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Application of micro-reactor chip technique for millisecond quenching of deuterium incorporation into 70S ribosomal protein complex

Tatsuya Yamamoto[a,b], Yoshihiro Shimizu[c], Takuya Ueda[c], Yoshitsugu Shiro[d,*], Makoto Suematsu[a,b,1]

[a] JST, ERATO, Suematsu Gas Biology Project, Tokyo 160-8582, Japan
[b] Department of Biochemistry, School of medicine, Keio University, Tokyo 160-8582, Japan
[c] Department of Medical Genome Science, Graduate School of Frontier Sciences, The University of Tokyo, 5-1-5, Kashiwanoha, Chiba 277-8562, Japan
[d] Biometal Science Laboratory, RIKEN SPring-8 Center, Harima Institute, 1-1-1 Kouto, Sayo, Hyogo 679-5148, Japan

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ABSTRACT

The hydrogen/deuterium exchange (HDX) method is useful to analyze kinetics of large macromolecular complexes, although its time resolution requires further improvement. A newly developed micro-reactor chip was made of polydimethylsiloxane with a 100-μm deep and wide microchannel. The channel in the chip has two mixing points of Y-shaped flow and allowed us to shorten time durations from the start to quenching for the HDX in 70S ribosome with high temporal resolution. This device enabled us to quench the deuterium incorporation at as early as 20 ms, detecting structural changes of individual ribosomal proteins in solution at the time scale comparable to a single reaction cycle for the peptide elongation. The profile of deuterium incorporation in individual proteins of the complex was superimposed on the X-ray crystal structure to depict the surface HDX map, revealing localization of protein movement in the ribosome. The current method serves as a useful method to visualize the regional movement of large macromolecules with high temporal resolution sufficient to examine protein dynamics.

1. Introduction

Hydrogen/deuterium exchange (HDX) study of proteins is a quantitative technique to examine protein dynamics and solvent accessibility through determining the exchange rates and ratios for the replacement of amide hydrogen with deuterium on a main chain of the protein of interests [1]. When combined with X-ray crystallography, the HDX analysis by mass spectrometry provides information of structural dynamics of proteins in solution, indicating functional movements and interactions in the macromolecular complexes. This technology thus enables us to develop new fields in protein dynamics and functional intermediates [2,3].

The *E. coli* 70S ribosome is a heterogeneous macromolecular complex (MW: 2.3 MDa) comprised of 55 proteins (these proteins are named as "S1, S2, ⋯" and "L1, L2, ⋯") which are assembled into the complicated and asymmetric structure by noncovalent intermolecular interactions with three ribosomal RNAs (rRNA). In previous studies, information collected by X-ray crystallography combined with cryo-electron microscopy provided evidence for structural differences in regional domains before and after the peptide elongation step [4–7]. However, such information is rather static, lacking in dynamic characterization of the movement of the domains and their spatio-temporal relationship to mechanisms for peptide elongation.

The HDX analysis supported by MALDI-TOF MS might serve as a potentially important method that overcomes the aforementioned limitation [8,9]. For rapid HDX measurements with reliable temporal resolution, several technical difficulties remain to be overcome: First, reducing amounts of individual reactants and proteins is crucial to achieve high temporal resolution. Such an approach was first performed in a previous study using multiple glass capillaries and connectors [10]. Second, reliable mixture of the reactants would be confirmed in the microreactor circuit. Finally, of great importance is accurate termination of the reaction in the circuit prior to sample transfer to MALDI-TOF MS. Taking these factors into accounts, we have herein developed a novel micro-reactor chip that made it possible to carry out accurate pulse labeling of deuterium and to achieve millisecond order of time resolution for reliable mixing and quenching the reactions, allowing us to analyze structural movement of 70S ribosome at the millisecond order in solution. The profile of deuterium incorporation into ribosomal proteins was superimposed on the X-ray crystal structure to depict the HDX surface map, revealing localization of protein movement in this

Abbreviations: HDX, hydrogen/deuterium exchange; MALDI, matrix-assisted laser desorption/ionization; MS, mass spectrometry; TOF, time of flight.

* Corresponding author. 1-1-1 Kouto, Sayo, Hyogo, 679-5148, Japan. Fax: +81 791 58 2817.
E-mail addresses: yshiro@riken.jp (Y. Shiro), msuem@sc.itc.keio.ac.jp (M. Suematsu).
[1] Tokyo 160-8582, Japan. Fax: +81 3 5363 3466.

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macromolecule ribosome at the time scale for the reaction cycle of peptide elongation.

2. Material and methods

2.1. Design of the micro-reactor chip

Micro-reactor chips have widely been utilized in device fields of chemistry to result in many advantages including down-sizing volumes of chemical reactants and shortening the reaction time. Furthermore, these methods allowed us to design the shape and length of flow path that guarantee the accurate control for starting and quenching chemical reactions as desired conditions [11].

We herein designed the micro-reactor chip as depicted in Fig. 1. Solutions mainly flow through channels with 100-μm depth and width, mixing with different solutions at two Y-shaped channels. After passing at these mixing sites, zigzag area (2.7 nL) with a path of 50-μm wide was placed to guarantee sufficient mixing of the samples flowing from the different paths. Such a micro-reactor chip was made of polydimethylsiloxane by Fluidware Technologies Inc., and the mixing efficiency was observed through a microscope (BX-41, OLYMPUS Corporation).

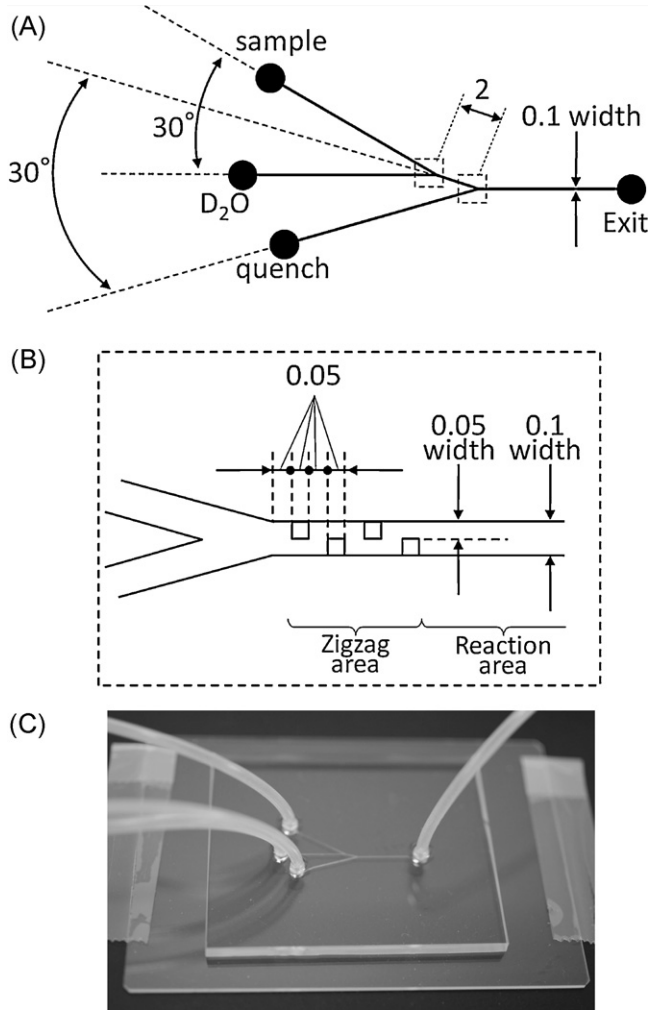

Fig. 1. Designed micro-reactor chip. A and B are drawings for designs of the micro-reactor chip and the mixing channel, respectively. C is an appearance picture of the micro-reactor chip. The unit of widths are mm.

2.2. Chemicals

Ribosome were purified from *E. coli* A19 by hydrophobic chromatography and ultracentrifugation with a sucrose cushion buffer [12], and were stored at −80 °C after resolving in 10 mM Tris buffer (pH 8.6) including 7 mM β-mercaptoethanol and 10 mM magnesium acetate. D_2O (99.9% atomic D) and acetic acid-*d* (99% atomic D) were purchased from EURISO-TOP and IsoTec, respectively. All other chemicals were of analytical grade.

2.3. Normal quenching of HDX

Deuterium incorporation was initiated by mixing 90 μL of D_2O with 9 μL aliquots of approximately 10 μM ribosome solution in 1.5-mL centrifuge tubes at 22 °C; the H:D atomic ratio was 1:10. The pH of mixture in reaction was 7.1 – 7.2, and the Mg^{2+} concentration was prepared as 5 mM. After 20, 60, 600, 1200, and 12000 seconds, 5 μL of the reaction mixture was removed, and the exchange reaction was quenched by adding 2 μL of 10% acetic acid (H:D = 1:10, pH 2.9) and freezing with liquid nitrogen. The HDX experiment for 200 min was a separate experiment using similar methods in previous studies [8,9].

2.4. Rapid quenching of HDX

Deuterium incorporation was initiated by mixing D_2O solution with ribosome solution at 22 °C in a designed micro-reactor chip; the H:D atomic ratio was 1:10. After 20 and 148 milliseconds, the reaction mixture was substantially quenched by mixing with 10% acetic acid (H:D = 1:10, pH 2.9); the reaction solution: acetic acid ratio was 5: 2. About 7 μL of substantial quenched solutions were frozen with liquid nitrogen. All solutions were flowed in micro-reactor by syringe pumps (Model 11 plus and PHD2000, HARVARD).

2.5. Mass spectrometry

A portion of quenched solution was mixed with 10 mg/mL 3,5-dimethoxy-4-hydroxycinnamic acid in 70% acetonitrile containing 0.2% trifluoroacetic acid, and loaded on the sample plate for MALDI. At three minutes after loading the sample plate under a pressure of 7 Pa, the plate was set up for MALDI-TOF mass spectrometer at 10^{-4} Pa.

All mass spectra were measured using MALDI-TOF mass spectrometer (Voyager DE-PRO, Applied Biosystems). Peaks were identified in a previous study [8], and overlapped peaks were deconvoluted to each peak as Gaussian type by the graph software. The spectrum was distributed over a wide range of masses, and hence was calibrated by separating into six regions: 3000 – 7000, 7000 – 11000, 11000 – 15000, 15000 – 20000, 20000 – 25000, and 25000 – 32000 m/z. The spectra of deuterated ribosomes showed similar peak patterns, and it was possible to detect 52 peak shifts associated with HDX. Thus, we could use MS to successfully analyze deuterium incorporation of 52 ribosomal proteins.

2.6. Analysis

All masses were read from the centroid values of each peak. Deuterium incorporation, D, was calculated by the following equation:

$$D = (M_t - M_{side})/(M_{100\%} - M_{side})$$

Where M_t is the mass of each protein after exposed time (t) to D_2O from starting H/D exchange. $M_{100\%}$ and M_{side} are fully deuterated masses of all residues and side chains only, respectively, at H:D = 1:10. Then, the exchange of side-chain protons is completely