Published in final edited form as:

Biomacromolecules. 2008 June ; 9(6): 1595–1603. doi:10.1021/bm800056r.

Modulation of Self-Association and Subsequent Fibril Formation in an Alanine-Rich Helical Polypeptide

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Abstract

Thermal unfolding, reversible self-association, and irreversible aggregation were investigated for an alanine-rich helical polypeptide, 17-H-6, with sequence [AAAEAAAAQAAAEAAQAAQ]₆. Dynamic light scattering, transmission electron microscopy, and thermal unfolding measurements indicate that 17-H-6 spontaneously and reversibly self-associates at acidic pH and low temperature. The resulting multimers have a compact, globular morphology with an average hydrodynamic radius ~10-20 nm and reversibly dissociate to monomers upon an increase to pH 7.4. Both free monomer and 17-H-6 chains within the multimers are α -helical and folded at low temperature. Reversible unfolding of the monomer occurs upon heating of solutions at pH 7.4. At pH 2.3, heating first causes incomplete dissociation and unfolding of the constituent chains. Further incubation at elevated temperature induces additional structural and morphological changes and results in fibrils with a β -sheet 2° structure and a characteristic diameter of 5-10 nm (7 nm mean). The ability to modulate association and aggregation suggests opportunities for this class of polypeptides in nanotechnology and biomedical applications.

Introduction

Nonnative protein aggregation is a common problem in biopharmaceutical production, purification, storage, and delivery,¹⁻⁶ and is linked to the progression of a number of neurodegenerative diseases.⁷⁻¹² In both cases, native or unfolded protein molecules assemble into stable aggregates that are at least putatively stabilized by nonnative secondary structures and interprotein contacts such as hydrogen bonding and hydrophobic contacts within and between β -sheets. Often, incompletely unfolded monomers are implicated as being more prone to aggregation than their native or folded counterparts.^{1,10,13-17} Nonnative aggregation is typically a multistage process, involving one or more conformational changes of individual monomer chains along with one or more assembly steps to create soluble or insoluble aggregates.¹³⁻¹⁷ In many cases, a nucleated-growth mechanism is implicated, in that some extent of reversible aggregation (hereafter termed self-association) or prenucleus formation occurs prior to the creation of stable, irreversible oligomers (hereafter denoted as aggregates) that can further grow by recruitment of monomers or other oligomers.^{14,18,19} In this context, prenucleus formation may be thermodynamically unfavorable or favorable and simply denotes the fact that the rate-limiting step for irreversible aggregate formation occurs after formation of a reversible oligomer.^{19,20} For natively folded proteins and polypeptides, monomer

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Supporting Information Available. DSC and DLS data analysis; two-state model fits; sedimentation velocity data; and additional TEM images. This information is available free of charge via the Internet at <http://pubs.acs.org>.

unfolding or misfolding is often implicated or assumed to precede reversible oligomer formation, but this is not strictly required for a general nucleation-and-growth mechanism.^{14,18,19} It is increasingly apparent that aggregates rich in β -sheet structure are an intrinsically stable state for even homopolymer polypeptide sequences²¹⁻²³ and that these structures are stabilized primarily by a combination of backbone-backbone hydrogen bonds, backbone-side-chain hydrogen bonds, and burial of hydrophobic side chains.²³⁻²⁷ For proteins and polypeptides that are able to fold stably as monomers, there also remain a number of open questions regarding the extent of unfolding and/or structural rearrangement that is required for stable aggregate formation.¹⁸ The role of electrostatic interactions is also incompletely understood. Acidic conditions are often used to promote aggregation by destabilization of folded monomers,²⁸⁻³¹ yet electrostatic interactions between monomers are net repulsive at low pH if there are a significant number of protonated basic residues.^{1,32,33} An improved understanding and characterization of unfolding/misfolding and aggregation in relatively simple polypeptide sequences may therefore provide insights into the roles of unfolding and interactions between specific amino acids in the more general field of protein aggregation.

In vitro aggregation of proteins is often influenced by a number of experimental factors, including primary structure, secondary structure, temperature, pH, and protein concentration.² The resulting aggregates are typically reported to be either amorphous precipitates or (ordered) fibrils.¹ In vivo aggregation occurs under a relatively restricted range of environmental conditions and is typically linked to mutations, to chemical degradation, such as oxidation, deamidation, or proteolysis, or to other factors that increase the misfolded protein concentration. In some cases, fibrillar or prefibrillar aggregates are associated with neurodegenerative diseases and may be detrimental.^{10,34,35} By contrast, aggregates are also of interest in nanotechnology fields through bottom-up approaches for the assembly of fibers employed in hydrogels and device applications.³⁶ Examples include self-assembly of fibrils to construct nanowires,^{37,38} and the use of fibrils from an IKVAV-containing peptide to promote cell adhesion.³⁹ Thus, in vitro polypeptide association and aggregation is not necessarily deleterious and can offer attractive avenues for designing desirable structures for material science applications.⁴⁰⁻⁴⁶ Independent of whether protein or peptide aggregates are formed via pathological pathways, elucidation of self-association mechanisms therefore will potentially have significant impact on design strategies for producing improved biomaterials and/or nanomaterials.

We have previously described a set of alanine-rich helical polypeptides with varied densities of reactive glutamic acid residues.^{47,48} These sequences were selected to create α -helical structures that could be chemically tuned to present biologically active ligands and/or to mediate self-assembly. A number of these polypeptide sequences were shown to be monomeric under physiological conditions but associated to some degree upon protonation of glutamic acid residues at acidic pH.⁴⁹ The present report focuses on a detailed investigation of monomer unfolding and aggregate assembly for a particular polypeptide sequence, [AAAEAAAAQAAAEAAQAAQ]₆. This sequence is denoted 17-H-6 and is from the family of sequences investigated previously that has the highest glutamic acid density (see also, Table 1).⁴⁷⁻⁴⁹ The high glutamic acid density of this and related sequences suggests the aggregation behavior of these molecules may be most easily promoted or suppressed under conditions where the glutamic acid side chains are neutral or charged, respectively. The association and assembly behavior were therefore characterized under both low pH and near-neutral pH so as to access aggregation-promoting and -suppressing conditions, respectively. Previous work focused primarily on characterization of the conformational properties of each of the sequences in Table 1. The results here provide a detailed characterization of the morphology, reversibility, and size of different associated or aggregated structures for 17-H-6, as well as the role of temperature and polypeptide charge state in modulating assembly. Table 1 illustrates that within this family of sequences 17-H-6 is intermediate in size and has a

relatively high glutamic acid density. The regularity of all of these sequences and their functional group placement may afford advantages in control of assembled structures, and perhaps in the organization of functional groups in fibrillar aggregates. Such control would permit the further elaboration of fibrillar scaffolds with other moieties, which would have potential application in nanotechnology.

Materials and Methods

Materials

17-H-6 has the sequence [AAAEAAAAQAAAEAAQAAQ]₆ and carries a His-tag (MGH₁₀SSGHIHMGGYGGMG) for ease of purification. The His tag was not removed from the polypeptide in order to maintain chemical functionality that could be useful for future functionalization of the observed aggregates. 17-H-6 was expressed in *E. coli* and purified using protocols described elsewhere.^{48,49} High purity polypeptide (>95%) was readily obtained, as confirmed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), reverse-phase high-performance liquid chromatography (HPLC), and amino acid analysis. The molecular weight of the purified polypeptide was confirmed via matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry. Yields of the expression were ~20-30 mg per liter of culture medium.

Two different buffers were used in the characterization of this polypeptide at low and neutral pH: 10 mM phosphate buffer saline (PBS), pH 7.4, containing 0.008 M sodium phosphate, 0.002 M potassium phosphate, 0.14 M sodium chloride, and 0.01 M potassium chloride (Pierce Biotechnology Rockford, IL); and 10 mM phosphate buffer, pH 2.3, containing 10 mM phosphoric acid, 0.14 M sodium chloride, and 0.01 M potassium chloride. All the buffers were prepared using Milli-Q grade water.

Circular Dichroic Spectroscopy

Circular dichroism (CD) data were collected using a Jasco (Easton, MD) J-810 spectropolarimeter equipped with a Jasco PTC-424S Peltier temperature controller. Quartz cells (1 mm or 1 cm; QS grade) were used depending on the concentration of the sample. All isothermal measurements were obtained with samples that were first equilibrated at initial temperature (5 °C) for at least 5 min. Isothermal spectra were recorded from 5 to 80 °C in increments of 5 °C, with a temperature scanning rate of 1 °C/min between temperatures. Spectra were recorded from 250 to 190 nm at a rate of 50 nm/min and were corrected by subtraction of the corresponding buffer spectrum. After spectra were collected at 80 °C, each sample was further incubated at 80 °C for 3 h. Spectra were then collected during cooling from 80 to 5 °C in 5 °C increments. The ellipticity values, Θ (mdeg), were converted to mean residue ellipticity values, $[\Theta]_{\text{MRE}}$ (deg cm² dmol⁻¹), using the molecular weight of the polypeptide and the cell path length.⁵⁰ Helical content was estimated by the equation proposed by Kennedy et al. (2002).⁵¹

Thermal unfolding curves were also constructed by recording ellipticity at 222 nm, Θ_{222} , every 1 °C during steady heating at a rate of 1 °C/min, and corrected by buffer subtraction. Apparent thermodynamic parameters for unfolding transitions were determined by fitting these ellipticity or the corresponding MRE values ($[\Theta]_{\text{MRE},222}$) using eq 1a, which assumes a two-state model with linear pre- and post-transition baselines and neglects the change in heat capacity upon denaturation.⁵²⁻⁵⁴

$$\Theta_{222}(T) = f_N(T) \Theta_{N,222}(T) + (1 - f_N(T)) \Theta_{U,222}(T) \quad (1a)$$

$$\Theta_{i,222}(T) = a_i T + b_i \quad (i=N \text{ or } U) \quad (1b)$$

$$f_N(T) = \exp \left\{ \frac{\Delta H_{\text{VH}}}{k_B} \left(\frac{1}{T_m} - \frac{1}{T} \right) \right\} \quad (1c)$$

In eqs 1a and 1b, $\Theta_{222}(T)$ is the ellipticity value at a particular temperature and 222 nm, k_B is the Boltzmann's constant, and T is the absolute temperature. $\Theta_{i,222}(T)$ denotes the ellipticity value at 222 nm for purely folded or unfolded 17-H-6, with a_i and b_i as temperature-independent constants; the subscripts N and U denoted folded (low-temperature) and unfolded (high-temperature) regimes. $f_N(T)$ is the fraction of molecules that are native or folded at a given T , T_m is the melting or midpoint unfolding temperature ($f_N = 0.5$ at T_m), and ΔH_{VH} is the van't Hoff enthalpy for unfolding, evaluated at T_m . Equations 1a-1c, were fit to experimental $\Theta_{222}(T)$ versus T data using nonlinear regression via the Sigma-Plot Version 9 software package (Systat Software Inc., San Jose, CA).

Differential Scanning Calorimetry

Differential scanning calorimetry (DSC) experiments were conducted using a VP-DSC (Microcal, Northampton, MA). Using the procedure described in Supporting Information, the differential power was converted to an excess apparent heat capacity at each temperature $C_{p,\text{app}}^{\text{ex}}(T)$. The calorimetric enthalpy of unfolding (ΔH_{cal}) was calculated from

$$\Delta H_{\text{cal}} = \int_{T_L}^{T_H} C_{p,\text{app}}^{\text{ex}}(T) dT \quad (2)$$

In eq 2, T_L denotes a temperature well below the start of any thermal transitions (i.e., where $C_{p,\text{app}}^{\text{ex}}(T)$ is essentially zero). T_H was selected as a temperature slightly higher than the end of the observable endotherm. The van't Hoff enthalpy (ΔH_{VH}) was calculated in the standard manner for a two-state transition;⁵⁵ additional details are provided in Supporting Information. Calorimetric and van't Hoff enthalpy values were calculated only for samples that were shown to be reversible upon repeated scanning of the sample.

Dynamic Light Scattering

Dynamic light scattering (DLS) measurements were performed with a Brookhaven (Holtville, NY) BI9000AT correlator and a BI200SM goniometer with a laser wavelength of 532 nm. Samples with a given concentration were prepared by directly dissolving the required amount of polypeptide in the buffer at pH 2.3. The samples were filtered using 0.22 μm Millex syringe driven filter unit (Millipore, Bedford, MA). The time correlation function for scattered intensity was measured at five different scattering angles: 45, 60, 90, 120, and 135°. The temperature of all samples was held constant at 20 °C, with at least 15 min of pre-equilibration prior to collecting light scattering data. The data were analyzed with the Brookhaven Instruments software package using the second-order cumulant fit. Details of the analysis can be found in Supporting Information. The resulting fits provided values for the average, effective hydrodynamic radius (R_h) and for the polydispersity index (p_2).

Analytical Ultracentrifugation

Sedimentation velocity experiments were conducted with a ProteomeLab XL-1 Protein Characterization Ultracentrifuge (Beckman Coulter, Fullerton, CA) using a standard, double sector centerpiece equipped with quartz windows and an An-60Ti rotor at 40000 rpm. The samples were prepared as described above for the DLS experiments and were equilibrated for 1 h at 20 °C prior to each experiment. Apparent sedimentation coefficients, s_{app} , were obtained by the ls-g*(s) method using Sedfit software.⁵⁶ Estimates of the average number ($\langle y \rangle$) of 17-H-6 chains per multimer were made from eq 3.⁵⁷

$$\langle y \rangle = \frac{M_{\text{agg}}}{M_{\text{mon}}} = \frac{s_{\text{app}} k_B T}{M_{\text{mon}} (1 - v\rho) D_{\text{app}}} \quad (3)$$

In eq 3, v is the partial specific volume of the polypeptide monomer (calculated from the amino acid composition), ρ is the density of the solvent, M_{agg} is the average molecular weight of the multimers, and M_{mon} is the molecular weight of the monomer (14770 Da).

Transmission Electron Microscopy

Transmission electron microscopy (TEM) images of negatively stained samples were taken on a Tecnai G2 12 TEM (FEI Company, Hillsboro, OR) at an acceleration voltage of 120 keV. For each sample, 3 μL of solution was placed on a carbon-coated copper grid and blotted after 2 min at room temperature. Each sample was stained with 2 μL of 1 w/v % uranyl acetate solution for 30 s prior to blotting. After blotting excess solution, samples were dried at room temperature at least overnight prior to imaging. All images were obtained at room temperature.

Results and Discussion

Temperature-Induced Conformational Changes of 17-H-6

Temperature-induced secondary structure changes were monitored by CD spectroscopy and recorded at 17-H-6 concentrations of 10 and 50 μM at each pH. Representative full wavelength spectra of 50 μM solutions during unfolding and refolding at pH 7.4 and at pH 2.3 are given in Figures 1 and 2, respectively; tabulation of representative data observed at 10 and 50 μM is provided in Table 2. At low temperatures for each pH, the CD spectra of 17-H-6 exhibit minima at both 222 and 208 nm. These are characteristic of α -helical folded structures and are consistent with other alanine-rich polypeptides with similar sequences.⁴⁷⁻⁴⁹ The helical contents (% helicity) of 17-H-6 at pH 7.4 and 2.3 at 5 $^{\circ}\text{C}$ were determined by evaluation of the $[\Theta]_{\text{MRE}}$ at 222 nm⁵¹ and are shown in Table 2. The values were near 60% of the theoretical maximum in both cases. As expected, these values are greater than the values for the shorter polypeptide, 17-H-3, and are lower than those obtained for 35-H-6.^{48,49} This is despite the slightly lower molecular weight of 35-H-6 and is due to the lower helical tendency of the fusion tag of 17-H-6 relative to that of 35-H-6. Slight differences between these helicity values and those reported previously for 17-H-6 are likely a result of differences in sample preparation techniques.⁴⁹

As the temperature is increased, the absolute intensity of the $[\Theta]_{\text{MRE}}$ values at 222 and 208 nm decreases and a blue shift in the minimum near 208 nm is observed under both pH conditions, together indicating unfolding of the polypeptide chains to a nonhelical conformation. Previous studies indicated similar transitions for other helical polypeptides under similar conditions, although one of the previously reported polypeptides did not show a complete transition and maintained its helical character up to 80 $^{\circ}\text{C}$ at low pH and isotonic conditions.⁴⁸ CD spectra before heating and after subsequent cooling were compared to assess the extent of reversibility of unfolding at acidic and neutral pH. $[\Theta]_{\text{MRE},222}$ values, measured at 5 $^{\circ}\text{C}$, are shown in Table 2 for samples before and after a heating-cooling cycle for pH 2.3 and pH 7.4. Also shown in Table 2 are the average % helicity values for each case. At neutral pH, the helical spectrum (and % helicity) was fully recovered after cooling from 80 $^{\circ}\text{C}$. This result was independent of polypeptide concentration and indicates the conformational transition is fully reversible under these conditions. However, at pH 2.3, the final $[\Theta]_{\text{MRE},222}$ values and average % helicity values were lower in magnitude than those before heating, and these disparities were greater at the higher concentrations of 17-H-6. These observations suggest a fraction of 17-H-6 molecules either did not refold upon cooling or refolded incompletely. The observation that the extent of irreversibility increased with increasing 17-H-6 concentration suggests that intermolecular interactions may slow or prevent

refolding upon cooling and that these interactions are stronger between unfolded than folded monomers. The observation that 17-H-6 is monomeric at pH 7.4 (see below) and exhibits fully reversible unfolding suggests the intermolecular interactions that lead to incomplete refolding are opposed by electrostatic repulsions when the Glu residues are charged.

No β -sheet structure is visually apparent in any of the CD spectra shown in Figures 1 and 2. It is possible that small amounts of β -sheet may be present but difficult to detect due to the presence of strong α -helical and/or coil CD signals after heating and cooling 17-H-6 at pH 2.3. In contrast, incubation of 17-H-6 (50 μ M) at 80 °C and pH 2.3 for a period significantly longer than three hours did result in additional structural changes. The spectra measured at selected time intervals are shown in Figure 3 and indicate measurable β -sheet after approximately 7.5 h at 80 °C.

Effect of pH on the Aggregation State of 17-H-6

Earlier investigations of 17-H-6 indicated that the polypeptide adopted a monomeric state under near neutral conditions (PBS buffer at pH 7.4), as shown by AUC at low concentrations and by non-denaturing poly(acrylamide) gel electrophoresis for higher concentrations.⁴⁹ In the present study, the concentration dependence of melting curves and the (apparent) thermodynamic parameters for unfolding at pH 7.4 and 2.3 were used to quantify the effect of Glu protonation on the self-association of 17-H-6. Ellipticity values at 222 nm were monitored during continuous temperature scanning at 1 °C/min to follow the loss of helical structure with increasing temperature. This is typically a slow enough temperature-scanning rate to allow reversible conformational changes to equilibrate.⁵⁸ However, it was sufficiently high to ensure samples spent less time at each elevated temperature than was possible in the measurements reported in Figures 1 and 2. This approach was employed to ensure the degree of reversibility in the temperature-scanning measurements was equal to or greater than those summarized in Table 2.

The melting curves (Θ_{222} versus T) were measured for different concentrations of 17-H-6 (1-100 μ M). Each curve was normalized by scaling it by its maximum (negative) value of ellipticity; typically this was the value at the lowest temperature. The resulting melting curves for 17-H-6 at pH 7.4 are shown in Figure 4, with calculated melting temperatures and van't Hoff enthalpies given in Table 3. Comparison of experimental data points and fits to the two-state model for a representative concentration is given in the Supporting Information (Figure S1); the experimental melting curves were well described by the two-state model in all cases reported here. The superposition of the melting curves given in Figure 4 indicates that there is no significant dependence of the unfolding transition on the concentration of 17-H-6. This is also indicated by the similarity in the values of the melting temperatures (35 ± 1 °C) and van't Hoff enthalpies (21 ± 1 kcal/mol) given in Table 3.

Similar melting experiments to those in Figure 4 were conducted at pH 2.3. The corresponding melting curves are shown in Figure 5a. Unlike the melting curves obtained at neutral pH, the data in Figure 5a show a systematic shift to higher temperatures with increasing concentration of 17-H-6. Fitted T_m values increased from about 47 to 58 °C with increasing concentration, with corresponding van't Hoff enthalpies from 36 to 56 kcal/mol (Figure 5b). Within the context of equilibrium unfolding models⁵⁹ and models of net-irreversible unfolding,⁶⁰ an increase in T_m with increasing polypeptide concentration indicates that the folded state is self-associated and that there is some degree of dissociation upon unfolding. Therefore, even though the melting curves for the highest 17-H-6 concentrations are not fully reversible at pH 2.3 (cf. Table 2), the shift of T_m with concentration clearly indicates self-association in the folded state. Based on equilibrium models of stoichiometric dissociation with unfolding,^{59,60} the observed concentration dependence of T_m for 17-H-6 appears too weak to be consistent with complete dissociation upon unfolding (see also discussion of estimated multimer stoichiometry below).

That notwithstanding, it is not possible to conclusively quantify the stoichiometry of the self-associated state via unfolding curves unless the unfolding transitions are equilibrated.⁶⁰ Therefore, it was deemed unreasonable to fit more sophisticated thermodynamic unfolding models that include self-association for the pH 2.3 data.

Effect of pH on Thermal Transitions in DSC

Thermal transitions were monitored via DSC to provide complementary information for unfolding and aggregation of 17-H-6 as a function of pH. Only relatively high concentrations (100 μ M) were employed due to limitations of instrument sensitivity at lower concentrations. Representative results are provided in Figure 6. The DSC curve for 17-H-6 at pH 7.4 showed a single broad peak (endotherm). Repeated scans with the same sample showed superimposable DSC profiles for pH 7.4 (not shown) and confirmed that unfolding was reversible under those conditions. Only a single endothermic peak was observed but was much broader than is typical of two-state unfolding.^{55,61} The ratio of ΔH_{VH} to ΔH_{cal} is much less than one (cf. Table 3) and clearly indicates that although eq 1 fit the unfolding curves from CD reasonably well, the unfolding of 17-H-6 is not two-state at pH 7.4. This is perhaps not unexpected based on work elsewhere,⁶² indicating the helix-to-coil transition for polypeptides is only weakly cooperative. Independent of that conclusion, the agreement at pH 7.4 between the transition temperatures and van't Hoff enthalpies (Table 3) determined by CD and DSC indicates that the two techniques detect the same transition, that is, unfolding of helix.⁶²

In contrast, at pH 2.3 the DSC profile is more complex, including two or more overlapping endothermic transitions (midpoints near 50 and 65 $^{\circ}$ C) followed by a large exotherm before the second endotherm is completed. The exotherm prevents accurate determination of van't Hoff or calorimetric enthalpies at pH 2.3, but the qualitative observation of multiple endotherms suggests unfolding is more complex than two-state. Repeated scans of the same sample showed that unfolding was incompletely reversible and became more so as the number of scans increased (Figure 7). The exotherms evident in all scans at pH 2.3 are indicative of an irreversible process and may correspond to a further structural change or a change in aggregation state that prevents 17-H-6 from refolding. The asymptotic downturn in the signal at about 80 $^{\circ}$ C is due to the precipitation of the polypeptide. The location and broad shape of the low-temperature endotherm are similar to the CD-monitored transition at 100 μ M in Figure 5a. Combined with the discussion of Figure 5 and the results below from light scattering, these observations suggest the lower-temperature endotherm in Figure 6 corresponds to the same unfolding and (incomplete) dissociation step(s) monitored by CD at pH 2.3. The higher-temperature endotherm indicates a transition that is not detected by CD, yet is relatively large in terms of the change in enthalpy for the transition. In the context of the discussion below, the high-temperature endotherm and exotherm indicate that at least two transitions, in addition to the unfolding of 17-H-6, may be involved in the conversion of 17-H-6 from globular multimers to fibrillar aggregates at elevated temperatures.

Characterization of the Aggregates Formed under Low pH Conditions

Dynamic light scattering (DLS) measurements were conducted to determine the diffusion coefficient and to estimate the average hydrodynamic radius (R_h) and polydispersity of the pH-reversible multimers present at low pH. A representative plot of the autocorrelation function as a function of delay time is provided in Figure 8a, along with the cumulant fit to the data (see also Supporting Information for additional details of DLS fitting). All samples were freshly prepared at room temperature and were not subjected to heating prior to measurement in DLS or AUC. Diffusion coefficients were determined from the slope of Γ versus q^2 for samples of folded 17-H-6 ($T = 20$ $^{\circ}$ C) over a range of polypeptide concentration; a representative Γ versus q^2 profile is shown in Figure 8b.

In separate experiments (data not shown), solutions of 17-H-6 prepared at pH 7.4 were characterized via DLS; no detectable multimers were present at pH 7.4. After adjusting the pH to 2.3, solutions of 17-H-6 showed multimer R_h and p_2 values that were essentially indistinguishable from those of samples originally prepared at pH 2.3. When the solution pH was readjusted to pH 7.4 for the same samples, no multimers were detected. An additional readjustment to pH 2.3 again yielded multimers. Together, these results indicate the multimers at pH 2.3 form spontaneously and are reversible via changes in pH.

The hydrodynamic radius of the multimers across all concentrations were ~10-20 nm, with relatively low values of polydispersity index for a given sample (p_2 values between 0.15 and 0.25, data not shown). AUC sedimentation velocity experiments were also performed at selected concentrations to complement DLS experiments. Size distributions from ls-g*(s) analysis as a function of 17-H-6 concentration at pH 2.3 and 20 °C are provided in the Supporting Information (Figure S2). The corresponding values of the sedimentation coefficients ranged over $3-5 \times 10^{-12}$ s. The results from DLS and AUC were combined using eq 3 to estimate $\langle y \rangle$, the average number of monomers per self-associated native multimer. The values of $\langle y \rangle$ at all polypeptide concentrations were $\sim 10^2$ within statistical uncertainty. Combined with the relatively low polydispersity of the samples, as noted above, and the observation that increasing 17-H-6 concentration significantly increases the effective T_m for unfolding, these results indicate that the folded multimers of 17-H-6 at pH 2.3 are relatively large and can reversibly unfold and dissociate to some extent with increasing temperature. Our results do not conclusively indicate why the multimers do not grow to larger sizes or significantly coalesce with each other. However, at pH 2.3, the block [AAAEQAAAAQAAAEAAQAAQ]₆ will be essentially uncharged (pK_a of Glu $\gg$ 2.3), while the His- tag will have a charge of +12, assuming all His residues are protonated. Therefore, it is reasonable to hypothesize that multimers are micellar to some extent, with the His- tags at the surface of the multimers and with the core of the multimers composed of the remainder of the polypeptide chains. There also may be significant exposure of the block [AAAEQAAAAQAAAEAAQAAQ]₆ at the multimer surface, as the His-tag constitutes only about 16% (by number) of the amino acids in 17-H-6.

The morphology of the aggregates formed at room temperature was assessed by TEM with negative staining; representative data are presented in Figure 9. The TEM images indicate globular species with diameters ~20-60 nm (Figure 9a). These dimensions are consistent with the DLS data considering the possible effects of interactions of the multimers with the surface of the TEM grid during sample preparation. In addition, the multimers appear to be able to assemble into nominally oriented structures as a common morphology on the surface of the TEM grid (Figure 9b). It is not clear whether this is an artifact of interactions with the grid or is indicative of multimer-multimer attractions that become significant at the elevated surface concentrations induced by drying the solutions onto the grid. However, considering the low polydispersity values from DLS, it appears likely these arrangements of globular aggregates were not present in solution. It is also possible that the appearance of some fibril-like structures may result from formation of β -sheet structure with the increasing concentration of 17-H-6 encountered during drying. Indeed, FTIR assays of higher concentration samples of other polypeptides of this general composition show increased contribution from β -sheet structures.
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TEM images were also obtained for pH 2.3 samples that were incubated at 80 °C for 1 and 18 h (Figure S3 and Figure 10, respectively). As opposed to the samples shown in Figure 9, the aggregates created by extended heating appear as long fibrils with a typical width of 5-10 nm. Fibrillar morphologies were also observed after heating at 80 °C for 1 h, although those fibrils were significantly shorter (Figure S3 in Supporting Information). The combination of the TEM data in Figures 9, 10, and S3 suggests that fibril formation can be accelerated via TEM sample

preparation and by incubation at elevated temperatures. Based on these results, the self-association of the reversible monomers may be followed by coalescence into high-concentration aggregates and the emergence of fibrillar structures. In addition, heating of the self-associated multimers facilitates the formation of both β -sheet conformation and fibrillar structures.

For many systems,^{14,17,18} detectable formation of β -sheet structure occurs prior to or concomitantly with formation of aggregates (e.g., fibrils or filaments). Within that context, it is perhaps surprising that fibrils of 17-H-6 are apparent in TEM prior to detectable β -sheet structures by CD spectroscopy. It is unclear based on the present data whether this indicates a fundamentally alternative pathway for fibril formation or is a case of differences in the location of the rate-determining steps for β -sheet versus fibril formation. Alternatively, it may simply indicate a limitation of solution CD spectroscopy for detecting β -sheet structure at early stages of fibril formation. As the ultimate secondary structure of the fibrils is β -sheet, it is reasonable to conclude that 17-H-6 fibrils do share the common feature of being stabilized by β -sheet formation.^{18,21-23,26}

Together, the results presented and discussed above suggest the qualitative picture for unfolding, self-association, and fibrillar aggregate formation depicted in Figure 11. 17-H-6 is monomeric and primarily folded (α -helical) at pH 7.4 and low temperatures; it unfolds reversibly in a non-two-state (weakly cooperative) manner to a nonhelical state at higher temperatures. At pH 2.3, 17-H-6 is folded and α -helical at low temperatures, but the folded monomers spontaneously self-associate to form stable multimers in solution. The multimers are globular and relatively large ($R_h \sim 10$ -20 nm), containing $\sim 10^2$ monomers. It is speculated that the particular morphology and size of the multimers may result from a balance between the burial of the hydrophobic, Ala-rich stretches in 17-H-6, and the exposure and solvation of the positively-charged His tags at the multimer surface. The multimers unfold and at least partially dissociate upon heating to temperatures $\sim 60^\circ\text{C}$. The unfolding transition is reversible, but refolding is in competition with one or more additional steps that instead convert 17-H-6 to fibrils. The conversion to fibrils is effectively irreversible and also appears to promote or be promoted by 17-H-6, adopting β -sheet secondary structures.

The stark difference in self-association and aggregation behaviors of the polypeptide at acidic and near-neutral pH is presumably mitigated by the difference in charge state of the Glu side chains in 17-H-6. At pH values significantly above the pK_a of Glu, 17-H-6 has a significant negative-charge density that is relatively uniform along its sequence, thus inhibiting association. Conversely, for $\text{pH} \ll pK_a(\text{Glu})$ 17-H-6 is essentially a large hydrophobic block (133 amino acids; containing an additional alanine at the end formed during the multimerization of the gene encoding 17-H-6 repeating unit after digesting with the type II restriction enzyme⁴⁸) with a short, positively-charged His tag (26 amino acids containing 12 histidines). The hydrophobic blocks presumably drive association in aqueous solution, mitigated to some extent by the charge-charge repulsions of the His tags. At longer times and conditions favoring monomer unfolding, the multimers can ultimately convert to fibrils with elevated β -sheet structure.

Conclusions

Modulation of the self-association and unfolding of an acidic, alanine-rich α -helical polypeptide has been demonstrated. Characterization of the associative behavior of the polypeptide via multiple techniques illustrates that the association behavior of the polypeptide is dramatically affected by variations in pH and that this variation yields aggregate structures of different morphologies. At room temperature and pH values significantly lower than the pK_a of the anionic glutamic acids residues, the polypeptide forms native multimers that contain

$\sim 10^2$ monomers. No such associative behavior is observed at neutral pH ($\gg pK_a$ of Glu). TEM characterization of the polypeptide at low pH indicates different supramolecular structures; globular aggregates form at room temperature and convert to fibrillar structures after annealing at elevated temperature. Changes in multimer morphology with increasing temperature and comparison with higher pH suggests the initial self-association at low pH is required for subsequent fibril formation and that some degree of unfolding of the polypeptide facilitates conversion to fibrils that adopt and are likely stabilized by β -sheet secondary structure. In contrast to most common models of fibril formation via nonnative aggregation, with these polypeptides, native multimer formation occurs as an *on-pathway* step in unfolding-mediated fibril formation. The polypeptide 17-H-6 provides an illustrative example of a system in which (reversible) association and (irreversible) aggregation can be controlled by pH-adjustable, charge-charge repulsion combined with temperature-modulated polypeptide unfolding to promote hydrophobic attractions. The balance of these factors permits the formation of qualitatively distinct nanostructures. Over the long-term, development of such approaches to tune the associative behavior of these types of macromolecules may permit their use in multiple areas in medicine and biotechnology. Micellar drug delivery systems could be obtained by the use of alanine-rich blocks in amphiphilic block copolymers; the conjugation of hydrophobic drugs to reactive glutamic acids could be employed to tune drug loading. Further development of macromolecules and procedures to control formation and chemical composition of the fibrillar systems formed at high temperatures could be used in the assembly and organization of organic and inorganic hybrid structures. Such investigations are underway.

Acknowledgment

This work was supported in part by grants from the National Institutes of Health (NIH) and the National Center for Research Resources (NCRR), a component of the NIH (1-P20-RR017716, 1-RO1-EB006006, and 5-P20-015588 (instrument facilities)). Its contents are solely the responsibility of the authors and do not necessarily represent the official views of NCRR or NIH. Preliminary studies were made possible by funding by the National Aeronautics and Space Administration (NA68-01923). W. M. Keck College of Engineering Electron Microscopy Laboratory at the University of Delaware is thanked. Jared Sharp and Dr. Robin S. Farmer are also thanked for the development of the polypeptide expression hosts and protocols.

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Figure 1. Full wavelength spectra of 17-H-6 during (a) unfolding and (b) refolding at 50 μ M polypeptide in pH 7.4 PBS. In each panel, spectra are shown for 5-80 $^{\circ}$ C in 5 $^{\circ}$ C increments.

Figure 2. Full wavelength spectra of 17-H-6 during (a) unfolding and (b) refolding at 50 μM polypeptide in pH 2.3 phosphate with 150 mM salt. In each panel, spectra are shown for 5-80 $^{\circ}\text{C}$ in 5 $^{\circ}\text{C}$ increments.

Figure 3.
Time course of random coil-β-sheet transition of 50 μM 17-H-6 at pH 2.3 at 80 °C.

Figure 4.
CD melting curves for 17-H-6 in pH 7.4 PBS buffer.

Figure 5. Characterization of the thermodynamic parameters of unfolding for 17-H-6 at pH 2.3. (a) CD melting curves and (b) two-state model thermodynamic parameters of 17-H-6 at pH 2.3; T_m (closed circles) and ΔH_{VH} (open circles).

Figure 6.
Comparison of DSC curves of 100 μ M 17-H-6 at different pH values; pH 7.4 (solid line) and pH 2.3 (dashed line).

Figure 7.
Consecutive DSC scans of 100 μ M 17-H-6 in pH 2.3 buffer.

Figure 8.

Representative plots of (a) change in autocorrelation function as a function of delay time represented by experimental data points (circles) and cumulant fit (solid line) and (b) angular dependence of relaxation time represented by experimental data points (circles) and linear fit (solid line) ($C = 50 \mu\text{M}$).

Figure 9.
TEM pictures of room temperature aggregates of 17-H-6 at pH 2.3. Scale bars: 100 nm.

Figure 10. TEM pictures of 100 μ M of 17-H-6 at pH 2.3 annealed at 80 $^{\circ}$ C for 18 h. Scale bars: (a) 200 nm (b) 50 nm.

Figure 11.

Schematic depiction of unfolding, self-association, and aggregation for 17-H-6. The upper portion of the scheme shows simple monomer unfolding and is operative at pH conditions where the Glu side chains are charged and monomer-monomer interactions are therefore repulsive. The remainder of the scheme applies at low pH, where Glu side chains are uncharged, and monomer-monomer interactions are significantly attractive even for folded molecules.

Table 1

Description of the Alanine-Rich Helical Polypeptides

notation	repeat sequence	MW (Da)	nominal spacing between Es (Å)
17-H-6	[AAAEAAAAQAAAEAAQAAQ] ₆	14770	17
17-H-3	[AAAEAAAAQAAAEAAQAAQ] ₃	8875	17
35-H-6	[AAAEAAQAAAEAAAEAAQAAQ] ₆	14159	35
65-H-2	[(AAAE) ₅ (AAAE)(AAAE) ₅] ₂	9889	65

Table 2
Mean Residue Ellipticity and Helicity Values for the Polypeptide under Various Conditions

pH	c (μM)	[θ] _{MRE, 222} at 5 °C prior to unfolding	% helicity (prior to unfolding)	[θ] _{MRE, 222} at 5 °C after refolding	% helicity (after refolding)
2.3	10	-37900 ± 100	63	-35700 ± 1100	59
2.3	50	-39300 ± 700	65	-29700 ± 300	50
7.4	10	-34000 ± 200	57	-35200 ± 100	59
7.4	50	-36000 ± 100	60	-37200 ± 1700	62

Table 3

Comparison of CD and DSC Data for 100 μ M 17-H-6

pH	T_m ($^{\circ}$ C) by CD	T_{max} ($^{\circ}$ C) by DSC	ΔH_{NH} (kcal/mol) by CD	ΔH_{NH} (kcal/mol) by DSC	ΔH_{cal} (kcal/mol) by DSC
2.3	57.2	62.9	55.9	N/A	N/A
7.4	35.7	37.8, 36.7 ^a	20.3	21.2	81.4

^a Second scan in the same run.