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Direct monitoring of DNA cleavages catalyzed by an ATP-dependent deoxyribonuclease on a 27 MHz quartz-crystal microbalance†

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Kinetic studies of enzymatic DNA cleavage reactions (the enzyme binding, hydrolysis along DNA strands, and then release of the enzyme from the completely hydrolyzed ssDNA) were carried out on a 27 MHz quartz-crystal microbalance.

Replication and transcription are key processes in the cell. In these processes, many kinds of proteins including enzymes interact with nucleic acids and express various functions. ATP-dependent deoxyribonucleases (DNase) are enzymes[1] that hydrolyze the phosphodiester linkage between deoxyribonucleosides in the presence of ATP and these enzymes are thought to be involved in the processes of genetic recombination and repair of radiation damage.[2] The ATP-dependent DNase from *Micrococcus luteus* (MW 160 kDa) shows the activity for single or double stranded linear DNA *via* an end-attachment 3′→5′ exonucleolytic processive mechanism, and shows no activity for circular DNA,[2] so it has been used as a tool for DNA manipulation to prepare circular DNA *in vitro*.[3] The reaction mechanism of DNA hydrolysis by this enzyme has been studied mainly in solution by measuring the accumulation of the acid-soluble hydrolysis products from radioisotope-labeled substrates as a function of time.[2] Despite several improvements, there are still some difficulties with this method, such as the requirement for radioisotope labeling of probes and for special techniques. For gaining insight into the total enzyme reaction mechanism, it is more useful to monitor *in situ* all of the reaction steps such as enzyme binding, hydrolysis along the DNA strand and release of the enzyme from the DNA on the same device.

In this communication, we report that the oligodeoxyribonucleotide-immobilized 27 MHz quartz-crystal microbalance (QCM) is a useful tool to detect directly and quantitatively each step of DNA cleavage in aqueous solution (see Fig. 1). The enzymatic cleavage could be detected in real time from the frequency changes responding to the mass changes on an Au electrode of a QCM. QCM has been employed recently for investigating various DNA interactions such as DNA hybridization and DNA–protein interactions.[4–6]

A schematic illustration of experimental setup and chemical structures of oligonucleotides is shown in Fig. 1. A 27-MHz QCM (8 mm diameter quartz plate and an Au electrode area of 4.9 mm^2), one side of which is sealed with silicon casing to avoid contact with ionic buffer solution, is commercially available from Initium Inc., Tokyo. The 27 MHz QCM is calibrated to decrease the frequency by 1 Hz in response to a mass increase of 0.62 ng cm^{-2} on the electrode.[5-7] † The frequency changes were followed by a universal frequency counter attached to a personal computer. Various oligodeoxyribonucleotides with different chemical structures were used as shown in Fig. 1B: the 3′-biotinylated dsDNA (51 bp) having a blunt end (**1**) and a 5′-15 base overhanging (**2**), and the 3′-biotinylated or 5′-biotinylated single strand DNA (**3** or **4**, respectively). These oligodeoxyribonucleotides were immobilized on a cleaned Au electrode of the QCM using a biotin–avidin linkage and a tetraethyleneglycol spacer according to previous papers.[5,6] The amount of immobilized dsDNA (**1**) was maintained at 60 ± 2 ng cm^{-2} (*ca.* 1.9 ± 0.1 pmol cm^{-2}). This corresponds to approximately 4% coverage of the Au surface, and this small coverage would give enough space for binding of a large enzyme molecule.[5,6] The amount of other immobilized DNAs was also maintained at 4% coverage.

Fig. 2 shows typical frequency changes of the immobilized-dsDNA (**1**) QCM as a function of time, responding to the addition of ATP-dependent DNase and ATP in the solution. In a curve (a), when 138 nM of DNase was injected at the first arrow, the frequency decreased (mass increased) gradually for 30 min due to the slow binding of DNase to the dsDNA (step 1). When an excess of ATP solution (0.5 mM) was added at the second injection of the curve (a), the frequency rapidly

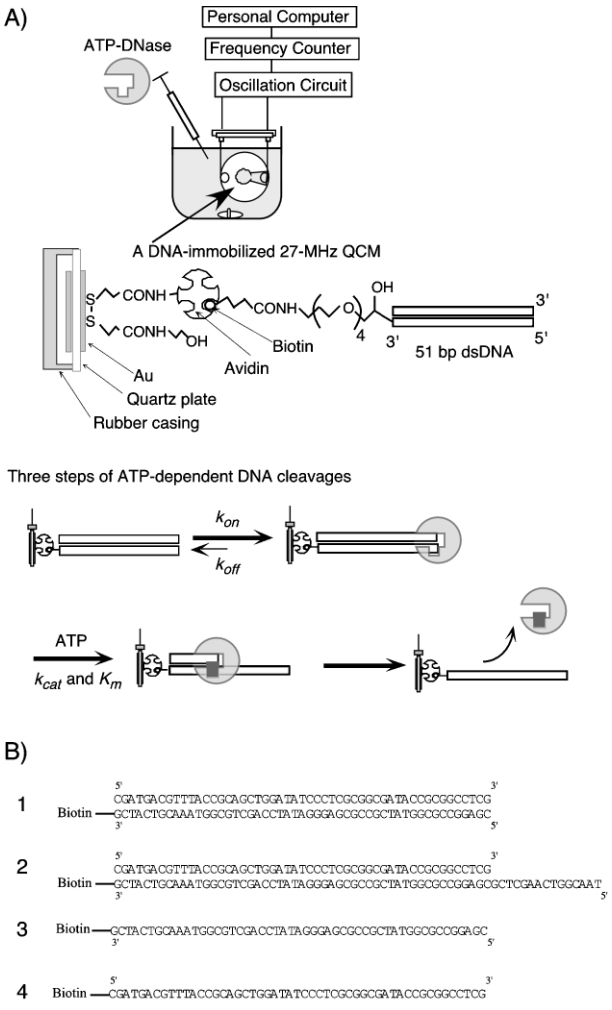

Fig. 1 (A) Experimental setup of enzymatic DNA cleavage reactions on a DNA-immobilized 27-MHz QCM in buffer solution. (B) DNA structures immobilized on the QCM.

† Electronic supplementary information (ESI) available: calibration methods of a 27 MHz QCM are provided. See http://www.rsc.org/suppdata/cc/b1/b109690g/

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Table 1 Kinetic parameters of ATP-dependent DNase for various DNA strands[a]

DNA	Binding process			Hydrolysis process		
	$k_{on}/10^3$ M^{-1} s^{-1}	$k_{off}/10^{-3}$ s^{-1}	$K_a/10^6$ M^{-1}	$k_{cat}/10^3$ s^{-1}	$K_m/10^{-6}$ M	$k_{cat}/K_m/10^6$ M^{-1} s^{-1}
1	8.0 ± 0.3	0.29 ± 0.01	28 ± 2	4.0 ± 0.1	15 ± 1	270 ± 5
2	7.9 ± 0.1	0.14 ± 0.01	56 ± 6	—	—	—
3	nd[b]	nd[b]	nd[b]	nd[b]	nd[b]	nd[b]
4	97 ± 7	4.9 ± 0.5	19 ± 5	0.7 ± 0.1	11 ± 1	64 ± 3

[a] pH 9.4, 66.7 mM glycine–NaOH buffer, 30 mM $MgCl_2$, 8.4 mM 2-mercaptoethanol, 0.1% Nonidet P-40 (ethylphenyl polyethyleneglycol), 30 °C. [b] Not detected.

increased (mass decreased) to the constant value (30 ± 3 ng cm^{-2} over the starting point) within a few minutes (step 2). This indicates that ATP-dependent DNase can hydrolyze dsDNA using the hydrolysis energy of ATP. As a control experiment, when adenosine 5′-γ-thiotriphosphate (ATPγS), which is not hydrolyzed by the enzyme, was added instead of ATP, no frequency changes could be observed. When ATP was present in advance and then enzyme was injected, the frequency simply increased (mass decreased) as shown in a curve (b) and reached the same mass decrease as shown in the curve (a). The decreased mass from the starting point was 30 ± 3 ng cm^{-2}, corresponding to 50 ± 5% of the immobilized dsDNA (**1**): one strand is completely hydrolyzed. Since ATP-dependent DNase is reported to hydrolyze dsDNA from the 3′-end,[2] the 3′→5′ hydrolysis occurs only at the upper strand of dsDNA because the 3′-end of the lower strand is blocked by a biotin–avidin linkage. Thus, the mass decrease in step 2 of the curve (a) reflects two processes: the complete 3′→5′ hydrolysis of the upper strand of immobilized dsDNA and the release of enzyme from the QCM after the hydrolysis. The frequency change in curve (b) also reflects the mass decrease due to the hydrolysis on the QCM. This slow frequency change may reflect the slow binding of enzyme to the dsDNA, since the hydrolysis process and the release of enzymes may proceed fast in the presence of excess ATP in the solution.

When the concentrations of the injected DNase were changed in the range of 14–56 nM in the absence of ATP, the binding of the enzyme showed a typical saturation curve. Binding and dissociation rate constants (k_{on} and k_{off}) and association constant (K_a, the ratio of k_{on} to k_{off}) of the enzyme for various DNA strands could be obtained from time courses of step 1 of the curve (a), according to the previous papers.[5,6] Results are summarized in Table 1. Comparing the kinetic parameters of the dsDNA having a blunt end (**1**) with those of the dsDNA having a 5′-overhanging end (**2**), the K_a value of (**2**) was 2-fold larger than that of (**1**) due to the decrease of the k_{off} value to half. Since the overhanging structure of (**2**) corresponds to the hydrolyzing process by the enzyme, the higher affinity of the enzyme for the overhanging structure than for the blunt end is reasonable for the processive and efficient hydrolysis due to the low dissociation rate constant (k_{off}). For the 5′-free ssDNA (**3**) structure that corresponds to the completely hydrolyzed dsDNA (**1**), the binding of the enzyme couldn't be observed in the same range of enzyme concentration. These results clearly reflect that the DNase can bind to the blunt end of dsDNA (**1**) and the K_a value increased 2-fold during the 3′→5′ hydrolysis process, and then the DNase is easily released from the completely hydrolyzed form of ssDNA (**3**). The DNase can bind to the 3′-free ssDNA (**4**) with a similar K_a value to that for the blunt end dsDNA (**1**), in which both k_{on} and k_{off} values for (**4**) are 10 times larger than those for (**1**). This indicates that the DNase can bind to the 3′-end whether single or double strand; however, the dsDNA structure is favorable for the stable enzyme–DNA complex due to the very slow binding and dissociation rate constants.

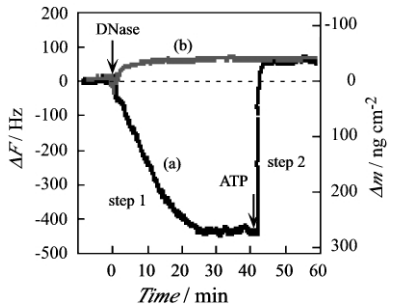

Fig. 2 Typical time courses of frequency changes of the immobilized-dsDNA (**1**)-QCM, responding to the addition of ATP-dependent DNase and ATP at arrows. (a) DNase was added first, and then excess ATP was added after the enzyme had bound. (b) DNase was added in the presence of excess ATP. Conditions: 66.7 mM glycine–NaOH buffer, pH 9.4, 30 mM $MgCl_2$, 8.4 mM 2-mercaptoethanol, 0.1% Nonidet P-40 (ethylphenyl polyethyleneglycol), 30 °C, [DNase] = 138 nM, [ATP] = 0.5 mM.

The hydrolysis process by the enzyme for dsDNA (**1**) and 3′-free ssDNA (**4**) was also investigated. When ATP concentrations injected in step 2 were changed in the range of 2.5 to 200 μM, the initial rates of step 2 showed the saturation behavior of Michaelis-Menten kinetics. The catalytic hydrolysis rate constant (k_{cat}), K_m for ATP, and apparent second-order rate (k_{cat}/K_m) are summarized in Table 1. The k_{cat}/K_m value for (**1**) was 4-fold larger than that for (**4**) due to the large k_{cat} and the constant K_m values for (**1**). Thus, the binding ability of ATP to the enzyme–DNA complex was independent of the DNA structure, and the dsDNA is hydrolyzed more efficiently than ssDNA.

In summary, the QCM is useful to detect kinetically and quantitatively each step of DNA cleavage reactions, such as enzyme binding, the hydrolysis process along DNA strands in the presence of ATP, and then the release of the enzyme from the completely hydrolyzed ssDNA, as mass changes on the same device. The QCM technique will be expanded to detect kinetically various DNA manipulations such as ligation and polymerization.

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Notes and references

1 A. Kornberg and T. Baker, *DNA Replication*, 2nd ed., p. 403, Freeman, New York, 1992.
2 (*a*) G. Cerio-Ventura, M. Fossato, A. Vellante, F. Palitti, P. M. Fasella and E. P. Whitehead, *Biochim. Biophys. Acta*, 1981, **652**, 283–293; (*b*) B. V. Dorp, M. TH. E. Ceulen, H. L. Heijneker and P. H. Pouwels, *Biochim. Biophys. Acta*, 1973, **299**, 65–81; (*c*) B. V. Dorp, M. TH. E. Caulen and P. H. Pouwels, *Biochim. Biophys. Acta*, 1974, **340**, 166–176.
3 H. Yamagishi, T. Tsuda, S. Fujimoto, M. Toda, K. Kato, Y. Maekawa, M. Umeno and M. Anai, *Gene*, 1983, **26**, 317–321.
4 J. Wang, M. Jiang and E. Palecek, *Bioelectrochemstry and Bioenergetics*, 1999, **48**, 477–480.
5 H. Matsuno, K. Niikura and Y. Okahata, *Chem. Eur. J.*, 2001, **7**, 3305–3312.
6 Y. Okahata, M. Kawase, K. Niikura, F. Ohtake, H. Furusawa and Y. Ebara, *Anal. Chem.*, 1998, **70**, 1288–1296.
7 (*a*) Y. Ebara, K. Itakura and Y. Okahata, *Langmuir*, 1996, **12**, 5165–5170; (*b*) G. Sauerbrey, *Z. Physik.*, 1959, **155**, 206–222.