Relative Influence of Hydrophobicity and Net Charge in the Aggregation of Two Homologous Proteins†

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ABSTRACT: A potentially amyloidogenic protein has to be at least partially unfolded to form amyloid aggregates. However, aggregation of the partially or totally unfolded state of a protein is modulated by at least three other factors: hydrophobicity, propensity to form secondary structure, and net charge of the polypeptide chain. We propose to evaluate the relative importance of net charge, as opposed to the other factors, on protein aggregation and amyloidogenicity. For this aim, we have used two homologous proteins that were previously shown to be able to form amyloid fibrils *in vitro*, the N-terminal domain of HypF from *Escherichia coli* (HypF-N) and human muscle acylphosphatase (AcP). The aggregation process from an ensemble of partially unfolded conformations is ca. 1000-fold faster for HypF-N than for AcP. This difference can mainly be attributed to a higher hydrophobicity and a lower net charge for HypF-N than for AcP. By using protein engineering methods, we have decreased the net charge of AcP to a value identical to that of wild-type HypF-N and increased the net charge of HypF-N to a value identical to that of wild-type AcP. Amino acid substitutions were selected to minimize changes in hydrophobicity and secondary structure propensities. We were able to estimate that the difference in net charge between the two wild-type proteins contributes to 20−25% of the difference in their aggregation rates. An understanding of the relative influences of these forces in protein aggregation has implications for elucidating the complexity of the aggregation process, for predicting the effect of natural mutations, and for accurate protein design.

Protein aggregation is a phenomenon with considerable relevance in cell biology (*1*, *2*), medicine (*3*−*5*), and biotechnology (*6*, *7*). Aggregation of peptides or proteins into amyloid fibrils is known to be associated with many human protein deposition diseases, such as neurodegenerative conditions and systemic amyloidosis (*3*−*5*). Protein aggregation is also known to occur naturally in nonpathological states when proteins fail to fold properly and consequently is actively combated by the cell (*1*, *2*). Furthermore, expression of proteins of biotechnological interest in bacterial cells often results in the aggregation of the expressed protein into inclusion bodies that accumulate intracellularly (*6*, *7*). Although the biophysical features underlying protein aggregation are still poorly understood, significant advances have recently been achieved. To aggregate, a protein must generally convert from its folded structure into a conformational state that is at least partially unfolded and prone to aggregation (*3*, *5*). Natural mutations associated with familial forms of amyloid diseases have been shown to reduce the stability of the folded state (*8*−*11*). Moreover, many proteins and peptides forming amyloid deposits *in vivo*, such as tau, α-synuclein, the amyloid β peptide, and the islet amyloid polypeptide, are natively unfolded (*5*). However, destabilization of the globular state of a protein with its consequent exposure of potentially interacting groups is not by itself sufficient to promote aggregation. Indeed, most of the natively unfolded proteins do not undergo amyloid aggregation (*12*). This indicates that there must be other factors promoting aggregation from a denatured state.

In agreement with this view, many natural mutations associated with familial forms of amyloid diseases facilitate self-assembly of the unstructured protein or peptide with which they are associated and do not cause alteration of protein processing (*13*, *14*). Recent studies have allowed three major factors to be identified as important parameters in the conversion of the partially or totally unfolded state of a protein into aggregates. These are a high hydrophobicity, a high propensity to convert from α-helical to β-sheet structure, and a low net charge (*15*−*24*). Most of the natively unfolded proteins are probably able to avoid aggregation as a consequence of their low hydrophobicity and high net charge (*12*). An understanding of the relative importance of these three parameters is fundamentally important not only for elucidating the mechanism of aggregation but also for correctly predicting the effect of mutations or other alterations on protein aggregation, especially when these involve counteracting effects on these parameters.

This work aims to quantify the role and relative importance of net charge with respect to hydrophobicity and secondary

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structure propensity. To achieve this result, two homologous proteins, namely, the N-terminal domain of *Escherichia coli* HypF protein (HypF-N)[1] and human muscle acylphosphatase (AcP), have been used. These proteins are not associated with amyloid diseases, but they are able to form amyloid fibrils *in vitro* under partially denaturing conditions (*25*, *26*). Despite their shared ability to convert into amyloid, HypF-N aggregates with a rate dramatically higher than that of AcP. Although they share similar propensities to convert from α-helical to β-sheet structure, HypF-N has a net charge lower and a hydrophobicity higher than those of AcP. By substituting charged amino acid residues in the sequence of AcP, we changed the net charge of this protein to a value identical to that of HypF-N. Subsequently, we compared the rates of aggregation of the AcP variants with that of wild-type (WT) HypF-N to evaluate the extent to which charge is responsible for the measured difference in the aggregation rate. The results show that hydrophobicity has a greater influence than net charge in the process of aggregation.

MATERIALS AND METHODS

Protein Production and Purification. Expression and purification of wild-type AcP and HypF-N and their variants were performed according to the procedure previously described (*27*). The QuikChange Stratagene kit was used for site-specific mutagenesis. All the AcP variants of this study have the cysteine at position 21 replaced with serine to avoid complexities arising from a free cysteine residue (*28*). DNA sequencing was used to ensure the presence of the desired mutations. Protein concentrations were measured by UV absorption using ϵ_{280} values of 1.49 and 1.25 mL mg^{-1} cm^{-1} for AcP and HypF-N, respectively. The mutants were designed to perturb the α-helical and β-sheet propensities and the hydrophobicity of the protein sequence as little as possible. The scale of β-sheet propensities of the various amino acids edited by Street and Mayo (*29*), the AGADIR algorithm (*30*), and the scale of hydrophobicities of the various amino acid residues edited by Roseman (*31*) were used to evaluate changes in β-sheet propensity, α-helical propensity, and hydrophobicity of the protein sequence following mutation, respectively.

Aggregation Kinetics. The rates of aggregation of the AcP variants were measured as previously described (*16*). In brief, WT AcP and mutated AcP variants were incubated at a concentration of 0.4 mg/mL in 25% (v/v) trifluoroethanol (TFE) and 50 mM acetate buffer (pH 5.5) at 25 °C. In each experiment, aliquots of 60 μL of the sample were mixed with 440 μL of 25 mM phosphate buffer (pH 6.0) containing 25 μM thioflavine T (ThT), at regular time intervals. A Shimadzu RF-5000 spectrofluorimeter with excitation and emission wavelengths of 440 and 485 nm, respectively, was used to determine ThT fluorescence values. Kinetic plots reporting the measured ThT fluorescence versus time were fitted to single-exponential functions to determine aggregation rate constants.

[1] Abbreviations: AcP, human muscle acylphosphatase; HypF-N, N-terminal domain of *E. coli* HypF; WT, wild type; CD, circular dichroism; TFE, trifluoroethanol; ThT, thioflavin T; ln k_{agg}, natural logarithm of the aggregation rate constant.

RESULTS

HypF-N and AcP Aggregate with Very Different Rates. The starting point of this work has been the observation that AcP and HypF-N aggregate with very different rates under identical denaturing conditions. Aggregation of AcP was initiated by incubation of the protein at a concentration of 0.4 mg/mL in 25% (v/v) TFE and 50 mM acetate buffer (pH 5.5) at 25 °C. By the time of the first acquisition of experimental data following this procedure, circular dichroism (CD) spectra and stopped-flow measurements show that the protein is substantially denatured, though exhibiting a considerable amount of α-helical structure (*16*). Within 1−2 h, AcP converts into aggregates that can be observed by electron microscopy as assemblies of granules or very short protofibrils ∼4 nm in width (*25*). The aggregates possess considerable β-sheet structure as revealed by CD and Fourier transform infrared spectroscopy, and have the ability to bind specific dyes such as Congo red and ThT (*25*). Mature amyloid fibrils form subsequently after some days (*25*). The first aggregational event, which is the conversion of the ensemble of partially denatured conformations into protofibrillar aggregates, occurs with no observable lag phase with a rate constant of 0.00093 ± 0.00004 s^{-1} under these conditions (*15*, *16*). HypF-N also aggregates under these experimental conditions, but with a rate constant of ca. 1.0 ± 0.5 s^{-1}, ca. 1000 times faster than that measured for AcP (unpublished result). Such a rapid aggregation results in the formation of aggregates which appear to have the ability to bind amyloid-specific dyes such as ThT (*26*). As for AcP, aggregation of HypF-N also occurs from an ensemble of partially denatured conformations, given the conditions employed. The observed difference in the aggregation rates of the two proteins therefore originates from intrinsic differences of the two protein sequences rather than from differences in conformational stability of the native state.

To investigate the origin of such a large difference in aggregation rate, the sequences of the two proteins have been compared, with particular consideration of those factors which were previously shown to be important determinants of the aggregation rate, i.e., hydrophobicity, propensities to form α-helical and β-sheet structure, and net charge. Figure 1 shows the α-helical, β-sheet, and hydropathy profiles of the AcP and HypF-N sequences. Despite differences in detail, the average propensities to form α-helical and β-sheet structure were similar in the two cases; using the scales considered in this analysis, the average propensities to form α-helical structure for HypF-N and AcP are 1.50 and 1.82, respectively, and the average propensities to form β-sheet for HypF-N and AcP are 0.65 and 0.69, respectively. The propensity to form both types of secondary structure is lower for HypF-N than for AcP. Since these propensities have opposite effects in the aggregation process, one can conclude that such slight differences between AcP and HypF-N are likely to determine a very minor, if not negligible, fraction of the observed difference in aggregation rate. Moreover, from the quantitative analysis carried out by Taddei et al. (*32*), specific differences between various portions of the sequence in the two proteins can account for a very small fraction of the gap existing between the aggregation rates of the two proteins.

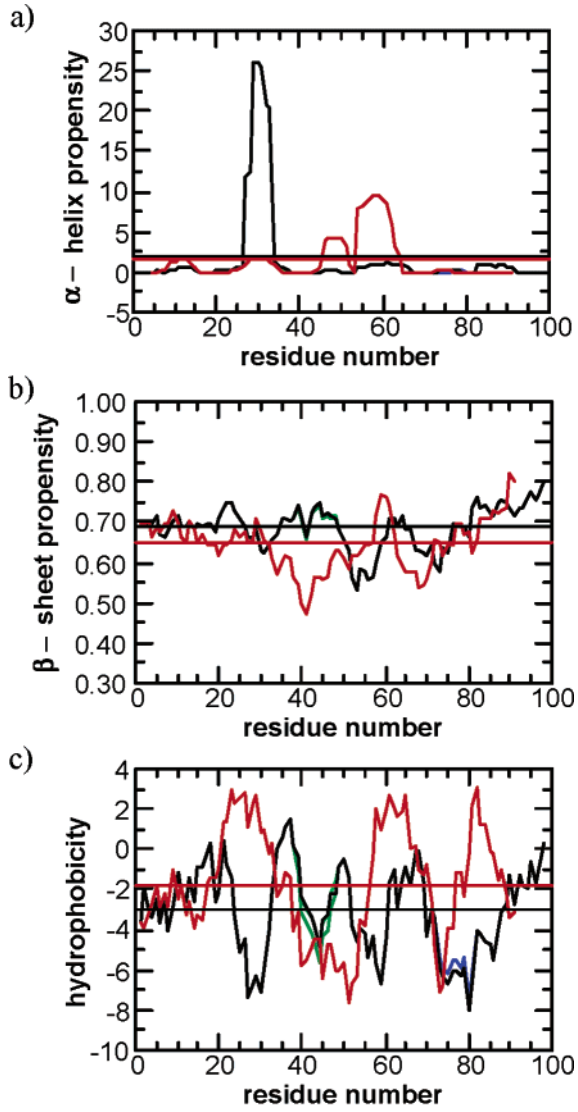

FIGURE 1: Comparison of α-helix propensity (a), *β*-sheet propensity (b), and hydropathy (c) profiles of HypF-N (red), WT AcP (black), and three representative AcP mutants, S43E/K44S/R77E (blue), S43E/K44S (light green), and S43E (dark green). The profiles were determined using (a) the AGADIR software (*30*), (b) the Street and Mayo scale (*29*), and (c) the Roseman scale (*31*). The profiles of WT and mutant AcP are largely superimposable. The horizontal lines in the three panels indicate the average α-helix propensities (a), *β*-sheet propensities (b), and hydrophobicities (c) of WT AcP and HypF-N. In panels a and b, large numbers indicate large α-helix propensity and *β*-sheet propensity, respectively. In panel c, less negative values indicate higher hydrophobicity.

By contrast, the two proteins differ considerably in their values of net charge and in their hydropathy profiles. At the pH value considered here, HypF-N and AcP have net charges of +1 and +5, respectively. The difference in hydrophobicity, determined using the Roseman scale (*31*), is evident when considering both the average values calculated on the entire sequence (−1.74 and −3.01, respectively) and the values of hydrophobicity for the peaks in the hydropathy profiles (Figure 1c).

Strategy. To elucidate whether the remarkable difference in the aggregation rates of the two proteins can be attributed to differences in net charge or hydrophobicity, we have chosen to decrease the net charge of AcP to +1 so that the difference in charge between the two proteins is eliminated.

Table 1: Aggregation Rates of AcP Mutants[a]

	net charge	ln k_{agg}
wild type	+5	−6.98 ± 0.04[b]
S43E	+4	−6.33 ± 0.12
Q52E	+4	−6.38 ± 0.13
S43E/K44S	+3	−5.62 ± 0.12
K44E	+3	−5.99 ± 0.13
Q52E/K57Q	+3	−6.57 ± 0.13
R77E	+3	−6.16 ± 0.12
S43E/K44S/R77E	+1	−5.57 ± 0.13
K44E/R77E	+1	−5.15 ± 0.12
Q52E/K57Q/R77E	+1	nd[c]

[a] All experimental errors reported in the table are standard deviations unless stated otherwise. [b] The best estimate and experimental error reported for the WT protein are the average value and standard error, respectively, obtained from nine independent measurements. [c] Data not determined because of aggregation of this mutant during purification.

Multiple amino acid substitutions involving residues on the protein surface have been used for this purpose. Elimination of the discrepancy existing between the hydrophobicities of the two proteins is undoubtedly more difficult to achieve because of the dramatic effect of multiple substitutions of hydrophobic residues on protein stability and the difficulty of leaving the secondary structure propensities unaltered when making such substitutions.

Eight variants of AcP were therefore produced with net charges ranging from +1 to +5 (Table 1). All the substituted residues are located on the surface of the protein, and they do not affect the stability of the native hydrophobic core. The two triple mutants of AcP we have produced have an overall charge of +1 (S43E/K44S/R77E and Q52E/K57Q/R77E). Importantly, their hydrophobicity is very similar to that of the WT protein since in both cases a charged residue is substituted with another charged one and a polar residue is first replaced with a negatively charged residue, and then replaces a positively charged residue in nearby positions. These mutations produce the same global effect as the K44E/R77E double mutant, where two positively charged residues are substituted with two negative ones. Similarly, the S43E/K44S and Q52E/K57Q double mutants and the R77E and K44E single mutants bring the net charge from +5 to +3 without affecting significantly the overall hydrophobicity of the sequence. In addition, all mutations were carefully chosen to minimize any change in secondary structure propensity (α-helical and *β*-sheet). α-Helical and *β*-sheet profiles of a number of mutated sequences were compared with those of the wild-type sequence. Only mutants with unaltered or slightly different profiles were selected for this study (see Figure 1). Since the change in the aggregation rate caused by mutations involving charged residues does not show a clear dependence on the position of the mutations (*15*), all the mutations considered here involve various positions along the sequence.

Correlation between the Net Charge and the Rate of Aggregation. Since it was shown, from the analysis of a group of AcP variants, that the aggregation rate under native conditions depends on the conformational stability of the native state (*33*), we determined the aggregation rate of the AcP variants under conditions in which the native states of the proteins are substantially disrupted. The presence of 25% TFE allows both denaturation and aggregation of the proteins to occur (*15*, *16*, *25*, *32*). As a consequence, the aggregation

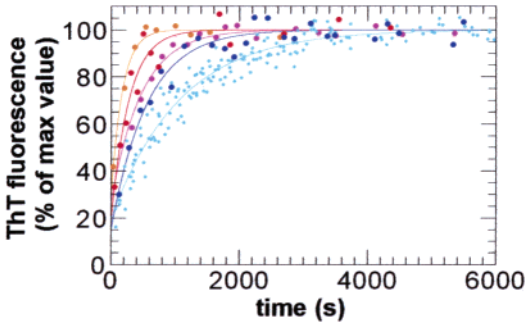

FIGURE 2: Aggregation of WT and four representative variants of AcP in the presence of 25% TFE and 50 mM acetate buffer (pH 5.5) at 25 °C, followed by ThT fluorescence. The AcPs are as follows: wild type (cyan, net charge of +5), S43E (blue, net charge of +4), R77E (purple, net charge of +3), S43E/K44S (red, net charge of +3), and K44E/R77E (orange, net charge of +1). The solid lines through the data points represent the best fits to single-exponential functions. The calculated rate constants for all variants are reported in Table 1.

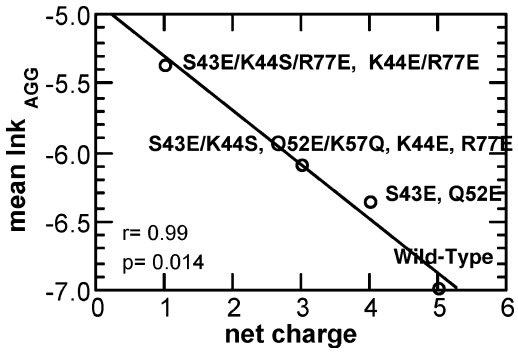

FIGURE 3: Aggregation rate vs net charge. The plots are constructed from the aggregation rates of the WT protein and the averages of mutants with the same net charge. Changes in net charge upon mutation are calculated at pH 5.5 assuming standard pK_a values for amino acid residues.

rate measured in all the experiments presented here is completely independent of the destabilization of the native state caused by the introduced mutations, allowing their effects on the aggregation process to be directly determined. Figure 2 shows the increment of ThT fluorescence resulting from the aggregation of WT AcP and some representative mutants under these conditions. The rate constants are obtained by fitting the data points to single-exponential functions. The resulting values for all studied variants are shown in Table 1.

Mutations can also exert a local effect, such as a specific electrostatic interaction, or interfere with other types of interactions. In addition, despite our efforts to keep the α-helical and β-sheet propensities unchanged, some mutations can lead to changes in these parameters that are more significant than those anticipated from the utilized algorithms. We therefore produced more than one mutant for each value of net charge so that an average value of their aggregation rates could be considered as a more accurate estimate. The mean aggregation rate is plotted against the net charge in Figure 3. The significance of the correlation between these two parameters is supported by a linear correlation coefficient of 0.99 and a p value of 0.014. The observed correlation indicates that a decrease in the positive net charge of the protein leads to an increase in the aggregation rate, in agreement with our previous study (*15*). The slope of the line that best fits the data is -0.40 ± 0.08, meaning that the aggregation rate increases by ca. 1.5 ± 0.1 per unit decrease in net charge. Remarkably, as the net charge of AcP approaches that of HypF-N, its aggregation rate is dramatically enhanced. The rate constant of aggregation at a charge of +1, i.e., that corresponding to WT HypF-N, can be estimated directly from interpolation on the straight line. This appears to be 0.0056 ± 0.0010 s^{-1}, in agreement with the average of the mutants of this study with a charge of +1 (0.0047 ± 0.0010 s^{-1}).

Table 2: Calculated Contribution of Net Charge Differences to the Difference in Aggregation Rate between AcP and HypF-N

	$\Delta\ln k_{\text{net charge}}$[a]	$\Delta\ln k_{\text{tot}}$[b]	charge weight (%)
S43E/K44S/R77E	1.41	6.98	20.2
K44E/R77E	1.83	6.98	26.2
mean of mutants with a charge of +1[c]	1.62	6.98	23.2
interpolation on the straight line	1.79	6.98	25.7

[a] Corresponding to $\ln[k^{\text{AcP}(+1)}] - \ln(k^{\text{AcP}})$, i.e., to the difference in the aggregation rates between AcP and HypF-N due to only the net charge difference. [b] Corresponding to $\ln(k^{\text{HypF-N}}) - \ln(k^{\text{AcP}})$, i.e., to the difference in the observed aggregation rates between AcP and HypF-N. [c] S43E/K44S/R77E and K44E/R77E.

To determine the extent to which the difference in charge existing between AcP and HypF-N contributes to the difference in the aggregation rates of the two proteins (charge weight), the following equation was used:

$$\text{charge weight} = \{\ln[k^{\text{AcP}(+1)}] - \ln(k^{\text{AcP}})\}/[\ln(k^{\text{HypF-N}}) - \ln(k^{\text{AcP}})] \times 100 \quad (1)$$

where k^{AcP}, $k^{\text{AcP}(+1)}$, and $k^{\text{HypF-N}}$ are the rate constants of aggregation for WT AcP, AcP variants with a charge of +1, and WT HypF-N, respectively. In this equation, the denominator, i.e., $\ln(k^{\text{HypF-N}}) - \ln(k^{\text{AcP}})$, is the difference in the observed aggregation rates of the two proteins, while the numerator, i.e., $\ln[k^{\text{AcP}(+1)}] - \ln(k^{\text{AcP}})$, represents the fraction of this difference arising from only charge differences. Utilization of eq 1 leads one to estimate that the relative importance of the net charge is approximately 20−26% with respect to other determining factors (Table 2). As described above, these factors are mainly hydrophobicity differences. They seem to contribute to ca. 74−80% of the gap in the aggregation rates existing between the two proteins.

A double mutant of HypF-N bearing the E55K and E87K mutations was also produced to convert the net charge of HypF-N from its natural value (+1) to that of WT AcP (+5) under the conditions that were tested. Unfortunately, this mutant is able to yield a remarkably smaller amount of aggregates than the WT protein, making it difficult to estimate the aggregation rate with reasonable accuracy. While we experienced these technical problems, it is clear that the few aggregates formed from this variant appear relatively quickly and remarkably faster than any variant of AcP. This finding confirms that net charge contributes to the aggregating potential of these proteins to a lower, albeit significant, extent than hydrophobicity.

DISCUSSION

The results obtained in this study add to our previous observations about the role of net charge in the process of

aggregation (*15*). In agreement with our previous study, aggregation propensity under partially denaturing conditions correlates inversely with the net charge in the various AcP variants. When the values of aggregation rates for mutants with the same net charge are averaged, a very good correlation with a low level of scatter of data points is obtained (Figure 3). This correlation allows important details in the mechanism of aggregation of AcP to be obtained. A generic phenomenon of repulsion between distinct protein molecules, rather then any specific electrostatic interactions such as salt bridges or local repulsions, is likely to be the mechanism through which the charge of the protein exerts its effect. When the net charge of the protein is high, the approach and interaction between distinct protein molecules are hindered by an overall effect of electrostatic repulsion. A decrease in the net charge leads to a reduction in the extent of such repulsions, contributing to an acceleration of the aggregation process. The linearity of the relationship between aggregation rate and net charge indicates that this electrostatic effect is similar in the various protein variants. Furthermore, the observed relationship holds true even if mutations at various positions along the protein sequence are used. The absence of any major position-specific effects along the protein sequence emphasizes the importance of global repulsions between protein molecules and rules out the possibility that the electrostatic effects are limited to only some residues or regions of the protein.

The main aim of the mutational study described here is to provide a quantitative estimate of the influence of charge, as opposed to other factors such as hydrophobicity, on the rate of protein aggregation. Cancellation of the difference in net charge existing between the slowly aggregating AcP and the rapidly aggregating HypF-N proteins only reduces the gap in the aggregation rate between the two proteins by 20−25%. This is evident when the net charge of AcP is reduced from +5 (the value of WT AcP) to +1 (the value of WT HypF-N). This indicates that the difference in hydrophobicity between the two proteins accounts for most of the difference in the aggregation rate. The relative importance of charge and hydrophobicity in protein aggregation can also be estimated using an equation that we edited from a detailed experimental study of AcP involving not only alterations of charge but also modification of hydrophobicity and α-helical and β-sheet propensities (*34*). Substitution of the amino acid residue with the highest value of hydrophobicity for that with a positive charge and the lowest value of hydrophobicity (such as Phe for Arg) leads to a theoretical increase in the aggregation rate, expressed as the natural logarithm of the rate constant [$\Delta(\ln k_{agg})$], of ca. 4.4 units (*34*). By contrast, any substitution of a negatively charged residue with a positively charged one induces a theoretical change in the aggregation rate of ca. 1.0 unit (*34*). This indicates that the maximum change in the aggregation rate that can be achieved with a single mutation that only changes charge without significantly affecting hydrophobicity is in theory 22% of that obtained by changing charge and hydrophobicity together. This estimate is in good agreement with that obtained from the comparison between AcP and HypF-N. Since the previously edited equation was found to be generically applicable (*34*), we believe these results are of general validity and not limited to the AcP and HypF-N systems.

Although contributions from charge repulsions appear to be less important than those involving hydrophobic interactions, the influence of charge factors on protein aggregation must not be underestimated. Natural β-sheet proteins use inward pointing charges, such as a lysine residue with its side chain located at the edge of a β-sandwich, to avoid edge to edge aggregation (*35*). This strategy is also recognized to be the most applicable in the prevention of aggregation in the *de novo* design of β-sheet proteins (*35*, *36*). Proteins that are unfolded under physiological conditions, i.e., natively unfolded proteins, have a total net charge that is generally higher, in addition to having a lower content of hydrophobic residues, than those of proteins that fold into globular structures (*12*). This is likely to be a strategy by which proteins that do not fold into globular structures avoid aggregation and remain soluble in the crowded environment of the cell. Recently, it has also been shown that the electrostatic interactions between the positive charges of the fibrils formed from a fragment of the Alzheimer amyloid β peptide and the negative charges on acidic proteins strongly promote the aggregation of the latter (*20*). In our previous work, we were also able to attribute the onset of some unexplained hereditary forms of amyloid diseases to the reduction in the net charge of the aggregating protein caused by the mutations associated with such diseases (*15*).

From a wider point of view, the analysis of the several mutations associated with various protein deposition diseases shows that mutations involving reduction of charge are as recurrent as those associated with an increase of hydrophobicity. This appears to be striking when one considers that, on a purely random basis, mutations involving a decrease in charge are expected to be considerably less frequent than those involving an increase in hydrophobicity (*37*). Moreover, the results obtained from this work show that alterations in charge produce changes in the aggregation rate of unstructured polypeptide chains considerably less marked than those associated with a change in hydrophobicity. This apparent contradiction could be due to the ability of molecular chaperones, such as heat shock protein 90 and other possible quality control mechanisms of the cell, to buffer the effects of the intrinsic genetic variations that are present in each species (*38*). The ability of molecular chaperones to bind preferentially to exposed hydrophobic groups, such as those newly generated from mutation (*39*), could offset the potential pathogenic effect of some of the mutations associated with an increase in hydrophobicity, therefore explaining the apparent imbalance between the effects of hydrophobic residues observed *in vitro* and those existing *in vivo*.

In conclusion, hydrophobic interactions appear to be more effective in protein aggregation than charge effects. This will help in clarifying the mechanism of protein aggregation and the identification of the driving forces in this apparently complex process. Despite differences in the relative importance of these two factors, the fact remains that both determinants are critical and need to be considered for understanding protein aggregation *in vivo* and the influence of amino acid composition, sequence, and substitutions in protein deposition diseases and protein design.

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