Electrostatic Effects in Filamentous Protein Aggregation

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ABSTRACT  Electrostatic forces play a key role in mediating interactions between proteins. However, gaining quantitative insights into the complex effects of electrostatics on protein behavior has proved challenging, due to the wide palette of scenarios through which both cations and anions can interact with polypeptide molecules in a specific manner or can result in screening in solution. In this article, we have used a variety of biophysical methods to probe the steady-state kinetics of fibrillar protein self-assembly in a highly quantitative manner to detect how it is modulated by changes in solution ionic strength. Due to the exponential modulation of the reaction rate by electrostatic forces, this reaction represents an exquisitely sensitive probe of these effects in protein-protein interactions. Our approach, which involves a combination of experimental kinetic measurements and theoretical analysis, reveals a hierarchy of electrostatic effects that control protein aggregation. Furthermore, our results provide a highly sensitive method for the estimation of the magnitude of binding of a variety of ions to protein molecules.

INTRODUCTION

Protein self-assembly into linear structures is a process that is crucial to biological function but also associated with the onset of disease. Examples of functional protein polymerization include the formation of actin (1) and tubulin filaments (2), whereas amyloid diseases (3) and sickle cell anemia (4) represent cases where protein polymerization can cause disease. It is known that electrostatic effects play a significant role in the formation and growth of amyloid fibrils and, a change in solution ionic strength is commonly reported to influence the rate of formation of amyloid structures (5–13). In some cases, the effects of salts on amyloid growth have been reported to follow to a good approximation the Hofmeister series (7,14), but in other cases they appear to reflect more closely the electroselectivity series (9,15). The large variety of reported effects is likely to stem from the complex nature of the interactions. Proteins are heteropolymeric polyelectrolytes that can carry many charges of both signs simultaneously at intermediate pH values, and therefore their interactions with any given type of ion are even more difficult to predict than for classical polyelectrolytes (16,17).

In an effort to develop a systematic basis for the understanding of electrostatic effects in the interactions leading to fibrillar protein aggregation, we have performed accurate kinetic measurements of the aggregation of a representative selection of peptides and proteins under solution conditions where electrostatic effects are well defined. This approach, combined with quantitative analysis based on physicochemical principles, not only reveals fundamental features that are independent of the particular protein under study, but can define a general strategy for probing the interactions of ions with proteins in a highly sensitive manner. Indeed, we show that this approach allows the detection of the ion binding at levels corresponding, on average, to less than one bound species per protein molecule.

To explore this approach using a well-defined configuration, we have studied systems that form amyloid fibrils under acid-denaturing conditions, where the proteins used in this study carry only positive charges. In addition, we probe specifically a single step in the complex mechanism of linear protein polymerization (18,19), namely the elongation of mature fibrils by addition of soluble precursors molecules. If a solution of amyloidogenic peptides is sufficiently strongly seeded, the elongation of the seed fibrils is the dominant process and primary and secondary nucleation processes can be neglected (19). This strategy hence allows a specific molecular level process to be measured under steady-state conditions where the accuracy of measurements can be increased simply through increased integration times. The elongation step is a bimolecular reaction between a growth-competent fibril-end and a monomeric precursor protein. Furthermore, both reaction partners in this system are well characterized and structural information is available from, for example, NMR (5), AFM (20), or cryo-electron microscopy (21) studies.

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This use of a preformed template for the polymerization reaction eliminates complications encountered for de novo polymerization that stem from the observation that different solution conditions can induce the formation of structurally very different aggregates (5,9). Indeed, the use of seed fibrils from the same batch in solution-state measurements, or even of a constant ensemble of fibrils in the case of biosensor measurements in a series of experiments at different ionic strengths, ensures that the observed differences in kinetics can be directly related to a modulation of the electrostatic forces acting between the fibril and the soluble precursor, as the fibril imposes the structure of the aggregate to the forces acting between the fibril and the soluble protein in most cases (22,23). Using this strategy, we find that for proteins with moderate charge density in solutions containing simple halide salts, a combination of Debye-Hückel theory and chemically nonspecific ion binding can quantitatively explain our kinetic data. For more highly charged proteins and more-complex ions, however, specific ion binding occurs and can influence the kinetic behavior very significantly.

METHODS

Proteins and chemicals

The SH3 domain of human phosphatidylinositol 3-kinase (PI3K-SH3) was expressed recombinantly as described previously in Zurdo et al. (5). Bovine insulin and all salts were purchased from Sigma (Dorset, UK). All proteins were dissolved in 0.01 M HCl. The concentrations of the protein solutions were for bovine insulin, 0.1–3 mg/mL; human PI3K-SH3, 0.02–0.67 mg/mL; human glucagon, 0.33–0.5 mg/mL; and human β-2 microglobulin, 0.17–0.2 mg/mL.

Kinetic measurements of amyloid fibril elongation

The experiments later shown in Fig. 2 in the main text were carried out using four different experimental methods that are described in detail below. The temperature at which the aggregation was monitored varied between 25 and 40°C for the individual experiments. Changes in temperature can have a strong effect on the aggregation rate (24), and here we used this fact to tune the elongation rate into the optimal dynamic range for each experimental technique. Although electrostatic effects are expected to be largely insensitive to small changes of temperature around ambient conditions, we investigated the temperature dependence of the electrostatic screening using two techniques, Thioflavin-T (ThT) and quartz crystal microbalance (QCM) measurements, which were used to probe insulin amyloid fibril growth.

Thioflavin-T fluorescence measurements

Seeded Thioflavin-T (ThT) fluorescence experiments with bovine insulin were performed at 35, 40, and 45°C in an Optima Fluostar platereader (BMG Labtech, Aylesbury, UK), using bottom fluorescence reading. We used low protein absorption 96-well plates with clear bottoms (Dow Corning, Midland, MI), sealed with clear or metallic tape. The excitation wavelength was 440 nm, with emission recorded at 480 nm. We used 0.01 mg/mL seed fibrils (sonicated for 10 min as described in Buell et al. (25)), 0.5 mg/mL protein monomer, and 0.2 mg/mL ThT. The fluorescence curves were scaled by normalization to the plateau level of fluorescence at the end of the experiment, to account for different final levels due to the sensitivity of ThT fluorescence on ionic strength. Then the initial part of the curves (after ~30 min when the system had equilibrated to the higher temperature) were fitted with a linear function and the slopes were taken to be proportional to the elongation rates.

Dynamic light scattering measurements and ζ-potential measurements

The dynamic light scattering (DLS) and ζ-potential measurements were carried out using a Zetasizer Nano ZS (Malvern Instruments, Malvern, UK). All reactions were initiated by mixing a homogeneous seed-fibril population with precursor monomers; this approach to measuring amyloid fibril elongation in a label-free manner in bulk solution avoids the common complications connected with light scattering experiments that are due to the difficulty in resolving polydisperse mixtures. The kinetics of insulin amyloid fibril elongation were measured at 45°C, ensuring that the reaction was sufficiently fast to avoid sedimentation of the fibrils formed which would interfere with the measurements, and also was sufficiently slow to be resolved within the time resolution of the instrument. As seeds, we used pregrown insulin amyloid fibrils that had been sonicated for 15 min (see Buell et al. (25) and Huang et al. (26)) and filtered using a syringe filter with 220-nm-pore size. The length distribution of the fibril suspension was measured by DLS and confirmed by AFM imaging to be very narrow (Fig. 1 a). Under the scattering conditions in our DLS setup and the aspect ratio of the seed fibrils, rotational diffusion can be neglected (27) and the autocorrelation function is monomodal to a very good approximation.

In this case, the apparent hydrodynamic radius obtained from a fit of the autocorrelation function to a single exponential, together with the thickness of the fibril suspension...
of the fibrils from AFM measurements, can be converted into an average length for the fibrils in a straightforward manner (28). Subsequently, 0.17 mg/mL of soluble insulin was added and the elongation of the seeds was monitored via the change in the apparent hydrodynamic diameter from ~60 to ~100 nm. According to AFM images, by the end of the measurements, the fibrils are 500–1000 nm in length (Fig. 1 b) and have left the regime (described above) where rotational diffusion can be neglected. However, if the individual measurements at different salt concentrations are performed in the same range of apparent hydrodynamic diameters, the times can be directly compared and are inversely proportional to the elongation rates.

The ζ-potential of sonicated fibrils and soluble insulin was measured in clear disposable ζ-cells (Malvern Instruments). The charge of the proteins was then calculated according to the relationship given in Chun and Lee (29). It should be noted here that the calculation of the surface charge according to this equation is only strictly valid for values of the ζ-potential ≤25 mV, corresponding to the electrostatic energy being less than or equal to the thermal energy k_BT. However, this restriction will not influence the conclusion that can be drawn from the finding that the values of the ζ-potential of the monomeric insulin and of the sonicated amyloid fibrils are very similar. The similarity of the ζ-potentials suggests that the charge densities of these two species are also very similar, and therefore that the protein does not change its protonation state significantly when incorporating into an amyloid fibril.

Quartz crystal microbalance measurements

Seed fibrils were prepared and attached to the surface of the quartz crystal microbalance (QCM) sensors as described in detail in Buell et al. (25). Briefly, bovine insulin and human β2-microglobulin fibrils were attached via cysteine residues in their protein sequences. For the attachment of PI3K-SH3 and glucagon, the amino-acid sequence does not contain any cysteine residues; we therefore attached sulfur-containing small molecules to the seed fibrils to allow the fibrils to interact with the gold surface. To this effect, we used cystamine (for PI3K-SH3) and 2-iminothiolane (for glucagon); details can be found in Buell et al. (25). The surface-bound seed fibrils were then repeatedly incubated with protein solutions with varying salt concentrations. The induced frequency shifts due to the elongation of the surface-bound fibrils were fitted with a linear function and the slope was taken to be directly proportional to the elongation rate. The experiments were performed at temperatures between 25 and 45°C.

Surface plasmon resonance measurements

Surface attachment of the insulin seeds was performed by methods analogous to those used for the preparation of QCM sensors (25). The elongation of the seed fibrils on the surface plasmon resonance (SPR) sensor was monitored at 40°C with a Biacore 3000 (GE Healthcare, Amersham, Little Chalfont, Bucks, UK). The seeds were repeatedly incubated with insulin monomer solution at varying salt concentrations and the rate of net shift in response units during an incubation is proportional to the rate of protein addition and therefore to the elongation rate, and was fitted with a linear function, similar to the QCM measurements.

RESULTS AND DISCUSSION

Quantification of amyloid elongation kinetics at steady state

To be fully able to probe the influence of ionic strength on the elongation rate of amyloid fibrils at acidic pH, the accuracy of the kinetic assay is crucial. To obtain robust data, we employed four methods that probe the growth of preformed fibrils (Fig. 1); two of these methods provide the possibility of monitoring the elongation of seed fibrils in bulk solution, namely, ThT fluorescence and DLS, and two methods monitor the steady-state growth of the fibrils along the surface of a biosensor, namely, QCM or SPR. In our ThT fluorescence measurements of the elongation rate of amyloid fibrils at different ionic strengths (Fig. 2 a), the measured fluorescence data were normalized to take into account the differences in fluorescence efficiency at different salt concentrations (see Methods). This phenomenon can be attributed to electrostatic screening of the repulsion between the positively charged dye molecules and the positively charged fibrils as well as changes in the fluorescence quantum efficiency of the bound dye.

To exploit an alternative and label-free kinetic assay in bulk solution, we developed a strategy to monitor the elongation of a homogeneous population of short (sonicated (26)) preformed fibrils by dynamic light scattering (Fig. 2 b; see Methods for details). This avoids the difficulties usually encountered in light scattering measurements of protein aggregation stemming from the highly polydisperse nature of aggregated protein samples.

Previous studies have shown that biosensing techniques provide a highly accurate means of measuring amyloid elongation rates, using QCM (30–32) or SPR (33), offering the ability to study the growth of a constant ensemble of fibrils under varying conditions. We used a protocol for the attachment of the fibrils to the gold-coated sensors that we have described in detail previously (25). The elongation of the fibrils was then monitored by measuring the decrease in resonant frequency (QCM; Fig. 2 c), or the shift in resonance angle of surface plasmon excitation (SPR response units; Fig. 2 d).

Role of Debye screening in amyloid fibril elongation

The data obtained in this study reveal a hierarchy of complexity in the effects that ions exert on amyloid fibril growth. At the most elementary level, a general trend is observed where the rate of elongation increases with ionic strength (Fig. 2 e), in accord with previous studies (5–13). The different experimental techniques used in this study have different optimal temperatures under which the measurements are most accurately carried out, ranging from 35 to 45°C (see Methods for details). To test whether a variation in temperature within this range influences the observed effects of NaCl on the fibril elongation rate, we carried out QCM and ThT experiments at different temperatures. Importantly, the data obtained from all four different experimental techniques throughout the temperature range explored are in excellent agreement, as can be seen from the data in Fig. 2 e).

To our knowledge, this is the first report that establishes quantitatively the equivalency of solution-based and
surface-based methods to study protein aggregation. Our results confirm that all four techniques are indeed measuring the same molecular process—the elongation of amyloid fibrils—and that this process is strongly influenced by the solution ionic strength. The growth kinetics of amyloid fibrils can have a strong temperature dependence (24), but our data show that its modulation by ionic species depends only weakly on temperature. An increase in the rate of a bimolecular association reaction between charged species (of equal sign) with increasing ionic strength is predicted by Debye-Hückel (DH) theory, and the reaction rate is predicted to vary exponentially with the square-root of the solution ionic strength (see, e.g., Moore (34)), as is observed here (Fig. 2e).

Indeed, the seminal contributions of Debye and Hückel (35) to the understanding of the interactions of charged particles (ions, molecules, or colloids) in solutions containing salts are still largely unchallenged and widely used. It has become clear over the last few decades, however, that this theory fails to account for many effects that are due to the presence of specific ions in solution, or do so only when several empirical fitting parameters are invoked (36). The deviations between simple theory and experiment are particularly striking for biologically relevant salt concentrations where electrostatic interactions are largely screened, and where other types of interactions such as dispersion or hydration forces can become important (37).

To account fully for the interaction characteristics of even the simplest colloidal systems, an attractive van der Waals potential has been added and combined with electrostatic repulsion by Derjaguin and Landau (38), and Verwey and Overbeek (39). We have recently shown that the attractive potential governing amyloid fibril elongation kinetics is dominated by very short-range forces, especially the hydrophobic effect (24).

To simplify the analysis of our kinetic data in this study, we used conditions (millimolar ionic strength) where simple DH theory is likely to be valid to a good approximation (40) in addition to the other effects observed. To illustrate the failure of such simple theories at higher ionic strength, we have extended a DH plot of insulin amyloid fibril elongation up to 100 mM NaCl as opposed to 20 mM in the experiments described above (Fig. 2f). Above ~20 mM, the slope of the DH plot decreases, indicating a decrease in relative screening efficiency with increasing ionic strength. Furthermore, to probe whether the fibril elongation rate increases monotonically with ionic strength, we have extended these experiments up to 1 M added NaCl (Fig. 2g). Interestingly, above 300 mM NaCl, the elongation rate decreases; this effect is likely due to a change in the stability of the soluble protein.
state of the protein (41,42), an effect that is not obtained from simple theories like the Debye-Hückel. We therefore focus, for the remainder of this study, on the low millimolar range of ionic strength (≤20 mM), where the simplest behavior is observed.

The measured increase in the reaction rate at low ionic strength is in agreement with the fact that the protein molecules and fibrils under study are positively charged in 10 mM HCl, and hence experience unfavorable electrostatic interactions that can be screened by ionic species in solution. To probe the overall charge state of both soluble and fibrillar insulin under these conditions, we measured the ζ-potential (see Methods) of sonicated fibrils and soluble protein in the absence of any added salt, and obtained values of +35.5 ± 2.0 mV for monomeric insulin and +40.0 ± 2.0 mV for sonicated fibrils (~100 nm in length). In the case of the soluble insulin molecules, we can estimate the surface charge of the molecule from the ζ-potential, with the effective hydrodynamic radius, \( r_p \), of the soluble protein (1.7 nm (43)), following the discussion in Chun and Lee (29),

\[
Q = 4\pi r_p (1 + \kappa r_p) \zeta_p,
\]

where \( \epsilon \) is the dielectric constant of the solution and \( \kappa \) is the inverse Debye screening length (~ 3 nm at \( I = 10 \) mM). This calculation yields a total surface charge of ~5.1 ± 0.3, a value close to the theoretical absolute charge of the molecule at this pH (+5.0, calculated under the assumption of independent pKₐ values for the different residues within the amino-acid sequence of the protein).

The combination of Debye-Hückel theory with a reaction-rate theory appropriate for charged species predicts that the slope of a plot of \( \log(\Phi) \) against \( \sqrt{I} \), where \( \Phi \) is the reactive flux (44) and \( I \) is the ionic strength of the solution, is proportional to the product of the effective charges of the two reacting species (see Appendix and Moore (34)). Fitting this model to the data shown in Fig. 2 e yields 10.0 ± 1.0 for the slope. Since the ζ-potential measurements reveal that the molecule at the fibril-end carries a similar charge to that of the soluble precursor, we can identify the geometric average of the charges of a monomer and a fibril-end as the effective charge of the protein, yielding a value of +3.12 ± 0.16. This effective charge entering the energy function that describes the barrier that the monomer has to overcome to attach to a fibril-end is smaller but of a similar order of magnitude to the total charge of the molecule (+5.0).

**Chemically nonspecific trends in the electrostatic modulation of elongation rates**

Having established the equivalence of the surface-based and bulk solution experimental techniques for the study of the effect of ions on amyloid fibril growth, we focus in the following on sensor-based measurements due to their rapidity, ease of use, and, in particular, the possibility to measure the steady-state growth of a fixed ensemble of fibrils for well-defined periods of time. This last feature allows the integration of the measured signal for achieving a better signal/noise ratio. We note here that, in this respect, linear protein aggregation represents an ideal system to probe subtle effects on the kinetics, because in other types of protein-protein interactions, such as dimerization, steady state cannot be reached. To explore effects beyond the screening mechanism described above, we performed equivalent experiments with different halide salts both with bovine insulin and with another protein, bovine PI3K-SH3, which has been shown to form amyloid fibrils under very similar solution conditions (5), where it carries a significantly higher net charge than insulin. It is known that, in the case of PI3K-SH3, electrostatic factors have a significant effect on the rate of fibril elongation (45). We tested a series of simple halide salts with monovalent and divalent cations. The results are shown in Fig. 3, a–c), where all the rates have been normalized by the elongation rate in 10 mM HCl in the absence of added salts. A graph of the logarithm of the rates

\[
\log\left(\frac{\Phi(I)}{\Phi(I_0) = 10^{-2}M}\right)
\]

as a function of the square-root of the total solution ionic strength (see Appendix for details) reveals a qualitatively very similar behavior between the two proteins, with PI3K-SH3 amyloid growth rates being more sensitive to changes in ionic strength, in agreement with its higher maximum net charge compared to insulin under these acidic conditions (+12.0 vs. +5.0). Most interestingly, however, for both proteins, the data points for the fibril growth rates fall into two classes: halides with divalent and trivalent cations lead to a distinctly weaker increase in elongation rate, at any given ionic strength, than halides of monovalent cations, with a more pronounced difference in the case of PI3K-SH3 compared to insulin. Therefore, the multivalent salts appear to be less efficient in screening the intermolecular repulsions that form part of the energy barrier for the bimolecular reaction under study (44).

This result differs from the prediction of simple Debye-Hückel (DH) theory, namely that the screening efficiency of a solution depends only on its total ionic strength, and this observation forms the basis for understanding the hierarchy of further phenomena that operate in addition to simple solution screening in protein systems. In Fig. 3 a, we show a fit to Eq. 10 (derived from simple DH theory; see Appendix), using the effective charge \( Q(I) = Q(I_0) \) as the only free parameter for the fit. We identify the characteristic length-scale in the free energy function, the transition state separation \( r^t (24,44) \), with the reported hydrodynamic radii for the two proteins (1.7 nm for insulin (43) and 2.4 nm...
for PI3K-SH3 (5)). A change of this distance will affect the fitted value for the effective charge; the maximum possible charge of the protein at pH 2.0, therefore, sets a physical upper limit to this transition-state separation, but the qualitative behavior is not affected by a change in $r_z$.

Fig. 3 shows clearly that the differential behavior of the distinct classes of salts is not captured by the simple DH theory. Even a more rigorous extension of this approach to include multivalent electrolytes (46) is not able to account for the experimental observations; the correction terms invariably lead to a greater screening ability of electrolytes with polyvalent ions, while experimentally, the opposite trend is observed in our data. This conundrum can be solved by considering explicitly, as shown below, the binding of ions to the protein, which leads to a change in the global charge of the protein, thereby altering the electrostatic repulsion between the protein and the fibril end without changing the nonelectrostatic contributions to the free energy landscape.

### Quantification of chemically nonspecific ion association to proteins

To probe the nature and magnitude of ion binding, we first considered effects that are present independently of the chemical nature of the anions. A physical mechanism that can operate in addition to screening in solution is the binding equilibrium of ions to the proteins with an overall positive charge, which leads to the modification of the effective charge of the protein molecules (see Appendix). Within this framework, the effective charge of the protein is a function of the solution ionic strength, leading to a more complex dependency of

$$\log \left[ \frac{\Phi(I)}{\Phi(I_0)} \right]$$

on $\sqrt{I}$ than that suggested by simple screening. Fig. 3, $b$ and $c$, shows the best fits of the variable charge model to the data sets for both proteins (mono- and divalent cations). We
considered two extreme cases: 1), of exclusively anion binding and 2), of exclusively divalent cation binding. Since we are performing global fits to the entire dataset, no degrees of freedom are available for adjusting the slopes of the individual lines on the Debye plot. Qualitatively, both models can account for the observed difference in influence on the elongation rate of the halides of mono- and divalent cations (details are given in the Appendix). Anion binding is more pronounced for the halides of monovalent cations as anion concentration at a given ionic strength is higher, and therefore more anions would be bound, lowering the electrostatic repulsion to a greater extent and leading to higher relative elongation rates. However, divalent cation binding will decrease the rate in the presence of these salts through an increase in effective charge.

As can be seen in Fig. 3b, however, the inclusion of anion binding alone yields relatively poor quantitative agreement with the data, particularly in the case of PI3K-SH3; for instance, it is not able to explain the large differences between the relative rates resulting from the presence of ions of different charge. The difference in anion concentration when monovalent or divalent halides are added to the samples is too small to account in a self-consistent way for the observed differences in screening through anion binding. Remarkably, however, chemically nonspecific binding of divalent cations yields significantly better fits (Fig. 3c). We conclude, therefore, that binding of divalent cations is necessary and indeed sufficient to account for our data set presented in Fig. 3. It is interesting to note that the binding of positive ions to a globally positively charged protein molecule can be readily rationalized as a consequence of the presence of neutral, polarizable patches on the protein even at acidic pH. Such binding has indeed been observed in the literature, in particular in the context of protein crystallization (47). The binding constants from the fits predict a maximal binding of 0.2 (insulin) and 0.8 (PI3K-SH3) divalent cations per protein molecule, on average, under the conditions of the study: a value <1 and consistent with the weak attachment to polarizable areas. Much experimental effort has been undertaken in the past in attempts to quantify binding of ions to proteins and to determine the effective charge of a protein. The methods used were initially conductometric (48) or potentiometric (49); later, ultracentrifugation (48) and capillary electrophoresis (49) were also used. A recent experimental advance is to measure the ζ-potential from dynamic light scattering in the presence of rapidly oscillating AC fields (29). The experiments presented in this article, using highly accurate measurements of steady-state elongation kinetics of amyloid fibrils, apart from being a systematic study of the influence of salt on amyloid formation, can also be regarded as an extension of the measurements of the effective charge of proteins toward higher sensitivities on minor perturbations. Most of these measurements involve transport properties (conductivity, electrophoretic mobility) and depend in a linear manner on the charge of the protein. The measurements of aggregation rates presented here rely on the exponential dependence of the aggregation rates on the square of the net charge of the protein that influences the aggregation reaction. This strong dependence allows us to resolve subtle differences in the overall charge state of a protein in the presence of different salts—something that would be very difficult to detect with the techniques mentioned above.

In addition, an analysis of the differences between the absolute charges of amyloidogenic proteins and their effective charges for aggregation can yield important insight into the aggregation mechanism. The general mechanisms of electrostatic screening and nonspecific cation binding are able to account quantitatively for the aggregation behavior of insulin and PI3K-SH3 in the presence of low concentrations of halide salts. However, some of the more complex effects discussed in the Introduction cannot be explained by taking only these chemically nonspecific mechanisms into account. Therefore, to relate the fundamental results obtained in this work to the existing literature on the effect of changes in ionic strength on the kinetics of protein aggregation, we have investigated the aggregation of two additional polypeptides as well as the effects of more complex salts.

Chemically specific electrostatic effects

We were able to confirm qualitatively the results described above for two additional polypeptides that both readily form amyloid fibrils in 10 mM HCl, namely, human glucagon and human β2-microglobulin; in both cases, divalent chlorides are less efficient in screening than monovalent chlorides (see Fig. 4, a and b). It has been reported, however, for both glucagon (9) and β2-microglobulin (8), that iodide ions are more efficient in accelerating amyloid growth than chloride ions, in contrast to the behavior described above for insulin and PI3K-SH3 where the chemical nature of the halide ion does not influence the behavior. We tested these findings with our methodology and confirmed these earlier reports. In addition, we found that even bromide ions, more similar in size and polarizability to chloride ions, (Fig. 4, c and d) are more efficient in accelerating the fibril elongation rate than chloride. The results in Fig. 4d clearly show that the difference in aggregation kinetics can be attributed to the different anions; a change in cation from Na⁺ to Li⁺ has no effect on the aggregation rate. Thus, we conclude that specific anion binding, which differs for the different halide ions, must play a significant role in these systems, as opposed to the combination of Debye screening and nonspecific cation binding described above.

To investigate this difference between insulin and PI3K-SH3 on the one hand and glucagon and β2-microglobulin on the other, we computed the linear charge density of the four proteins by dividing the total charge of the protein by
the number of amino acids in its sequence. The resulting values are for bovine insulin, $+5/51 = 0.10$; bovine PI3K-SH3, $+12/84 = 0.14$; human glucagon, $+5/29 = 0.17$; and human $\beta 2$-microglobulin, $+18/100 = 0.18$. The proteins that show resolvable differences in the effects of chloride, bromide, and iodide are those with the highest average charge density. Bromide and iodide ions are more polarizable than chloride ions and therefore have a stronger tendency to associate with the positive alkylammonium groups on proteins, such as the N-terminus and protonated lysine side chains, according to HSAB theory (51). However, it appears that the extent to which this association can manifest itself in, for example, protein-protein interactions, may be determined not only by the polarizability of the anion, but also by the global charge density of the protein.

A further increase in the complexity in the effects of ions on protein behavior emerges through the examination of the role of the oxyanions nitrate and sulfate on the elongation of PI3K-SH3 fibrils (see Fig. 4 e, where the effects of these two salts are compared with those of NaCl). We find that both of these more complex ions have a much greater effect on the kinetics of fibril elongation than the simple halides and, in agreement with studies of other proteins, that sulfate is by far the most efficient anion in accelerating amyloid growth (9). Indeed, only very low millimolar concentrations of this anion could be used in our experiments (Fig. 4 e), as PI3K-SH3 is susceptible to amorphous aggregation in the presence of sulfate concentrations $>10$ mM. For comparison, only concentrations $>100$ mM of NaCl induce significant amorphous aggregation (5). This effect can be attributed to tight binding between the sulfate ion and the protein, and this association equilibrium is likely to depend significantly on the particular protein under study. In addition, such tight binding of ions with nonspherical structure and charge distribution is more likely to change the structure of the soluble precursor protein and therefore to affect non-electrostatic contributions to the free energy landscape of the protein (Fig. 4 f).

In addition to being able to screen electrostatic interactions, ionic species can also influence reactions in aqueous solution via their effects on the properties of solvent water. Although it has been shown that ions, even at concentrations much higher than those used here, do not significantly alter the structure of bulk water (52), the structure at the interface with the protein can be altered significantly by millimolar concentrations of ions. This effect shows a strong dependence on the nature of the ion (53): the higher the polarizability of a halide ion, for example, the larger the effects on the interfacial water (53). Given that hydrophobic effects contribute significantly to the energetics of the aggregation reaction (24), such interfacial effects can strongly influence the kinetics. The extent of this effect will depend on structural and sequence parameters of the protein, and, indeed, may contribute to the differences observed between insulin and PI3K-SH3 on the one hand, and glucagon and $\beta 2$-microglobulin on the other.

Under physiological conditions (neutral pH, $\sim 100$ mM ionic strength, multitude of simple and complex ions) protein- and ion-specific effects such as the ones identified in Fig. 2 g and Fig. 4 are likely to modulate significantly the electrostatic interactions between proteins, in addition to simple Debye-Hückel screening and nonspecific ion binding. However, the approach that we outline in this article, starting from the simplest possible mechanisms and increasing the complexity of the additional effects in a stepwise manner, is a powerful strategy for establishing a full description of the complex interactions between ions and charged macromolecules in a physiological context.
CONCLUSIONS
We have demonstrated that accurate measurements of the steady-state kinetics of a well-defined molecular step driven by protein-protein association, the elongation step in amyloid growth, enables the identification of the fundamental principles that govern the behavior of proteins in the presence of low concentrations of inorganic ions. We find in particular that a crucial contribution is given by the nonspecific ion binding of divalent cations with a high charge density to protein molecules, thereby modifying their overall effective charge. This process operates in parallel with simple Debye-Hückel type screening in solution, and is crucial to account for the less efficient screening of halides with multivalent cations compared to that of monovalent cations. In addition to these chemically nonspecific electrostatic effects, specific ion effects can play a role in the electrostatics of protein interactions, which depend on factors such as the charge density and chemical nature of both the ions and the protein molecules. The experimental toolkit and theoretical framework presented in this work allow these effects with increasing complexity to be unraveled and shed light more generally on the effects of ions on protein-protein interactions. This is important for the functional roles of proteins and also their involvement in disease through the formation of aberrant protein polymers of the type studied in this work.

APPENDIX: ELECTROSTATICS WITH VARIABLE CHARGES

We start with the rate equation of amyloid elongation, that we have recently reported (44):

$$\Phi = \Gamma e^{-\beta \Delta G^i}. $$

The frequency factor

$$\Gamma = \frac{Dr_{eff}C}{1 + (Dr_{eff}C)\tau_K}$$

was derived using polymer theory (44), and is independent of ionic strength. The free energy barrier $\Delta G^i$ can be decomposed into electrostatic $G^i_e$ and nonelectrostatic $G^i_{ne}$ parts. We model the electrostatic contribution as a Debye-screened Coulomb potential $U_C$:

$$G^i_e = G^i_{ne} + G^i_e = G^i_{ne} + \frac{z_i z_2 e^2}{4\pi\varepsilon_0 \varepsilon_r r^3} e^{-\kappa r}. $$

(1)

If this expression for the free energy is substituted into the rate equation for amyloid fibril growth, we obtain the following expression for the natural logarithm of the rate as a function of ionic strength (for $r^i < \kappa^{-1}$):

$$\log(\Phi) = \log(\Gamma) - \beta(\Delta G_{ne}^i + \Delta U_C^i) + \sqrt{\frac{2N_A}{\varepsilon_0^i e^2 (k_B T)^3}} \frac{e^3}{4\pi} z_1 z_2 \sqrt{I} = \text{const.} + \chi z_1 z_2 \sqrt{I}, $$

(2)

with the numerical value of the constant $\chi$.

$$X = \sqrt{\frac{2N_A}{\varepsilon_0^i e^2 (k_B T)^3}} \frac{e^3}{4\pi} = 0.075 \frac{m^{3/2}}{\text{mol}^{1/2}} $$

at 298 K; this is the same expression as that obtained by combining the activity coefficients of the reactants and the transition state (34). The usual convention is to use $\log_{10}$ and the units of concentrations are mol/L. Carrying out these conversions yields

$$X = 1.03 \frac{m^{1/2}}{\text{mol}^{1/2}}. $$

This description, which assumes constant charges, cannot even qualitatively account for the kinetic data we have acquired, however. We therefore include chemically nonspecific ion binding. The charge $z_i$ of monomer and fibril-end in the presence of a salt with the general formula $A^{n+}B^{-}$ can be written as

$$z_i = e(z_0 + in_i K_i^{+}[A^{n+}] - n K^{-}[B^{-}]), $$

(3)

$$\approx e(z_0 + in_i K_i^{+}[A^{n+}] - n K^{-}[B^{-}]^2), $$

(4)

where the $K$ are association constants, the $n$ are the number of binding sites, and the concentrations are in square brackets. The value $z_0$ is the net charge of the protein that influences the aggregation reaction in the absence of ion binding. For small products of the binding constant and the ion concentration, $K[X] \ll 1$, the approximation is valid. We do not introduce a separate binding constant for hydronium ions other than that for direct protonation, as this contribution is constant for all cases. The resulting net charge of the protein is Debye-screened, as detailed above. As the concentration of each ionic species is directly related to the ionic strength, and this relationship depends only on the valency of the cation (for monovalent anions, such as halides), we can use the ionic strength as the only independent variable for the elongation rate. Now we express the concentrations of the ionic species as a function of the total ionic strength of the solution, $I$. There is a unique relationship between $I$ and $|X|$ for each of the valencies. For halides of monovalent cations, this relationship yields

$$[A^{+}] = I - I_0; \quad [B^{-}] = I, $$

(5)

$$Q_1(I) = e z_0 + e I(K_1 - K_=) - e I_0 K_1, $$

(6)

where $I_0$ is the concentration of HCl. For halides of divalent cations, we obtain

$$[A^{2+}] = \frac{I - I_0}{3}; \quad [B^{-}] = \frac{2(I - I_0)}{3} + I_0, $$

(7)

$$Q_2(I) = e z_0 + e I \left( \frac{2}{3} K_2 - \frac{2}{3} K_- \right) - e I_0 \left( \frac{2}{3} K_2 + \frac{1}{3} K_- \right). $$

(8)

Rewriting the expression for the electrostatic repulsion gives

$$\Delta G_e^i = \frac{Q(I)^2}{4\pi\varepsilon_0 \varepsilon_r r^3} e^{-\kappa_0 \sqrt{I} r}, $$

(9)

where $\kappa_0 \sqrt{I} = \kappa$ and in order to simplify this expression, we consider the normalized rates

$$\frac{\Phi(I)}{\Phi(I_0)} = \frac{Q(I)^2}{4\pi\varepsilon_0 \varepsilon_r r^3} e^{-\kappa_0 \sqrt{I} r} - \frac{Q(I_0)^2}{4\pi\varepsilon_0 \varepsilon_r r^3} e^{-\kappa_0 \sqrt{I} r}. $$

(10)
We use this expression to fit the data in Fig. 3 of the main text for anion binding alone and for cation binding alone. To obtain an understanding of the qualitative effect of anion and cation binding on a plot of

\[ R(I) = \log \left( \frac{\Phi(I)}{\Phi(I_0)} \right) \]

against \( \sqrt{I} \), we calculate the derivative \( \frac{\partial R(I)}{\partial I} \):

\[ \frac{\partial R(I)}{\partial I} = \frac{Q e^{-\kappa_0^2 I}}{4\pi\kappa_0^2 r^2} \left( \frac{Q e^0 r^2}{2\sqrt{I}} - 2 Q \frac{\partial Q}{\partial I} \right). \] (11)

We see that anion binding (\( \partial Q/\partial I < 0 \)) will increase the slope in the case of the salts of monovalent cation relative to the salts of the divalent cations as there are more anions present in a solution of a monovalent cation at a given ionic strength. Conversely, cation binding (\( \partial Q/\partial I > 0 \)) will reduce the slope. In principle, both anion and cation binding could qualitatively explain the data set. However, global fits (see Fig. 3) show that the data can be quantitatively explained by binding of divalent cations.

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