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A Rapid Flow Mixer with 11-μs Mixing Time Microfabricated by a Pulsed-Laser Ablation Technique: Observation of a Barrier-Limited Collapse in Cytochrome *c* Folding

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For many proteins with more than ~100 residues, the earliest event in folding dynamics is the collapse of polypeptides, which occurs within ~100 μs and leads to the formation of compact intermediates that eventually convert to native states.[1a−d] The mechanism of the collapse is therefore essential to understand the conformational search of proteins;[2] however, the limit in the time resolution of available experimental methods precluded us from conducting a detailed analysis of the event. In this study, we developed a new flow mixer by a femtosecond laser ablation technique and achieved a mixing time of 11 μs. This mixer enabled us to demonstrate a single-exponential kinetics in the initial collapse of cytochrome *c* (cyt *c*) initiated by solution mixing.

Cyt *c* from horse heart is a globular protein with 104 amino acids and a covalently attached heme, which is surrounded by three major helices termed the N-terminal, C-terminal. and 60's helices.[3] The native cyt *c* possesses a compact size with a radius of gyration (R_g) of 13.9 Å.[1a] In contrast, the acid unfolded cyt *c* at pH 2.0 is expanded to an R_g of 24.3 Å.[1a] A single tryptophan (Trp59) of cyt *c* emits strong fluorescence in the unfolded state, which is quenched in the native state by heme located in the vicinity of Trp59. Roder et al. observed Trp59 fluorescence in the folding process initiated by rapid solution mixing, and detected an initial decrease in intensity that was fitted by a single-exponential curve with a time constant of ~60 μs.[4a] The phase was interpreted to be the collapse that proceeds in a barrier-limited manner with the specific structural changes. The time-resolved SAXS measurements indicated that the collapsed conformation possesses an R_g of 20.5 Å.[1a] These observations suggest a specific collapse as the initial dynamics of cyt *c* folding.[4b]

In contrast to the above proposal, a nonspecific collapse in the folding of cyt *c* was also proposed. Englander et al. observed the structural changes of nonfolding fragments of cyt *c* with a time resolution of several milliseconds, and detected the burst phase whose amplitude was the same as that of the full length protein.[4c] They concluded that the collapse is caused by nonspecific hydrophobic interactions triggered by the changes in solvent conditions. Saigo et al. observed the non-exponential decay of Trp59 fluorescence of full-length cyt *c* by using a cryogenic stopped-flow device, which is rather consistent with the nonspecific collapse.[4d]

It should be noted that a large part of the initial phase observed by Roder et al. with a time constant of ~60 μs is hidden in the observation dead time of their device (~45 μs).[4a] There remains a possibility that the collapse at room temperature initiated by solution mixing might also contain a non-exponential process. Observation of the collapse at room temperature with an improved time resolution is required.

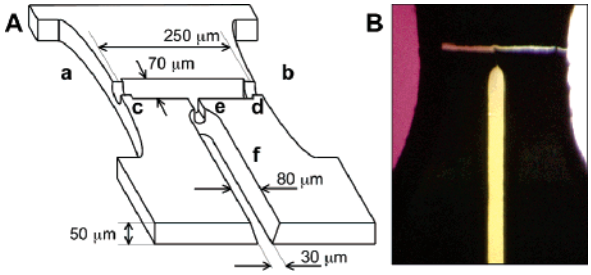

Figure 1. (A) An illustration of a mixing plate microfabricated by the pulsed-laser ablation technique on a stainless plate. An equal volume of two solutions from channels a and b pass through gaps c and d, which help create nonlaminar flows *before* the mixing gap e. The mixed solution flows into the observation channel f. The bottom width of the channel between gaps c and d is less than 10 μm. (B) A top view of the mixing channel upon mixing of BCP at pH > 7 (left) and an acidic buffer (right).

To improve the time resolution, we developed a new mixing strategy, in which rapid mixing is achieved by creating nonlaminar flows *before* the collision of two solutions. The previous strategy for rapid mixing was pioneered by Regenfuss et al.[5a] and was widely adopted in recent studies.[5b−d] In their strategy, mixing is achieved by introducing two solutions into a narrow channel and by creating a turbulent flow *after* the collision of the two solutions. This method can reproduce a mixing time of ~100 μs but is difficult to achieve a mixing time of less than several ten's of microseconds. The newly developed strategy utilizes a channel depicted schematically in Figure 1A. The two solutions are supplied from channels a and b, and pass through gaps c and d, which create nonlaminar flows before the mixing gap e, thus enabling rapid mixing.

To construct the mixing channel on a stainless plate, we utilized a femtosecond laser ablation technique, which allows for a nonthermal processing of a stainless plate by irradiation of many photons in a short duration.[6a,b] The method enables the fabrication of a narrow channel with a 20~30 μm width and rugged surfaces that help create nonlaminar flows. We searched for an optimal design for fast mixing, and reached the dimensions shown in Figure 1A. The velocity of the mixed solution in the observation channel f was 7.1 mm/ms. Rapid mixing is demonstrated in a photograph showing a discoloration reaction of bromocresol purple (BCP) after a pH jump (Figure 1B). The color change of BCP was completed at the top of the observation channel, which corresponds to a mixing time of ~10 μs (Supporting Information). A plate with similar

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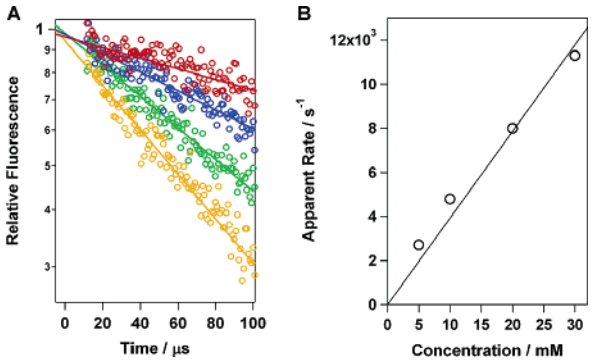

Figure 2. (A) Semilogarithmic plots of the relative intensity changes of NATA fluorescence. The red, blue, green, and orange circles represent data obtained at the final NBS concentrations of 5, 10, 20, and 30 mM, respectively. The intensity changes were fitted by single exponentials (lines). (B) The apparent rate constants of the quenching reaction as the function of the NBS concentration. The second-order rate constant estimated by a linear fitting of the plot was 3.9 (±0.2) × 10^5 M^{-1}·s^{-1}, and is consistent with the reported value.[5d]

dimensions but without gaps c and d cannot achieve a mixing time of less than 50 μs (not shown).

We used the quenching reaction of *N*-acetyl-L-tryptophane-amide (NATA) fluorescence by *N*-bromosuccinimide (NBS) for quantitative estimations of the mixing time.[5d,7] The excitation light at 280 nm was focused on the sample flow, and the fluorescence image at 350 nm was created on CCD. We measured the fluorescence intensity changes of NATA after mixing with several concentrations of NBS (Supporting Information). Figure 2A shows a semilogarithmic plot of the relative fluorescence. The decay of NATA fluorescence could be fitted by single exponentials (lines) as expected for the pseudo first-order kinetics. The fitted lines for the different NBS concentrations intersected at the intensity corresponding to the unreacted NATA solution and at the time representing the initiation point of the mixing. The delay between this point and the first observable point corresponds to the dead time and was 11 μs. The apparent rate constants for the quenching reaction plotted as the function of the NBS concentration was linear (Figure 2B) and substantiate the accurate mixing. Thus, the device achieved one of the fastest mixing times ever developed for kinetic observation of biological reactions.[5a−e]

To monitor the collapse in the folding of cyt *c*, we observed the intensity changes of Trp59 fluorescence after the pH jump. The folding of cyt *c* was induced by pH-jumps from 2.0 to 4.5 and 6.0 (Supporting Information). As shown in Figure 3, the observed kinetics could be fitted by single exponentials within the *S*/*N* ratio. No burst phase amplitude was detected. The rate constants at pH 4.5 and 6.0 were 1.17 (±0.04) × 10^4 s^{-1} and 2.16 (±0.08) × 10^4 s^{-1}, respectively. The results are consistent with those obtained by Roder et al. and demonstrate the absence of non-exponential kinetics in the collapse of cyt *c* initiated by solution mixing at room temperature.[4a]

It was pointed out that exponential kinetics does not necessarily demonstrate a single barrier;[8a,b] however, various other observations suggested the barrier-limited collapse in cyt *c*.[5d,8c−e] The exponential change in the Soret absorption band was observed after rapid mixing[8c] and photochemical triggering of cyt *c* folding,[8d] although the observed changes in heme coordination states might be rate limited by the ligand binding to heme. The expansion dynamics of cyt *c* after a temperature jump was exponential, supporting the exponential dynamics for the reverse collapse under the assumption of the two-state kinetics.[8b,e] The distance between a labeled fluorophore and heme in unfolded cyt *c* possesses two minima,

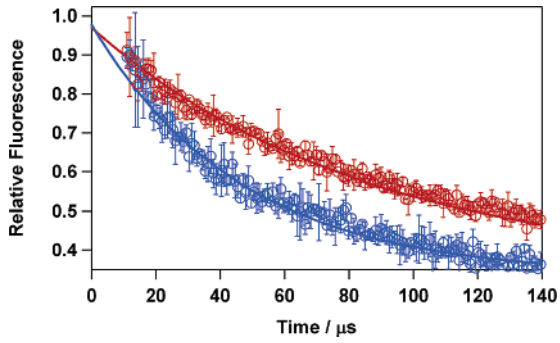

Figure 3. The refolding kinetics of acid unfolded cyt *c* after rapid pH jumps to pH 4.5 (red) and 6.0 (blue) monitored by Trp59 fluorescence. The lines are the data fits to single-exponential curves. The relative fluorescence intensity was determined by flowing the acid unfolded cyt *c* in the same mixing cell as the intensity standard (Supporting Information).

supporting the energy barrier separating them.[5d,8f] The collapse is exponential at room temperature as demonstrated in this study but becomes non-exponential at subzero temperatures.[4d] The difference might be explained by the temperature dependency of conformational relaxation time as suggested by Saigo et al.[4d]

In summary, the exponential kinetics was observed for the initial folding phase of cyt *c*, which strongly suggested the barrier-limited collapse. The developed mixer can be combined with various spectroscopic and scattering techniques for the analysis of protein folding and other fast events.

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Supporting Information Available: Experimental details. This material is available free of charge via the Internet at http://pubs.acs.org.

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