major peak at 1657 cm⁻¹ indicative of α-helix/turns/bends, and a small peak corresponding to random structure at 1645 cm⁻¹ (21).

As the pH* of the fibril suspension is increased to pH* 3.6, only very small and subtle changes are observed in the spectrum; between pH* 3.6 and 7.6, however, a number of significant spectral changes are evident. The most pronounced is a decrease in the intensity of the peak at 1645 cm⁻¹ with a concomitant increase in those at 1657 cm⁻¹ and 1645 cm⁻¹, indicating a partial loss of ordered β-sheet structure. In addition, there is a subtle shift in the maximum of the β-sheet peak to higher frequencies indicative of an overall weakening of the hydrogen-bonding network in the fibril (39). Deconvolution of the spectra at pH* 1.6 and 7.6 was performed to estimate the secondary structural content of the fibrils (see Fig. S1). These analyses indicate that the fibrils at pH* 1.6 contain 75% β-sheet structure, but that those at pH* 7.6 contain only 58% of such structure, indicating a relative decrease in β-sheet structure of 23%. This standard type of analysis assumes identical extinction coefficients for the different structural elements, and has been shown to be subject to absolute errors of 2.5–4% in structural content (21).

The behavior of the fibrils below pH 8.0 was further examined by comparing the resistance of the fibrils to dissolution by guanidinium thiocyanate (GdnSCN) (Fig. 3 B). Fibrils were incubated at between pH 2.0 and 8.0 in the presence of increasing concentrations of the denaturant for 48 h before ultracentrifugation. The fraction of insulin in the supernatant was again used as a measure of fibril dissociation. At pH 2.0, the fibrils appear to undergo a single dissociative transition with a midpoint of 3.75 M GdnSCN. As shown in Fig. 4 A, the fibrils at pH 4.0 are only slightly destabilized relative to those at pH 2.0; at pH 6.0 and 8.0, however, the fibrils are substantially destabilized, with dissociation midpoints of 2.5 M and 1.5 M GdnSCN, respectively. These data fit well to the linear polymerization model of Oosawa and Kasai (34), yielding fibril stabilities of ΔG = −56.3 ± 1.2, −55.4 ± 1.6, −43.2 ± 0.9, and −33.3 ± 0.6 kJ/mol at pH 2.0, 4.0, 6.0, and 8.0, respectively, where the quoted uncertainties correspond only to the error in fitting.

Hexameric insulin has previously been shown to be completely dissociated by 0.25 M GdnHCl (a weaker denaturant than GdnSCN) and insulin to be mostly monomeric at 2 M GdnHCl, even at pH 7.4 where oligomers are maximally stable, and a high concentration of 2 mg/ml (40). This justifies application of a model involving an equilibrium between monomeric and fibrillar insulin. In addition, similar m-values (related to the apparent slope) were obtained at each pH, ranging from 7.9 kJ/mol/M (pH 6) to 9.8 kJ/mol/M (pH 4), suggesting a similar change in solvent-accessible surface area in each case.

Remarkably, the stability of the fibrils determined here at pH 6.0, close to the predicted isoelectric point of the protein (5.5), is very similar to the fibril stability determined in the absence of charge by analysis of the effects of electrostatic interactions as detailed above (Fig. 2 B; ΔG0 = −39.4 ± 1.3 kJ/mol). To explore the origin of the dramatic decrease in fibril stability between pH 2.0 and 8.0, the fibril stabilities were considered as a function of the relative intensities of the main β-sheet peak in the FTIR spectra (Fig. 4 B). The resulting correlation coefficient of r = −0.99, p < 0.01, demonstrates that fibril stability is strongly related to the fraction of β-sheet structure within the fibrils, and therefore is strongly influenced by the interactions within the cross-β network.

Unlike the high pH regime, above pH 8.0, in which we observe fibril dissociation, the changes in fibril structure and stability between pH 2.0 and 8.0 cannot be modeled...
in terms of simple charge repulsion, given that the net charge of the protein molecule changes from positive to negative in this pH range. Furthermore, between pH 2.0 and 8.0 the unfolding of a discrete proportion of β-sheet structure within the fibrils indicates that local rather than global changes may govern, at least in part, the changes observed in this pH range. In this case, the disruption of one or more ionic interactions in association with the increase in the pH from 2.0 to 8.0, could trigger a change in fibril structure that impacts on the stability of the fibrils. Based on the distribution of pK\textsubscript{a} values for amino-acid side chains within the context of folded proteins (29), any of the four glutamic acid or two histidine side-chain moieties are capable of participating in an ionic interaction that could be disrupted in this pH range.

If, therefore, a glutamic acid or histidine side chain forms a salt bridge at pH 2.0 with a partially buried group of opposite charge, disruption of this interaction as the solution pH increases will result in a partial reorganization of the structure to solvate the resulting highly energetically unfavorable buried charge. Our observation from FTIR that the β-sheet content of the fibrils decreases from 75% (pH 2.0) to 57% (pH 8.0), indicates that between eight and nine hydrogen bonds within the total sequence length of 51 residues are disrupted to accommodate this structural reorganization. In support of this speculation, we note that the breakage of 8–9 hydrogen bonds with an energy of 2.1–6.3 kJ/mol per bond is similar in magnitude to the energetic penalty for the burial of a charge in the interior or the protein structure (~21 kJ/mol (41)).

The kinetics of insulin fibril dissociation is dependent on the ionization state of a single side chain

After this analysis, we returned our attention to the pH range in which dissociation readily takes place by examining the kinetics of insulin fibril dissociation. Such dissociation was induced by the addition of sodium hydroxide and monitored by following the decrease in solution turbidity during the course of the reaction (Fig. 4A). These kinetic data were then analyzed by fitting to a double-exponential function (see Materials and Methods). The two decay rates (k\textsubscript{1}) differ by almost an order of magnitude and appear to be directly proportional to each other (R = 0.99) with a proportionality constant of 7.6 ± 0.2 (Fig. 4B). An analysis of the temperature dependence of the two rate constants at pH 11.3 reveals that, within the temperature range 15–50°C, both of these processes are, to a very good approximation, governed by Arrhenius-type behavior (Fig. 4C).

This observation does not exclude deviations from classical Arrhenius behavior—i.e., changing heat capacity, outside this temperature range, as is commonly seen in the case of protein folding over larger temperature ranges (42). Arrhenius-type behavior has previously been observed in amyloid formation (43,44). Based on these data, the
enthralic activation barriers calculated for the fast and slow
dissociation rates are 55 ± 2 kJ/mol and 54 ± 4 kJ/mol,
respectively. The similarity of these two values strongly
suggests that the fast and slow rate constants for the disso-
ciation reaction monitor the same fundamental chemical
event. These activation energies are, interestingly, similar
in magnitude to, but smaller than, the activation energy
for the reorganization of insulin molecules that occurs as
they attach to the fibril ends under optimal conditions for
growth at pH 2.0 where ΔH^growth = 102.5 kJ/mol (44).

As shown in Fig. 4 B, the dissociation rates have a strong
dependence on the final solution pH. A plot of log k against
pH (Fig. 4 B) shows linear regions with gradients of 0.98 ±
0.05 for the slow phase and 0.91 ± 0.05 for the fast phase,
indicating that both rates are approximately inversely
proportional to the number of hydrogen ions free in solution.
A comparison of the results with a modified mathematical
model for rationalizing the pH dependence of protein dena-
turation (see the Supporting Material) shows that the
gradient of close to unity suggests that the kinetics of insulin
fibril dissociation are regulated by the charge state of a single
amino-acid residue.

Further to this conclusion, the analysis stipulates that the
pK_a of this residue must be higher than the pH range for
which log k displays a linear dependence on pH, indicating
that the pK_a of the amino acid must be above 12.0. This
finding suggests a critical role under these highly destabiliz-
ing conditions for the unique arginine residue (pK_a 12.3) at
position B22, whereby this residue may participate in a salt
bridge capable of kinetically stabilizing the fibrils against
rapid dissociation. The pK_a values of both lysine and tyro-
sine side chains, however, have been observed to exceed
12.0 within the folded context of some proteins, and as
such we cannot rule out that one of the four tyrosine residues
or the unique lysine residue in the sequence may account for
our observations.

CONCLUSION

Many polypeptides and proteins with variable primary
sequences can assemble into amyloid fibrils; different solu-
tion conditions, however, are often required to tune the
chemical properties of the amino-acid side chains, such as
their charge, to facilitate this conversion (45). We have
demonstrated in this article that, even once formed, the
stability of fibrillar species, typically highly resistant to
dissociation, is susceptible to the pH-induced ionization of
side-chain moieties. The stability of fibrils composed of the
protein insulin prepared at pH 2.0 and then suspended
in buffers of various pH, can be divided into three different
pH regimes. Insulin fibrils are highly stable at low pH values
(2.0–4.0). At pH values in the range 4.0–8.0, however, the
disruption of specific electrostatic interactions results
in structural reorganization and a decrease in size of the
β-sheet network, with a concomitant decrease in the thermo-
dynamic stability of the fibrils. Above this pH range the
increasingly negative net charge on the insulin molecules
drives substantial dissociation of the fibrils as a result of
increasing electrostatic repulsive forces.

Our data demonstrate in addition that specific interactions
between side-chain groups, such as salt bridges, can influ-
ence the structure and stability of the fibrils in a rational
manner. Within the context of globular proteins, ionic pair-
ing interactions are capable of stabilizing native folds rela-
tive to the unfolded state both thermodynamically and
kinetically (46–49). The data presented here show that
specific electrostatic interactions may also play a similar
role within the architecture of amyloid fibrils.

Globular proteins have a well-defined three-dimensional
structure under their native conditions, encoded by their
amino-acid sequence, which has been optimized (to varying
extents) by evolution (50) to guarantee reliable folding. This
structure is destabilized outside the relatively narrow
working pH range of the protein. In contrast, amyloid fibrils
may be formed from the same protein under a variety of
conditions. For example, insulin may form amyloid fibrils
both at acidic and at neutral pH values, resulting in struc-
tures that are morphologically distinct (51). Indeed, tech-
niques such as AFM and cryo-EM have shown multiple
strains of insulin amyloid fibrils to coexist within the
same solution (37).

The persistence of the observed fibril types implies that
they are likely to be some of the most thermodynamically
stable structures under the conditions that promote their
formation. In agreement with this idea, in this article we
observed the fibrils to be maximally stable under their
growth conditions. This suggests that these conditions
favor the more specific stabilizing interactions such as salt
bridges, which drive the assembly process, and subse-
quently contribute to the thermodynamic stability of the
final structure. The electrostatic repulsion model discussed
in this study is coarse-grained, involving no such specific
interactions, and thus does not describe the behavior close
to the fibril-forming conditions where specific interactions
dominate. However, at high pH values where specific
stabilizing interactions have been disrupted, we have found
the soluble-fibril protein equilibrium to be well explained
by principles commonly applied in the field of colloid
science (35).

The correlation observed in this study between β-sheet
content and fibril stability supports the idea (13) that the
highly ordered (52) cross-β array of hydrogen bonds within
amyloid fibrils represents an essential requirement for their
very high stability. Remarkably, the energy required to
unfold the native state of insulin molecules in solution
(44) is fivefold lower than that required to dissociate insulin
molecules from amyloid fibrils under identical conditions.
This finding indicates that insulin is capable of making
more stable intermolecular interactions than the intramolec-
ular ones that stabilize its globular fold and suggests that the
fibrillar state can be thermodynamically more stable than the native state.

**SUPPORTING MATERIAL**

Additional information, three equations, and four figures are available at http://www.biophysj.org/biophysj/supplemental/S0006-3495(11)00515-7.

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