Supporting information

"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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Sample preparation

All chemicals were purchased as the highest grade available and used without further purifications except for cytochrome *c* (cyt *c*). Cyt *c* from horse heart (Sigma) was treated with ferricyanide to oxidize the residual ferrous form and was purified on Sephadex G-25 column. The unfolded cyt *c* was prepared by dissolving the native protein in deionized water and by adjusting pH to 2.0 using 1-N solution of HCl.

Rapid-mixing cell

A mixing channel was fabricated on a stainless plate (SUS304) of 0.05 mm thickness by using femtosecond laser ablation technique (Fig. 1A of the main text). Pulsed laser irradiations with wavelength of 800 nm, pulse width of 100 fs and oscillating frequency of 1 kHz (Hurricane, Spectra Physics) were focused on the stainless plate. The machining conditions were programmed into a numerical control machine (FANUC Power Mate, Kataoka Seisakusyo). A polarization of the laser was adjusted to be perpendicular to a scanning direction to reduce the roughness of side surfaces of channels. The laser power was ~3 mW for producing the narrowest channel with a width of 20~30 μm, and was ~150 mW for producing wider channels. The rapid mixing cell was constructed by placing the mixing plate between two quartz plates with a thickness of 2 mm. Two solutions to be mixed were concurrently introduced to the mixing cell by a mechanical syringe pump (PHD22/2000, Harvard Apparatus).

Time-resolved fluorescence measurements

The time-resolved fluorescence detection system was based on the rapid mixing cell. A 500-W deep UV lamp (UXM501-MA, Ushio), a cold mirror (Nihonshinkukogaku) and a band pass filter (λ_{max} = 282 nm, Nihonshinkukogaku) were used for the fluorescence excitation at 280 nm. The excitation light was focused on the observation channel of the cell from the off-axis direction. A fluorescent emission from the entire observation channel was collected by a UV-achromatic lens with a focal length of 80 mm (UDL-30-80P, Sigma Koki) and focused onto a cooled CCD camera (NTE/CCD-512-TK, Roper Scientific). Two types of optical filters, a long pass filter (325-nm cut off, Asahi Spectra) and a band pass filter (λ_{max} = 360 nm, Sigma Koki), were placed in front of CCD. The total exposure time of CCD was typically 540 s for one measurement. All kinetic measurements were performed at room temperature. The same experimental setting was used for

the observations of the quenching reaction of N-acetyl-L-tryptophanamide (NATA) and of the folding reaction of cyt *c*.

Along with the fluorescence images for the mixing process of two parent solutions (I_{raw}), two types of control images were obtained. The background images (I_{bg}) were obtained by mixing buffers without fluorescent samples. The standard images (I_{stand}) for the correction of spatial non-uniformity in the excitation light were obtained by mixing one of the parent solutions containing fluorescent molecules, either the unfolded cyt *c* or NATA, and buffers used to dissolve the fluorescent molecules. The relative fluorescence images, I_{rel}, were calculated according to equation (1):

$$I_{rel} = (I_{raw} - I_{bg}) / (I_{stand} - I_{bg}) \quad (1).$$

The final fluorescence intensity at one time point was obtained by averaging the fluorescence intensities of I_{rel} along a cross section of the observation channel. The standard deviation of I_{rel} along the cross section was also calculated and used as an intensity error. The relative fluorescence intensity of unity, calculated through the above procedure, corresponds to the fluorescence intensity of either the unfolded cyt *c* or unreacted NATA. The time axis was calculated from the pixel number of CCD by considering the flow line speed of the sample. The time zero was temporally set at the initial point of the fluorescence image of the observation channel, which was later corrected by subtracting 11 μs as will be explained below.

Determination of the mixing dead time

First, the mixing dead time of the cell was roughly estimated by visually examining the discoloration reaction of bromocresol purple (BCP) under an optical microscope (Fig. 1B of the main text). The solution of BCP is colored blue-violet at pH more than 6.8, and turns to greenish yellow at pH less than 5.2. The solution of concentrated BCP whose pH was adjusted at above 7 and 200-mM acetate buffer at pH 4.5 were continuously supplied to the mixing cell at the volume ratio of 1:1. The flow line speed of the mixed solution in the observation channel was 7.1 mm/ms, which was calculated from both the cross section of 2,800 μm^2 and the solution flux of 1.2 ml/min. Since the color of the channel turned yellow at the top of the observation channel, we roughly estimated the mixing time to ~10 μs based on the solution flux and the estimated volume of the channel between the mixing point and the first detectable point.

Second, the quenching reaction of NATA fluorescence by N-bromosuccinimide (NBS) was used for the quantitative assessment of the mixing time (Fig. 2A of the main text). We measured the fluorescence intensity change by mixing 1-mM NATA solution with 10, 20, 40, 60 mM of NBS solution at a volume ratio of 1:1. The both solutions were buffered at pH 6.0 using 100-mM phosphate. The decay curves of NATA fluorescence in the time range within 100 μs were fitted by a single-exponential curve, in which the initial amplitude and the apparent rate constant were the only variables. The standard deviations of the fluorescence intensity data were used as weighting parameters for the fitting. The fitted curves for the kinetic traces obtained at different NBS concentrations intersected at the relative fluorescence intensity of about one, which corresponds to the intensity of unreacted NATA signal. The crossing point of the fitted curves represents the real initiation point of the mixing reaction. The delay between this point and the first observable point corresponds to the dead time for the device and was 11 μs. In the data presented as Fig. 2A and Fig. 3 of the main text, the time axis was presented by subtracting 11 μs from the values calculated by setting the first observable point of the observation channel as zero.

The available experimental data supported that the flow pattern of samples in the region between the mixing point and the first observable point is non-laminar, since the efficient mixing is achieved in this region (Fig. 1B of the main text). Furthermore, the flow pattern in the observation channel can be considered as uniform, since the observed decay kinetics of the NATA fluorescence at the different concentrations of NBS can be consistently analyzed by the single exponential curves (Fig. 2A of the main text).

Observation of cyt* c *folding

The folding reactions were initiated by a two-fold dilution of the acid denatured cyt *c* solution at pH 2.0 with 200 mM acetate and 200 mM phosphate buffer to the final pH of 4.5 and 6.0, respectively (Fig. 3 of the main text). The final protein concentration was determined by using the optical absorbance at 409 nm for the native protein (ε_{409} = 106 mM^{-1} cm^{-1}), and was 750 μM. The fluorescence intensity of Trp59 (I) in the time range within 140 μs were fitted by a single-exponential curve described in equation (2):

$$I = I_0 \cdot \exp(- k \cdot t) + I_f \qquad (2),$$

where I_0, k, t, and I_f represent amplitude, rate constant, time after the mixing corrected by subtracting 11 μs as explained above, and final intensity, respectively. The standard deviations of the fluorescence intensity data were used as weighting parameters for the fitting. The fitted values for I_0 were 0.626 (0.006) and 0.64 (0.01) at the final pH of 4.5 and 6.0, respectively. The values for k were 11700 (400) s^{-1} and 21600 (800) s^{-1} at pH 4.5 and 6.0, respectively. The values for I_f were 0.34 (0.01) and 0.334 (0.005) at pH 4.5 and 6.0, respectively. The values in parenthesis denote standard deviations obtained from the fitting.

The goodness of the fitting was verified by examining the χ^2 values. For the datum obtained at pH 4.5, the χ^2 value of the above single exponential fitting was 525. The fitting of the same datum based on the standard double exponential function with five fitting parameters gave the same χ^2 value of 525. Thus, the fitting based on the single exponential function was enough for the datum at pH 4.5. In the case for the datum obtained at pH 6.0, the χ^2 value of the single exponential fitting was 206. The fitting based on the double exponential function gave the χ^2 value of 184. Since the improvement in the χ^2 value was small, we concluded that the single exponential fitting is the simpler and better approximation of the collapse process observed at pH 6.0.