Pulse Frequency-dependent Regulation of Lysozyme Reactivity under Pulsed Ultrasound Irradiation

Takayoshi Kawasaki, Momoko Toyoda, and Yoshio Okahata*
Department of Biomolecular Engineering and Frontier Collaborative Research Center, Tokyo Institute of Technology, B-53, 4259 Nagatsuta-cho, Midori-ku, Yokohama 226-8501

(Received March 16, 2009; CL-090267; E-mail: yokahata@bio.titech.ac.jp)

Hydrolytic reactivities of hen egg white lysozyme under 0.05–0.5 MHz pulsed ultrasound irradiation (base frequency: 1 and 14.5 MHz) could be controlled by a pulse frequency of ultrasound.

In functions of enzyme proteins, a considerable number of studies have shown that structure fluctuation is of significant importance.[1] While molecular motions of proteins and enzymes take on a wide range of time constants, domain motions that perform a crucial role in the enzyme function have time scales in the range of 10^{-7} to 10^{-3} seconds (10^{3}–10^{7} Hz).[1b] Slower time constants correspond to the larger domain motions. In this context, we have applied ultrasound irradiation to artificially control the conformational motion of enzymes, and we examined the influence of ultrasound on enzyme activities. As a result, we demonstrated that the activity of thermolysin, a metalloprotease that has been well researched with regard to the relationship between molecular fluctuation and its reactivity,[2] was modulated with continuous 0.1–0.3-MHz ultrasound irradiation depending on its frequency.[3] Furthermore, we showed that the DNA polymerization activity of the Klenow fragment from *E. coli* can also be modulated with continuous 0.8-MHz ultrasound.[4] These phenomena are mainly caused by the ultrasound perturbation of enzymatic molecular motion that has an important effect on the formation of the enzyme–substrate complex.

However, there is a huge gap between the frequencies of ultrasound used in previous studies [10^{4}–10^{6} Hz ($=s^{-1}$)] and the time scale of conformational motion [10^{-9}–10^{-3} s ($=10^{3}$–10^{9} s^{-1} in frequency)] and turnover number (10^{-3}–$10\,s^{-1}$) of enzymes. This time gap remains to be explored. And in this study, we propose a novel approach to this issue. If large and slow molecular motion could be achieved by the sum of fast and small sub-motions of polypeptides as mentioned by Hammes,[5] the middle range pulse of fast perturbations would cause slower motion of large domain. In particular, we applied pulsed ultrasound herein to control protein properties by not only the base frequency of the ultrasound but also the pulse frequency for the precise modulation of enzyme activities.

For instance, the molecular fluctuations around the active site of lysozyme with a time scale both from picoseconds to nanoseconds and from microseconds to milliseconds are important for its function.[6] In the present study, we investigated the influence of 0.05–0.5-MHz pulse (corresponding to the middle-range motion of domains in proteins) of 1- and 14.5-MHz base (corresponding to the high-speed motion of side chains or small domains) ultrasound irradiations on the lysozyme reactivity. With these pulsed ultrasounds, we aim to control enzyme functions with the total slower large domain motion (Figure 1A).

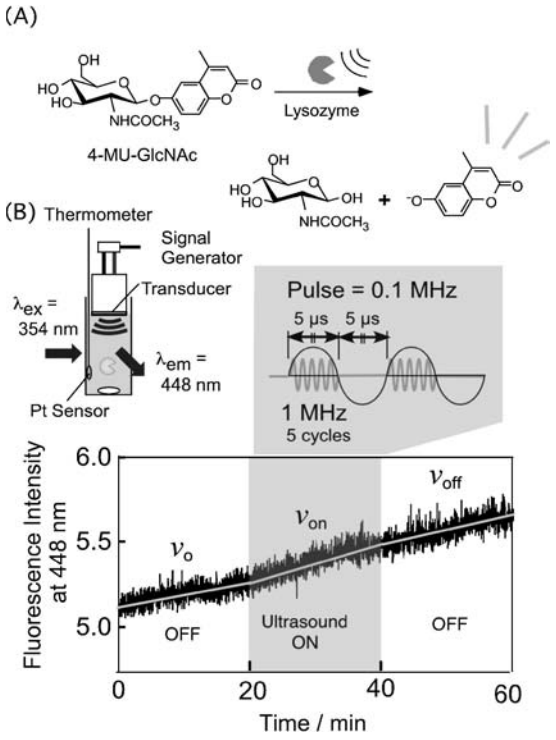

Figure 1. (A) A schematic illustration of the hydrolysis reaction of 4-methylumbelliferyl *N*-acetyl-β-D-glucosaminide (4-MU-GlcNAc) by lysozyme, and (B) the experimental setup and a typical time course of the hydrolysis followed by fluorescent emission at 448 nm under 0.1 MHz pulsed ultrasound (5 ms intervals of on/off) of the 1-MHz base frequency.

As an ultrasound generator, we used a piezoelectric element (7BB-20-6L0, Murata Mfg., Kyoto, Japan). The piezoelectric element was coated with a silicone-curing agent. The oscillation characteristics of ultrasound generators that we used were evaluated by a 27-MHz quartz crystal resonator as the ultrasound receiving equipment. We used two ultrasound generators with resonance frequencies at 1 and 14.5 MHz, respectively.[3] The lysozyme from chicken egg white was purchased from Sigma (L6876) and used without further purification. 4-Methylumbelliferyl *N*-acetyl-β-D-glucosaminide (4-MU-GlcNAc) was used as a substrate of the lysozyme hydrolysis reaction. The amount of umbelliferone, a fluorescent hydrolytic product, was monitored at $\lambda_{em} = 448$ nm ($\lambda_{ex} = 354$ nm) with a fluorescence spectrometer (FP-6300, Jasco, Tokyo, Japan) (Figure 1B). A $4 \times 1 \times$ 1-cm acryl cell was used as a reaction cell. The ultrasound generator was placed on top of the reaction solution in the cell, and 0.05–0.5-MHz pulsed signals were generated by a function

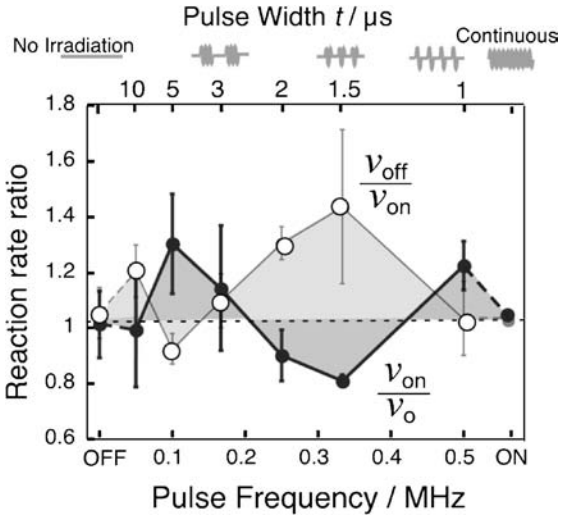

Figure 2. Pulse frequency dependency on reaction rate ratio (v_{on}/v_o and v_{off}/v_{on}) of the hydrolysis of 4-MU-GlcNAc catalyzed by lysozyme under the irradiation of pulsed 1-MHz ultrasound. [lysozyme] = 10.5 μM, [4-MU-GlcNAc] = 342 μM, 20 mM MES, pH 6.0, 20 °C.

synthesizer (WF1956, NF Corp., Tokyo, Japan) and applied to the ultrasound generator. The pulse width was just half of one cycle. Therefore, ultrasound was generated at half time of the total irradiation time at each condition.

As shown in Figure 1B, the pulsed ultrasound was applied to the reaction solution for 20 min after a 20-min reaction without ultrasound. Reaction monitoring was maintained for more than 20 min after stopping the ultrasound irradiation. The 0.1-MHz pulsed ultrasound indicates the 5 μs intervals of on/off of 1-MHz base ultrasound irradiation, as shown in inserted illustrations of Figures 1B and 2. Effects of the pulsed ultrasound irradiation were examined by comparing three reactive velocities: v_o, before the irradiation; v_{on}, under the irradiation; and v_{off}, after the irradiation. The reaction solution was stirred and the solution temperature was controlled by a circulator and monitored in 1/1000 °C. There was little change in temperature (within ±0.01 °C) under the ultrasound irradiation.

Figure 2 shows the pulse frequency dependency on the relative rate velocity under ultrasound against the initial velocity (v_{on}/v_o), and on the relative rate velocity after ultrasound irradiation against the rate velocity under irradiation (v_{off}/v_{on}) with 1 MHz as the base ultrasound frequency. Continuous ultrasounds have little effects on the lysozyme activity. Although 30% enhancement in the relative activity was found at the 0.1-MHz pulse frequency by the 1-MHz ultrasound irradiation (v_{on}/v_o), the reactivity was reduced by about 20% by the ultrasound around at the 0.3-MHz pulse. On the other hand, the v_{off}/v_{on} values show clearly opposite changes against v_{on}/v_o values. This means that the reactive velocity returns to the initial velocity after stopping the pulse ultrasound irradiation. In other words, the pulsed ultrasound effect is completely reversible in the enzymatic reaction. With a 14.5-MHz ultrasound generator, similar pulsed ultrasound dependency was observed (Figure S1).[9] The 1- and 14.5-MHz ultrasounds may produce the same effect on the enzyme property.

The pulse length dependency on lysozyme reactivity with 0.1-MHz pulse ultrasound (14.5 MHz) irradiation was also investigated. It was demonstrated that the half time of one cycle is the most effective pulse length under our conditions (Figure S2).[9] This may come from the harmonic oscillation feature of the enzymatic motion mode excited by the pulse ultrasound.

If the ultrasound irradiation serves as a stirrer or a thermal source of a molecular level, it is easy to understand the ultrasound effect as the reaction activator. However, the fact that the pulse irradiation at a specific frequency rather suppresses the enzymatic reaction refutes these hypotheses and is intriguing in the following two points. One is a possibility of the precise control of the enzymatic reaction. For the simple regulation of the enzymatic reaction, temperature control should be the simplest method. However, the pulse-frequency dependent reactivity control of the specific reactions will allow the total regulation of whole chemical reaction series under the existence of multiple enzymes and/or substrates. The second point is related to the time correlation of the molecular motion and the function of enzyme. Although some studies show the strict relationship between the specific molecular movement and the catalytic mechanism of the enzymatic reaction in the same time scale,[7,8] it is difficult to monitor the temporal sequences of enzymatic motion and the reaction steps.

In this study, we combined two time scales by using pulsed ultrasound with the middle-range pulse (sub MHz) of the base high frequency ultrasound (1 and 14.5 MHz). We speculate that these vibrational perturbations would correspond to the time constants of domains and side chains motions of the enzyme. Furthermore, the sum effect of a pulsed ultrasound causes a total molecular motion of large domains that have crucial roles at the substrate binding, the product release and/or catalytic mechanism. In this case, a pulsed ultrasound irradiation methodology will provide a novel technique to study the time series variations of the enzymatic hierarchical motions based on their function.

References and Notes

1 a) A. Fersht, *Structure and Mechanism in Protein Science—A Guide to Enzyme Catalysis and Protein Folding*, W. H. Freeman and Company, New York, **1999**. b) M. Daune, *Molecular Biophysics—Structure in Motion*, Oxford University Press, **1999**.
2 S. Kidokoro, Y. Miki, K. Endo, A. Wada, H. Nagao, T. Miyake, A. Aoyama, T. Yoneya, K. Kai, S. Ooe, *FEBS Lett.* **1995**, *367*, 73.
3 T. Kawasaki, Y. Hoshino, Y. Ishizu, Y. Mizushiro, Y. Okahata, *Chem. Lett.* **2005**, *34*, 1602.
4 Y. Hoshino, T. Kawasaki, Y. Okahata, *Biomacromol.* **2006**, *7*, 682.
5 G. G. Hammes, *Biochemistry* **2002**, *41*, 8221.
6 S. Mine, S. Tate, T. Ueda, M. Kainosho, T. Imoto, *J. Mol. Biol.* **1999**, *286*, 1547.
7 T. Kamiyama, K. Gekko, *Biochim. Biophys. Acta* **2000**, *1478*, 257.
8 C. J. Falzone, P. E. Wright, S. J. Benkovic, *Biochemistry* **1994**, *33*, 439.
9 Supporting Information is available electronically on the CSJ-Journal Web site, http://www.csj.jp/journals/chem-lett/index.html.

Published on the web (Advance View) April 25, 2009; doi:10.1246/cl.2009.536