Direct Monitoring of DNA Polymerase Reactions on a Quartz-Crystal Microbalance

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Replication and transcription are key processes in the cell. DNA polymerase is one of the responsible enzymes for replication and repair of DNA along the sequence of a template strand.[1] The reaction mechanism of polymerases has been studied mainly by Benkovic et al. by measuring the accumulation of RI-labeled products as a function of time, which has been achieved by the combination of gel electrophoresis with a stopped-flow/quenched-flow technique and by measuring time-resolved fluorescent spectroscopy.[2] Despite several improvements, these techniques still have some difficulties such as the requirement of isotope-labeling of probes and of special techniques. To know the total enzyme reaction mechanism, it is more useful to monitor *in situ* all of the reaction steps such as enzyme binding, elongation along the template, and release of enzymes from the template on the same device.

In this paper, we describe that the template/primer-immobilized 27-MHz quartz-crystal microbalance (QCM) is a useful tool to detect directly and quantitatively each step of polymerase reactions in aqueous solution (see Figure 1). QCMs are known to provide very sensitive mass measuring devices in aqueous solution, and their resonance frequency is proved to decrease linearly upon the increase of mass on the QCM electrode in a nanogram level.[3−7] Three steps of (1) binding of polymerase to the primer of the immobilized DNA on the QCM (mass increase), (2) elongation of complementary nucleotides along the template (mass increase), and (3) release of the enzyme from the polymerized DNA (mass decrease), could be observed continuously from time dependencies of frequency changes of the QCM.

A 27-MHz QCM (8-mm diameter of a quartz plate and an area of 4.9 mm^2 of Au electrode) is commercially available from Showa Crystal Co., Chiba, Japan, which is calibrated to change frequency by 1 Hz responding to the mass increase of 0.62 ng cm^{-2} on the electrode.[5] The one side of Au electrode of the QCM was sealed with silicon casing to avoid contact with the ionic buffer solution.[3−5] The frequency changes were followed by a universal frequency counter (Hewlett-Packard Co., Tokyo) attached to a Macintosh Power Book 170, Apple Co. (see Figure 1). Oligonucleotides having both a primer (25 mer) and a template [Template I: $(TTTTC)_3$ or Template II: $(TTTTC)_{10}$] were immobilized on a cleaned Au electrode of the QCM using a biotin−avidin method according to previous papers.[4] The immobilized amount of the biotinylated Template I or II was maintained to be 120 ± 10 and 180 ± 10 ng cm^{-2} (ca. 6.0 ± 0.5 pmol cm^{-2}), respectively. The amount corresponds to ca. 15% coverage of the surface, and this small coverage would give enough space for binding of a large enzyme molecule.

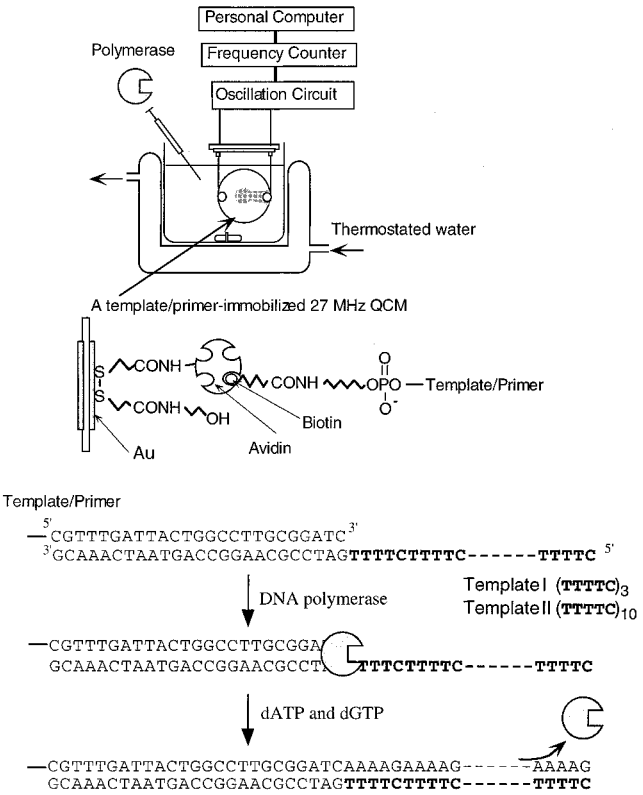

Figure 1. Experimental setup of a polymerase reaction on a template/primer-immobilized 27-MHz quartz-crystal microbalance (QCM) in buffer solution.

The Klenow fragment of DNA polymerase I from *Escherichia coli* was chosen as a polymerase because it is expected to show only polymerase activity from 5′ to 3′ along the template in this experiment.[1,8]

Figure 2 shows typical frequency changes as a function of time of the template I $(TTTTC)_3$-immobilized QCM, responding to the addition of the Klenow fragment (Mw 68 000, TaKaRa, Co.,

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(8) Polymerase I (*Escherichia coli*) usually has the 5′ → 3′ exonuclease activity for double strands and the 3′ → 5′ exonuclease activity for single strand, as well as the 5′ → 3′ polymerase activity. The Klenow fragment has the strong 5′ → 3′ polymerase activity and the weak 3′ → 5′ exonuclease activity. Since the 5′-primer is immobilized on the QCM in our system, only the polymerase activity is expected to be observed.

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Table 1. Mass Changes and Rate Constants of Each Polymerase Reaction Catalyzed by Klenow Fragment on a QCM[a]

	step 1		step 2		step 3	
template length	binding amount of polymerase/ng (pmol) cm^{-2}	K_d[b] nM	elongation amout of DNA/ng (pmol) cm^{-2}	k_{cat}[c]/s^{-1}	released amount of polymerase/ng (pmol) cm^{-2}	k_{off}[d]/s^{-1}
$(TTTTC)_3$	140 ± 10 (2.0)	3 ± 1	40 ± 5 (6.0)	8 ± 1	140 ± 10 (2.0)	0.02 ± 0.01
$(TTTTC)_{10}$	150 ± 10 (2.0)	3 ± 1	120 ± 10 (6.0)	8 ± 1	140 ± 10 (2.0)	0.02 ± 0.01

[a] Conditions: 30 °C, pH 7.8, 20 mM Tris, 10 mM $MgCl_2$, [Klenow fragment] = 7.0 pmol per 8 mL, [monomers] = 520 nmol per 8 mL. [b] Obtained from saturation binding curves by changing enzyme concentrations. [c] Obtained from the initial slopes by changing monomer concentrations. [d] Obtained from the initial slopes.

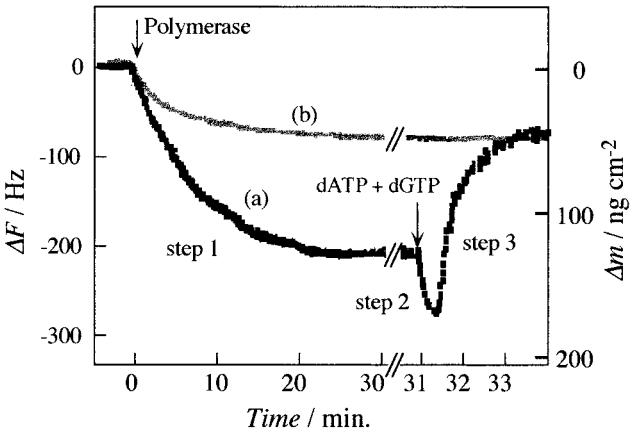

Figure 2. Typical time courses of frequency changes of the Template I $(TTTTC)_3$-immobilized QCM, responding to the addition of polymerase (Klenow fragment) and monomers (dATP and dGTP). (a) Polymerase was added at first, and then excess monomers were added after the enzyme bound. (b) Polymerase was added in the presence of excess monomers. Reaction conditions: 30 °C, pH 7.8, 20 mM Tris buffer, 10 mM $MgCl_2$, 40 mM KCl, [Klenow fragment] = 7.0 pmol per 8 mL, [dATP] = [dGTP] = 520 nmol per 8 mL.

Shiga, Japan) as a polymerase and/or dATP and dGTP as complementary monomers in the aqueous solution. In curve a, when polymerase was injected at 7.0 pmol per 8 mL of a cell, the frequency decreased (mass increased) gradually for ca. 30 min due to the slow binding of polymerase on the primer (step 1). The binding amount at equilibrium was 140 ± 10 ng (2.0 pmol) cm^{-2}, which indicates one polymerase binds per ca. 3 template/primer chains, since 6.0 pmol (120 ± 10 ng cm^{-2}) of DNA was immobilized on the QCM. When monomers were added excessively at the second injection of the curve a (520 nmol per 8 mL), the mass rapidly increased within 1 min (step 2, Δm = 40 ± 5 ng cm^{-2}) and then rapidly decreased to the constant value (step 3, Δm from the starting point is 40 ± 5 ng cm^{-2}). The elongated mass of 40 ng cm^{-2} (6.0 pmol) corresponds to the immobilized amount (6.0 pmol) of the single strand of the template $(TTTTC)_3$. The mass decrease at step 3 (140 ± 10 ng cm^{-2}) corresponds well to the mass increase due to the polymerase binding of step 1 (140 ± 10 ng cm^{-2}). When a nonsense solution of dCTP and dTTP was injected instead of the complementary monomers as a second injection, no changes in the frequency were observed. Thus, the mass increase at step 2 indicates elongation along the template, and the mass decrease at step 3 means the release of the enzyme from the completely polymerized DNA. After the release of the bound enzyme, the resulting mass increase was consistent with the mass increase at step 2 [40 ng cm^{-2} (6.0 pmol)]. The obtained results are summarized in Table 1.

When monomers of dATP and dGTP had existed in advance and then polymerase was injected at the arrow, the mass simply increased as shown in curve b and reached the same mass increase as shown in curve a. Thus, the frequency change apparently reflects the mass increase due to the elongation on the QCM. This slow frequency change may reflect the slow binding of polymerase to the template since the elongation process and the release of enzymes may proceed very fast in the presence of excess monomers.

When the long template II $(TTTTC)_{10}$ was employed at the same conditions of curve a, both the binding amount (step 1) and the release amount (step 3) of polymerase were similar to those for Template I (see Table 1). However, the mass increase due to the elongation (step 2 and the final mass increase) became ca. 3 times larger corresponding to the 3.3 times longer length of the template.

When the concentration of polymerase increased in the range of 0.7−14 pmol per 8 mL in curve a of Figure 2, the binding amount (step 1) showed a simple saturation curve against the injected concentrations. From a reciprocal plot, the dissociation constant K_d was obtained to be 3 ± 1 nM independent of the template length (see Table 1). When the monomer concentrations injected at step 2 were changed in the range of 0.5 to 10 nmol per 8 mL, the initial slope of step 2 increased linearly and the apparent elongation rate constant k_{cat} could be obtained to be 8 ± 1 s^{-1} independent of the template length. The release rate constant k_{off} could be obtained from the initial slope of step 3 to be 0.02 s^{-1} (see Table 1).

Kinetic values of K_d = 200 nM, k_{cat} = 3.8 s^{-1}, and k_{off} = 0.1 s^{-1} have been obtained in the combination of a very long $(dA)_{1000}$-$(dT)_{10}$ template/primer and the same Klenow fragment by using the rapid quenching gel electrophoresis.[2b] The smaller K_d = 5−8 nM has been obtained in the combination of the short template[9] similar to ours and the same Klenow fragment by using both the rapid quenching gel electrophoresis[2c] and time-resolved fluorescent method.[2f] Kinetic values obtained by our QCM methods were very consistent with those obtained by the conventional methods.

In summary, the QCM is useful to detect kinetically and quantitatively each step of polymerase reactions such as the binding of the enzyme, the elongation rate, and the release of the enzyme from the completely polymerized DNA as the mass changes. This is the first example to detect kinetically and quantitatively the binding, catalysis, and release processes of polymerase reactions on the same device.

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(9) The template: 3′AGCGTCGGCAGGT**TCCCAAA**5′. The primer: 5′-TCGCAGCCGTCCA3′.