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Revealing Fast Structural Dynamics in pH-Responsive Peptides with Time-Resolved X-ray Scattering

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Abstract

Many biomaterials can adapt to changes in the local biological environment (such as pH, temperature, or ionic composition) in order to regulate function or deliver a payload. Such adaptation to environmental perturbation is typically a hierarchical process that begins with a response at a local structural level and then propagates to supramolecular and macromolecular scales. Understanding fast structural dynamics that occur upon perturbation is important for rational design of functional biomaterials. However, few nanosecond time-resolved methods can probe both intra- and intermolecular scales simultaneously with a high structural resolution. Here, we utilize time-resolved X-ray scattering to probe nanosecond to microsecond structural dynamics of poly-L-glutamic acid undergoing protonation via a pH jump initiated by photoexcitation of a photoacid. Our results provide insights into the protonation-induced hierarchical changes in packing of peptide chains, formation of a helical structure, and the associated collapse of the peptide chain.

Graphical Abstract

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ASSOCIATED CONTENT

Supporting Information

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Detailed experimental methods, SAXS characterization and titration curves, solvent subtraction and global analysis routines, pH-jump magnitude estimation, kinetic dependence analysis on magnitude of pH change and peptide lengths, reconstruction of structural contributions to TRXSS signals, and comparison of intermolecular interaction peak location in TRXSS and static SAXS data. (PDF)

Notes

The authors declare no competing financial interest.

INTRODUCTION

Natural and synthetic biomaterials are often required to adapt to environmental changes in order to perform specific functions.¹ For example, biomaterials for drug release are often rationally designed to release a payload only in response to specific environmental conditions, such as pH, temperature, or salt composition, and this action can be triggered and completed on timescales of seconds.^{2,3} Biomaterial responses to environmental stimuli often originate from a hierarchical structural transformation that begins with chemical alterations at the molecular level (e.g., by protonation of functional groups in peptides or ion-induced folding of nucleic acids as utilized in DNA origami materials) and then propagates to the macro-molecular scale by affecting intermolecular interactions.^{2,4} A detailed understanding of dynamic structural responses to stimuli is important for the rational design of stimuli-responsive and adaptive biomaterials, in particular for biomedical and nanodevice applications.^{2–6} However, capturing structural dynamics of biomolecules on multiple length scales and time scales remains a challenge for commonly used optical time-resolved techniques, which typically rely on molecular vibrational and electronic transition signatures in UV to IR regions and provide only indirect information about the hierarchical structure. Here, we utilize time-resolved X-ray scattering to gain insights into structural dynamics of biomaterials in response to environmental changes on multiple length scales (nanometers to 100 nm) and timescales (10^{-9} to 10^{-3} s) simultaneously.

Poly-L-glutamic acid (PGA) is among the most common components of engineered adaptive biomaterials.⁴ At low pH, PGA adopts an alpha helix configuration as the glutamic acid (Glu) residues that comprise the peptide are protonated.^{7,8} However, when the Glu residues become deprotonated, the helical structure becomes destabilized due to the electrostatic repulsions between negatively charged carboxylate groups of Glu in PGA. Consequently, the peptide is constrained to adopt an unfolded extended coil conformation. The ability to adapt the conformation in response to a pH change has been utilized in several applications, including pH-responsive gates for water membranes, drug delivery micelles, and therapeutic hydro-gels.^{4,9–12} Questions remain, however, with regard to the dynamics of helix formation, specifically, whether there is structural evidence for nucleation precursors prior to helix formation and what is the role of electrostatic interactions in the helix formation processes. Moreover, questions remain regarding the structural reorganization between macromolecules following protonation, and whether the intramolecular interactions affect these intermolecular dynamics in hierarchical structures. Finally, it has been posited that there is a fundamental speed limit for structural intra- and intermolecular reorganization

because of energy barriers and solvent interactions, although these limits are not well characterized.^{3,7} Answers to these questions are not only beneficial for understanding the physics of helix formation but would also be important for rational design of stimuli-responsive biomaterials.^{5,7,13–15}

The formation of the helix structure in PGA was previously studied by optical spectroscopic methods, such as UV-circular dichroism (CD)^{7,16–18} and Förster resonance energy transfer,¹⁹ and its dynamics were studied by photoinduced temperature-jumps and pH-jumps in conjunction with IR spectroscopy.^{7,8,17} Winkler et al. have identified that a 20-mer PGA peptide adopts an extended conformation at pH 6 with an end-to-end distance of ~ 31 Å, whereas at pH 4, the peptide adopts a significantly more compact conformation with the end-to-end distance of only ~ 24 Å.¹⁹ Volk et al. conducted helix unfolding experiments by photoinducing temperature jumps (T-jumps) and probing the resulting structure with IR spectroscopy.⁸ These T-jump experiments indicated that the dynamics are nonexponential and include a multistep unfolding process. In addition, Hamm and Donten followed the dynamics of helix folding by carrying out photoinduced pH-jump experiments with IR probes in a series of PGA peptides and found that the helix formation kinetics depended on peptide length, providing further evidence for nucleation–propagation in helix formation.⁷ They also reported that the helical content of completely protonated PGA peptides is ~ 30 – 50% , so the chain adopts a structure consisting of alpha helices connected by random coil segments, rather than a single helical structure.

In this work, we utilize photoinduced pH-jumps coupled to time-resolved X-ray solution scattering (TRXSS) to directly probe the dynamics of secondary structure formation in a series of PGA peptides of different lengths 20, 50, 100, and 200 residues, which were chosen to enable a comparison with previous IR studies.⁷ TRXSS provides a direct structural probe for protein tertiary and secondary structure and therefore can provide complimentary information to that derived from IR studies, especially in the tracking of protein dynamics.^{20,21} Moreover, X-ray solution scattering is a product of both intermolecular interactions between peptides (the structure factor) and the internal peptide structure (the form factor) and hence can be used to concurrently monitor both inter- and intramolecular organization in solution. In addition, TRXSS experiments are typically less intrusive to the protein sample as they do not require deuteration of the protein that may impact thermodynamics of the system.²² We have previously shown that TRXSS provides a structural probe that, in conjunction with photoinduced pH-jumps, provides insights into dynamics of nonphotoactive systems on timescales of μs to ms .²³ Here, we show that TRXSS coupled with pH-jumps is a viable method for observing dynamics of intra- and intermolecular dynamics on nanosecond timescales.

METHODS

Sample Preparation.

Peptide samples were obtained either from Sigma-Aldrich for the longest peptide (P4761, average ~ 200 repeating units) or Alamanda Polymers for shorter peptides PLE20, PLE50, and PLE100 (20, 50, and 100 units, respectively). The peptides were dissolved to a concentration of 2 mg/mL in an aqueous solution. For time-resolved experiments, the

aqueous solution also contained 8 mM *o*-nitrobenzaldehyde (*o*-NBA) as the photoacid generator. No other salts were added in order to avoid buffering or charge-screening effects, except for HCl which was used to correct pH to the desired level as measured by a calibrated pH electrode. Solutions were prepared and syringe filtered immediately prior to performing the scattering experiments. All time-resolved experiments were performed on solutions with initial pH = 6.0.

TRXSS pH Jump Experiments.

The TRXSS experiments were carried out at BioCARS 14-ID-B beamline at the Advanced Photon Source (APS). Details of the X-ray scattering setup and generic TRXSS pump-probe data acquisition methodology at BioCARS have been previously published.^{20,21,23,24} Full experimental details are available in the Supporting Information.

RESULTS AND DISCUSSION

Static solution scattering experiments were carried out at the DND-CAT beamline 5-ID-D at the APS at Argonne National Laboratory. Comparison of scattering results for different pH values are shown in Figure 1a. At pH 4.5, the Glu residues in PGA are protonated and, therefore, the peptide adopts a folded and helical conformation. Hence, the small-angle X-ray scattering (SAXS) curve follows the expected behavior for dilute monodispersed peptides, that is, scattering intensity increases from large to small transferred momentum value, q , culminating in a plateau region toward $q \approx 0$, without peaks in the low q (small angle) region ($q < 0.1 \text{ \AA}^{-1}$). However, as pH values approach neutrality (i.e., pH ≈ 7), the carboxylate group ($-\text{COOH}$) in each Glu deprotonates to $-\text{COO}^-$ carrying a negative charge, leading to repulsion between side chains that affects the intra- and intermolecular peptide conformations. The intramolecular repulsion results in a loss of the helical structure, which is pronounced in a form factor change that leads to decreased scattering intensity at the wide angles region ($0.1 < q < 1 \text{ \AA}^{-1}$) as the pH increases (Figure 1a). Meanwhile, the negative charges at the side chains of PGA also result in the repulsion between neighboring peptides so that they maintain characteristic distances from each other. The resulting intermolecular packing results in a structure factor that appears as a peak in the small angle region with peak position, q_{max} , between 0.02 and $0.04 \text{ \AA}^{-1}$, depending on the length of the peptide at a constant concentration (2 mg/mL), as shown in Figure 1b. The change in q_{max} appears to be in accordance with previous observation for polyelectrolyte solutions in the dilute regime. In this regime, q_{max} is expected to decrease with increase in molecular weight at equal weight concentration because of the formation of a locally ordered structure that is close packed.^{25–29} The characteristic distances between chains can simply be calculated by the relation, $d = 2\pi/q_{\text{max}}$, where d is the characteristic distance between the chains.²⁹ We found that characteristic distances correspond to 14, 15, 18, and 20 nm for the peptide lengths of 20, 50, 100, and 200 residues, respectively.³⁰ It should be noted that for the long peptides (200 residues), the behavior of the peptide chain may approach the semidilute regime, in which chains begin to intercalate each other and may affect the trend in interchain distances; however, q_{max} would still be indicative of the distance between the chains. Distance trends may also be affected by the fact that at the same pH, different length chains may have different degrees of protonation that would lead to different effective charge distribution.

The static scattering curves also provide insights into the degree of peptide chain protonation and helical content by observing the trends of the scattering signals in the SAXS and wide-angle X-ray scattering (WAXS) regions, respectively, as a function of pH. The integration of the WAXS region, which is free of the intermolecular structural peak and, therefore, representative of pure helix formation and protein collapse, demonstrates a clear sigmoidal behavior as a function of pH in the range of $4 < \text{pH} < 7$ with an inflection point at $\text{pH} \approx 5.5$, with little helical formation above pH 6. In contrast, integration of the SAXS region contains information from the interpeptide structural peak that is lost as pH decreases, indicating that protonation of the chains is occurring throughout the entire tested pH range, even at $\text{pH} > 6$ (see titration curves in Figures S1 and S2 in the Supporting Information). Such behavior suggests that although the degree of protonation changes continuously with pH, the formation of helices ensues only once a significant portion of the chain is already protonated, in accordance with previously reported 40% protonation threshold observed from UV-CD spectroscopy.⁷

Time-resolved experiments were carried out by utilizing photoinduced pH jumps to trigger peptide folding, and the ensuing structural dynamics were probed with TRXSS at Beamline 14ID (BioCARS) of the APS by previously published procedures.²⁰ In brief, samples containing PGA peptides and α -NBA photoacid at pH 6 were infused into a temperature-controlled capillary flow cell kept at 25 °C. The pH jump was achieved by protons released from α -NBA, which is a photoacid, by using 300 nm light excitation with 7 nm laser pulses (full width at half-maximum). In order to maximize the scattering signal changes due to the α -helix folding, the initial pH was chosen based on the titration curves discussed above. The magnitude of the pH jump was found to be ~ 0.5 pH units, a pH change that is limited by the solubility of the photoacid and laser power (see estimation of pH section in the Supporting Information). The PGA solution was then probed by X-ray pulses at specific time delays following the laser excitation to produce scattering patterns on a Rayonix MX340-HS detector placed behind the sample. Difference scattering curves were produced by azimuthal integration and subtraction of the scattering patterns obtained at positive time delays from the scattering patterns obtained at a negative time delay (5 μs prior to laser interaction with the sample). The sample was refreshed so that each laser/X-ray pair of pulses had the same initial fresh sample conditions (i.e., same initial pH, photoacid concentration, and peptide protonation state see methods in the Supporting Information). The solvent contribution, resulting from a ~ 1 K temperature rise due to laser absorption in the sample, was removed from the TRXSS difference curves by using the standard procedure of fitting the pure buffer TRXSS signals at the wide angle portion of the signals (see Supporting Information for details).^{20,23,31} Such a small T-jump associated with the photoacid excitation is not expected to affect the peptide structural dynamics.⁷ Finally, the APS synchrotron was operating in a 324-bunch mode during this experiment, from which pulse trains were extracted using a mechanical chopper leading to an effective time resolution of ~ 250 ns (see Supporting Information).

The protein-associated TRXSS difference signals for the PGA with 200 residues are shown in Figure 2a. At 250 ns, the earliest measured time point, the signal already displays a negative feature in the SAXS region ($0.02 < q < 0.07 \text{ \AA}^{-1}$). At later delay times, a positive feature gradually appears in the scattering difference signals in the WAXS region ($0.07 < q <$

0.5 Å⁻¹), which causes the negative peak in the SAXS region to rise, while the relative amplitude of the negative dip remains largely unchanged. As both SAXS and WAXS regions appear to increase uniformly in intensity with time delay, the signal from the regions was integrated together to track the progression of the reaction as shown in Figure 2b. The integration indicates an interconversion between an intermediate and a final state, with the former appearing on the shortest timescale <250 ns, converting into the final state on μ s timescale and plateauing at >10 μ s.

To further investigate the structural dynamics of the system, we performed global analysis based on singular value decomposition (see Supporting Information for details), which clearly demonstrated that the entire TRXSS time series can be described by only two components implying that only two species are observed in the experiment. The data was globally fitted with a simple sequential kinetic model described as follows

where U represents unfolded ground state, I_p is the intermediate observed at the earliest time point, F_p is the folded state observed at later time delays, and τ is the time scale of interconversion between I_p and F_p . Population dynamics and species-associated differences obtained from the global analysis are shown in Figure 2c,d.

The main feature apparent in the TRXSS signal from the intermediate protonated species (I_p) is a sharp negative peak indicative of the loss scattering intensity in the SAXS region. The location and shape of this feature are correlated with those of the peak associated with interchain packing that was observed in the static SAXS experiments around 0.02–0.04 Å⁻¹ at neutral pH as shown in Figure 1a,b (see comparison between static SAXS and TRXSS signals in the Supporting Information). Therefore, we assign the loss of scattering intensity in this region to the loss of the correlated inter-PGA spacing. This result agrees with the expectation that protonation of the peptide chains will lead to neutralization of the negative charges and, therefore, result in a disappearance of the repulsive Coulombic interactions between chains, which results in diffusion of the peptides relative to each other and loss of correlated structure in the solution. The magnitude of the negative peak shows little evolution at longer timescales, implying that residue protonation and subsequent peptide chain relaxation are all completed by ~250 ns and no further protonation occurs beyond this timescale. This assignment is further strengthened by agreement with the timescales expected for photoacid generation from *o*-NBA (20 ns) and peptide chain motion (~100 ns) as reported in previous studies.^{7,32,33} Furthermore, the assignment is consistent with the expected timescale for the disappearance of the interparticle correlations predicted by the center of mass diffusion, based on previously reported diffusion coefficients.³⁴ The lack of significant WAXS signal for the I_p state implies that the protonation of PGA and loss of Coulombic interactions does not cause an immediate collapse of the chain into an alpha helix, but rather that the chains first adopt an intermediate disordered state that is unfolded prior to helix formation (i.e., the major contribution to the change in scattering intensity is associated with the change in the intermolecular structure factor).

The final protonated state (F_p) species that arises on the timescale of $1.8 \pm 0.1 \mu s$ appears as an increase in scattering intensity, which is apparent in both the SAXS and WAXS regions. The significant increase in intensity in the WAXS region ($q > 0.07 \text{ \AA}^{-1}$) indicates that this state comprises the majority of alpha helix formation and associated collapse of the peptide chain (see Figure 2e). We note that this state is, therefore, a combination of two structural contributions: (1) the loss of intermolecular organization and (2) helix formation and chain collapse to a compact state. These contributions can be decomposed into associated structural signals, which are shown in the Supporting Information, Figure S9. From the comparison of the two contributions, it is apparent that the I_p species contributes the majority change in the intermolecular structure factor, which is followed by evolution of internal molecular changes that mostly appear in the F_p species. Therefore, although both structure and form factors contribute to the scattering signals, the two evolve on different timescales allowing us to decouple their contributions.

Given that the present experiments achieve only partial protonation of the peptide chains, it is likely that the helices are not formed throughout the entire peptide chain, but rather localized inhomogeneously across the polypeptide. Such behavior has been observed previously even in completely uncharged alanine-based peptides,³⁵ (that have also been observed to possibly form polyproline-like helical structures³⁶) and is more likely in the case of PGA because of the repulsive interactions between the remaining unprotonated side chains. Once the signal saturates at $1.8 \mu s$, it remains constant until diffusion of the protons leads to disappearance of the signals at timescales longer than 10 ms. The scattering difference curve of F_p was compared to differences calculated from the static solution scattering data at different pH values in order to verify that it captures all conformational changes as a result of the pH change and to estimate the degree of the pH jump. The comparison of TRXSS and static differences is shown in Figure 2e. The good agreement between the time resolved data and the static differences from data at pH = 5.9 and pH = 5.4 suggest that the F_p state encompass the entire conformational change expected from the pH jump, including helix formation and loss of intermolecular repulsive interactions, and that the degree of pH jump is ~ 0.5 pH units. We note that the increase of pH jump magnitude to ~ 1.0 pH unit did not change the timescale of F_p formation, likely because of the small change in helical content between the two pH-jump magnitudes as evident from the titration curves (see Supporting Information).⁷

TRXSS experiments were also carried out on the shorter length peptides, including 20, 50, and 100 units, and similar kinetics, involving appearance of an intermediate state I_p on sub-100 ns time scale followed by conversion to a final F_p state, were observed in all cases (see the Supporting Information). However, the timescale of helix formation showed a degree of dependence on the chain length (see Figure 3) following a trend of the helix formation time constants of 750 ± 200 , 600 ± 200 , 1300 ± 200 , and 1800 ± 100 ns for peptide lengths of 20, 50, 100, and 200 residues (see the Supporting Information for details). Although for the longer peptide chains, the helix formation is significantly delayed from the protonation of Glu residues, in shorter peptides, the helix formation reaches a time constant limit of 600–700 ns at ~ 50 residues. This behavior has previously been observed by Hamm and Dönten in transient IR experiments and is also apparent in our current experiments.⁷ Different folding time for different peptide lengths was previously explained based on a

nucleation–propagation mechanism, where helical nucleation is considered as a local process, whereas formation of the helix proceeds via propagation across the peptide chain and therefore depends on the chain length.

CONCLUSIONS

In conclusion, our study shows that TRXSS coupled with pH jumps can track protein conformation dynamics following protonation on ultrafast timescales, including events such as peptide chain diffusion, intermolecular disordering, and secondary structure formation. As a complimentary technique to optical spectroscopies, TRXSS has the benefit of being sensitive to multiple length scales and, therefore, captures both intra- and intermolecular structural dynamics that would not otherwise be visible by vibrations or chromophores. This advantage allows us to directly observe propagation of events from the molecular level, such as protonation, to the macroscopic level, such as the loss of correlated peptide structure in the solution. Understanding the dynamics of such intermolecular interactions are particularly important in the rational design of stimuli-responsive hierarchical biomaterials, where dynamic conformational changes govern the mechanism of payload release. TRXSS also provides significant insights into intramolecular interactions, and in our current study, its results further support a nucleation–propagation mechanism for helix formation, as evidenced by the dependence of helix formation lifetime on the peptide chain length.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Static X-ray solution scattering results for PGA. (a) Log-log plot of X-ray solution scattering data for 200 unit PGA at different pH values. The inset shows the plots on a linear scale. (b) SAXS data for peptide lengths of 20, 50, 100, and 200 residues at pH 7. The inset presents the characteristic distances between chains because of intermolecular repulsion at 2 mg/mL concentration as a function of peptide length calculated from the SAXS peak location.

Figure 2.

TRXSS results from photoinduced pH jump experiment on 200 residue PGA. (a) Time series of the scattering difference signals at different time delays. (b) Integrated TRXSS signal as a function of time delay. (c) Species-associated population dynamics of the intermediate and final species following protonation. (d) Species-associated scattering differences derived from global analysis. (e) Comparison of final species-associated difference F_p signal with difference signal derived from static scattering curves at pH = 5.9 and pH = 5.4.

Figure 3. Comparison of integrated WAXS integrated TRXSS signal kinetics for different peptide lengths—200 units (black) and 20 units (green). (Inset) Comparison of extracted lifetimes from kinetic analysis of WAXS-integrated TRXSS signal for different length peptides.