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Slow Folding-Unfolding Kinetics of an Octameric β-Peptide Bundle

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Abstract

β-Peptide foldamers offer attractive frameworks for examining the effect of backbone flexibility on the dynamics of protein folding. Herein, we study the folding-unfolding kinetics of a β-peptide, Acid-1Y,[1] which folds in aqueous solution into an octameric bundle of peptides in a conformation known as the 14-helix. Acid-1Y is comprised exclusively of β-amino acids, which differ from α-amino acids by the addition of a single methylene into the backbone. We aim to understand how the additional degree of freedom and increased backbone flexibility in the β-amino acid affect folding dynamics and to measure folding rates of this octameric β-peptide. Previously, we found that the T-jump induced relaxation kinetics of a monomeric β-peptide that forms a monomeric 14-helix occurred on the nanosecond time scale[2] and are noticeably slower than a similar alanine-based α-helical peptide.[3] Additionally, in comparison to similar α-helices, the relaxation rates showed a weaker dependence on temperature. Here, we find that the T-jump induced relaxation kinetics of the octameric β-peptide occurs on an even slower time scale (minutes) and the unfolding relaxation rates show a large dependence on temperature. These differences indicate that folding energy landscapes of β-peptide secondary and quaternary structure are markedly distinct from one another and also from their α-helical counterparts.

Over the years there has been considerable interest in the design of non-natural quaternary structures with well-defined oligomerization states[1,4–10] because they have the potential for novel biological applications. Herein, we aim to characterize the folding and unfolding kinetics of a previously developed octameric β-peptide helical bundle of known crystallographic structure and high thermodynamic stability.[1] This 12-residue β³-peptide, Acid-1Y (Figure 1), was designed to form a 14-helix (the helical conformation formed from β³-peptides) with alternating cationic β³-homoornithine and anionic β³-homoaspartic side chains arranged on one helical face to support salt bridge formation, and β³-homoleucine residues on a second face to support side chain packing for oligomer formation. The third face consists of β³-homoglutamic acids and β³-homotyrosines. β³-Homotyrosine is frequently used as a probe for β-peptide concentration determination. Circular dichroism (CD) experiments of Acid-1Y revealed a cooperative self-association process thought to involve a minimally structured monomeric state that oligomerizes into a more highly structured state.[1] The β-peptide bundle was crystallized and its structure determined using

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data that extend to 2.3 Å resolution. The peptide forms a well-folded octamer (dimer of tetramers) with 14-helical conformation stabilized by electrostatic interactions between the cationic and anionic acid side chains and the hydrophobic packing of nonpolar β^3-homoleucine residues.

Previously, we characterized the folding kinetics of a 15-residue β^3-peptide 14-helix monomer.[2] The T-jump kinetics of this β^3-peptide occurred on the 0.5 µsec time scale and were noticeably slower than those of similar alanine-based α-helical peptides.[3] Additionally, in comparison to analogous α-helices, the rates showed a weak dependence on temperature. Here we examine the rate of formation of folding/unfolding of Acid-1Y, whose structure is stabilized by long-range hydrophobic forces. We show the folding occurs in a two-step manner – the first involving association of monomers into an intermediate in which the secondary structure has already formed. This slowly interconverts to the final, octameric folded form on the minute time scale.

RESULTS and DISCUSSION

Secondary structural and thermodynamic characterization, assessed by circular dichroism (CD) spectroscopy, confirms concentration-dependent self-association. To ensure that the β-peptide we synthesized behaved the same as the previously reported Acid-1Y we performed CD experiments, recording spectra (from 195 – 260 nm) of Acid-1Y at various concentrations at room temperature. The CD spectrum of Acid-1Y shows large changes in helical structure, indicated by increases in the magnitude of the mean residue ellipticity at 208 nm (MRE_{208}) at [Acid-1Y] ranging from 8 – 63 µM (Figure 2). Acid-1Y is nearly fully associated beyond 63 µM as indicated by equivalent spectra beyond this concentration. Together these results are consistent with the formation of a stable organized structure. This corroborates previous results indicating that Acid-1Y demonstrates concentration-dependent self-association and equilibrates between a minimally structured monomeric state and a more highly structured oligomeric state.[1]

The changes in the concentration-dependent CD spectra of MRE_{208} can be described by a monomer-octamer equilibrium (Figure 2).[11,12] In very good agreement with Goodman et al., we determined ln K_a equal to 82.9 (ln $K_a = 82.5 \pm 1.8$ was reported previously). Also consistent with reported data, our temperature-dependent CD melting curves (Figure S1) exhibit a concentration-dependent increase in T_m, again indicating concentration-dependent self-association. These data assure consistency in our samples and experimental conditions in preparation for kinetic experiments.

Temperature-jump, CD, and tyrosine fluorescence spectroscopy demonstrates identical relaxation kinetics

We first attempted to examine the folding of Acid-1Y with laser-induced T-jump IR spectroscopy, but found that the folding reaction was too slow to resolve in the submillisecond time regime using this technique. We therefore used conventional CD and fluorescence spectroscopy to measure its folding and unfolding kinetics. Since we know the stability of Acid-1Y is dependent on concentration and temperature we used both of these variables to vary the fraction of the foldamer in the octameric state and to characterize its association/folding and dissociation/unfolding.

We used CD in conjunction with a simple manual temperature jump setup to measure the association kinetics of 60 µM Acid-1Y. We have shown that at this concentration the β-peptide is fully folded (Figure 2) and our T-melt data validates its concentration dependence and reversibility (Figure S1), both requirements for T-jump experiments. The octamer is dissociated/unfolded by heating the foldamer sample to 90 °C and the destabilized β-peptide

is allowed to quickly cool to 12 °C; the equilibrium between the unfolded and folded states is then monitored as a function of time (Figure 3, black markers). The time-resolved MRE_{208} can be described by a single-exponential function with a relaxation rate, k or τ^{-1}, of 2.75×10^{-3} s^{-1} ($\tau = 364$ s).

We were able to resolve only a fraction of the CD amplitude on the time scale accessible to this experiment; approximately 90% of the total change in ellipticity occurred within the 10–15 s dead time of the manual T-jump. This suggests that the unresolved change in CD signal within the dead time can be attributed to a rapid initial conformational transition associated with helix formation. The monomeric Acid-1Y β-peptide has a relatively low degree of secondary structure formation as assessed from its CD signal[1] (Fig. S1) at the initial temperature, so most of the helix formation must be attributed to self-association rather than monomeric helix formation, which occurs much more rapidly than the times scale (~15 s to 1 h) probed here. The final folding step of Acid-1Y, reported in Figure 3, occurs on a much slower timescale, and near complete secondary structure formation precedes this step. Thus, we describe the process that occurs during this rate-determining step (r.d.s.) as the annealing of a partially to fully assembled helical oligomer that rearranges into the final native octamer conformation. The relaxation kinetics of Acid-1Y occurs on a significantly slower timescale, about a billion-fold slower, than what was demonstrated for a monomeric 14-helical β-peptide (hundreds of ns - μs timescale)[2]. The folding is also much slower than that of comparable α-helical bundles containing between two and six helices per bundle, which occur on millisecond timescales.[13–19]

Acid-1Y has no tryptophans (Trp), but contains two tyrosines (Tyr), which turned out to be sensitive fluorescence probes for monitoring folding/association transitions. Upon excitation at 277 nm, the folded oligomer has a strong fluorescence emission band at 303 nm, which increases by ~15% upon dissociation/unfolding (perhaps due to loss of a Tyr-Tyr quenching interaction). Tyr-fluorescence relaxation experiments were performed using the same steps taken for CD kinetics. Like the CD data, the fluorescence-detected kinetic data (Figure 3, blue markers) for a 90 - 12 °C T-jumps also fits well to a single-exponential decay function with $k = 2.75 \times 10^{-3}$ s^{-1}. However, in this case, essentially all of the amplitude is accounted for by a single exponential decay (Figure 3, red dashed line). This finding shows that, unlike its CD spectrum, the fluorescence spectrum of Acid-1Y does not report on the initial process, and reflects primarily the second phase that coincides with the r.d.s. In summary, CD and Tyr-fluorescence T-jump relaxation kinetics are consistent, both demonstrating very slow kinetics with rates nearly a billion times slower than the rate of 14-helix formation we observed.[2]

Acid-1Y association and dissociation does not reveal concentration dependence

To assess the concentration dependence of Acid-1Y association/folding kinetics, Tyr-fluorescence T-jump experiments were carried out at several different peptide concentrations (Figure 4). In these experiments samples underwent T-jumps from 90 to 22 °C. Multi-exponential fitting of the data suggests that the folding process is primarily single-exponential; in addition to a major phase with a time constant $\tau = 60 – 80$ s, accounts for about ¾ of the amplitude, there is a minor phase with τ ~400 – 800 s (Figure 4, inset). However, in marked contrast to the equilibrium concentration dependence of association, the rate of folding remains essentially constant over the 3.33-fold concentration range (60 to 200 μM) studied (Figure 4). By contrast, if the kinetics of association followed the same 8th order process of the equilibrium measurement, we would expect to see a rate increase of 3.33^8 or 15,120. Even if a dimer were formed in the rate-determining step, we would expect a rate increase of 3.33^2 or 11-fold. These data indicate that a change in association state does not occur in the r.d.s. Instead, the step reported by the fluorescence measurement must

reflect the reorganization of a pre-associated intermediate, which occurs at a rate slower than the association of the monomers to form this complex. This slow step leads to the native octamer, either directly or through a set of subsequent steps that are more rapid than the r.d.s. The idea that the slow step is a rearrangement is consistent with the slow time scale of the relaxation.

Tyr-fluorescence kinetics was also used to study the dissociation/unfolding of Acid-1Y (Figure 5). In these experiments the concentration was jumped from one where the β-peptide is fully folded to one where it is unfolded (from 60 to 3, 6, 12, and 20 μM [Acid-1Y], respectively), and the Tyr-fluorescence was observed over time. The kinetic traces fit to a double-exponential function with a minor fast phase (τ ~40 – 80 s) accounting for 10–20% of the total amplitude followed by a major phase with τ ranging from 230 to 630 s (Figure 5, inset). The relatively modest concentration dependence of the rate constants is consistent with a mechanism dominated by dissociation of the octamer with minor contributions from partially dissociated intermediates or remodeling steps.

Acid-1Y association and dissociation demonstrates Arrhenius temperature dependence

To study the temperature dependence on the association of Acid-1Y we evaluated the T-jump relaxation kinetics of a 60 μM sample that was jumped from 90 °C to various final temperatures, 8, 12, 22, and 42 °C. The linear dependence of the rate constants as a function of temperature indicates slight Arrhenius behavior, with a calculated activation energy, E_a, of 3.5 ± 0.7 kcal/mol (Figure 6). This is consistent with the fact that in the transition state for the folding of Acid-1Y the enthalpically favorable secondary structure has been already formed. Furthermore, the small E_a indicates that most of the enthalpically favorable packing of the core residues has already occurred as the conformational ensemble reaches the transition state. The slow rate of the folding is, thus, largely due to entropic contributions associated with the immobilization of the backbone and core side chains.

Next, the temperature-dependent dissociation kinetics were examined by monitoring the equilibration of β-peptide samples that underwent concentration jumps from 60 - 6 μM at various temperatures (Figure 7). Data were fit to exponential growths and the determined rates demonstrate temperature-dependent behavior with rates increasing with temperature. Arrhenius plots (Figure 8) illustrate that the temperature dependence of the calculated rate constants in the unfolding direction has a much steeper slope and a larger dependence on temperature relative to the folding rate constants corresponding to an E_a of 17.8 ± 0.9 kcal/mol. This increased enthalpy of activation is similar to what we would expect for the unfolding of globular domains. The encountered enthalpic energy barrier is 5-fold larger in the unfolding direction than in the folding direction. In comparison to the value previously reported for the 14-helix formation of a β-peptide monomer, $E_a = 6.8 ± 1$ kcal/mol,[2] the apparent enthalpies of activation for (un)folding of Acid-1Y is quite different ($E_a = 3.5 ± 0.7$ and 17.8 ± 0.9 kcal/mol in the folding and unfolding directions respectively). This finding highlights the differences between the processes measured in the two systems, which involve monomeric helix formation for the β-peptide monomer, but core packing in the case of the octameric peptide.

Conclusions

Equilibrium measurements demonstrate that Acid-1Y is a stable 14-helical β[3]-peptide that undergoes cooperative self-association into an octamer from an unstructured monomer.[1] We have demonstrated that the kinetics of quaternary structure formation of this β-peptide, like many globular proteins, is a slow process[20]. The secondary structure forms in the fast phase that leads to a highly helical oligomeric intermediate. This intermediate(s) then is converted into the native octameric structure through a very slow annealing process. It is also possible

that multiple intermediates occur at timescales faster than the r.d.s. Further study is necessary to determine the exact number of monomers in the intermediate state, although the lack of concentration dependence of the observed rate constants indicated that the r.d.s. is not coupled with a change in the oligomerization state.

The near concentration-independent kinetic behavior further supports the proposal that initial helix association is not the limiting step in the folding/association of Acid-1Y, and we can rule out the folding of the monomeric helix being the rate-determining step because that process occurs in the nano to microsecond time scale.[3,21] Instead, the rate-determining step is the annealing of an initial relaxed assembled complex to form the native octameric structure.

The Arrhenius process seen in the rate-determining step of Acid-1Y folding reports on the nature of the rate-determining kinetic barrier. The lack of a large enthalpy in the folding direction is consistent with the fact that secondary structure is largely formed in the unresolved fast step. Furthermore, the larger enthalpy in the unfolding direction suggests that the tight packing of this octamer is broken in the r.d.s. In conclusion, the tight packing of the core, which includes the precise docking of the individual helices and immobilizing sidechain rotamers, likely contributes to its very slow kinetic behavior. Furthermore, the extra degree of freedom in the backbone of β-peptide foldamers could also contribute to slower kinetics as this added flexibility could increase the entropic penalty for folding.

In summary, our results suggest that while the thermodynamic properties of Acid-1Y closely resemble those of natural α-helical bundle proteins, the kinetics and folding energy landscape of this quaternary β-peptide 14-helix may be distinctly different. Future implications for kinetic foldamer studies are of broad interests as they provide new information into the folding kinetics of foldamers beyond the secondary structure level. As we elucidate the folding behaviors of foldamers we gain further insight into the understanding of protein misfolding diseases, protein-foldamer interactions, and structure prediction and open up new avenues for the discovery, design, and development of novel materials and peptidomimetics.

METHODS

Acid-1Y Preparation

Acid-1Y was synthesized on a 25 μM scale using Fmoc ((9H-fluoren-9-yl)methoxycarbonyl) solid phase peptide chemistry. 4-(Bromomethyl) phenoxymethyl (or bromo-Wang) polystyrene resin (Novabiochem; 100–200 mesh; 0.76 mmol/g substitution) was used for support of β3-amino acids. All β3-amino acids were purchased from PepTech Corporation, with the exception of Fmoc-(S)-β3-Orn(Boc)-OH, which was prepared using general methods of homologation[22] from the Fmoc-(S)-Orn(Boc)-OH α-amino acid. At the start of the synthesis the bromo-Wang resin was swelled in 100% dimethylformamide (DMF, Fisher Scientific) for about one hour and thoroughly rinsed (about 6 times for 30 seconds each), this was followed by the loading of the first amino acid, Fmoc-L-β-homoaspartic acid (OtBu). Resin loading was achieved by treating the resin in DMF with 3 equivalents of both the β-amino acid and diisopropylethylamine (DIEA, CHEM-IMPEX International), and 0.3 equivalents of Cesium Iodide (CsI, Sigma Aldrich). After completion the resin was rinsed well with DMF, DCM, and DMF again and the loaded resin's terminal Fmoc protecting group was deprotected using 5% piperazine (Sigma-Aldrich). Each deprotection step was carried out in a CEM MARS5 microwave assisted reaction synthesizer at 70 °C, 400 W, 50% power, 1 min ramp, 6 minutes hold, with mixing. The solution was drained and this deprotection procedure was repeated twice more. At the final deprotection step the resin was rinsed 6 times for 30 seconds each with DMF. The next β3-

amino acid, in the reversed sequence, was then coupled to the resin using 3 equivalents of amino acid, 2.9 equivalents of 2-(6-chloro-1H-benzotriazole-1-yl)-1,1,3,3-tetramethylaminium hexafluorophosphate (HCTU, Novabiochem) activator, and 6 equivalents of DIEA for 10 minutes in the microwave at 70 °C (400 W, 50% power, 1 min ramp, with mixing). The resin was washed 3 times each (approximately 30 seconds for each rinse) with DMF, dichloromethane (DCM, Fisher Scientific), and DMF again. This step was followed by deprotection of the Fmoc group, as described above with the addition of 2% 1,8-Diazabicyclo[5.4.0]undec-7-ene (DBU, Aldrich) to the 5% piperazine in DMF. The subsequent coupling and deprotection steps were repeated as described for the remaining residues in the peptide sequence.

Following deprotection of the final residue the β-peptide was cleaved from the resin using a cocktail of 2:2:2:94 H_2O:TIS (triisopropyl silane):anisole:TFA (trifluoroacetic acid) (all obtained from Sigma-Aldrich) for 2 hours at room temperature. The peptide was collected separately from the resin and was precipitated using a cold ethyl ether/hexanes mixture (50/50, v/v). The precipitate was dried on the lypohilizer and was then purified using reversed-phase high performance liquid chromatography (HPLC, Waters 2996) on a Vydac peptide C18 prep column. The mass of the β-peptide product was verified by matrix-assisted laser desorption/ionization mass spectrometry (MALDI-TOF-MS, Bruker Microflex LRF). All peptide samples were prepared by directly dissolving purified lyophilized peptide solids into respective buffers. β-Peptide concentrations were determined optically using tyrosine (Tyr) UV-vis absorbance. Circular dichroism (CD) experiments (excluding CD kinetics) were completed in phosphate buffer (0.01 M NaH_2PO_4, 0.20 mM NaCl, pH 7.0). All kinetic experiments were performed in phosphate buffer with no added salt (0.01 M NaH_2PO_4, pH 7.0).

CD Measurements

Wavelength-dependent and temperature-dependent CD spectra, both at various concentrations, were collected on an AVIV CD Spectrometer (Model 410) at 25 °C using a 1 mm sample cuvette. The following settings were used for wavelength dependent scans: continuous mode, 1 nm bandwidth, 1 nm wavelength step, and 4 sec averaging time. The concentration dependence of the molar residue ellipticity (MRE) at 208 nm was determined by a least-squares fitting of the total peptide monomer concentration, $[peptide]_{total}$, as a function of the experimentally determined MRE (MRE_{obs}) using the following equation:[1,11,12]

$$[peptide]_{total} = \left\{ \frac{(MRE_{obs} - MRE_{mon})(1/K_a)}{n(MRE_{nmer} - MRE_{mon})\left[1 - \left(\frac{MRE_{obs} - MRE_{mon}}{MRE_{nmer} - MRE_{mon}}\right)\right]^n} \right\}^{1/(n-1)}$$

where MRE_{mon} represents the MRE of the β-peptide monomer, MRE_{nmer} = represents the MRE of the β-peptide oligomer, and n is the oligomerization state, equal to 8 for octamer formation.

Thermal unfolding (T-melt) experiments, where MRE at 208 nm was monitored from 1 – 97 °C, were acquired using the Peltier temperature control module provided with the Aviv CD instrument under the following settings: 1 nm bandwidth, 2 °C temperature step, 1.5 min equilibration time, 4 sec averaging.

CD relaxation kinetic data were collected using a 2 mm cuvette where MRE at 208 nm was monitored as a function of time. CD settings were as follows: 22 °C, 6 sec averaging, 3 nm

bandwidth, and 3 nm slitwidth. In manual T-jump experiments, a moderately fast cooling step was achieved by heating the sample to 90 °C and quickly injecting the hot solution from a syringe (also equilibrated to 90 °C) through a cooled 20 μm ID stainless steel HPLC tube into the CD cuvette for measurement. The HPLC tubing (20 cm long) was placed in a jacket and cooled to 12 °C by circulating water from a refrigerated water bath. Data were analyzed by least square fitting to single- or double-exponential functions.

Fluorescence Kinetic Experimentation

The relaxation kinetics of Acid-1Y was measured by a time-resolved fluorescence technique where the relaxation process was initiated by a manual T-jump. This was achieved using the same manual T-jump procedure used for CD kinetics, except that in these series of experiments the final temperatures varied. The cooled solutions were injected into a 4×4 mm cuvette equilibrated at the desired temperature a PTI fluorescence spectrometer (Birmingham, NJ). Excitation and emission wavelengths were set to 303 ± 1 nm and 277 ± 1 nm respectively; the excitation bandwidth ranged between 1 and 2 nm, and was 4 nm for the emission. Temperature control of the observation cuvette was maintained by a circulating water bath. The observed kinetic spectra, for these association experiments, were fit to single- or double-exponential decays.

To study the dissociation of Acid-1Y the octamer was quickly destabilized by rapid manual dilutions of the peptide sample from 60 - 3, 6, 12, or 20 μM final [Acid-1Y]; we then monitored the unfolding of this β-peptide by measuring the time-resolved Tyr-fluorescence. The remaining procedure was the same as described above and the observed kinetic spectra were fit to double-exponential functions.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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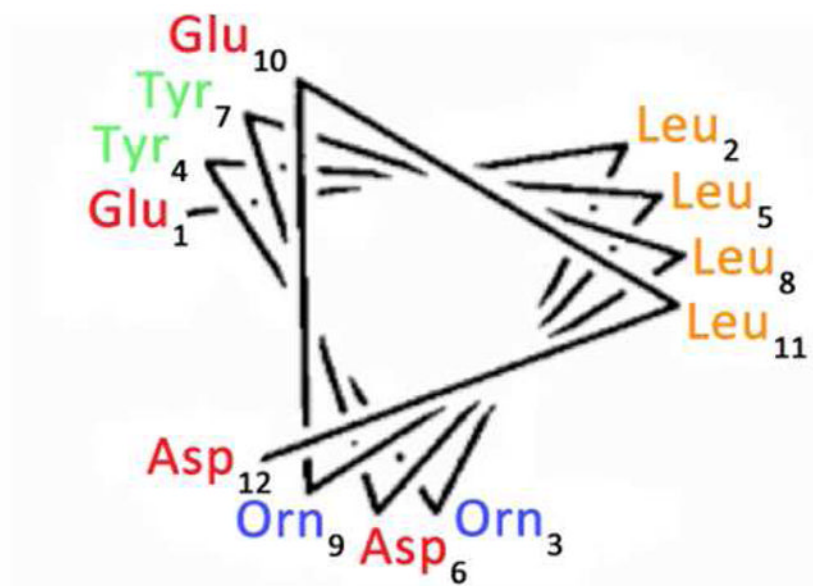
Figure 1.
Sequence of Acid-1Y as a 3-helical wheel. All residues are β^3-amino acids and the colors blue, red, green, and orange indicate cationic, anionic, aromatic, and hydrophobic amino acids, respectively. The sequence is β^3Glu-β^3Leu-β^3Orn-β^3Tyr-β^3Leu-β^3Asp-β^3Tyr-β^3Leu-β^3Orn-β^3Glu-β^3Leu-β^3Asp, and the terminal amine and carboxylate are unblocked.
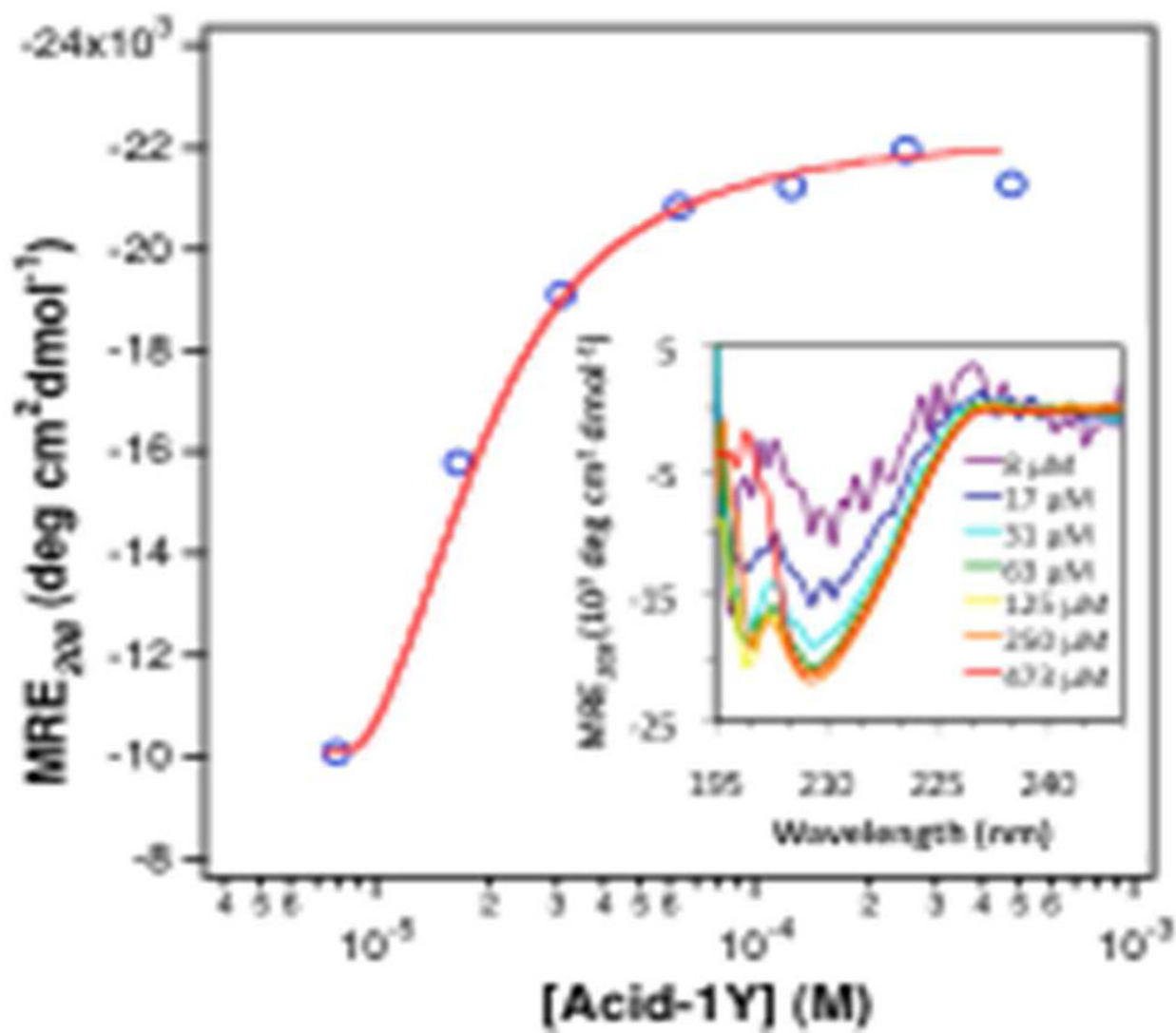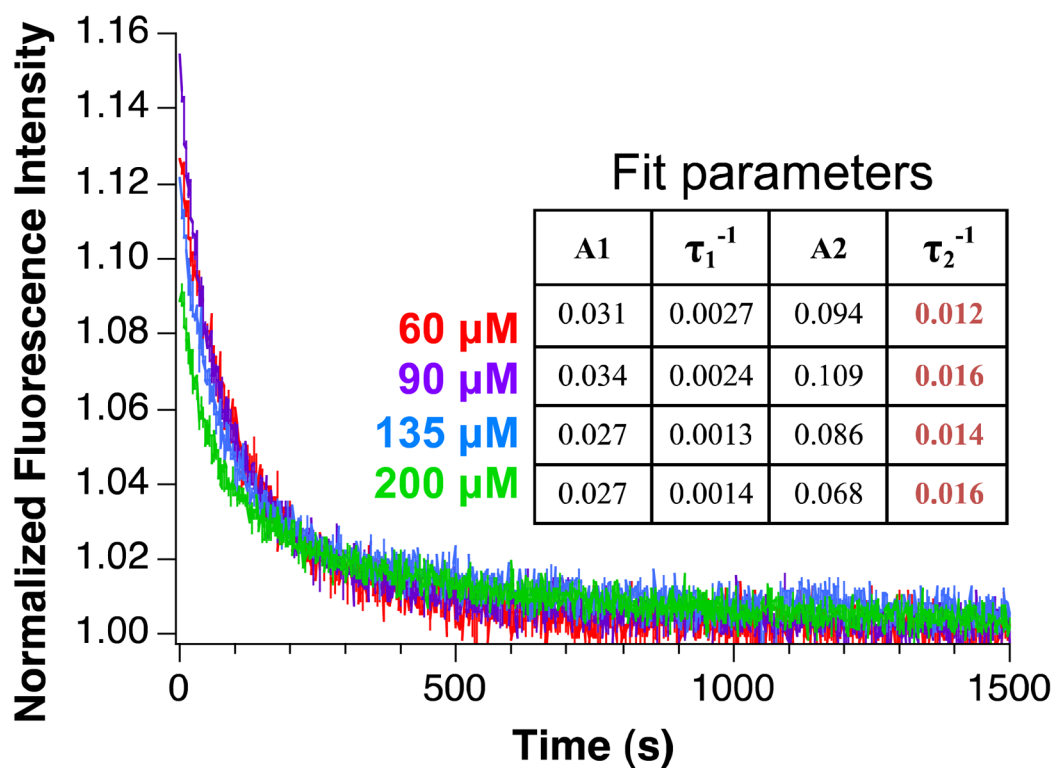
Figure 2.
MRE_{208} (deg cm^2 $dmol^{-1}$) as a function of Acid-1Y Molar concentration (plotted on a logarithmic scale) demonstrates concentration-dependent self-association. These data were analyzed using the equation, described in the experimental section, in the form of a monomer-octamer equilibrium; $MRE_{mon} = -10100$, $MRE_{nmer} = -22200$, $n = 7.9$, and $\ln K_a = 82.9$.
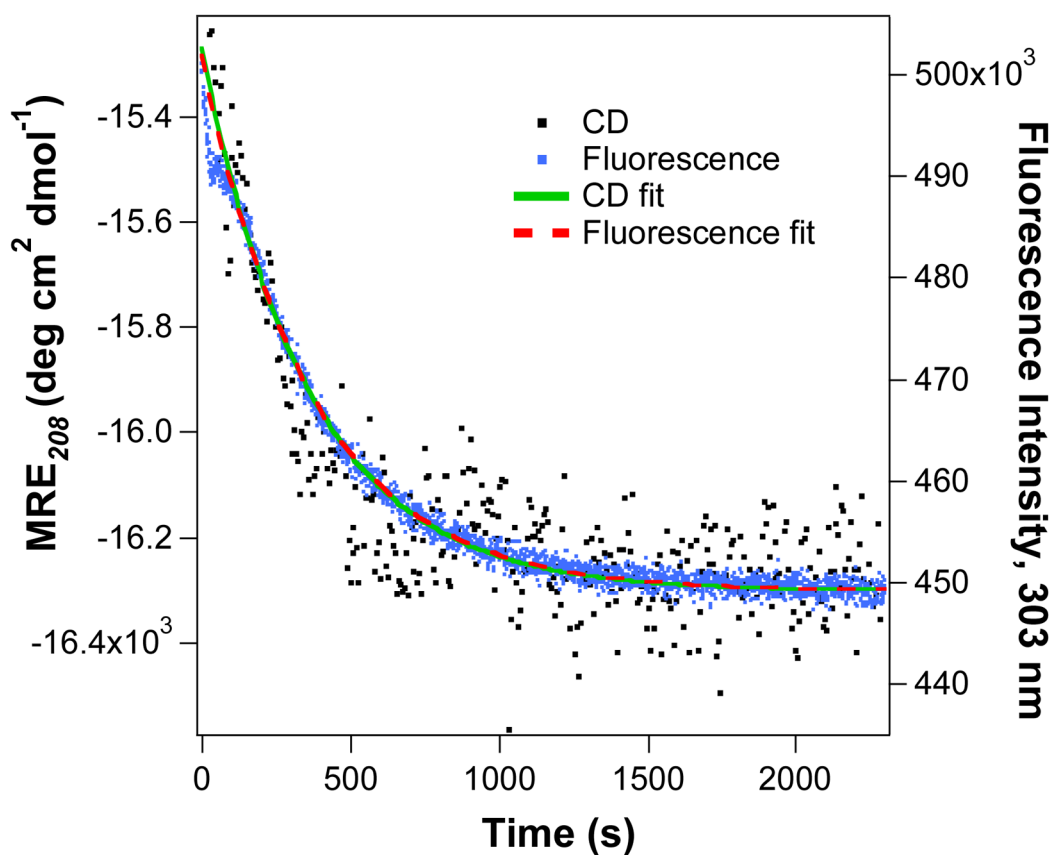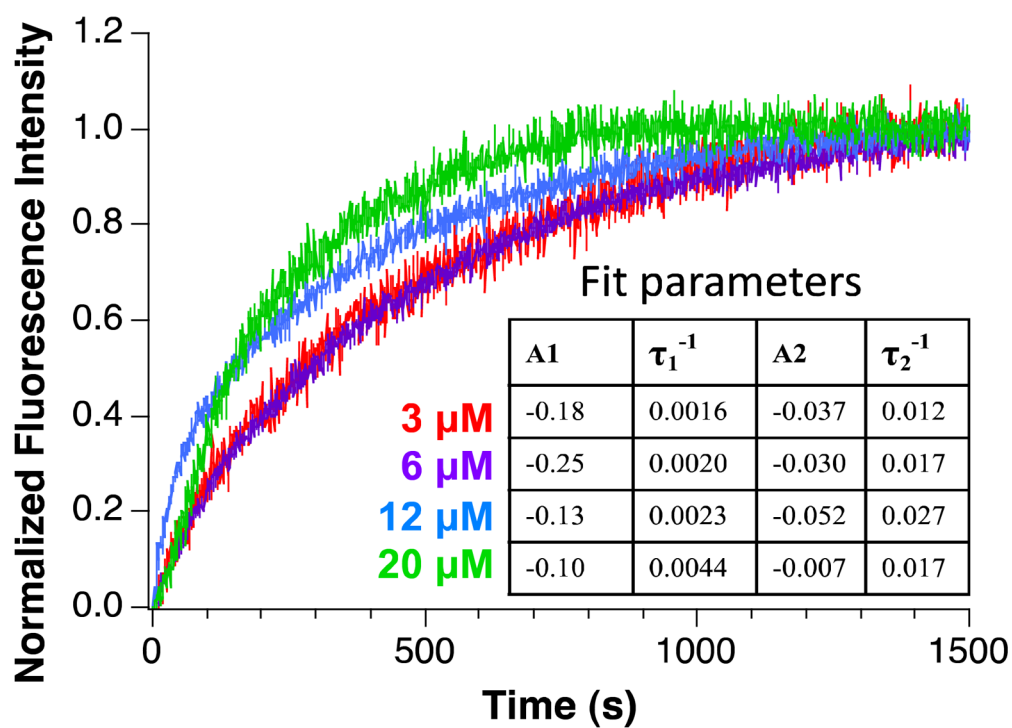
Figure 3.
Association of 60 µM Acid-1Y induced by a manual T-jump from 90-12 °C monitored by CD (black dots) and Tyr fluorescence (blue dots). Each kinetic trace was fit to a single-exponential decay, and the rate constants (k) were both determined to equal 0.00275 s^{-1} (relaxation time, τ, is 364 seconds or 6 minutes). The fits for the CD and Tyr fluorescence are represented by the green solid and red dotted lines, respectively.
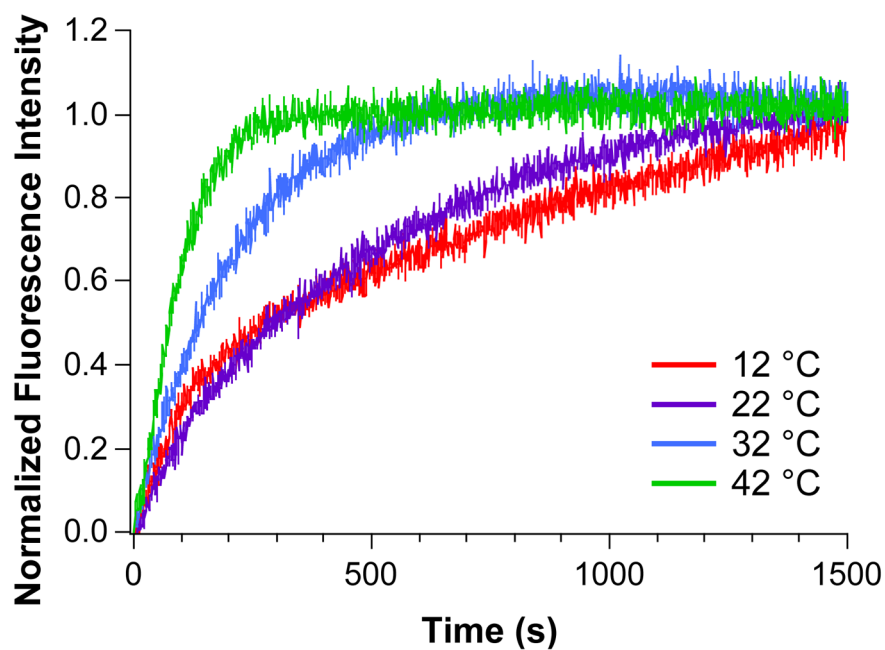
Figure 4.
T-jump-Tyr fluorescence association from 90 - 22 °C of 60, 90, 135, and 200 μM Acid-1Y. Data were fit to double exponentials, however; this process is primarily single-exponential, with about ~75% of the amplitude (A2) being contributed by the fast phase (τ_2^{-1}). The other amplitude (A1) process is about 5 to 10-fold slower (τ_1^{-1}) and only contributes ~25% of the amplitude. Calculated amplitudes and rate constants (in sec^{-1}) are shown in the table inset.

Figure 5.
Concentration-jump-Tyr-fluorescence dissociation data for 60 µM Acid-1Y diluted to 3, 6, 12, and 20 µM final concentrations at 22 °C. These data were fit to a double-exponential equation. The calculated rate constants as a function of [Acid-1Y], µM are tabulated in the inset.
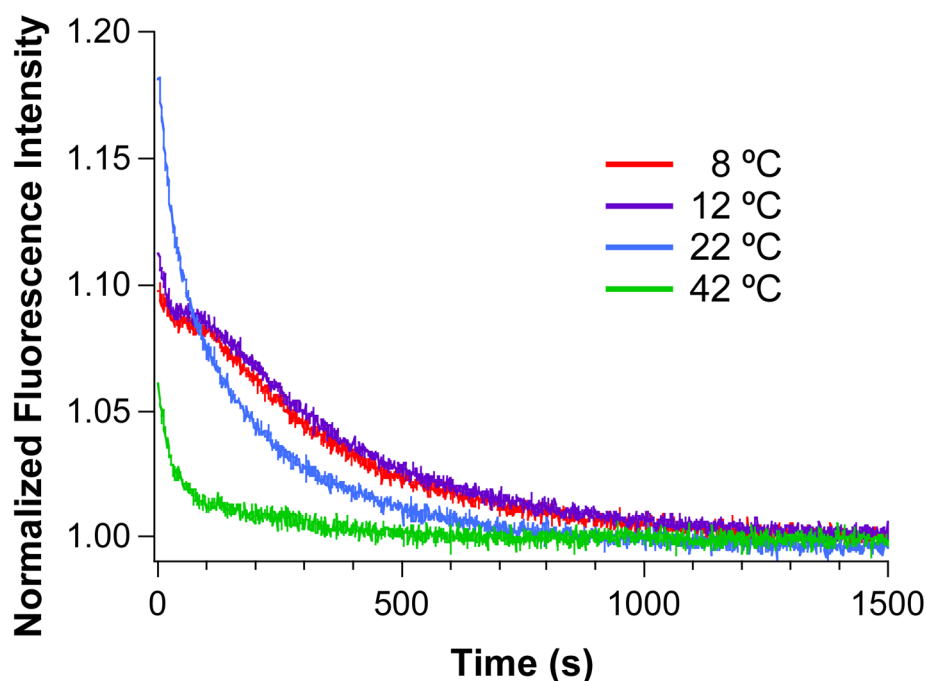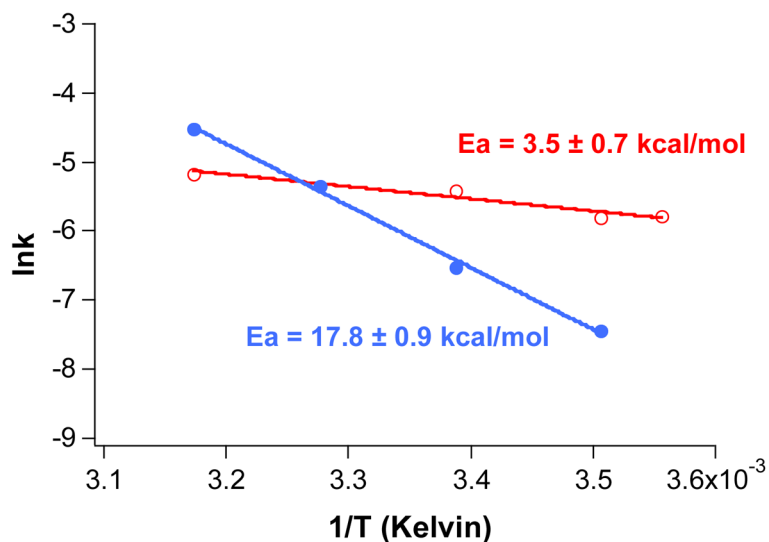
Figure 6.
T-jump-Tyr-fluorescence association of 60 μM Acid-1Y at various final temperatures; 90 - 8, 12, 22, and 42 °C, respectively. These data were best fit to a single-exponential function (excluding the points within the first minute which were uncertain due to incomplete equilibration of temperature; these points were therefore excluded from the fits). These data demonstrate slight temperature-dependent Arrhenius behavior with an enthalpy of activation, E_a, of 3.5 ± 0.7 kcal/mol (Figure 8).

Figure 7.
Concentration-jump-Tyr-fluorescence dissociation of 60 - 6 µM Acid-1Y dilutions at various temperatures, these data were collected at 12, 22, 32, and 42 °C. Peptide samples were manually and rapidly diluted to the respective desired final concentrations and fluorescence kinetics was measured at the noted temperatures. Data were fit to exponential growths. The determined rates demonstrate Arrhenius temperature-dependent behavior with a calculated activation energy (E_a) equal to 17.8 ± 0.9 kcal/mol (Figure 8).

Figure 8.
Temperature-dependent Arrhenius behaviors of T-jump-Tyr-fluorescence association (red) and dissociation (blue). Association rates demonstrate slight Arrhenius behavior with an enthalpy of activation equal to 3.5 ± 0.7 kcal/mol and with an intercept of 0.065. Temperature-dependent Tyr-fluorescence dissociation data (blue) demonstrates a much steeper Arrhenius temperature-dependence with $E_a = 17.8 \pm 0.9$ kcal/mol (intercept = 24). Markers represent observed data and solid lines represent the best fits for the data.