Pulsed Ultrasound Effect on DNA Polymerase Reaction Monitored on a QCM

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DNA polymerase reaction under pulsed ultrasound irradiation was investigated on a DNA-immobilized quartz-crystal microbalance (QCM), and the 240-kHz ultrasound irradiation pulsed at $20\,s^{-1}$ increased the polymerization reactivity owing to the increase of the stability of the DNA/polymerase/monomer complex, although the continuous irradiation decreases the stability.

Structural motions of enzymes are important for their functions. Over the past few decades, a considerable number of studies have been made on molecular dynamics of enzymes in catalytic processes.[1,2] However, time scale correlations of the function and the motion of these molecular movements of enzymes are still not clear. For instance, the structural fluctuation of enzymes in pico- to nanosecond time scale has been considered as essential to catalytic function.[3] At the same time, Daniel et al. demonstrated that these fast enzyme dynamics of some enzymes do not govern catalytic reactions directly.[4,5] On the other hand, the significance of micro- to millisecond time scale motion has been also noted.[6–8]

In order to focus on these relatively slow enzyme motions, we have utilized ultrasound at several tens to several hundreds of kHz to modulate these molecular motions and control enzyme activities. For instance, in the DNA polymerase reaction catalyzed by Klenow fragment (KF) on a DNA-immobilized quartz-crystal microbalance (QCM), stabilities of the reactive complex and the reaction rate were modulated with 80-kHz ultrasound.[9] Furthermore, we demonstrated reaction controllability of thermolysin with 100–150-kHz and 500–900-kHz ultrasound.[10] Therefore, we speculated that artificial external vibration in the time scale range of 1 μs to 0.1 ms (10^3–10^6 Hz) could perturb the molecular fluctuation of the enzyme.

However, the time scale of the ultrasound frequency used in these studies (10^5 Hz) is more than three orders of magnitude higher than the catalytic turnover number of KF ($1–100\,s^{-1}$). This drives us to the question whether these time scales interrelate with each other. And if there are some correlations between them, how can we deal with them? To address this issue, we used pulsed ultrasound here. We hypothesize that the ultrasound frequency would correspond to the molecular fluctuation of the enzyme, on the other hand, the pulse frequency would respond to the reaction kinetics. In this study, KF reactivity was monitored on a QCM system under pulsed ultrasound irradiations. We used ultrasound at 240 kHz as a base. The 240 kHz is also an effective frequency. DNA chain elongation activity dependency on pulse frequency was investigated.

The experimental system used here is identical with that used in the previous study (Figure 1),[9] and experimental procedure has been mentioned elsewhere.[10,11] A PZT ($Pb(Zr_xTi_{1-x})O_3$) ultrasound oscillator (6-mm diameter) was attached to the bottom of a stirring bar to generate the pulsed ultrasound from the top of the aqueous solution (Figure 1A). The pulse signal

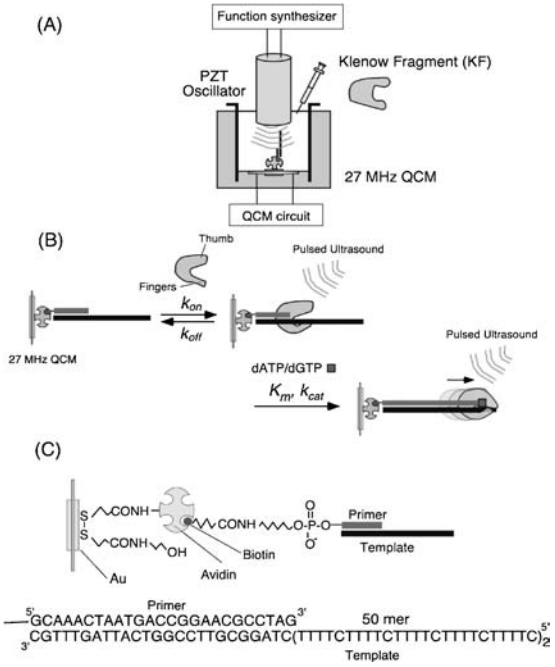

Figure 1. (A) An experimental setup of polymerase (Klenow Fragment, KF) reactions on a template/primer-immobilized 27-MHz quartz-crystal microbalance (QCM, Affinix Q4) under ultrasonic irradiation (240 kHz) from a PZT oscillator, (B) the reaction scheme and kinetic parameters obtained in this work, in which KF structures are drawn as an open form before binding, a thumb-closed form at the DNA/KF complex, and a thumb- and fingers-closed form at the ternary (DNA/KF/monomer) complex, according to refs 16–18, and (C) the illustration of DNA-immobilization by biotin–avidin interaction, and the sequence of the template/primer DNA.

of 240 kHz sine waves with $2\,V_{p\text{-}p}$ amplitude was regulated by a function synthesizer (WF1956, NF Co., Yokohama). The electric power consumption in our condition was below 1.7 mW, and cavitation could not be observed. Although ultrasound physically causes frequency shift on the QCM, surface condition absolutely restituted to the initial state after ultrasound irradiation. One pulse length of ultrasound was fixed at 10 ms each, and the interval time was modulated to be corresponding to a set pulse frequency. Therefore, a 100-Hz pulse frequency irradiation corresponds to the continuous ultrasound irradiation in this study.

Figure 2 shows typical frequency changes as a function of time of the primer/template-immobilized QCM, responding to the addition of KF as a DNA polymerase or dATP/dGTP = 1/4 as complementary monomers under the presence and absence of pulsed ultrasound irradiation ($60\,s^{-1}$) in the aqueous solution. When KF was injected at the first arrow (54 nM), the frequency decreased (mass increased) gradually for ca. 20 min because of the slow binding of polymerase on the primer/template.[11] This binding process of KF to the DNA was hardly

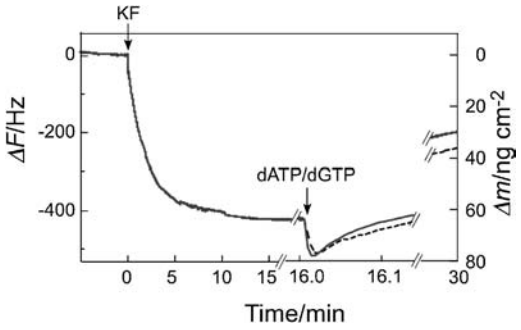

Figure 2. Typical time courses of frequency changes of the primer/template-immobilized QCM, responding to the addition of KF and monomers (dATP/dGTP = 1/4), under absence (dashed line) and presence (solid line) of the pulsed ultrasound irradiation ($60\,s^{-1}$ pulse of 240 kHz). 20 °C, pH 7.5, 10 mM Tris buffer, 7 mM $MgCl_2$, 0.1 mM DTT, [KF] = 54 nM, [dATP] = 112 mM, [dGTP] = 28 μM.

affected by the ultrasound irradiation under these conditions. When dATP/dGTP monomers were added excessively at the second injection, the mass rapidly increased within 1 min owing to the elongation of DNA along the template and then slowly decreased owing to the release of KF from the polymerized DNA (Figure 2). The ultrasound irradiation seems to affect the initial slope of the elongation step. And this slope of the rapid mass increase corresponds to the initial elongation rate (v_o) of KF along the template. The DNA elongation process obeys to the Michaelis–Menten kinetics.[11] Therefore, the kinetic parameters, K_m and k_{cat}, of the polymerization process on the DNA can be determined by changing the monomer concentration as previously reported.[9,11]

Figure 3 shows the pulse frequency dependency on KF activity and kinetic parameters. Although the 240-kHz continuous ultrasound irradiation at 100 pulse s^{-1} inhibited the activity (the apparent second-order rate constant, k_{cat}/K_m) by half, 1.4-fold enhancement of reactivity was achieved by 20 pulse s^{-1} ultrasound compared to non-ultrasound conditions (OFF) as shown in Figure 3. These activity changes of k_{cat}/K_m depending on the pulse frequency mainly seem to follow the K_m values. In previous studies, we have demonstrated that ultrasound may affect the monomer binding process to the DNA/KF complex of enzymatic reactions.[9] The present results also reveal that the pulsed ultrasound would modulate the monomer-binding feature with molecular motions of the enzyme in the DNA/KF complex in the time scale of the pulse frequency.

It is widely believed that the conformational motions of KF are critically important for enzymatic reaction.[12,13] The thumb-shaped domain grips DNA substrate, and then the large conformational motion of the finger-shaped domain, open to closed conformation, would occur by incoming dNTP monomer.[14,15] The time scale of these conformational changes of KF is in the same order (several to several tens of s^{-1})[16–18] as the pulse frequency applied here.

In conclusion, we speculate that the ultrasound pulse could affect the conformational motion of the enzyme synchronously, then the function of the enzyme would be modulated through the substrate binding and/or pyrophosphate release that require the structure changes. Pulsed ultrasound would be a novel methodology for controlling the properties of polypeptide/enzyme. The

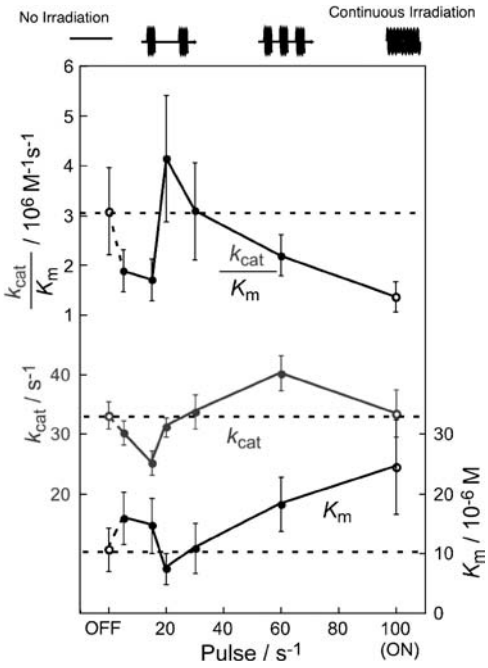

Figure 3. Pulse frequency dependency of k_{cat}/K_m, k_{cat}, and K_m of DNA polymerization of KF. 20 °C, pH 7.5, 10 mM Tris buffer, 7 mM $MgCl_2$, 0.1 mM DTT, [KF] = 14–57 nM, [dATP] = 112 mM, [dGTP] = 28 μM.

study on the effect of the 27-MHz oscilation of the QCM itself is currently under investigation.

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