Monitoring of EcoRV Digestions on a DNA-immobilized Quartz Crystal Microbalance

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Kinetic parameters of DNA digestions by EcoRV such as binding and dissociation rate constants of enzymes to DNA (k_{on} and k_{off}) and the site-specific cleavage reaction (k_{cat}) could be obtained using a DNA-immobilized quartz-crystal microbalance (QCM).

Restriction endonucleases are well known as enzymes that strictly recognize and hydrolyze specific sequences on double-stranded DNA. Type IIP restriction endonucleases such as EcoRV, which is a homodimer of a 29 kDa monomer, and recognizes a palindromic 5′-GATATC-3′ sequence, and cleaves in the presence of Mg^{2+}, have been widely used as biotechnical tools.[1,2] The enzyme reactions have been followed conventionally by the use of radioactively labeling substrates and gel-electrophoresis to obtain a radiolabeled cleaved product, and kinetic parameters are obtained from Michaelis–Menten kinetics.[3,4] Since DNA is cleaved by EcoRV in the presence of Mg^{2+}, it is difficult to separate the enzyme-binding process and the enzyme cleavage process by these conventional methods. In the presence of Ca^{2+} ions in vitro, EcoRV has been reported to be able to bind specifically to DNA but cannot cleave the DNA recognition site.[5]

In this communication, we apply a DNA-immobilized 27-MHz quartz-crystal microbalance (QCM) to monitor the site-specific DNA digestion reactions catalyzed by EcoRV. A QCM is a very sensitive mass-measuring device in aqueous solutions, and their resonance frequency has been shown to decrease linearly with increasing mass on the QCM electrode at a nanogram level.[6,7] By using the QCM technique, we can follow the formation (k_{on}) and decomposition rate constants (k_{off}) of the enzyme–substrate (ES) complex and then the following cleavage reaction rate constants (k_{cat}) as mass changes on the QCM (Figure 1A).

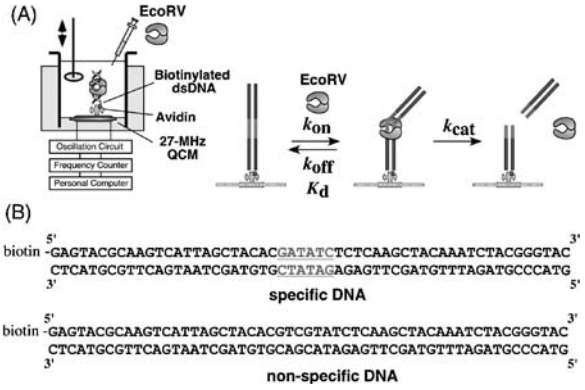

Figure 1. (A) Experimental setup for DNA cleavage reactions catalyzed by EcoRV on a DNA-immobilized 27 MHz quartz-crystal microbalance (QCM) in aqueous solutions and (B) DNA structures immobilized on an avidin–QCM surface.

E. coli strain carrying plasmids pHisRV and pLBM4422 were used to overproduce the EcoRV endonuclease.[8] The EcoRV was purified as described previously.[9] AFFINIX Q4 was used as a QCM instrument (Initium Co., Ltd, Tokyo: http://www.initium2000.com).[6,7] The 27-MHz QCM was calibrated to change frequency by -1 Hz, responding to the mass increase of 0.098 ng cm^{-2} on the electrode for 55-bp dsDNA. The factor of -0.098 ng cm^{-2} per 1 Hz was also applied for EcoRV binding to the DNA on the 27-MHz QCM. Biotinylated DNA and complementary DNA were commercially available, and these duplexes (shown in Figure 1B) were immobilized on an avidin-immobilized QCM plate according to previous paper.[6,7] The immobilized amount of dsDNA was maintained at 19 ± 1 ng cm^{-2} (ca. 55 ± 2 fmol cm^{-2}) and corresponds to approximately 1% coverage of the Au surface (4.9 mm^2) that is enough space for binding of a large enzyme molecule.

Since EcoRV is known to bind to the specific site of DNA but not to cleave it in the presence of Ca^{2+} ions,[5] we firstly observed the binding process of EcoRV to the DNA in the presence of Ca^{2+} ions. Figure 2A shows typical frequency decreases (mass increase) of the DNA-immobilized QCM cell as a function of time, responding to the addition of EcoRV with different concentrations in the presence of 5 mM Ca^{2+}. EcoRV did not bind to the nonspecific DNA but bound to the specific DNA with increasing its concentrations.

The EcoRV binding process to DNAs is described by eq 1. The amount of the DNA/EcoRV (ES) complex formed at time t is given by eqs 2 and 3. The relaxation time (τ) of EcoRV binding is calculated from curve fitting QCM frequency decreases at various concentrations according to eq 3. EcoRV binding and dissociation rate constants (k_{on} and k_{off}) obtained from the slope and intercept of Figure 2B are summarized in Table 1.

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

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

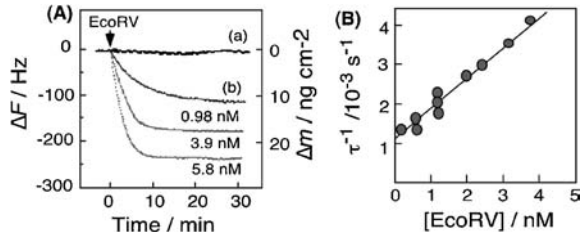

Figure 2. (A) Binding behaviors of EcoRV to (a) the nonspecific DNA and (b) the specific DNA depending on the EcoRV concentrations in the presence of 5 mM Ca^{2+} (10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM DTT, 5 mM Ca^{2+} ions, 25 °C). (B) Linear reciprocal plots of relaxation time (τ) against EcoRV concentrations according to eq 4 in the text.

Table 1. Kinetic parameters for binding and catalytic processes of EcoRV to the specific DNA on the 27 MHz QCM[a]

Metal ion	Binding process			Cleavage process
	$k_{on}/10^5\ M^{-1}\ s^{-1}$	$k_{off}/10^{-3}\ s^{-1}$	K_d/nM	k_{cat}/s^{-1}
Ca^{2+}	7.6	1.1	1.5	—
Mg^{2+}	7.4	1.2	1.6	0.6

[a]10 mM Tris-HCl, 150 mM NaCl, pH 7.5, 1 mM DTT, 5 mM $CaCl_2$ or $MgCl_2$, 25 °C. k_{on} and k_{off} were obtained from eq 4 and k_{cat} were obtained from eq 7.

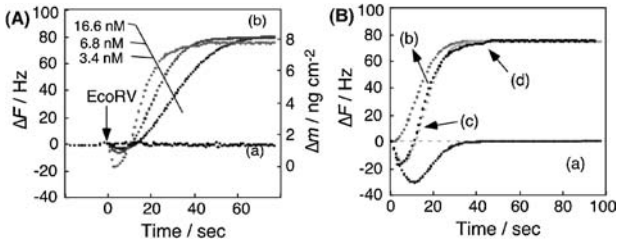

Figure 3. (A) Typical time courses of frequency changes of (a) the nonspecific DNA- and (b) the specific DNA-immobilized QCM, responding to the addition of different concentrations of EcoRV (3.4–16.6 nM) in the presence of Mg^{2+} (10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM DTT, 5 mM $MgCl_2$, 25 °C). (B) (a) The calculated time dependence of [DNA/EcoRV] as shown in eq 6 in the text, (b) the calculative time dependence of [cleaved DNA] as shown in eq 7 in the text, (c) the fitted curve obtained from simultaneous equations of 6 and 7, and (d) the experimental curve at [EcoRV] = 16.6 nM and [DNA] = 19 ng cm^{-2} on a QCM.

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

$$1/\tau = k_{on}[\mathrm{EcoRV}]_0 + k_{off} \quad (4)$$

As shown in Figure 3A, in the presence of Mg^{2+} ions, the complicated frequency changes were observed by the addition of various concentrations of EcoRV. The enzyme did not bind to the nonspecific DNA even in the presence of Mg^{2+} ions. In curves for the specific DNA, the frequency once decreased in several seconds in response to the addition of EcoRV into the cell. Following that, the frequency rapidly began to increase and reached to a constant value of +90 Hz (−8.8 ng cm^{-2}) within 1 min. The primary frequency decrease was caused by the enzyme binding to the recognition sequence of the target DNA; the following frequency increase (mass decrease) can be attributed to the enzymatic digestion of the target DNA. When the higher concentration of the enzyme was added, both faster and larger frequency decreases (mass increase) due to the ES complex formation and the larger slope of the frequency increase (mass decrease) due to the cleavage reaction were observed. The amount of cleaved DNA (8.8 ± 0.8 ng cm^{-2}) corresponded to ca. 50% mass of the QCM-immobilized DNA (19 ± 1 ng cm^{-2}), which is reasonable since the recognition sequence is located near the center of the immobilized DNA sequence (see Figure 1B).

The enzymatic catalytic process of EcoRV is shown by eq 5. In sigmoidal curves, the time dependence of Δm reflects both the formation of the ES (DNA/EcoRV) complex (eq 6) and cleaved DNA (eq 7). Curves (a) and (b) in Figure 3B are calculative curves from eqs 6 and 7, respectively, where the curve (c) is the fitted curve from the simultaneous equations both eqs 6 and 7 with the experimental curve (d) at [EcoRV] = 16.6 nM and [the specific DNA] = 19 ng cm^{-2} on a QCM.[10] Both curves were well consistent with each other. When relaxation rates (τ^{-1}) were plotted against [EcoRV], k_{on} and k_{off} values were obtained from the linear plot of eq 4. The dissociation constant (K_d) of the DNA/EcoRV complex was obtained from k_{off}/k_{on}. The cleavage rate constant (k_{cat}) was obtained from the fitting of eq 7. The obtained kinetic parameters are summarized in Table 1.

$$\mathrm{DNA} + \mathrm{EcoRV} \underset{k_{off}}{\overset{k_{on}}{\rightleftarrows}} \mathrm{DNA/EcoRV} \xrightarrow{k_{cat}} \text{cleaved DNA} + \mathrm{EcoRV} \quad (5)$$

$$[\mathrm{DNA/EcoRV}]_t = ([\mathrm{DNA/EcoRV}]_{max} - [\text{cleaved DNA}])\{1 - \exp(-t/\tau)\} \quad (6)$$

$$[\text{cleaved DNA}] = k_{cat}\int [\mathrm{DNA/EcoRV}]_t dt \quad (7)$$

In the presence of Ca^{2+} ions, although EcoRV has been known to bind to target DNA but not cleave the DNA strand,[5] it has been disputed whether the binding kinetics in the presence of Ca^{2+} as an analog is consistent with those in the presence of Mg^{2+} as a real cofactor. However, most experimental techniques have not determined all of the kinetic parameters simultaneously because it is difficult to detect both the amount of the ES complex and the cleaved DNA product with the same physical signal.[3,4] The QCM method separately examines enzyme binding and cleavage by following the ES complex as the mass changes. The binding (k_{on}) and dissociation (k_{off}) rate constants and dissociation constants (K_d) in the presence of Ca^{2+} ions were very well agreed with those in the presence of Mg^{2+} ions. Therefore, we suggest that when EcoRV cleaves DNA it would thermodynamically form the same transition state in the presence of Mg^{2+} and Ca^{2+} ions. The k_{cat} value was consistent with that obtained from a gel electrophoresis ($k_{cat} = 0.6\ s^{-1}$).[3]

In conclusion, we have determined all the kinetic parameters (k_{on}, k_{off}, K_d, and k_{cat}) for two-step DNA cleavage reactions by EcoRV, which were dependent on divalent cations using a DNA-immobilized 27-MHz QCM. This is the first example of investigating both kinetically and quantitatively the binding and the following cleavage processes of EcoRV reactions in situ on the same device.

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