



α -helix formation rate of oligopeptides at subzero temperatures

Zhi-jie Qin¹, Akio Shimizu², Jinsong Li¹, Masamichi Ikeguchi³, Masaji Shinjo¹ and Hiroshi Kihara^{1,4}

¹Department of Physics, Kansai Medical University, 2-5-1, Shin-Machi, Hirakata, Osaka 573-1010, Japan

²Department of Environmental Engineering for Symbiosis, Soka University, 1-236 Tangi-cho, Hachioji, Tokyo 192-8577, Japan

³Department of Bioinformatics, Soka University, 1-236 Tangi-cho, Hachioji, Tokyo 192-8577, Japan

⁴Present address: Hinomoto Gakuen Institute, 890, Koro, Koderama-Cho, Himeji, Hyogo 679-2151, Japan

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In 1999, Clarke *et al.* ((1999) Proc. Natl. Acad. Sci. USA 96, 7232–7237) reported that the nucleation rate of α -helix of oligopeptide AK16 is as slow as 60 ms. In the present study, we measured the nucleation rate of oligopeptide, C17 (DLTDDIMCVKKILDKVG, corresponding to α -helical region of 84th to 100th amino acids of bovine α -lactalbumin) using the same method as Clarke *et al.* We found only initial bursts of the increase of α -helices at temperatures higher than -50°C in the presence of 70% methanol. The result with AK16 was the same as Clarke *et al.* reported. We also found that the folding rate of polyglutamic acid is too fast to be detected by the stopped-flow apparatus at 4°C . These results demonstrate that the α -helix formation rates in C17, AK16 and polyglutamic acid are shorter than the dead time of the stopped-flow apparatus (6 ms).

Key words: alpha-helix, protein folding, stopped-flow, subzero temperature

In 1996, Ballew *et al.*¹ reported that, according to laser T-jump (up) measurements from the cold denatured state, apomyoglobin is folded in three phases; the single α -helix (helix A) formation (1 μs), the collapse of α -helices (A, G

and H; 5–17 μs) and folding to its final conformation (0.5 s), and emphasized that the initial collapse of protein folding took place in the μs or even faster timescale. In 1999, Clarke *et al.*² reported a rate of α -helix formation as slow as 60 ms in the case of oligopeptide AK16 (composed of 16 amino acid residues) by means of the GuHCl concentration-jump, using a stopped-flow method. They attributed the difference to the laser T-jump study in which Ballew *et al.* employed only shifted equilibrium. The initial state already included small fractions of α -helices, and therefore, the rate that Ballew *et al.* observed was not the nucleation rate of α -helix but rather the propagation rate of the α -helix. They also measured the rate of α -helix formation of polyglutamic acid and poly-L-lysine, and reported that the rates of such long polypeptides are observable but faster than those of short peptides. As to the reason, they speculated that the long peptides have some nuclei of α -helix even in the 4 M GuHCl.

We recently found that the initial step of α -helix formation in bovine β -lactoglobulin finished within the dead time of the stopped-flow apparatus (6 ms) at 4°C ³. This result was reproduced with other proteins such as SH3 domain⁴, FHA domain⁵ and equine β -lactoglobulin⁶. Our results cannot be understood if the initial α -helix formation rate is as slow as Clarke *et al.* reported. Therefore, we started measuring the nucleation rate of oligopeptide, C17 (DLTDDIMCVKKILDKVG), α -helical part of 84th to 100th amino acids of bovine α -lactalbumin⁷. We found only initial bursts of the increase of α -helices at temperatures higher than -30°C in the presence of 70% methanol. A similar experiment was conducted with AK16 peptide, as

Abbreviations: GuHCl, guanidine hydrochloride; PBS, phosphate buffer; CD, circular dichroism

Corresponding author: Hiroshi Kihara, Hinomoto Gakuen Institute, 890, Koro, Koderama-Cho, Himeji, Hyogo 679-2151, Japan.
e-mail: kiharah1234@gmail.com

Clarke had used in his experiment. In addition, we measured the folding rate of polyglutamic acid at 4°C. The result only showed the initial burst. These results strongly indicate that the α -helix formation rates in the case of C17 and AK16 peptides and polyglutamic acid are faster than the dead time of the stopped-flow apparatus (6 ms). The difference between our and Clarke's data is discussed.

Materials and methods

Materials

C17 and AK16 peptides were synthesized on 9050 peptide synthesizers (PerSeptive). Fluorenylmethyloxy-carbonyl (Fmoc) amino acids were coupled onto the PAL resin (PerSeptive) so that the resultant peptides have an amide group at the C terminus⁷. For coupling the Cys residue, N-Fmoc-S-acetamidomethylcysteine was used, leaving the S-acetamidomethyl group in the peptides. The peptides were purified by reverse-phase HPLC (Waters μ Bondasphere C18-100 Å 19×150 mm)⁷.

Polyglutamic acid was purchased from Sigma (Product No. P-1818, Mol. Weight; 1,500–3,000), and was used without further purification.

GuHCl was of ultrapure reagent grade from ICN Biomedicals, Inc. (Lot 2345B). Its concentration was calibrated by refractive index measurements. All other reagents were of guaranteed reagent grade for this study.

Temperature was controlled by a ULT-80 controller (NESLAB). Viscosities were measured by a syringe viscometer and calibrated by standard solution (JS 5 and JS 20 of Nihon Grease Co.).

Stopped-flow Apparatus

The stopped-flow device was constructed specially for high viscosity and low temperature use in collaboration with UNISOKU Inc⁴. The mixer combined one of two mixing units (4-jet and 6:1 mixer). Its dead time was estimated to be 6 ms based on a test reaction of DCIP reduction by ascorbic acid at 4°C⁸. The mixing was sufficient within the limits for the viscosity being less than 200 m Poise and the temperature higher than -40°C.

CD measurements.

CD measurements were performed with a spectropolarimeter specially designed by Unisoku Inc. In refolding experiments, measurements were repeated and accumulated to obtain a good signal/noise ratio. The averaged data were normalized to give molar ellipticity.

Results

C17 peptide does not take α -helical conformation in water between pH 2.0 and 12.4, as shown in Figure 1⁷. However, this peptide takes α -helical conformation with the addition of 20% trifluoroethanol, as shown in Figure 2. The peptide

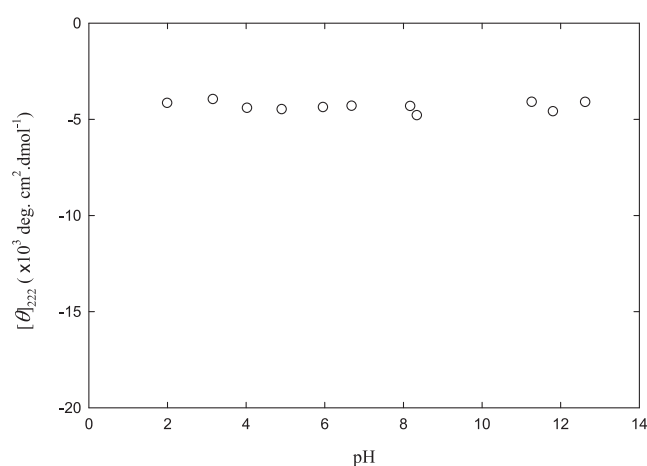


Figure 1 CD value at 222 nm of C17 peptide in water as a function of pH.

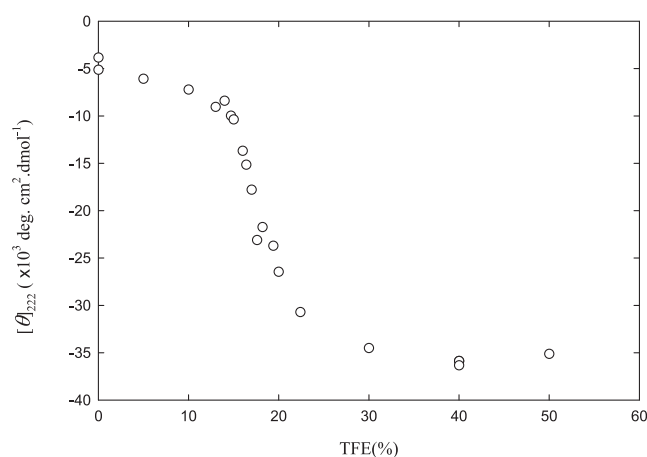


Figure 2 CD value at 222 nm of C17 peptide in 1 mM citrate buffer at pH 2.0 as a function of trifluoroethanol. 5°C.

also takes stable α -helical conformation with the addition of methanol at higher than 50%, as shown in Figure 3. In Figure 4(a), CD spectra of C17 peptide in the phosphate buffer are shown as a function of added GuHCl at -40°C. The GuHCl-induced unfolding transition of C17 peptide is shown as a function of GuHCl concentration in Figure 4(b). As seen in the figure, the peptide was unfolded with the midpoint of ca. 2 M of GuHCl.

We then performed GuHCl concentration jump experiments using C17 peptide. Above -30°C, we could only see the burst phase (Fig. 5). This indicates that the folding rate of α -helices is faster than the dead time of the stopped-flow apparatus (6 ms). We decreased the temperature to as low as -50°C, but failed to observe any detectable time course except for the initial burst (data not shown).

We repeated the denaturant-concentration jump experiments with AK16 peptide and the results are shown in Figure 6. As the quantity of peptide was limited, we could not obtain a good signal/noise ratio, as in the case of C17. How-

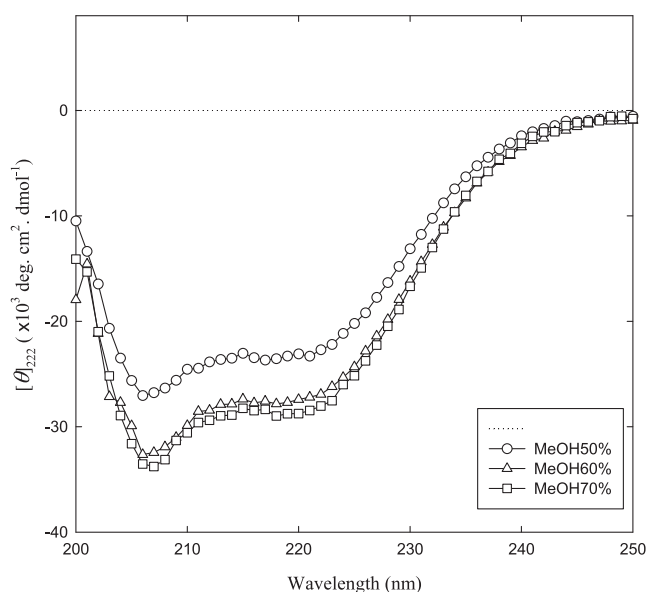


Figure 3 CD spectra of C17 peptide in various concentrations of methanol. pH 2.3, 0.01 M phosphate buffer, 20°C.

ever, we can clearly say that no slow phases (slower than 6 ms) were observed within an experimental error of 5%.

We also conducted pH jump experiment with polyglutamic acid, the results of which are shown in Figure 7. In both cases, no detectable time courses were observed.

Discussion

Difference of α -helix formation rate

From the experiments reported above, it is clear that C17 and AK16 peptides fold to the α -helical conformation very rapidly. This is in good agreement with other observations

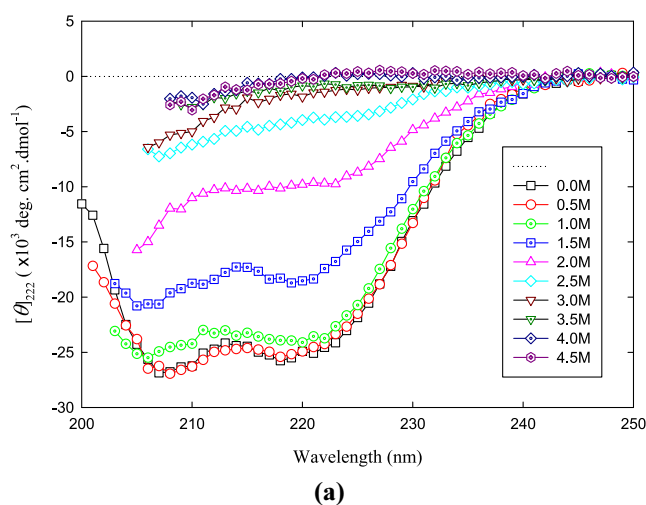
of protein folding; bovine³ and equine⁶ β -lactoglobulins, src and fyn SH3 domains⁴, Rad53 and Ki67 FHA domains⁵ and many other proteins⁴ took the α -helix-rich intermediate within the dead time of the stopped-flow apparatus (6 ms) at above -20°C . Apomyoglobin also took its α -helix-rich intermediate within the dead time of the stopped-flow apparatus (unpublished results). This is in apparent contradiction with the results reported by Clarke *et al.*² One interpretation of this discrepancy is that the rate of α -helical formation is highly dependent on the sequence investigated. Clarke *et al.* also reported that the α -helix formation rate of polyglutamic acid and poly-L-lysine were also in the observable time-scale. In contrast, however, we could not see any detectable changes of polyglutamic acid folding as shown in Figure 7.

One possible explanation is mixing insufficiency of the mixer used by Clarke *et al.* Actually, when we use a conventional mixer for the GuHCl-concentration jump, we often observed time courses due to insufficient mixing. For this reason we used a more sophisticated mixer in our stopped-flow machine.

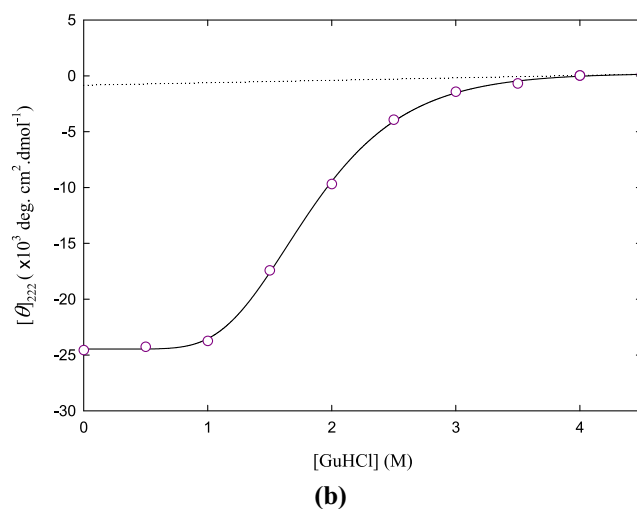
α -helix formation rate investigated by other methods

There are several reports on the helix-coil transition rate investigated by temperature-jump and NMR methods^{9–15}. The results may be summarized as: (a) fast step of ca. 20 ns^{9,10}, and (b) slower step of ca. 140–220 ns at 300 K^{10–12}. Gai reported that the slower process shows a monotonic temperature dependence with an activation energy of 15.5 kcal/mol¹⁰. If we employ this value, we might expect a rate of 140 μs at -30°C , or 210 μs at -38°C . In order to monitor with our stopped-flow method, the system would need to be cooled down as low as -60°C (6.5 ms). This shows our results are consistent with the temperature-jump study.

Thus, we have no other explanations except that Clark's data must be due to artifacts from insufficient mixing.



(a)



(b)

Figure 4 (a) CD spectra of C17 peptide in 0.01 M phosphate buffer in the presence of 70% methanol, pH 2.0 as a function of GuHCl at -40°C . (b) GuHCl-induced unfolding transition curve of C17 peptide in the presence of 70% methanol at -40°C .

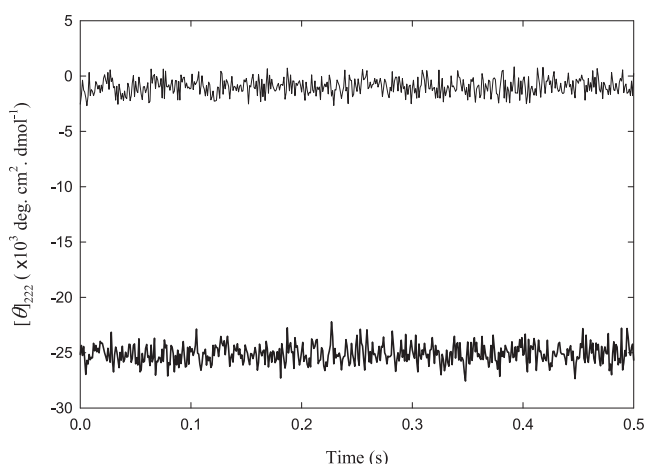


Figure 5 Kinetics for α -helix formation rate of C17 peptide in 0.01 M phosphate buffer in the presence of 70% methanol at -30°C . The reaction was initiated by a concentration jump of GuHCl from 3.5 M to 0.5 M by 1:6 dilution. The upper curve represents the initial CD_{222} (CD value at wavelength of 222 nm) level of C17 peptide in 3.5 M GuHCl from which the folding was initiated.

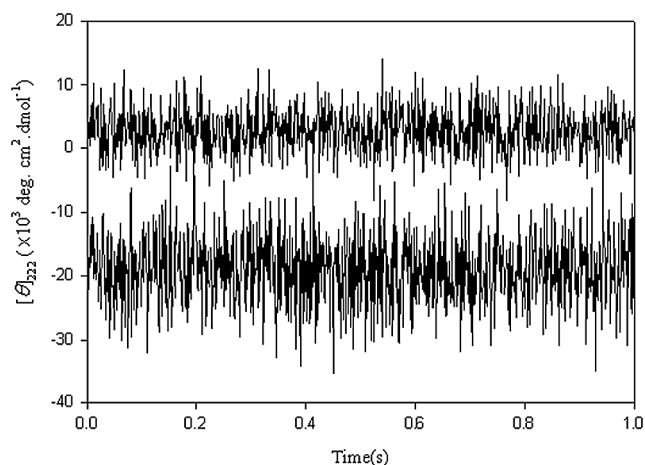


Figure 6 Kinetics for α -helix formation rate of AK16 peptide in 0.01 M phosphate buffer in the presence of 70% methanol at -35°C . The reaction was initiated by a concentration jump of GuHCl from 4.5 M to 0.64 M by 1:6 dilution. The upper curve represents the initial CD_{222} (CD value at wavelength of 222 nm) level of AK16 peptide in 4.5 M GuHCl from which the folding was initiated.

Helix-nucleation or helix propagation

There might be another argument regarding the nature of the present finding for rapid phase. That is, the rapid phase we observed might be due to helix-propagation and not helix nucleation. To clarify this point, we developed a statistical theory for the helix formation in the denatured solvents, and published it recently¹⁶. The paper clearly demonstrated that the helix in highly-concentrated denaturant is zero or one in oligopeptides¹⁶, and indicates that the experimentally observed process in this paper is due to helix nucleation rather than helix propagation.

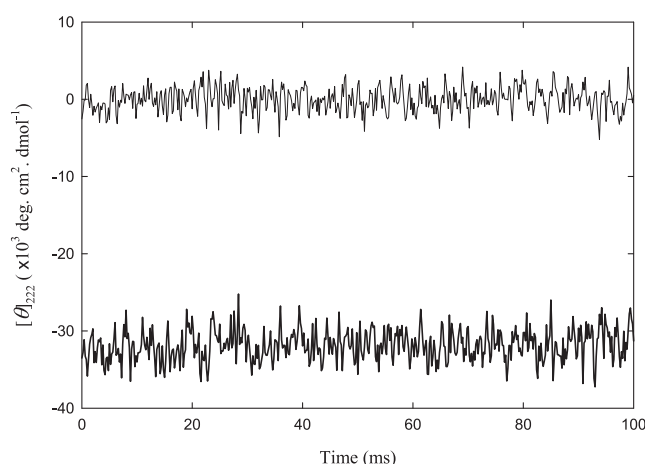


Figure 7 Kinetics for α -helix formation rate of polyglutamic acid in 0.01 M phosphate buffer at pH 8.0 at 4°C . The reaction was initiated by a pH jump from pH 8.0 to pH 3.3 by 1:1 mixing with sodium acetate buffer (pH 2.0). The upper curve represents the initial CD_{222} level of polyglutamic acid at pH 8.0 from which the folding was initiated.

Kinetics of poly-L-glutamic acid folding

As shown in Figure 7, we observed only the burst phase of poly-L-glutamic acid folding. This means that the poly-L-glutamic acid was folded within the dead time of the stopped-flow apparatus (6 ms). This is in sharp contradiction with the paper by Clarke *et al.*² who reported various steps; two rate-limiting steps ($600\text{--}690\text{ s}^{-1}$ and $150\text{--}180\text{ s}^{-1}$, corresponding to 1.4–1.8 ms and 5.6–7.1 ms) at 222 or 226 nm and a step of 24 s^{-1} (42 ms) at 196 nm.

The kinetics of the helix-coil transition of poly-L-glutamic acid were further investigated by two other groups^{17,18}. Causgrove and Dyer¹⁷ used a laser-induced pH-jump method with infrared light as a probe, and observed several phases. The slowest changes were in the order of 10 ms, but the signals were not dependent on the probe wavelength or sample conditions. Then they concluded the slow process is not due to changes in peptide structure or side chain deuteration, but is most likely due to the cooling of the excitation volume¹⁷. They also observed multi-phases; the fastest phase occurred within 40 ns, and this process was lengthened to as long as 625 ns, which is within the dead time of our stopped-flow apparatus. Kimura *et al.*¹⁸ also reported the kinetics of the folding of poly-L-glutamate. They reported that the helix was formed within 100 μs with IR monitoring, but ca. 250 ns or faster by CD monitoring. Thus, the folding of poly-L-glutamic acid was reportedly faster than 6 ms (the dead time of our stopped-flow apparatus) except in the results of Clarke *et al.* These results also imply that the results by Clarke *et al.* might be due to artifacts from their instruments.

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