Effect of Ultrasound on DNA Polymerase Reactions: Monitoring on a 27-MHz Quartz Crystal Microbalance

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Effects of ultrasound irradiation on DNA polymerase (Klenow fragment, KF) reactions were studied on the template/primer DNA-immobilized quartz crystal microbalance (QCM). Under ultrasound irradiation, binding of KF to the DNA was suppressed due to the decrease of the binding rate constant (k_1) and the increase of the dissociation rate constant (k_{-1}). The catalytic elongation rate (k_{cat}) was increased, but the stability of the KF/DNA/monomer ternary complex (K_m) was decreased by the ultrasound irradiation. Ultrasound effects are discussed in correlation with the conformation changes of domain structures in KF.

Introduction

Conformational motions of proteins have been discussed as being essential for their functions.[1] Nevertheless, few evidence of this has been demonstrated.[4−7] For instance, a catalytic turnover number of dihydrofolate reductase (DHFR) is close to the time scale of the conformational motion of a loop domain that covers the substrate and coenzyme binding sites of DHFR ($35\ s^{-1}$).[8] Furthermore, the hinge motion of the active site of this enzyme is controlled with static pressure.[9] This means that an artificial perturbation can alter the enzyme structure. An ultrasound wave, a periodic pressure fluctuation, is also expected to control the enzyme characteristics as a dynamic perturbation. Linear polymers generally absorb a wide range of sound and ultrasound waves (10^3 to 10^6 Hz) through mechanical relaxations according to the molecular weight and relaxation mode.[10−12] Proteins, as biopolymers, also show ultrasound absorption in aqueous solution relating to conformational changes of biopolymers.[13,14] Therefore, a sound−ultrasound wave in the range of 10s to several 100s of kilohertz would perturb loop and domain motions of enzymes.[15,16] Also, these forced conformational motions of enzymes would affect the enzyme activity itself.

In this letter, we report how ultrasound affects the binding process of DNA polymerase to DNA templates and the elongation process of DNA polymerase on the DNA-immobilized 27-MHz quartz crystal microbalance (QCM). DNA polymerases were expected to be a good candidate to study the ultrasound effect, because their rate-limiting step is thought to be not the chemical phosphodiester bond-forming step but dynamic conformational changes during both the binding to DNA and elongation steps.[17−19] The Klenow fragment (KF; Mw 68 000; TaKaRa, Co.; Shiga, Japan) of DNA polymerase I from *Escherichia coli* was chosen as a DNA polymerase, because we have already completed kinetic studies of each step (binding and dissociation of KF to the DNA substrate, elongation of DNA chains, and release from the polymerized DNA) of this DNA polymerization reaction on a 27-MHz QCM.[20,21] QCMs are known to provide very sensitive mass measuring devices in aqueous solution, and their resonance frequency is observed to decrease linearly upon the increase of mass on the QCM electrode on the nanogram level.[20−25] We have applied the QCM system to investigate various biomolecular interactions such as DNA hybridization,[26] DNA−protein interactions,[27−29] enzyme reactions on DNA strands,[30−32] and enzyme reactions on polysaccharides.[33,34]

Experimental Section

A schematic illustration of the experimental setup and the reaction schemes are shown in Figure 1. An Affinix Q^4 was used as a QCM instrument (Initium Co. Ltd, Tokyo, http://www.initium2000.com) that has four 300-μL cylindrical cells (10-mm i.d.) equipped with a 27-MHz QCM plate (8-mm-diameter quartz plate and 4.9-mm^2 Au electrode) at the bottom of the cell and an up-and-down stirring bar in a temperature-controlled system.[20,21,26−34] The 27-MHz QCM was calibrated to change frequency by −1 Hz, responding to the mass increase of 0.62 ng cm^{-2} on the electrode.[26−34] The oligonucleotide consisting of a 5′-biotinylated primer and a template $(TTTTC)_{10}$ was immobilized on a clean Au electrode of the QCM using the biotin−avidin method according to the previous paper (Figure 1B).[20,21] The immobilized amount of the biotinylated primer/template was maintained at 42 ± 5 ng cm^{-2} (ca. 5.0 ± 0.1 pmol cm^{-2}). The amount corresponds to ca. 10% coverage of the surface, and this small coverage would give enough space for binding of a large enzyme molecule.

A PZT ($Pb(Zr_xTi_{1-x})O_3$) ultrasound oscillator (6-mm diameter) was attached to the bottom of the stirring bar in the Affinix Q4 to generate the ultrasound from the top of the aqueous solution (see Figure 1A). The bottom surface of the oscillator fluctuates between 2 mm and 4 mm away from the quartz crystal plate surface for stirring. The frequency (80 kHz) and amplitude (2 V_{p-p}) of the square wave were regulated by a function synthesizer (WF1956, NF Co., Yokohama). Since the loop and domain motions of proteins are thought to be perturbed in 10s to several 100s of kilohertz, we chose the 80-kHz ultrasound in this experiment.[15,16]

We believe that the weak ultrasound irradiation does not cause a simple temperature effect, since the temperature of the reaction solution was confirmed to increase just 0.01 °C by the highly sensitive thermosensor.

The power of the ultrasound in this study is too small to measure the properties of the acoustic field in the reaction cell directly. Only a laser Doppler vibrometer could detect the oscillation of the oscillator used here, and the amplitude was about 1 nm with an AC signal (2 V_{p-p}). Furthermore, the oscillator faced the QCM plate at a distance

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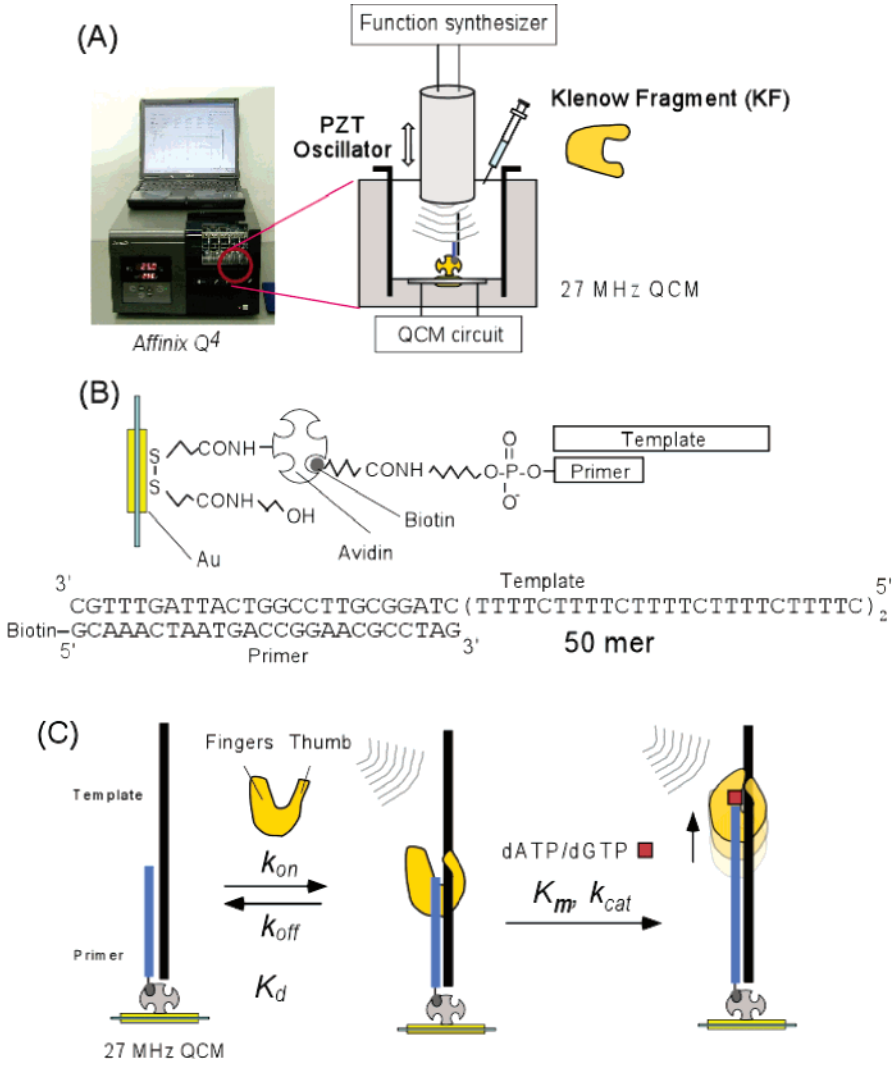

Figure 1. (A) An experimental setup of polymerase (Klenow fragment, KF) reactions on a template/primer-immobilized 27-MHz quartz crystal microbalance (QCM, Affinix Q4) under ultrasonic irradiation (80 kHz) from a PZT oscillator. (B) Chemical structures of the template/primer DNA. (C) The reaction scheme 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 17−19.

of 2 mm at the nearest point, and the oscillator attached to the bottom of the stirring bar is pumping in 4 Hz with 2-mm distance for solution stirring. Considering the wavelength of the acoustic wave used here (ca. 1.9 cm), the cell size (1 cm ϕ), and the power, it seems reasonable to suppose that a standing wave plays only a minor role in the reaction even if the standing wave of the ultrasound (including the harmonic overtone) exists in the cell.

We also confirmed from the frequency changes that DNA double strands on the QCM were not dehybridized, nor did they change their conformations, largely by ultrasound irradiation. This fact reveals that the avidin−biotin interaction that connects the primer DNA to the QCM is also maintained in the same condition.

Results and Discussion

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 in the aqueous solution with the ultrasound irradiation (80 kHz, 2 V_{p-p}) off and on. When KF was injected at the first arrow (54 nM), the frequency decreased (mass increased) gradually for 15 min due to the slow binding of polymerase on the primer/template.[20,21] Both the

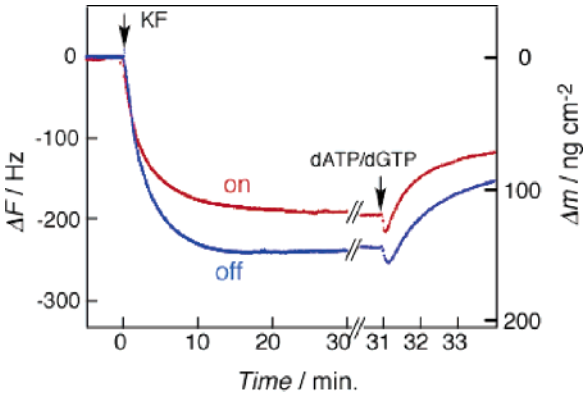

Figure 2. Typical time courses of frequency changes of the primer/template-immobilized QCM, responding to the addition of KF and monomers (dATP/dGTP = 1/4), under off and on of the ultrasound irradiation (80 kHz). 20 °C, pH 7.5, 10 mM Tris buffer, 7 mM $MgCl_2$, 0.1 mM DTT, [KF] = 54 nm, [dATP] = 112 mM, [dGTP] = 28 μM.

binding rate and the binding amount of KF under ultrasound seem to be slower and smaller, respectively, than those of the nonirradiated condition. When dATP/dGTP monomers were added in excess at the second injection, the mass rapidly

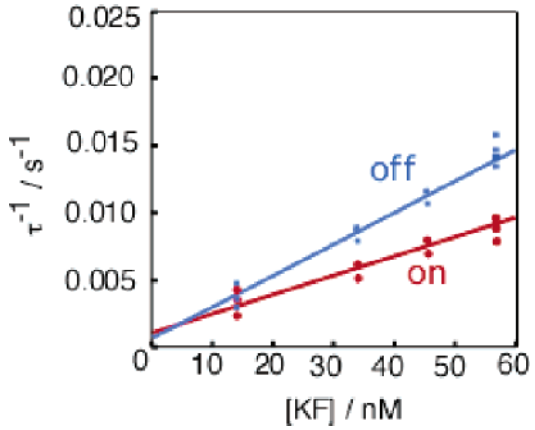

Figure 3. Plots of relaxation time (τ) against KF concentrations at the binding process according to eq 4 in the text, under off and on of the ultrasound irradiation (80 kHz). 20 °C, pH 7.5, 10 mM Tris buffer, 7 mM $MgCl_2$, 0.1 mM DTT, [KF] = 14−57 nM, [dATP] = 112 mM, [dGTP] = 28 μM.

Table 1. Kinetic Parameters of Polymerase Reactions with Ultrasound Irradiation (80 kHz) Off and On[a]

	binding process			catalytic elongation process		
ultra-sound	k_1 10^5 M^{-1} s^{-1}	k_{-1} 10^{-4} s^{-1}	K_d 10^{-9} M	K_m 10^{-7} M	k_{cat} s^{-1}	k_{cat}/K_m 10^{-5} M^{-1} s^{-1}
off	2.2	4.5	2.0	9.0	10	1.1
on	1.5	9.7	6.7	17	22	1.3

[a] 20 °C, pH 7.5, Tris buffer, 7 mM $MgCl_2$.

increased within 1 min, due to the elongation of DNA along the template, and then slowly decreased, due to the release of KF from the polymerized DNA.[20,21] The ultrasound irradiation seems to slightly affect the initial slope of the elongation step.

The binding process is determined by eq 1. The amount of the DNA/KF complex formed at time t is given by eqs 2−4.[20,21]

$$\mathrm{DNA} + \mathrm{KF} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \mathrm{DNA/KF} \tag{1}$$

$$[\mathrm{DNA/KF}]_t = [\mathrm{DNA/KF}]_\infty \left\{1 - \exp\left(\frac{-t}{\tau}\right)\right\} \tag{2}$$

$$\Delta m_t = \Delta m_\infty \left\{1 - \exp\left(\frac{-t}{\tau}\right)\right\} \tag{3}$$

$$\tau^{-1} = k_1[\mathrm{KF}] + k_{-1} \tag{4}$$

The relaxation time (τ) of KF binding was calculated from the curve fitting of the first QCM frequency changes at different KF concentrations (14−57 nM), and linear correlations were obtained with ultrasound irradiation both off and on, as shown in Figure 3. The binding and dissociation rate constants (k_1 and k_{-1}) and dissociation constant ($K_d = k_{-1}/k_1$) were obtained according to eq 4, and the results are summarized in Table 1.

In the binding process of KF to the primer/template DNA, the ultrasound irradiation increased the K_d value threefold from 2.0×10^{-9} M to 6.7×10^{-9} M (see Table 1). This is explained by both the decrease of the binding rate constant (k_1) and the increase of the dissociation rate constant (k_{-1}) by the ultrasound irradiation. Li and co-workers reported from X-ray crystallography that DNA polymerase I from *T. aquatics* opens a thumb domain and closes it when the DNA substrate is bound (see Figure 1C).[19] Thus, the ultrasound irradiation may stabilize

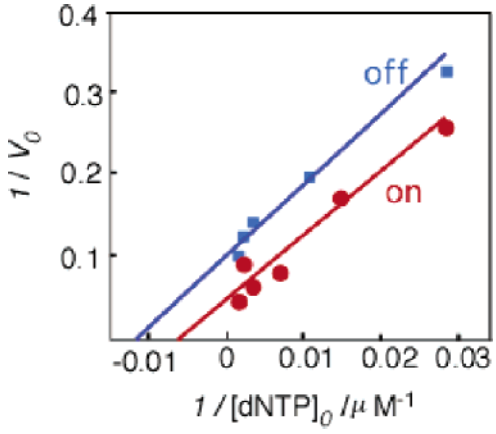

Figure 4. Lineweaver−Burk plots at the catalytic elongation process under off and on of the ultrasound irradiation (80 kHz). 20 °C, pH 7.5, 10 mM Tris buffer, 7 mM $MgCl_2$, 0.1 mM DTT, [KF] = 54 nM, [dATP] = 10−600 μM, [dGTP] = 2.5−125 μM.

the open style, and this causes the decrease of the affinity to the DNA substrate, due to the decrease of the binding rate constant and the increase the dissociation rate constant.

When dATP/dGTP monomers were added in excess at the second injection, the mass rapidly increased within 1 min and then slowly decreased to the constant value (see Figure 2). When only dGTP monomer was injected into the solution, the mass increasing profile was not obtained with or without ultrasound irradiation. Because the template used here has the TTTT-sequence at the 3′-terminus, the polymerization process requires dATP monomer. This fact indicates that the mass increasing profile corresponds to the polymerization process. Furthermore, we may say that ultrasound did not negatively affect the monomer recognition property of the DNA polymerase.

The slope of the rapid mass increase corresponds to the initial elongation rate (v_o) of KF along the template. The DNA elongation process is expressed in the Michaelis−Menten equation as shown in eqs 5−7, where DNA′ indicates elongated DNA.[20,21]

$$\mathrm{DNA/KF} \overset{K_m}{\longleftrightarrow} \mathrm{DNA/KF/dNTP} \xrightarrow{k_{cat}} \mathrm{DNA'/KF} \tag{5}$$

$$v_o = \frac{k_{cat}[\mathrm{DNA/KF}]_0[\mathrm{dNTP}]}{K_m + [\mathrm{dNTP}]_0} \tag{6}$$

$$\frac{1}{v_0} = \frac{K_m}{k_{cat}[\mathrm{DNA/KF}]_0}\frac{1}{[\mathrm{dNTP}]} + \frac{1}{k_{cat}[\mathrm{DNA/KF}]_0} \tag{7}$$

When concentrations of (dATP/dGTP = 4/1) were increased in the range [dATP] = 10−600 μM, the initial elongation rate (v_0) showed saturation behavior according to eq 6. The dissociation constant of dNTP monomers (K_m) and the elongation catalytic rate constant (k_{cat}) were obtained from the reciprocal plot of Figure 4 according to eq. 7, with the ultrasound irradiation off and on. The results are summarized in Table 1.

In the elongation process, the ultrasound irradiation increases both the K_m of dNTP monomers from 9.0×10^{-7} M to 17×10^{-7} M and the k_{cat} from 10 s^{-1} to 22 s^{-1}. As a result, the apparent elongation activity (k_{cat}/K_m) seems not to be affected by the ultrasound (see Table 1). During the elongation of a single nucleotide, polymerase I has been reported to close tightly a fingers domain to form a stable ternary complex and open again after a formation of a chemical bond (see Figure 4).[19] The rate-limiting step of the elongation is thought to be not the chemical

phosphodiester bond formation nor the monomer binding, but the dynamic conformation change of the ternary DNA/KF/monomer complex.[17,18] The ultrasound wave seems to accelerate the motion of the ternary complex, and as a result, the stability of the ternary complex decreases (K_m increases), but the catalytic rate constant (k_{cat}) increases. When the reaction temperature was increased from 20 °C to 30 °C, the K_m value was decreased and k_{cat} value increased. Thus, the temperature rise simply increases both the binding ability and the catalytic elongation rate of the enzyme.[21] The ultrasound effect could not be explained simply by the temperature effect.

In conclusion, we investigated effects of the ultrasound irradiation on each step of KF polymerase reaction (the binding process of KF to the DNA substrate (k_1 and k_{-1}), and the elongation process (K_m and k_{cat}) on the 27-MHz QCM. The ultrasound wave around 80 kHz would affect the motion of the thumb and fingers domains of KF; as a result, the binding process of KF to the DNA substrate is decreased, but the catalytic elongation process is accelerated. We believe that these sound/ultrasound effects on enzyme activities will launch novel techniques of step-specific control of enzyme functions.

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