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

Hisao Matsuno, Kenichi Niikura, and Yoshio Okahata*[a]

Abstract: Catalytic reactions of DNA polymerase I from *E. coli* (Klenow fragment, KF) were monitored directly with a template/primer (40/25- or 75/25-mer)-immobilized 27-MHz quartz-crystal microbalance (QCM). The 27-MHz QCM is a very sensitive mass-measuring device in aqueous solution, as the frequency decreases linearly with increasing mass on the QCM electrode at the nanogram level. Three steps in polymerase reactions which include 1) binding of DNA polymerase to the primer on the QCM (mass increase); 2) elongation of complementary nucleotides along the template (mass increase); and 3) release of the enzyme from the completely polymerized DNA (mass decrease), could be monitored continuously from the time dependencies of QCM frequency changes. The binding constant (K_a) of KF to the template/primer DNA was $10^8\,\text{M}^{-1}$ ($k_{on} = 10^5\,\text{M}^{-1}\,\text{s}^{-1}$ and $k_{off} = 10^{-3}\,\text{s}^{-1}$), and decreased to $10^6\,\text{M}^{-1}$ ($k'_{on} = 10^4\,\text{M}^{-1}\,\text{s}^{-1}$ and $k'_{off} = 10^{-2}\,\text{s}^{-1}$) for completely polymerized DNA. This is due to the 10-fold decrease in binding rate constant (k_{on}) and 10-fold increase in dissociation rate constant (k_{off}) for completed DNA strands. K_a values depended slightly on the template and primer sequences. The kinetic parameters in the elongation process (k_{cat} and K_m) depended only slightly on the DNA sequences. The repair process during the elongation catalyzed by KF could also be monitored in real time as QCM frequency changes.

Keywords: DNA replication · kinetics · Klenow fragment · quartz-crystal microbalance

Introduction

Replication and transcription are key processes in the cell. DNA polymerase is one of the key enzymes responsible for replication and repair of DNA along the sequence of a template strand. In genetic engineering, DNA polymerase has also been used in DNA sequencing. DNA polymerase I is a multifunctional enzyme involved in the replication and repair of DNA in vivo. In addition to its polymerase activity, which catalyzes the template-directed extension of a primer DNA strand, the enzyme also processes distinct $3' \rightarrow 5'$ and $5' \rightarrow 3'$ exonuclease activities on a single 10 kDa polypeptide chain. The large (Klenow) proteolytic fragment (KF) of DNA polymerase I[1] has been elucidated by a variety of stereochemical,[2] structural,[3] and enzymatic analyses.[4–9] The KF has both $5' \rightarrow 3'$ polymerase and $3' \rightarrow 5'$ exonuclease activities. The reaction mechanisms of DNA polymerase I and KF have been extensively studied by Benkovic et al. by the measurement of the accumulation of radioisotope-labeled products as a function of time. This has been achieved by the combination of gel electrophoresis with a stopped-flow/quenched-flow technique, and by time-resolved fluorescent spectroscopy.[4] These techniques still have some difficulties, despite several improvements, for example, the requirement of isotope labeling of probes and of special techniques. To understand the complete enzyme reaction mechanism, it is more useful to monitor all the reaction steps such as enzyme binding, elongation along the template, and release of enzymes from the template in situ with the same device.

In a preliminary paper, we reported that a 27-MHz quartz-crystal microbalance (QCM) is a useful tool in the detection and quantitative analysis of each step in polymerase reactions in aqueous solution.[10] QCMs are very sensitive mass measuring devices in air and aqueous solution, and their resonance frequency has been proved to decrease linearly with increasing mass on the QCM electrode at a nanogram level.[11–13] When the template/primer oligonucleotides were immobilized on a QCM plate, three steps such as 1) polymerase binding to the primer (mass increase); 2) elongation of complementary nucleotides along the template (mass increase), and release of the enzyme from the completely polymerized DNA (mass decrease) could be observed continuously from time dependent frequency changes (see Figure 1).

[a] Prof. Y. Okahata, H. Matsuno, Dr. K. Niikura
Department of Biomolecular Engineering
Tokyo Institute of Technology, 4259 Nagatsuda
Midori-ku, Yokohama 226-8501 (Japan)
Fax: (+81) 45-924-5836
E-mail: yokahata@bio.titech.ac.jp

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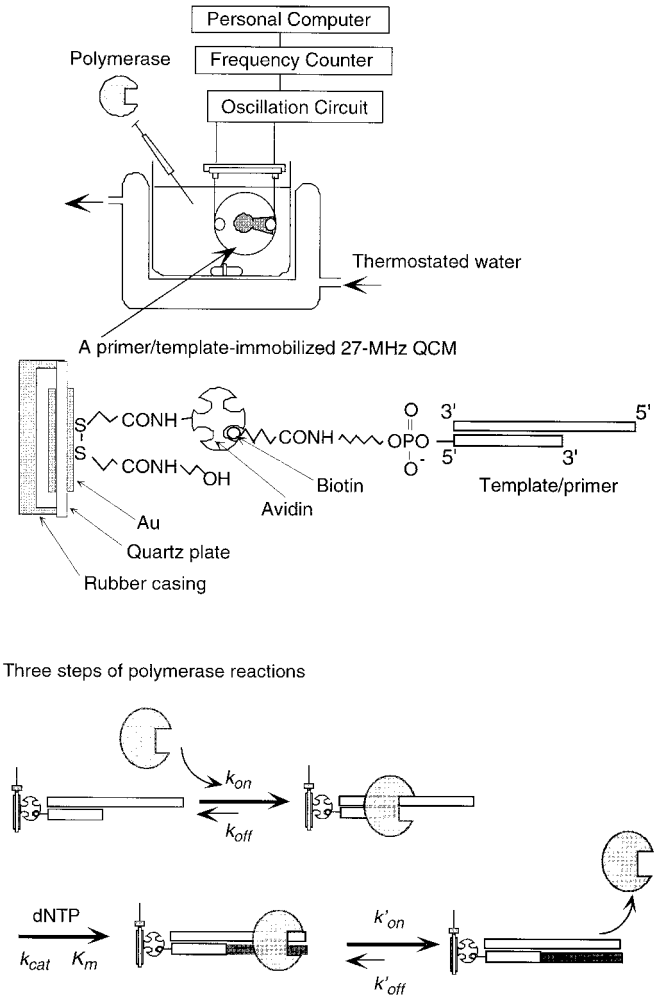

Figure 1. Experimental setup of DNA polymerase (KF) reactions on a template/primer-immobilized 27 MHz quartz-crystal microbalance (QCM) in buffer solution.

In this paper, we report precise kinetic parameters for each step of polymerase reactions by a study of the change in the length and sequences of the template/primer strand on the QCM, and change in concentrations of monomers and enzymes in the solution. In the binding process of KF to the template/primer DNAs, the binding amount (Δm), the binding (k_{on}) and dissociation rate constants (k_{off}), and binding constants (K_a) were obtained. In the following elongation step, Michaelis–Menten parameters (k_{cat} and K_m) for enzyme reactions could be obtained. In the release process of the enzymes from the polymerized DNA, the dissociation (k'_{off}) and rebinding rate constants (k'_{on}), and binding constants (K'_a) were obtained. A repair process of polymerase KF was also observed from frequency changes. The 27-MHz QCM used in this study has a sensitivity of 0.6 ng cm^{-2} of mass change per 1 Hz of frequency decrease. This sensitivity is large enough to detect the binding of enzyme and the elongation process. A schematic experimental set-up and a reaction scheme are shown in Figure 1. Prepared and immobilized template/primer DNAs are summarized in Figure 2.

Results and Discussion

Figure 3 shows typical frequency changes as a function of time of the template/primer **1**-immobilized QCM with the addition of Klenow fragment (68 kDa) as a polymerase and/or dATP and dGTP as complementary monomers in the aqueous solution. In curve A), the frequency decreased (mass increased) gradually for approximately 15 min, after the polymerase was injected initially (7.0 pmol per 8 mL of cell), as a result of the slow binding of polymerase onto the primer (step 1). The binding amount at equilibrium was 140 ± 3 ng (2.1 pmol) cm^{-2}, and this indicates that one polymerase binds for approximately every three template/primer strands, since 6.0 pmol (120 ± 10 ng cm^{-2}) of DNA was immobilized on the QCM. It was confirmed that the Klenow fragment hardly bound to a simple single-stranded DNA and also hardly bound to a avidin surface without DNAs (data not shown). When monomers (dATP and dGTP) were added in excess at the second injection point of the curve A) (520 nmol per 8 mL), the mass rapidly increased within 1 min (step 2, $\Delta m = 28 \pm 3$ ng cm^{-2}) and then rapidly decreased to a constant value (step 3, Δm from the starting point is 30 ± 5 ng cm^{-2}). The increased mass of 30 ng cm^{-2} (6.0 pmol) corresponds to the immobilized amount

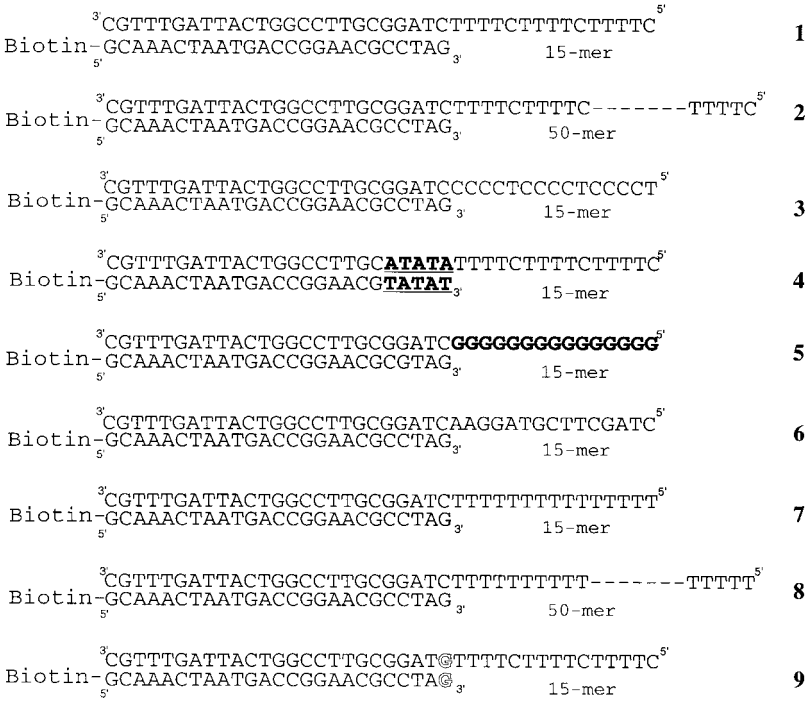

Figure 2. Template/primer structures immobilized on an avidin–QCM.

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(6.0 pmol) of a single strand of the template **1**. The mass decrease at step 3 (140 ± 3 ng cm^{-2}) is almost identical to the mass increase with polymerase binding in step 1 (140 ± 3 ng cm^{-2}). When a solution of noncomplementary bases of dCTP and dTTP (instead of complementary monomers) was injected as the second injection, no changes in the frequency were observed. Thus, the mass increase at step 2 indicates elongation along the DNA template, and the mass decrease at step 3 signifies the release of the enzyme from the completely polymerized DNA. After the release of the bound enzyme, the final mass increase was consistent with the mass increase at step 2 [30 ng cm^{-2} (6.0 pmol)] from template polymerization.

When monomers of dATP and dGTP were added prior to addition of polymerase (injected at the arrow Figure 3) the QCM mass simply increased as shown in curve B) and reached the same mass increase as the curve A). Thus, the frequency change is consistent with the mass increase owing to the KF-mediated elongation of DNA 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.

Each step of these polymerase reactions observed in the curve A) (binding of polymerase, elongation along the template, and enzyme release from the newly polymerized DNA) was studied kinetically with changes in the sequences of the template/primer strands, polymerase concentrations, and dNTP monomer concentrations.

The effect of the DNA template/primer sequence and length on the binding process of the Klenow fragment: The KF binding to the template/primer (step 1 of the curve A) in Figure 3) is described by Equation (1). The amount of the DNA/KF complex formed at time t after injection is given by Equations (2) – (4).

$$\mathrm{DNA}+\mathrm{KF} \underset{k_{off}}{\overset{k_{on}}{\rightleftharpoons}} \mathrm{DNA/KF} \quad (1)$$

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

$$\Delta m_t = \Delta m_\infty\{1-\exp(-t/\tau)\} \quad (3)$$

$$\tau^{-1} = k_{on}[\mathrm{KF}]+k_{off} \quad (4)$$

The relaxation time (τ) of KF binding is calculated from curve fitting the QCM frequency changes at various KF concentrations. KF binding and dissociation rate constants (k_{on} and k_{off}) could be obtained from the slope and intercept of the plot of τ^{-1} against KF concentration. The binding constants (K_a) could also be obtained from the ratio of k_{on} to k_{off}.

Figure 4A shows typical time courses for KF binding to the template/primer **1** with changes in the KF concentration. KF-binding amounts increase with the increase in KF concentrations. Similar time courses with KF concentrations were observed for the other template/primers shown in Figure 2; the binding and dissociation rate constants (k_{on} and k_{off}) were obtained according to Equation (4) (Figure 4B). The results are summarized in Table 1, together with

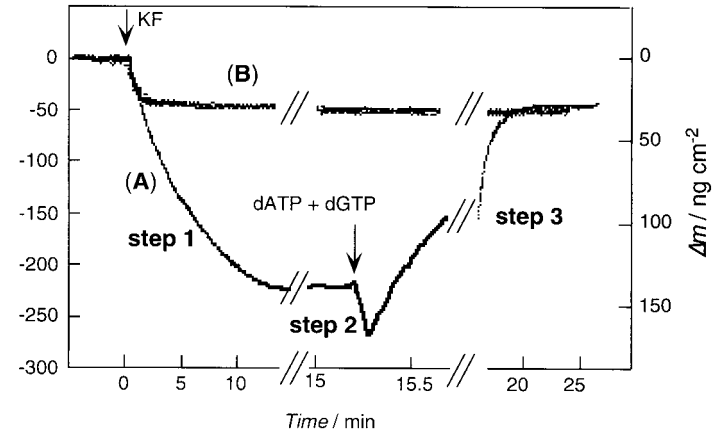

Figure 3. Typical time courses of frequency changes of the template/primer **1**-immobilized QCM, responding to the addition of DNA polymerase (KF) and monomers (dATP and dGTP). A): KF was added at first and then excess monomers were added after the enzyme bound. B): Polymerase was added in the presence of excess monomers. (10 mM Tris-HCl, pH 7.4, 10 mM $MgCl_2$, 40 mM KCl, 30 °C, [KF] = 7.0 pmol per 8 mL (0.88 nM), [dATP/dGTP 4:1] = 520 nmol per 8 mL (65 μM)).

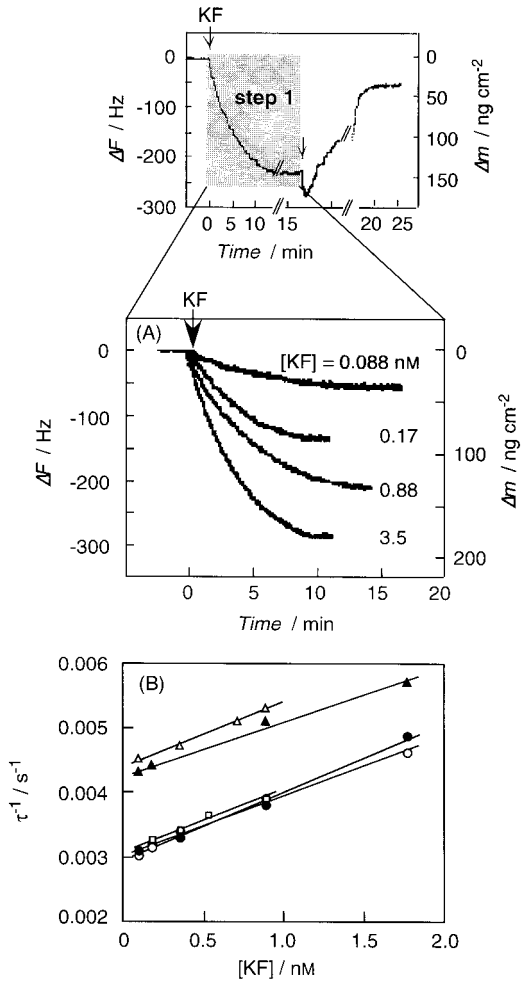

Figure 4. A) Binding behaviors of KF to the template/primer **1** dependent on the KF concentrations. (10 mM Tris-HCl, pH 7.4, 10 mM $MgCl_2$, 40 mM KCl, 30 °C, [KF] = 0.7 – 28 pmol in 8 mL (0.088 – 3.5 nM)). B) Linear reciprocal plots of relaxation time τ against KF concentration according to Equation (4). (●), template/primer **1**; ○, template/primer **2**; □ template/primer **3**; ▲ template/primer **4**; △, template/primer **5**.

Table 1. Amount of binding (Δm), binding and dissociation rate constants (k_{on} and k_{off}), and binding constants (K_a) of KF on various template/primer termini.

Template/ primer[a]	Δm[b]/ng^{-1} [pmol]cm^{-}	k_{on}[c]/10^6 M^{-1}s^{-1}	k_{off}[c]/ 10^{-3}s^{-1}	K_a[d]/ 10^8M^{-1}
1	140 (2.1)	1.0	3.0	3.3
2	135 (2.0)	0.94	3.0	3.1
3	140 (2.1)	0.99	3.1	3.2
4	130 (1.9)	0.87	4.2	2.1
5	50 (0.74)	1.0	4.4	2.3

[a] Sequences are shown in Figure 2. [b] [KF] = 7.0 pmol per 8 mL (0.88 nM). [c] Obtained from Equations (2) – (4). [d] Ratio of k_{on} to k_{off}.

the QCM binding amount (Δm) when KF was injected at 7.0 pmol in 8 mL cell (0.88 nM).

In the case of templates/primers **1**–**3**, the KF binding amounts (Δm) were almost constant (135 – 140 ng cm^{-2}), and were found to be independent of the length (15- or 50-mer) and sequence (TTTTC or CCCCT) of the template. The k_{on} and k_{off} values were almost the same for templates **1**–**3**. This indicates that KF binding is independent of the sequence and length of the DNA template. As an exception, when the template comprises oligo dG_{15} **5**, the QCM Δm value decreased to one third (50 ng cm^{-2}) and K_a also decreased, because of the increasing k_{off} value. The continuous oligo dG chains are known to aggregate in aqueous solution.[14] Although KF is reported to be negatively affected by template/primer secondary structure compared with other polymerases,[15] the aggregated dG_{15} chains may obstruct the enzyme binding to the template/primer. Actually, in this case the subsequent elongation reaction along the template **5** (step 2) could not be observed when an excess of monomers was added (data not shown).

When template/primer **4** (no GC pairs) was employed, the K_a value decreases slightly (2.1×10^8 M^{-1}) owing to the slightly larger k_{off} value compared with templates/primers **1**–**3**. The QCM Δm value is unchanged. The large molecular motion and weak hydrogen bonding of AT base pairs at the primer end compared with those of GC pairs accounts for this observation. It has been reported that the exonuclease part of the Klenow fragment binds to the template/primer with AT rich or mismatched sequences, although the active polymerase component binds to the regular template/primer.[16]

Effect of enzyme concentration on the DNA elongation process: Figure 5A shows the entire reaction process when KF was added at the first arrow at different concentrations (0.088, 0.17, and 0.88 nM in solution) and excess monomers were added at the second arrow. The DNA elongation (steep frequency decreases after the second injection) was approximately 30 ng cm^{-2} independent of KF concentrations. Figure 5B shows the reaction process when the monomers were added at different times prior to binding equilibration for KF (0.88 nM added in the solution). In these cases, the elongation amounts were also approximately 30 ng cm^{-2} independent of the time of the monomer additions, and these results

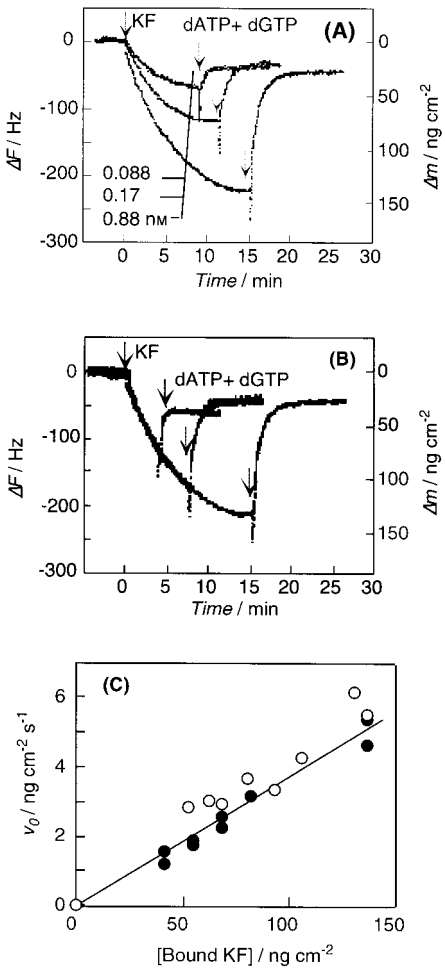

Figure 5. Elongation processes dependent on A) the KF concentration in the bulk solution (0.088, 0.17, and 0.88 nM), and B) the KF concentration bound on the template/primer part: A) dNTP monomers added after the equilibrium of the KF binding. B) dNTP monomers added before the equilibrium of the KF binding. (10 mM Tris-HCl, pH 7.4, 10 mM $MgCl_2$, 40 mM KCl, 30 °C). C) Plot of initial elongation rates (v_0) just after the monomer injections against the concentrations of the bound KF on the template/primer obtained from the mass increase after the KF injection in A) (●) and the mass increase after the KF injection of B) (○).

indicate that DNA elongation was completed along the template. The amount of KF bound on the template/primer and the initial rate (v_o) of DNA elongation just after enzyme injection were calculated from the QCM mass increases after enzyme injection and the slope of the QCM mass increase observed just after monomer injection in Figure 5A and 5B, respectively. The v_o values were plotted against the concentration of bound KF (Figure 5C). The elongation rate increased linearly with the bound amount of KF on the DNA template. This indicates clearly that the enzyme catalyzing the elongation is the bound KF form on the template, and not KF in bulk solution.

Effect of template/primer sequence and length on the DNA elongation process: Elongation reactions along the DNA template (step 2 of the curve A) in Figure 3) were initiated by addition of complementary monomers (dNTP) after formation of the DNA/KF complex on the QCM. The elongation

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process is expressed simply by the Michaelis–Menten reaction between the DNA/KF complex and added monomers as shown in Equations (5)–(7) where DNA′ indicates elongated DNA.

$$\mathrm{DNA/KF} \overset{K_m}{\rightleftharpoons} \mathrm{DNA/KF/dNTP} \xrightarrow{k_{cat}} \mathrm{DNA'/KF} \quad (5)$$

$$v_0 = \frac{k_{cat}[\mathrm{DNA/KF}]_0[\mathrm{dNTP}]}{K_m + [\mathrm{dNTP}]_0} \quad (6)$$

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

The QCM resonance frequency was defined as the zero position at the point of addition of monomer solution; the frequency changes were then recorded with time. When the initial concentration of DNA/KF was fixed at 13 pM in the solution (0.24 pmol cm^{-2} on the template **1**), the elongation processes occurred after the injection of various concentrations of dATP/dGTP = 4/1 in the solution (Figure 6A). The initial elongation rate (v_0) increased with the addition of monomers, and Figure 6B shows the plot of v_0 against dNTP concentrations. From the reciprocal plots (Figure 6C), the dissociation constants of dNTP monomers (K_m) and the elongation catalytic rate constants (k_{cat}) were obtained from the slope and intercept [Eq. (7)]. Michaelis–Menten parameters for the template/primer **1** are summarized in Table 2, together with the elongation amount and the results obtained

Table 2. Amount of elongation (Δm), dissociation constant of monomers (K_m) and catalytic elongation rate constant (k_{cat}) in the DNA/KF complex on the QCM.[a]

Template/primer[a]	Δm/ng [pmol] cm^{-2}	$K_m/10^{-6}$ M	k_{cat}/s^{-1}
1	28 (6.0)	20	10
2	83 (5.4)	22	10
3	26 (5.6)	17	8
5	0 (0)	–	–
6	23 (4.9)	24	9
7	9 (1.9)	nd[c]	nd[c]
8	12 (2.6)	nd[c]	nd[c]

[a] [DNA/KF complex] = 13 pM in the solution (0.24 pmol cm^{-2} on the template/primer). [b] Sequences are shown in Figure 2. [c] Since the elongation amounts were small, kinetic parameters could not be obtained.

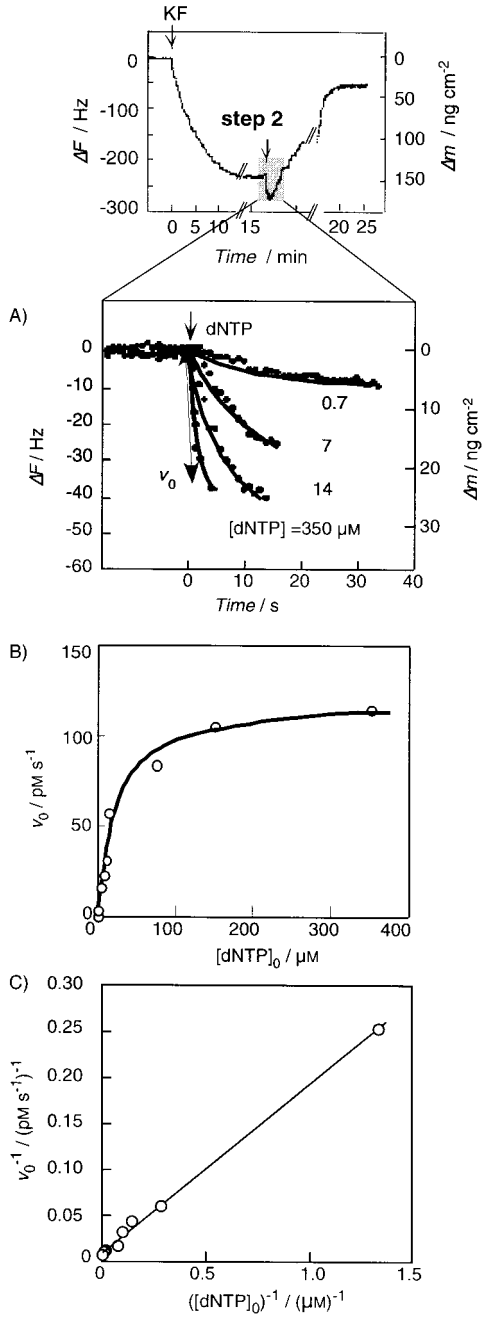

Figure 6. A) Time courses elongation processes on the template/primer **1**-immobilized QCM, dependent on monomer concentrations (0.7–350 μM, dATP/dGTP 4:1). B) Saturation behaviors of initial elongation rates (v_0) against dNTP concentrations. C) Lineweaver–Burk plots according to Equation (7) (10 mM Tris-HCl, pH 7.4, 10 mM $MgCl_2$, 40 mM KCl, 30 °C, $[\mathrm{DNA/KF}]_0$ = 13 pM in the solution (0.24 pmol cm^{-2} on the QCM)).

for various template/primer structures (**2**–**8**). The elongation amount (Δm) of the template **1** was 28 ng cm^{-2} (6.0 pmol cm^{-2}), consistent with the immobilized amount of DNA template (6.0 pmol). Elongation was shown to be completed along this template. The QCM Δm value (83 ng cm^{-2}, 5.4 pmol cm^{-2}) of the template **2** was three times greater than for template **1**, nearly consistent with its 3.3 times longer template length. The slightly smaller than expected value for **2** may be because KF cannot completely elongate along the long template of **2**. The QCM Δm values for the template/primer strands **3** and **6** were very consistent within error, and independent of the DNA template sequence. When the template sequence was comprised of only dT (n = 15- or 50-mer) in **7** and **8**, however, the elongation amounts were smaller than expected. Apparently polymerase KF is not able to slip completely along the DNA template because of the weak hydrogen bonds between AT base pairs at the elongation part.

When reasonable QCM Δm values were obtained, K_m and k_{cat} values were independent of the sequence and length of the templates **1**–**3** and **6**. Thus, KF can recognize all dNTP monomers similarly, and KF readily polymerizes along these immobilized templates. The molecular recognition of monomers was readily facilitated by the complementary template.

Benkovic et al. have reported kinetic values of $K_d = 200$ nM in the binding process and $k_{cat} = 3.8\ s^{-1}$ in the elongation process for combinations of a very long radioisotope-labeled $dA_{1000} \cdot dT_{10}$ template/primer and DNA polymerase I using rapid quenching gel electrophoresis.[5] Smaller $K_d = 5-8$ nM values have been obtained for combinations of short radioisotope-labeled templates similar to template **6** and the same Klenow fragment using both rapid quenching gel electrophoresis[6] and time-resolved fluorescent methods.[9] Kinetic values obtained by our QCM methods compare well with those obtained by these conventional methods.

Effect of template/primer sequence and length on the KF dissociation processes: KF dissociation from the elongated DNA duplexes (step 3 of the curve (A) in Figure 3) were observed as frequency increases (mass decreases) on the QCM following the elongation process. The resonance frequency of the QCM was defined as the zero position at the time of elongation completion, and the frequency increase was recorded with time. In Figure 7A, the KF dissociation process was monitored at different KF concentrations using the template/primer **1** on the QCM.

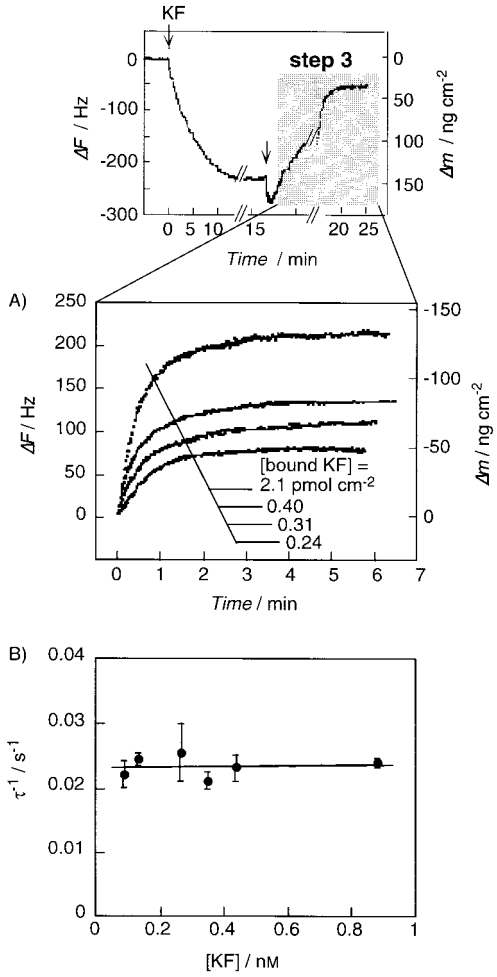

Figure 7. A) Release behavior of KF from the completely polymerized terminus **1** dependent on the bound amount of KF on the template. B) Linear reciprocal plot of relaxation time (τ) against KF concentration according to Equation (11) (10 mM Tris-HCl, pH 7.4, 10 mM $MgCl_2$, 40 mM KCl, 30 °C, [KF] = 0.10, 0.13, 0.17, and 0.88 nM added in the solution, [dNTP] = 14 μM).

The dissociation process is described by Equation (8). The amount of DNA/KF complex left at time t is given by Equations (9) – (11).

$$\mathrm{DNA/KF} \underset{k'_{on}}{\overset{k'_{off}}{\rightleftharpoons}} \mathrm{DNA+KF} \quad (8)$$

$$-[\mathrm{DNA/KF}]_t = -[\mathrm{DNA/KF}]_\infty\{1-\exp(-t/\tau')\} \quad (9)$$

$$-\Delta m_t = -\Delta m_\infty\{1-\exp(-t/\tau')\} \quad (10)$$

$$\tau'^{-1} = K'_{on}[\mathrm{KF}] + k'_{off} \quad (11)$$

The dissociation and rebinding rate constants (k'_{off} and k'_{on}) and binding constants (K'_a) for the dissociation process could be obtained similarly to that for step 1. These values were obtained by changing the template/primer sequences, and the results are summarized in Table 3. The rebinding rate constants (k'_{on}) calculated from the slope [Eq. (11)] could not be obtained precisely because of the very small slope values observed in step 3 (Figure 7B).

Table 3. Released amount (Δm), dissociation and rebinding rate constants (k'_{off} and k'_{on}) and binding constants (K'_a) of KF from various template/primer termini.

Template/ primer[a]	Δm[a]/ ng^{-1} [pmol] cm^{-2}	k_{on}'[c]/ 10^6 M^{-1} s^{-1}	k_{off}'[c]/ 10^{-3} s^{-1}	K_a'[d]/ 10^8 M^{-1}
1	140 (2.1)	0.1	23	0.04
2	135 (2.0)	0.1	26	0.03
3	140 (2.1)	0.1	21	0.05
6	135 (2.0)	0.1	25	0.04

[a] Sequences are shown in Figure 2. [b] [DNA/KF complex] = 0.88 nM in the solution (2.1 pmol cm^{-2} on the template/primer). [c] Obtained from Equations (9) – (11). [d] Ratio of k'_{on} to k'_{off}.

Released amount, dissociation, and rebinding rate constants of KF were also independent of the sequence and length of templates **1** – **3** and **6**, as well as the binding process of step 1 (see Table 1). In the case of template/primer **1**, rebinding rate constants ($k'_{on} = 0.1 \times 10^6$ M^{-1}s^{-1}) in step 3 were approximately ten times smaller than binding rate constants ($k_{on} = 1 \times 10^6$ M^{-1}s^{-1}) in step 1. On the contrary, dissociation rate constants ($k'_{off} = 23 \pm 3 \times 10^{-3}$ s^{-1}) in step 3 were approximately seven times larger than dissociation rate constants ($k_{off} = 3.0 \pm 0.5 \times 10^{-3}$ s^{-1}) in step 1. As a result, binding constants ($K'_a = 0.04 \times 10^8$ M^{-1}) of KF to the complete terminus in step 3 were approximately 100 times smaller than those ($K_a = 3 \times 10^8$ M^{-1}) for the incomplete terminus in step 1. Thus, KF has a stronger binding ability to an incomplete terminus of the template/primer than to the completely polymerized terminus, and KF can release from the completely polymerized terminus as shown in curve A) in Figure 3. The larger K_a value for KF to the incomplete terminus is explained by the larger k_{on} value and the smaller k_{off} value in step 1 compared with those values for step 3.

Monitoring the repair of mismatched bases by the Klenow fragment: One important role of DNA polymerase I in vivo is the repair of DNA strands using the $3' \rightarrow 5'$ exonuclease activity as well as the $5' \rightarrow 3'$ polymerase activity.[2] A time

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course for QCM frequency changes is shown in Figure 8 when KF and dATP/dCTP monomers were added to template **9** with a mismatched GG base pair at the KF binding site (shown by outlined letters in Figure 2).

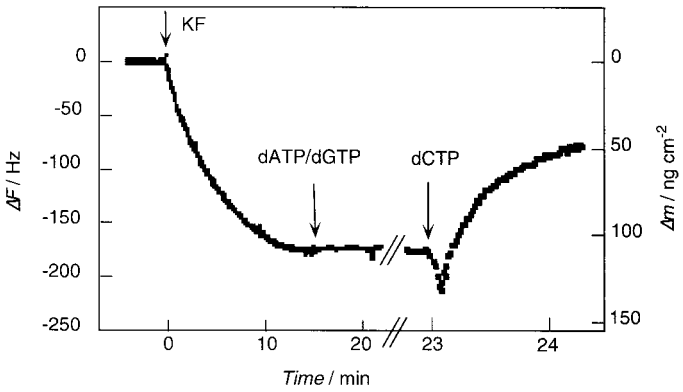

Figure 8. Time course of frequency changes of the template/primer **9**-immobilized QCM with the mismatched GG base pairs at the KF binding site, and the response to the addition of 0.88 nM of KF, 65 μM of dATP/dGTP 4:1 monomers, and then 8.7 μM of dCTP monomer. (10 mM Tris-HCl, pH 7.4, 10 mM $MgCl_2$, 40 mM KCl, 30 °C).

The binding amount of KF to the template/primer **9** was 110 ng cm^{-2}, which is smaller than the 135–140 ng cm^{-2} observed with the complementary template/primer end **1**–**3**, as a result of the GG mismatch bases (see Table 1). In the presence of only dATP/dGTP monomers that are complementary to the template sequence, polymerization did not occur. However, when dCTP was added, the general polymerization and subsequent release of enzyme were both observed with the usual time course A) in Figure 3. This indicates that dCTP is required for polymerization of the template **9** in addition to complementary dATP and dGTP monomers. Thus, KF removes the mismatched G base from the primer using the $3' \rightarrow 5'$ exonuclease activity, when KF binds to the template **9**, and remains on the template with the free G base. When only dATP and dGTP monomers were added, KF could not polymerize along the template because of the lack of the dCTP monomer that is complementary to the free G base of the template. When dCTP was added in addition to dATP and dGTP, the general polymerization proceeds along the template **9**. This process indicates the repair process of KF with its intrinsic exonuclease activity.

The effect of Mg^{2+} ions on the KF enzyme reactions: DNA polymerases generally require divalent metal ions such as Mg^{2+} ions for enzymatic activities. The role of the Mg^{2+} ions can be clearly monitored using the QCM method. Figure 9A shows the time course of frequency changes of the template/primer **1** in the absence of Mg^{2+} ions in solution (10 mM Tris-HCl, pH 7.4, 40 mM KCl, 30 °C), after addition of KF and then dATP/dGTP monomers. In the absence of Mg^{2+} ions, KF could bind to the template, however, the following elongation reaction did not proceed even in the presence of excess monomers. When $MgCl_2$ solution was added at the third arrow in Figure 9A, the usual elongation and release of KF could be observed. In Figure 9B, KF was added

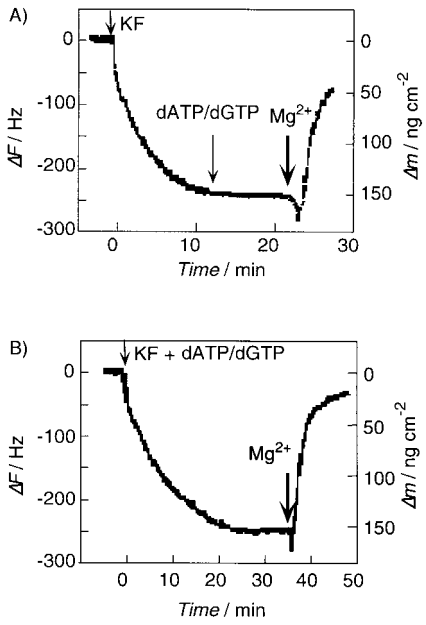

Figure 9. Time courses of frequency changes of the primer/template **1**-immobilized QCM, showing the effect of Mg^{2+} ions on polymerization by KF. A) In the absence of Mg^{2+} in the solution, 0.88 nM of KF and then 65 μM of dATP/dGTP 4:1 monomers were added at each arrow, and then 25 μM of $MgCl_2$ was added to initiate the polymerase reaction. B) In the absence of Mg^{2+} in the solution, both 0.88 nM KF and 65 μM dATP/dGTP 4:1 monomers were first added, and then 25 μM $MgCl_2$ to initiate the polymerase reaction. (10 mM Tris-HCl, pH 7.4, 40 mM KCl, 30 °C).

at the arrow in the presence of monomers without Mg^{2+} ions. However, only KF binding was observed, with no subsequent elongation reaction. When $MgCl_2$ solution was added at the second arrow, the usual elongation and release of KF were both observed. It was also confirmed that the addition of a $MgCl_2$ solution did not affect the oscillation. Thus, KF can bind to the template/primer without Mg^{2+} ions, but cannot catalyze the elongation reaction. This is very consistent with the reported general mechanism of DNA and RNA polymerases: the 3′-end OH-group of the primer cannot attack the γ-phosphate group of dNTP without chelation of Mg^{2+} ion to the β- and γ-positions of the base phosphates.[4–9]

Conclusion

We have determined all kinetic parameters for a three-step DNA polymerization by a Klenow Fragment using a template/primer-immobilized 27-MHz QCM: KF binding of the template/primer with $K_a = 10^8\,M^{-1}$ ($k_{on} = 10^5\,M^{-1}s^{-1}$ and $k_{off} = 10^{-3}\,s^{-1}$) independent of both primer and template sequences (step 1); Michaelis–Menten parameters of KF in the elongation process ($K_m = 10^{-5}$ M for monomers $k_{cat} = 10\,s^{-1}$, and $k_{cat}/K_m = 10^6\,M^{-1}s^{-1}$) (step 2), and KF can easily release from the completed strands with $K'_a = 10^6\,M^{-1}$ ($k'_{on} = 10^4\,M^{-1}s^{-1}$ and $k'_{off} = 10^{-2}\,s^{-1}$) (step 3). This is the first example of investigating both kinetically and quantitatively the binding, catalysis, and release processes of DNA polymerase reactions in situ on the same device.

We believe that the QCM system is highly sensitive in the detection of in situ enzyme reactions on a DNA strand

without any labeling. This system will be applied to other DNA/RNA enzyme reactions such as ligation and restricted enzyme reactions, and also to many reactions or recognition processes of biomolecules such as protein–substrate and protein–protein interactions.

Experimental Section

Materials: The Klenow fragment (KF) of DNA polymerase I from *Escherichia coli* and dNTP were purchased from TaKaRa, Tokyo. Avidin from egg white was from Nacalitesque, Kyoto. *N*-Hydroxysuccinimide and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride were from DOJINDO, Tokyo. Single-stranded oligonucleotides (template chains) and biotinylated oligonucleotides (primer chains) were purchased from Sawady Technology, Tokyo. All other reagents were from Nacalitesque and used without further purification.

27-MHz QCM and its calibration: A 27-MHz, AT-cut QCM is commercially available from Ukou Electronics, Saitama, Japan. The diameter of its quartz plate is 8 mm and Au electrodes are deposited on both sides (diameter: 2.5 mm, area: 4.9 mm^2).[10, 12] One side of the quartz crystal was sealed with silicon rubber, maintaining it in an air environment to avoid contact with the ionic aqueous solution, while the other is exposed to aqueous buffer solution. A cased 27-MHz QCM was connected to an oscillation circuit designed to drive the quartz in aqueous solution (see Figure 1). The frequency changes were followed by a universal frequency counter (Hewlett Packard Japan, Tokyo, model 53131A) attached to a microcomputer system (Macintosh Power Book 170, Apple Japan, Tokyo). The Sauerbrey's Equation (12) was obtained for the AT-cut shear mode QCM[11] where ΔF is the measured frequency change [Hz], F_0 the fundamental frequency of the QCM [27×10^6 Hz], Δm the mass change [g], A the electrode area [4.9 mm^2], ρ_q the density of quartz [2.65 $g\,cm^{-3}$], and μ_q the shear modulus of quartz [2.95×10^{11} $dyn\,cm^{-2}$]. The noise level of the 27-MHz QCM was ±1 Hz in buffer solution at 30 °C, and the standard deviation of the frequency was ±2 Hz for 2 h in buffer solution at 30 °C.

$$\Delta F = -\frac{2F_0^2}{A_m\sqrt{\rho_q \mu_q}}\Delta m \qquad (12)$$

When the QCM is employed in an aqueous solution, Equation (12) cannot be simply applied due to interfacial liquid properties (i.e., density, viscosity, elasticity, conductivity, and dielectric constant),[17] thin film viscoelasticity,[17] electrode morphology,[17, 18] and the mechanism of acoustic coupling[19] impact on the QCM oscillation behavior. Therefore, we calibrated our cased 27-MHz QCM in the aqueous solution (pH 7.9, 10 mM Tris-HCl, 0.2 M NaCl, at 20 °C).

A respective amount of polymer solution was cast (0.1–15 $\mu g\,cm^{-2}$), or LB film of lipid monolayers was deposited (0.04–4 $\mu g\,cm^{-2}$), or 10–30-mer oligonucleotides with SS groups were directly immobilized (10–60 $ng\,cm^{-2}$) on the bare Au electrode side of the cased QCM plate. A linear relationship was observed between the deposited amount of mass and the frequency decrease of the QCM both in the air phase and in the aqueous solution, independent of deposition methods and chemical compounds. The slope of this curve showed that a frequency decrease of 1 Hz corresponded to a mass increase of 0.61 ± 0.1 $ng\,cm^{-2}$ on the QCM electrode. This means that the Sauerbrey's Equation can be applied in the aqueous solution limited to these experimental conditions. When the long chain ssDNA such as 200–300-mer was immobilized on the QCM, however, the linear correlation could not be obtained in the aqueous solution owing to the effect of the flexible conformation of the long DNA chains on the electrode.

Immobilization of DNA duplexes on a 27-MHz QCM: Oligonucleotide duplexes were formed by mixing the biotinylated primer (25-mer) and template (40- or 75-mer) in 200 mM NaCl and 10 mM Tris-HCl, pH 7.8, boiled for 1 min and then cooled to room temperature over 3 h. The DNA duplexes prepared are shown in Figure 2. The biotinylated oligonucleotide duplexes were immobilized on a cleaned Au electrode of the QCM using an avidin–biotin method according to previous papers (Figure 1).[10, 12] The immobilized amounts of 40/25-mer and 75/25-mer oligonucleotide duplexes were controlled to 120 ± 1 and 185 ± 1 $ng\,cm^{-2}$ (ca. 6.0 $pmol\,cm^{-2}$), respectively. These amounts correspond to approximately 15% coverage of the surface of the Au electrode, and this small coverage provided enough space for binding of a large enzyme molecule.

Enzyme reactions on a template/primer-immobilized QCM: A template/primer-immobilized QCM was soaked in aqueous solution (8 mL, 30 °C, 10 mM Tris-HCl, pH 7.4, 10 mM $MgCl_2$, 40 mM KCl) and the resonance frequency of the QCM was defined as zero after equilibrium. Frequency changes corresponding to the addition of enzyme and/or dNTP monomers were followed with time. The solution was stirred to avoid any effect of diffusion of enzyme and monomers, and the stirring did not affect the stability and magnitude of frequency changes.

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