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# Direct Observation of the Multistep Helix Formation of Poly-L-glutamic Acids

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α-Helix is a basic building structure of proteins. Accordingly, the kinetics of helix formation[1−6] have been the subject of intensive discussion. The helix-coil (HC) transition theory assumes two elementary steps in the helix formation: the rate-limiting formation of the first helical turn (nucleation) followed by the fast addition of helical residues to the ends of the existing helix (propagation).[1,7] However, molecular dynamics simulations suggested that the HC transition involves other dynamical events such as the accumulation of the $3_{10}$-helix or turn.[3] Huang et al. reported a multiexponential helix relaxation using the temperature-jump FTIR method in the nanosecond time domain,[4] and Kinnear et al. indicated the intermediate conformation in the helix relaxation in vacuo.[5] Clarke et al. observed the slow helix formation in the millisecond time domain using the stopped-flow CD method.[6] We report here a new dynamical event involved in the helix formation of poly-L-glutamic acids (PGAs) using the microsecond-resolved CD and FTIR spectroscopies.

Our strategy to investigate the helix formation includes two novel aspects. First, we developed kinetic observation systems for the forward helix formation initiated by the rapid mixing of two solutions within 50 $\mu$s.[8] Second, the helix formation was monitored by both CD and FTIR spectroscopies. FTIR can quantitate the total amount of helix,[9] while CD can differentiate conformations such as the $3_{10}$-helix[10] and the lengths of helical segments.[11] These techniques enabled us to characterize the detailed dynamics of PGAs during the helix formation.

We first selected 34-residue PGA (average 4.4 kDa, distribution 2−7 kDa, Sigma). PGA adopts random coils when the side chains are charged at pH 8.0 and monomeric helices when the side chains are mostly neutral at pH 4.9.[12] Hence, the helix formation was initiated by pH and pD (corrected for $D_2O$ by adding 0.4 to the pH-meter reading) jumps from 8.0 to 4.9 for the kinetic CD and FTIR measurements, respectively.

To investigate the growth of the total helical content, we utilized FTIR spectroscopy and monitored the amide I′ line that is sensitive to the conformations of peptides.[2b,4,9] The dotted line in Figure 1a is the difference FTIR spectrum generated by subtracting the initial spectrum at pD 8.0 from the final spectrum at pD 4.9 and indicates the depletion of random coils and the increase of helices at 1665 and 1628 cm$^{-1}$, respectively.[13] The relative area of the helical line to the total area of the amide I′ indicates ∼32% of helix formation in the final state. The kinetic difference spectrum obtained at 100 $\mu$s after the pD jump (thick line) is coincident with that of the final state, indicating that PGA establishes the final helical content within 100 $\mu$s.

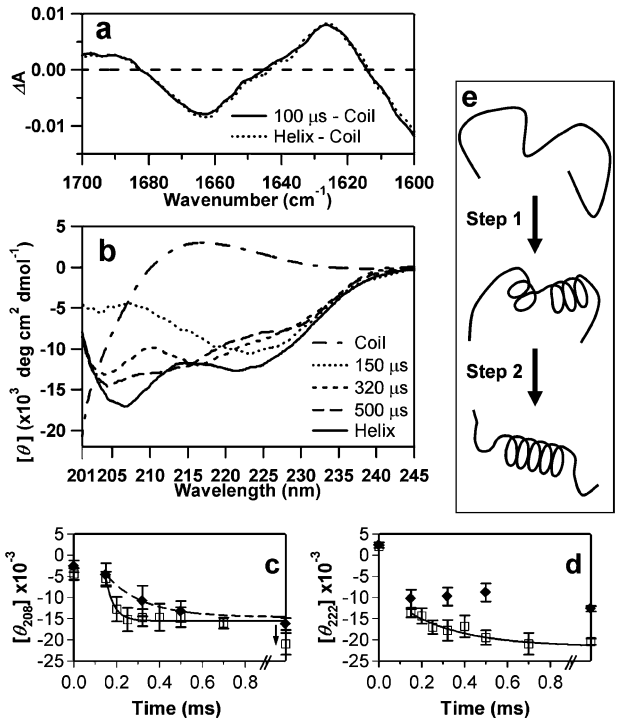


***Figure 1.*** The helix formation processes of 34- and 190-residue PGAs. The PGA solution containing 100 mM phosphate at pH 8.0 (pD for FTIR measurements) and 100 mM KCl was rapidly mixed with 10 mM HCl with a 1:1 volume ratio to a final pH of 4.9 at 20 °C for the subsequent spectroscopic measurements. (a) Difference FTIR spectra of 34-residue PGA (2.2 g L$^{-1}$). The dotted and thick lines are generated by subtracting the initial spectrum at pD 8.0 from the final spectrum at pD 4.9 and from the time-resolved spectrum at 100 $\mu$s, respectively. (b) The time-resolved CD spectra of 34-residue PGA (1.1 g L$^{-1}$). (c) and (d) The time-dependent changes in the molar ellipticities observed at 208 nm (c) and 222 nm (d). ◆ and □ indicate 34- and 190-residue PGAs, respectively. The lines represent the single-exponential fits to the respective data. The rate constants are 4000 ± 600, >5000, and 3600 ± 400 s$^{-1}$ for 34-residue PGA at 208 nm, 190-residue PGA at 208 nm, and 190-residue PGA at 222 nm, respectively. (e) Schematic drawing of the helix formation process for 34-residue PGA. The formation of short helices (step 1) is followed by their elongation (step 2).[15]

In contrast to the fast change of amide I′, the time-resolved CD signal of 34-residue PGA for the same pH jump continues to develop after 100 $\mu$s (Figure 1b).[14] The filled diamonds in Figure 1c and d illustrate the time courses of the ellipticities at 208 and 222 nm ([$\theta_{208}$] and [$\theta_{222}$]), respectively, both of which are characteristic of α-helices. While [$\theta_{222}$] develops more than 80% of the final amplitude within 150 $\mu$s, [$\theta_{208}$] recovers most of the final amplitude after 150 $\mu$s with a rate constant of 4000 ± 600 s$^{-1}$. The complete development of the two negative bands takes about 1 ms (thick line in Figure 1b). Therefore, the helix formation

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of 34-residue PGA comprises two distinct steps: the rapid (<100 $\mu$s) step detected in amide I′ and $[\theta_{222}]$, and the slower (~1 ms) step detected in $[\theta_{208}]$.

We exclude several possibilities as the origin of the two-step dynamics. The dimerization of PGA is unlikely, because the CD kinetics were almost invariant in the concentration range from 0.55 to 2.2 g $L^{-1}$ (data not shown). We rule out the involvement of the $3_{10}$-helix, because the CD spectrum at 150 $\mu$s is distinct from that of the $3_{10}$-helix.[10] The molecular weight distribution of the 34-residue PGA cannot account for the observed dynamics, because an additional CD measurement on the PGA sample having the fixed chain length (95% of 34-residue PGA) indicates the similar spectral changes and the time constant (Supporting Information).

To explain the FTIR and CD results consistently, we consider an intermediate conformation consisting of several short α-helices. It has been calculated that the intensity of $[\theta_{208}]$ is weak in short helices composed of less than 10 residues.[11] The intensity ratio of $[\theta_{208}]$ relative to $[\theta_{222}]$ at 150 $\mu$s ($[\theta_{208}]/[\theta_{222}] = 0.45 \pm 0.17$) corresponds to a helix consisting of $5 \pm 1$ residues.[11] At the same time, the total helix contents estimated at 150 $\mu$s are 26 and 32% by $[\theta_{222}]$[11c] and amide I′,[9] respectively, and correspond to 9−11 residues. We thus interpret that the initial intermediate contains two helices composed of ~5 residues (Figure 1e). Because the final CD spectrum indicates the formation of helices consisting of more than 10 residues, the slower step after 100 $\mu$s corresponds to the "elongation" of the short helices to the longer helix.

The helix formation process is more complex for longer PGA. The FTIR measurements on the same pD jump of ~190-residue PGA (average 25 kDa, distribution 15−30 kDa) revealed the fast formation of ~16% helix within 100 $\mu$s and the slower formation of ~19% helix with a rate constant of $3900 \pm 500$ $s^{-1}$ (Supporting Information). The $[\theta_{208}]/[\theta_{222}]$ ratio at 150 $\mu$s estimated from the CD measurements (□, Figure 1c and d) is $0.40 \pm 0.16$ and indicates the formation of short helices. The next phase is the elongation of short helices as indicated in the growth of $[\theta_{208}]$ with a rate constant of more than 5000 $s^{-1}$. $[\theta_{222}]$ also grows after 150 $\mu$s with a rate constant of $3600 \pm 400$ $s^{-1}$, which is consistent with the slower phase observed in the FTIR results. $[\theta_{208}]$ does not reach the equilibrium value within 0.7 ms. Thus, 190-residue PGA requires another phase that takes more than 1 ms to reach the equilibrium state having the helix content of ~36%.[18]

The observed helix formation dynamics of PGA are in marked contrast to those of alanine-based polypeptides that show fast relaxations with time constants of 10−100 ns.[2,4] We suppose that the difference might be explained by the strong propensity of protonated glutamic acid for hydrogen bonding. After the pH jump, PGA likely forms intramolecular hydrogen bonds between the side chains and between the backbone amides and the side chains[19] probably before the formation of the helix nucleation sites. The intramolecular hydrogen bonds might trap PGA into kinked conformations and block the propagation of helices until 100 $\mu$s (Figure 1e). The more complex helix formation observed for 190-residue PGA suggests the larger numbers of the intramolecular hydrogen bonds in the intermediate.

The current results are different from those by Clarke et al., who observed the slow helix formation of the 4.4 kDa PGA having a polydispersity with a time constant of ~2 ms using the stopped-flow CD method.[6] We also examined the helix formation of PGA under the pH jump condition utilized by Clarke et al. (from 8.0 to 3.3); however, the result was almost identical to that presented in Figure 1b. The $[\theta_{222}]$ value develops within 150 $\mu$s, which is in contrast to the slow helix formation reported by Clark et al.

In conclusion, we demonstrated that the helix formation process of PGA involves at least two dynamical steps, which are the formation of short helices and their elongation. We hypothesize that the observations are caused by the hydrogen bonding between protonated glutamic acids. We suggest that the amino acids which can form hydrogen bonds might indicate similar properties in the folding processes of actual proteins.

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**Supporting Information Available:** The time-resolved CD spectra for 34-residue PGA without a polydispersity and the time-resolved difference FTIR spectra for 190-residue PGA (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

## References

(1) Schwarz, G. *J. Mol. Biol.* **1965**, *11*, 64.
(2) (a) Dyer, R. B.; Gai, F.; Woodruff, W. H.; Gilmanshin, R.; Callender, R. H. *Acc. Chem. Res.* **1998**, *31*, 709. (b) Williams, S.; Causgrove, T. P.; Gilmanshin, R.; Fang, K. S.; Callender, R. H.; Woodruff, W. H.; Dyer, R. B. *Biochemistry* **1996**, *35*, 691. (c) Thompson, P. A.; Muñoz, V.; Jas, G. S.; Henry, E. R.; Eaton, W. A.; Hofrichter, J. *J. Phys. Chem. B* **2000**, *104*, 378. (d) Lednev, I. K.; Karnoup, A. S.; Sparrow, M. C.; Asher, S. A. *J. Am. Chem. Soc.* **2001**, *123*, 2388.
(3) (a) Tirado-Rives, J.; Jorgensen, W. L. *Biochemistry* **1991**, *30*, 3864. (b) Takano, M.; Yamato, T.; Higo, J.; Suyama, A.; Nagayama, K. *J. Am. Chem. Soc.* **1999**, *121*, 605. (c) Wu, X.; Wang, S. *J. Phys. Chem. B* **2001**, *105*, 2227.
(4) (a) Huang, C. Y.; Getahun, Z.; Wang, T.; DeGrado, W. F.; Feng, G. *J. Am. Chem. Soc.* **2001**, *123*, 12111. (b) Huang, C. Y.; Getahun, Z.; Zhu, Y.; Klemke, J. W.; Degrado, W. F.; Gai, F. *Proc. Natl. Acad. Sci. U.S.A.* **2002**, *99*, 2788.
(5) Kimmear, B. S.; Hartings, M. R.; Jarrold, M. F. *J. Am. Chem. Soc.* **2001**, *123*, 5660.
(6) Clarke, D. T.; Doig, A. J.; Stapley, B. J.; Jones, G. R. *Proc. Natl. Acad. Sci. U.S.A.* **1999**, *96*, 7232.
(7) Poland, D.; Scheraga, H. A. *Theory of Helix-Coil Transitions in Biopolymers*; Academic Press: New York, 1970.
(8) (a) Akiyama, S.; Takahashi, S.; Ishimori, K.; Morishima, I. *Nat. Struct. Biol.* **2000**, *7*, 514. (b) Akiyama, S.; Takahashi, S.; Kimura, T.; Ishimori, K.; Morishima, I.; Nishikawa, Y.; Fujisawa, T. *Proc. Natl. Acad. Sci. U.S.A.* **2002**, *99*, 1329.
(9) Siebert, F. *Methods Enzymol.* **1995**, *246*, 501.
(10) Toniolo, C.; Polese, A.; Formaggio, F.; Crisma, M.; Kamphuis, J. *J. Am. Chem. Soc.* **1996**, *118*, 2744.
(11) (a) Woody, R. W. Circular dichroism of peptides. *The Peptides*; Academic Press: New York, 1985; Vol. 7, p 15. (b) Chen, Y. H.; Yang, J. T.; Chau, K. H. *Biochemistry* **1974**, *13*, 3350. (c) Yang, J. T.; Wu, C. S. C.; Martinez, H. M. *Methods Enzymol.* **1986**, *130*, 208.
(12) (a) Holtzer, A.; Hawkins, R. B. *J. Am. Chem. Soc.* **1996**, *118*, 4220. (b) Olander, D. S.; Holtzer, A. *J. Am. Chem. Soc.* **1968**, *90*, 4549.
(13) The system for the time-resolved FTIR measurements was constructed by setting the rapid mixing cell at the focal point of the FTIR microscope (BioRad, FTS-575C/UMA500). The mixing cell with $CaF_2$ windows was reported previously[8b] and possesses an observation channel (100 $\mu$m × 100 $\mu$m cross section) and 50 $\mu$s of a mixing time at the flow rate of 16.8 m $s^{-1}$. The spectra were the average of 50 scans at a resolution of 4 $cm^{-1}$.
(14) The time-resolved CD spectra were obtained as described previously,[8a] although the mixing cell used for the FTIR measurements was used with quartz windows.[8b] The spectra were the average of at least five scans obtained at 0.5 nm resolution using JASCO dichrograph (J-720).
(15) The numbers of the 5-residue helices at 150 $\mu$s estimated from the kinetic CD spectra are 2 and ~20 for 34- and 190-residue PGAs, respectively. In contrast, the numbers of the helical segment in equilibrium can be estimated as 1.3 and 3.3 for 34- and 190-residue PGA, respectively, using eq 50 of ref 16 and HC parameters, $s = 1.35$ and $\sigma = 0.01$, for protonated glutamic acid.[17] Therefore, the numbers of the short helical segment kinetically formed in the intermediate state are larger than the numbers of the equilibrium helical segments.
(16) Nagai, K. *J. Chem. Phys.* **1961**, *34*, 887.
(17) (a) Maxfield, F. R.; Alter, J. E.; Taylor, G. T.; Scheraga, H. A. *Macromolecules* **1975**, *8*, 479. (b) Wójcik, J.; Altmann, K.-H.; Scheraga, H. A. *Biopolymers* **1990**, *30*, 121. (c) Holtzer, A. *J. Am. Chem. Soc.* **1994**, *116*, 10837.
(18) We calculated the helical content on the basis of the FTIR spectrum. The equilibrium helix content estimated from $[\theta_{222}]$ and eq 8 in ref 11c for 190-residue PGA is 58% and does not coincide with the estimation (36%) based on the FTIR spectrum. This may be caused by the difference in the detectable conformations between FTIR and CD.
(19) Laskowski, M.; Scheraga, H. A. *J. Am. Chem. Soc.* **1954**, *76*, 6305.

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