Long Range Polymer Chain Dynamics of Structured and Unstructured Polypeptides Probed by PEF

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Introduction

In 1969 Leventhal outlined that in order for proteins to fold on a biological timescale, the polypeptide backbone cannot pursue a folding pathway in which every backbone conformation is sequentially searched. Instead of viewing a protein as a sequence of amino acids along a chain, a protein should be thought of as an ensemble of amino acid subsets, recently referred to as *Foldons*, where the residues fold cooperatively.[1] The notion of *Foldons* was supported in recent studies that showed that subsets of ca. 20 amino acids exist in partially folded proteins which exchange amide protons on similar timescales suggesting that they belong to a same *Foldon*.[2] As an illustrative example, a polypeptide chain containing 100 residues, where each residue can adopt one of three possible conformations, and each conformation takes 1 ps to probe, can have its folding time reduced from (3^{100}×1 ps =) 1.6×10^{28} years to a much more reasonable ((100/20)×3^{20}×1 ps =) 17 ms by considering the backbone as a series of *Foldons* rather than an uninterrupted sequence of amino acids. Although the *Foldon* size has been determined for partially folded polypeptides, it has yet to be characterized along the folding pathway, starting from a random polypeptide coil and ending with a fully structured polypeptide. Beside the *Foldon* size, another equally important parameter is the dynamics of folding within a *Foldon*, which define the rate at which each *Foldon* folds into a structured conformation.

To help answer these questions the Fluorescence Blob Model (FBM) was applied to analyze the fluorescence decays of a series of structured and unstructured pyrene-labeled poly(glutamic acid)s (Py-PGAs) in solution. Much in the same way as a protein is divided into *Foldons*, the framework of the FBM requires that a polypeptide backbone be divided into small sub volumes, referred to as *blobs*, where the volume of a blob is defined by the volume probed by an excited pyrene during its lifetime. Since the mobility of a pyrene moiety is limited to that of the amino acid it is covalently bound to, the *blob* volume is defined by the local volume an amino acid can probe and is reported as N_{blob}, the number of amino acid residues encompassed in the volume of a single *blob*. By analyzing the fluorescence decays of helical and coiled Py-PGAs, the N_{blob} value can be studied at specific times along the folding pathway of a polypeptide yielding valuable information on how the *blob* volume changes as folding occurs. In addition to N_{blob}, the FBM also yields k_{blob}, the rate constant for encounters between pyrenes, or the amino acid residues, within a given *blob*. For an unstructured PGA, k_{blob} represents a quantitative measure of the dynamics of the polypeptide backbone which defines the motion of amino acids as they diffuse together to form a higher order structure.

Experimental

Pyrene-labeled poly(glutamic acid)s (Py-PGAs): Poly(*L*-glutamic acid sodium salt) (P*L*GNa, M_n = 121300 g·mol^{-1}, *DP* = 803, *PDI* = 1.02) and poly(*D*,*L*-glutamic acid sodium salt) (P*DL*GNa, M_n = 118400 g·mol^{-1}, *DP* = 784, *PDI* = 1.06) were labeled with 1-pyrenemethyl amine yielding Py-P*L*GA and Py-P*DL*GA, respectively. In total, seven Py-P*L*GA polymers were prepared with 3 to 18 mol% (f_{Py}) of the glutamic acid residues labeled with pyrene, and five Py-P*DL*GA polymers with f_{Py} ranging from 6 to 12 mol%.

Steady-State Fluorescence (SSF): Fluorescence spectra were acquired with a Photon Technology International steady-state fluorometer equipped with a xenon arc lamp. The samples were prepared with pyrene concentrations of ca. 2.5 μM which were then purged with N_2 to remove oxygen and sealed in a quartz cell before being excited at 344 nm and scanning the emission from 350 to 600 nm. The I_E/I_M ratio was calculated by integrating the fluorescence signal from 372 to 378 nm for the monomer (I_M) and from 500 to 530 nm for the excimer (I_E).

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1. a) Levinthal, C. (**1968**) *J. Chim. Phys. 65*, 44-45. b) Levinthal, C. (**1969**) *Spectroscopy in Biological Systems. Proceedings University of Illinois Bulletin*, University of Illinois Press, Urbana, IL, pp 22-24.
2. Englander, S. W., and Mayne, L. (**2014**) *Proc. Natl. Acad. Sci. 111*, 15873-15880.

Time-Resolved Fluorescence (TRF): Samples were prepared in a similar manner as for the steady-state fluorescence measurements. The fluorescence decays of pyrene were acquired using an IBH TC-SPC spectrofluorometer in conjunction with a NanoLED-340 laser. The samples were excited at 344 nm and the fluorescence decays were monitored at 375 and 510 nm for the monomer and excimer decays, respectively. The monomer and excimer decays were globally fitted using the Fluorescence Blob Model (FBM).
Circular dichroism (CD): The molar ellipticity was determined with a Jasco J-715 spectropolarimeter, CD spectra were averaged over ten scans from 400 to 250 nm using a 0.10 mm pathlength quartz cell containing a Py-PGA solution in DMF or DMSO with a pyrene concentration of ca. 2.5×10^{-3} M. A solvent background correction was applied to each spectrum.

Results and Discussion

PGA Conformation in Solution: The conformation of Py-P*L*GA and Py-P*DL*GA were determined using CD measurements in both DMF and DMSO. The presence of secondary structure was determined by measuring the molar ellipticity [θ] using the ^1B_b band of pyrene bound to the PGA backbone, which has been shown to reliably describe the backbone conformation of Py-PGA.[3] The [θ] values obtained in DMF and DMSO are plotted as a function of pyrene content in Figure 1. As f_{Py} increased, [θ] was also expected to increase with increasing content of secondary structure of the backbone as seen for Py-P*L*GA in DMF and DMSO. Py-P*L*GA is expected to adopt an α–helical conformation in DMF,[4] which is supported by the relatively high [θ] values in Figure 1. Although DMSO was expected to disrupt the secondary structure of PGA,[5] the upward trend in [θ] with increasing f_{Py} indicates that some α–helical domains still exist for Py-P*L*GA in DMSO, and the chain must adopt a partially helical conformation. The stereo irregularity along the Py-P*DL*GA backbone disrupts the formation of secondary structure resulting in random coil conformations in both DMF and DMSO as seen by the near zero [θ] values in Figure 1. The helical Py-P*L*GA in DMF representing a folded polypeptide, the partially helical Py-P*L*GA in DMSO representing a partially folded polypeptide intermediate, and the randomly coiled Py-P*DL*GA in DMF and DMSO representing a polypeptide in the initial stages of folding were then studied using pyrene excimer fluorescence (PEF) to probe chain dynamics along the folding pathway of a polypeptide.

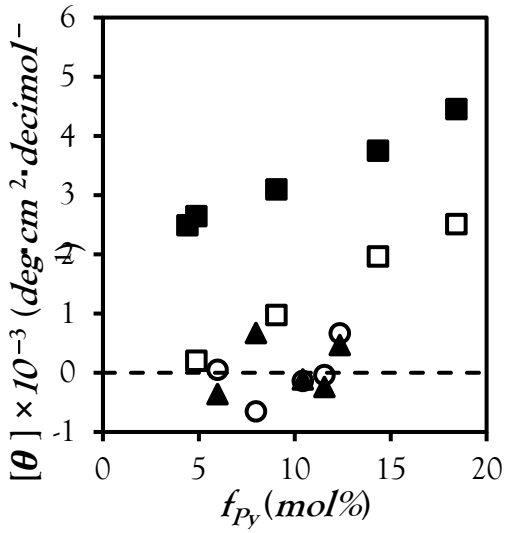

Figure 1. Average molar values of the ^1B_b band of pyrene for Py-P*L*GA in DMF (■) and DMSO (□) and Py-P*DL*GA in DMF (▲) and DMSO (○). The solid and dashed lines are added to guide the eyes for the molar ellipticity of Py-P*L*GA and Py-P*DL*GA, respectively.

Steady-State Fluorescence: The SSF spectra for Py-P*L*GA in DMF are presented in Figure 2A. They display the typical sharp peaks of the monomer (I_M) around 375 nm and an increasing excimer emission (I_E) around 475 nm for samples labeled with increasing amounts of pyrene. The amount of excimer formed was quantified with the I_E/I_M ratio, where a higher value indicates more excimer formation. Figure 2B shows that the I_E/I_M ratios for the helical Py-P*L*GA are larger than its coiled counterpart Py-P*DL*GA in DMF. The difference in excimer formation was attributed to the difference in coil density between the helical and coiled conformations. The compact helical backbone brings the pyrene pendants closer to each other resulting in more excimer formation compared to the coiled backbone. Surprisingly, the I_E/I_M ratios of Py-P*DL*GA in

[3]. Shoji, O., Okumura, M., Kuwata, H., Sumida. T., Kato, R., Annaka, M., Yoshikuni, M., and Nakahira, T. (**2001**) *Macromolecules 34*, 4270–4276.
[4]. Matsumoto, M., Watanabe, H., and Yoshioka, K. (**1970**) *Biopolymers 9*, 1307-1317.
[5]. Yamaoka, K., and Ueda, K. (**1982**) *J. Phys. Chem. 86*, 406-413.

DMSO overlapped with those in DMF as seen in Figure 2C. Since DMSO is about twice as viscous as DMF, the I_E/I_M ratio was expected to be lower in the more viscous DMSO since the dynamics of excimer formation should scale with the inverse of viscosity.[6] However, a previous study has shown that the efficiency of excimer formation for the pyrene-derivative used to label PGA is about twice as high in DMSO compared to DMF.[7] As a result, the change in solvent viscosity and efficiency of excimer formation cancel one another out, resulting in same I_E/I_M ratios in DMF and DMSO. Consequently, the decrease in the I_E/I_M ratios obtained for Py-P*L*GA in DMSO compared to those in DMF must be due to the loss of some of the P*L*GA helical structure in DMSO, further supporting the results obtained by CD measurements.

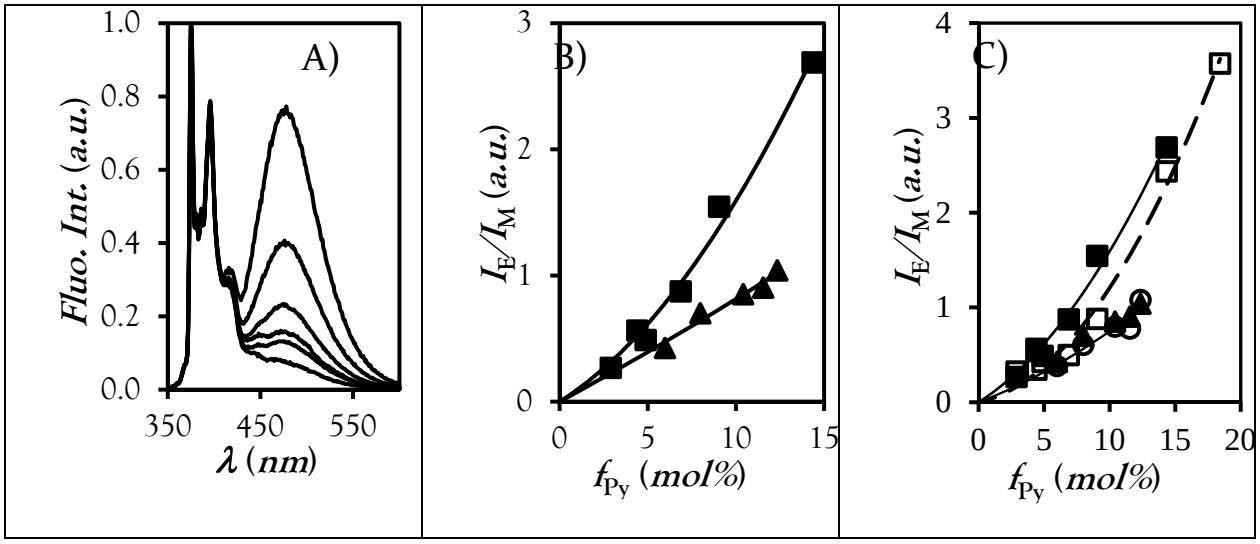

Figure 2. A) Steady-state fluorescence spectra of Py-P*L*GA in DMF, B) the I_E/I_M ratios of Py-P*L*GA (■) and Py-P*DL*GA (▲) in DMF, and C) the I_E/I_M ratios of Py-P*L*GA in DMF (■) and DMSO (□) and Py-P*DL*GA in DMF (▲) and DMSO (○). The lines for DMF (solid) and DMSO (dashed) were added to guide the eyes.

Size of a Folding Unit: The monomer and excimer decays were globally analysed with the FBM to obtain N_{blob}, the average number of glutamic acid residues contained within a *blob*. Since pyrene is bound directly to the glutamic acid, N_{blob} represents the number of glutamic acid residues which are able to encounter one another on the time scale of backbone motion. In other words, N_{blob} should represent the number of residues which cooperatively diffuse together during the folding of a polypeptide. As seen in Figures 3A and B, the N_{blob} values remained constant as a function of pyrene content indicating that N_{blob} reflects the properties of PGA and its value is not influenced by the addition of pyrene. N_{blob} was found to equal 21 ± 1 glutamic acid residues for Py-P*L*GA in DMF, 18 ± 1 residues for Py-P*L*GA in DMSO, and similar values of 10 ± 1 and 11 ± 2 residues for Py-P*DL*GA in DMF and DMSO, respectively. The larger N_{blob} value of 21 for the helical Py-P*L*GA in DMF compared to that of 10 for the coiled Py-P*DL*GA is consistent with the relatively high density of the helical backbone, which brings the glutamic acid residues close together in space. As expected, the partially helical Py-P*L*GA in DMSO exhibits an N_{blob} value that is between those obtained for the helical and random coil conformation. These results suggest that as a polypeptide folds, initially the blob size starts rather small at 10

6. Farhangi, S., and Duhamel, J. (**2016**) *Macromolecules 49*, 6149-6162.
7. Hall, T. (**2012**) Unpublished, MSc. Thesis, University of Waterloo, Waterloo, ON, Canada.

residues and progressively increases as folding occurs to reach a value of 18 units for a partially folded chain and continues to increase to a maximal *blob* size of 21 units for a fully folded chain. Interestingly, the N_{blob} values for the partially and fully helical PGA match closely the reported *Foldon* size of ca. 20 amino acid residues of proteins probed through hydrogen-exchange studies.[2] This good agreement supports the notion that a polypeptide *blob* may indeed be equivalent to a protein *Foldon*.

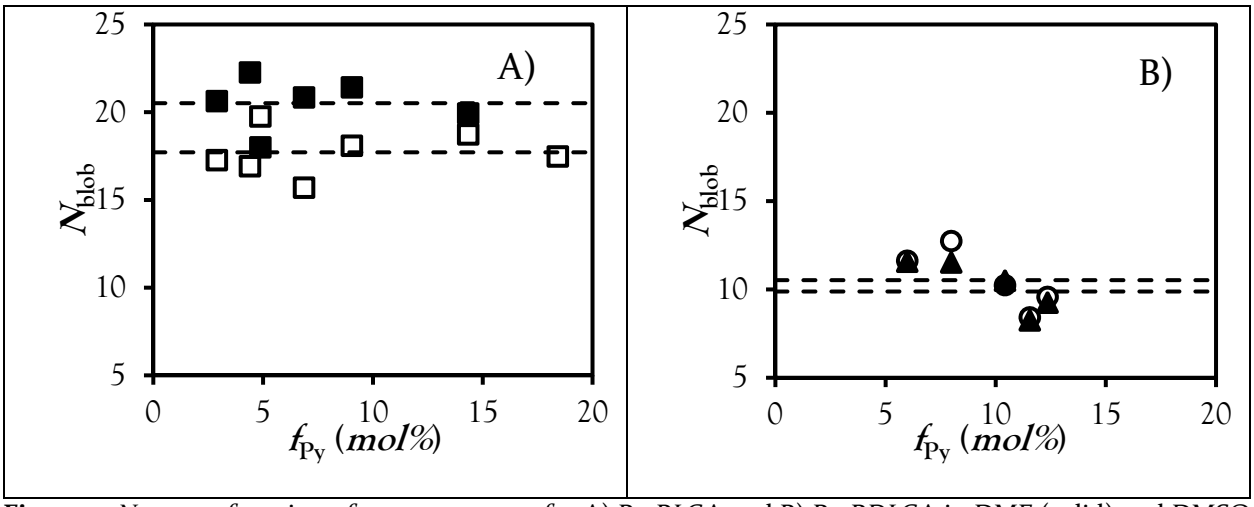

Figure 3: N_{blob} as a function of pyrene content for A) Py-P*L*GA and B) Py-P*DL*GA in DMF (solid) and DMSO (hollow). The dashed lines represent the averages.

Folding Dynamics: In addition to the *blob* size, the FBM analysis was also able to retrieve k_{blob}, the average rate constant of encounter between two pyrenes within a *blob*. Although k_{blob} took similar values regardless of the PGA conformation as seen in Figures 4A and B, k_{blob} represented two different molecular processes in the context of structured and unstructured PGA. For helical PGA, the backbone is locked in place on the timescale of fluorescence and k_{blob} reports on the sidechain dynamics of the glutamic acid residues. More interestingly for the coiled PGA, in order for two pyrenes to encounter within a *blob*, the PGA backbone must first undergo Brownian motion which implies that k_{blob} reflects the rate of encounter between the glutamic acid residues located inside a *blob*. In the case where k_{blob} represents backbone dynamics, $(k_{blob})^{-1}$ yields the timeframe of encounter between two glutamic acid residues located within a *blob*. For Py-P*DL*GA in DMF, k_{blob} equals 0.014 ± 0.002 ns^{-1} which in turn yields an encounter time of 70 ± 8 ns for each of the 10 residues encompassed within a *blob*. Applying the same methodology as for Leventhal's paradox, the characteristic time (τ_p) taken by each glutamic acid residue to probe one of three conformations would be given by $(k_{blob} \times 3^{N_{blob}})^{-1}$, which yields an order of magnitude x for the characteristic time ($x = log_{10}(\tau_p)$) of –12.0 ± 0.7. This result predicts that τ_p is in the picosecond range, matching the expected dynamics of polypeptides.[1,2]

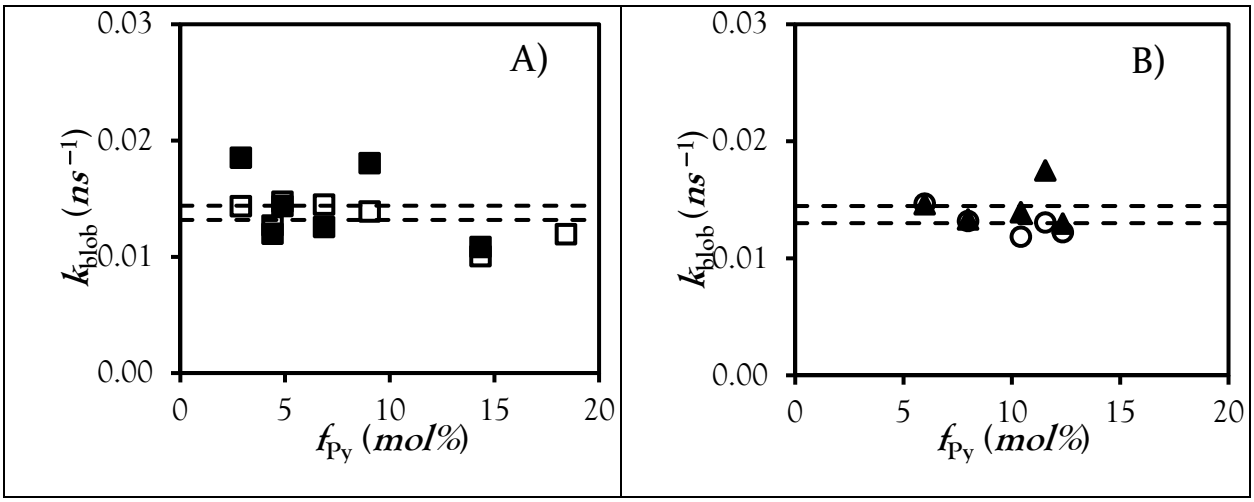

Figure 4: k_{blob} as a function of pyrene content for A) Py-P*L*GA and B) Py-P*DL*GA in DMF (solid) and DMSO (hollow). The dashed lines represent the averages.

Conclusions

A series of poly(glutamic acids)s were labeled with pyrene and their structure and dynamics were studied in DMF and DMSO. The stereo irregular structure of Py-P*DL*GA was found to adopt a coiled conformation in both DMF and DMSO, while Py-P*L*GA was found to adopt an α–helical conformation in DMF and a partially helical conformation in DMSO. Analysis of the fluorescence decays of the Py-PGA constructs yielded a *blob* size of 21 for the helical conformation, 18 for a partially helical conformation, and 10 for a random coil. This result suggests that as PGA folds into a helix from a random coil, the *blob* size is expected to increase from 10 to 21 as the coil collapses into a folded conformation. The *blob* size of the helical PGA was found to match the reported *Foldon* size of ca. 20 for proteins. In addition to the *blob* size, the dynamics of glutamic acid residues within a *blob* was also determined. Using k_{blob} for the coiled PGA, the characteristic time taken by a glutamic acid residue to probe a given conformation within a *blob* was found to be on the order of 1 ps, matching the dynamics expected for a folding protein. The fact that N_{blob} of PGA matched the *Foldon* size, and that k_{blob} reports on dynamics expected for a folding protein suggests that the parameters obtained from the FBM can be confidently used to model the dynamics of a polypeptide as folding occurs.