Kinetic Studies of Sequence-Specific Binding of GCN4-bZIP Peptides to DNA Strands Immobilized on a 27-MHz Quartz-Crystal Microbalance

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ABSTRACT: Specific protein−DNA interaction was studied quantitatively by using a highly sensitive 27-MHz quartz-crystal microbalance (QCM). Biotinylated DNA double strands (21 bp, having a CRE site of $^{5'}$ATGACGTCAT$^{3'}$) were immobilized on an avidin-bound QCM surface, and sequence-specific binding of bZIP 56-mer peptides (having both the basic region for binding and the leucine zipper region for dimerization) to the DNA strand on the QCM was observed. The binding amount (Δm) at the nanogram level and kinetic parameters such as association constants (K_a) and binding and dissociation rate constants (k_1 and k_{-1}) could be obtained from time courses of QCM frequency decreases. A bZIP peptide as a dimer was observed to bind sequence-specifically to one DNA strand having a CRE site. K_a values of ss-bZIP, in which the leucine-zipper region of bZIP was substituted by a Cys−Cys linkage, were largely decreased, and the sequence selectivity also disappeared. K_a values obtained by the QCM method showed good agreement with those obtained from the conventional gel mobility shift assay or from circular dichroism spectrum changes. When the specific sequence of the CRE site of DNA strands was partly changed, K_a values decreased by about a half due to the increase of the dissociation rate constant (k_{-1}) independent of the binding rate constant (k_1).

Specific interactions between proteins and DNA are of fundamental importance for understanding how genetic regulatory proteins bind at promoter and enhancer sites and affect gene transcription (*1*). It is known that such sequence-specific proteins have some similar DNA binding domains that are classified as motifs. The yeast transcription factor GCN4 has the basic leucine-zipper (bZIP) motif. The motif corresponds to a 56-mer peptide of the C-terminal sequence 225−281 and forms a short region of predominantly basic amino acid residues immediately preceding the leucine-zipper domain that acts as a dimerization unit. The bZIP 56-mer peptide is a simple α-helix-based DNA binding motif, and it has been reported to recognize the DNA sequence by itself (*2*−*9*). A cylindrical structure of a bZIP monomer is shown in Figure 2. X-ray crystallography confirms that the leucine-zipper region forms a homodimer of parallel α helices in the coiled-coil region and the two N-terminal basic regions fit into the major groove of specific DNA recognition sites of the sequence $^{5'}$ATGACGTCAT$^{3'}$ (CRE site) (*2*−*4*). Electrostatic effects and hydrogen bonding of Lys, Arg, and Asn residues in the basic region are thought to be important for specific binding to the CRE site. The effect of the dimerization of bZIP on DNA binding has been studied by preparing a chemically modified bZIP peptide: the leucine-zipper region is exchanged with a covalently bonded Cys−Cys linkage (ss-bZIP) (*10*) or a cyclodextrin-adamantane interaction (*11, 12*).

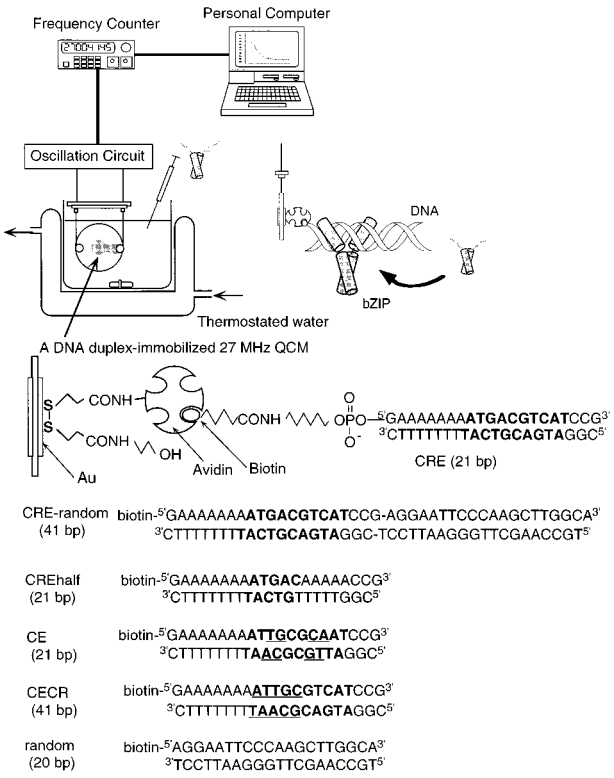

FIGURE 1: A schematic illustration of a 27-MHz QCM measurement system and chemical structures of immobilized DNA strands on the Au electrode (4.9 mm^2 area) of the QCM.

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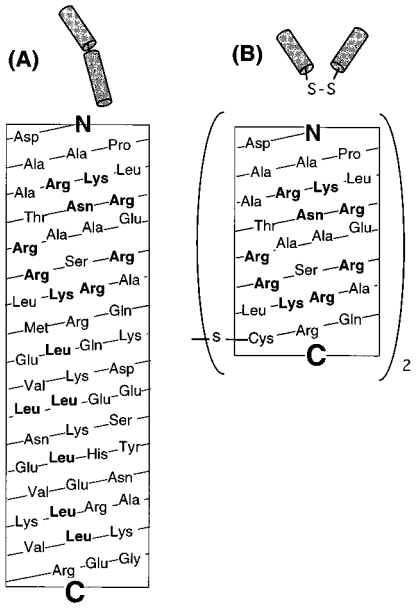

FIGURE 2: Cylindrical structures of (A) bZIP monomer and (B) ss-bZIP.

The binding behavior of bZIP peptides or GCN4 proteins to DNA strands has been studied mainly by gel mobility shift assay (*5*−*12*). This technique is widely used in molecular biology; however, it has some difficulties with regard to quantitatively detecting interactions. DNA or peptides are usually labeled either directly or indirectly with fluorescent or radioactive molecules, and it takes a relatively long time to analyze the results. The results are often qualitative, and there can be uncertainty in knowing the absolute binding amount, the time course of the reaction, and the kinetic factors.

In this paper, we report quantitative kinetic studies of specific binding of bZIP to a duplex DNA immobilized on a highly sensitive 27-MHz QCM in aqueous solution (see Figure 1). A QCM is known to provide very sensitive mass-measuring devices in the gas phase (*13*, *14*) and in aqueous solution (*15*, *16*). Its resonance frequency decreases linearly upon the increase of mass on the QCM electrode at the nanogram level (*17*). From the time course of the frequency decrease (mass increase), due to the binding of bZIP to the DNA-immobilized QCM, the binding amount (Δm), the association constant (K_a), and the binding and dissociation rate constants (k_1 and k_{-1}) can be obtained. Kinetic studies were carried out by changing (i) the immobilization amount of DNA on the QCM surface, (ii) the binding conditions of temperatures and ionic strength, (iii) the bZIP structures to ss-bZIP in which a leucine-zipper region was substituted by a covalently bonded Cys−Cys linkage, and (iv) the DNA sequences on the QCM.

We (*14*, *16*) and other researchers (*13*, *15*) have been using 5−9-MHz QCMs as molecular detectors or biosensors. The 27-MHz QCM used in this study is about 10 times more sensitive than the conventional 9-MHz QCM (*14*, *16*) and has a sensitivity of 0.6 ng cm^{-2} of mass change per Hertz of frequency decrease (*18*, *19*). This sensitivity is enough to detect a mass change of 50−100 ng cm^{-2} obtained by the binding of relatively small bZIP 56-mer peptides. We also compared our QCM results with those obtained by a gel mobility shift assay.

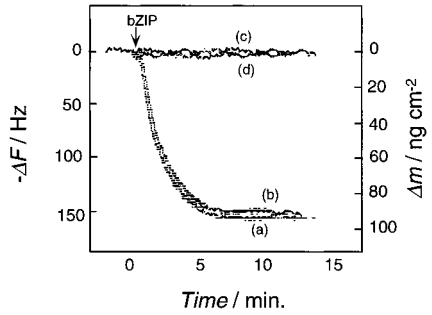

FIGURE 3: Typical time courses of frequency decreases of DNA-immobilized 27-MHz QCMs, responding to the addition of peptides in the aqueous solution: (a) CRE (21 bp) + bZIP, (b) CRE-random (41 bp) + bZIP, (c) random (20 bp) + bZIP, and (d) CRE (21 bp) + ss-bZIP (20 °C, pH 7.5, 20 mM Tris-HCl, 2 mM EDTA, 10 mM $MgCl_2$, 0.2 M KCl, [bZIP] = 0.2 μM, [ss-bZIP] = 0.1 μM).

RESULTS AND DISCUSSION

A method for immobilizing DNA strands is shown in Figure 1. At first the avidin molecules were covalently bound on carboxyl groups on one side of the Au electrodes (4.9 mm^2) of a 27-MHz QCM. Avidin molecules were confirmed from the frequency decrease (mass increase) of the QCM to cover the electrode surface as a Langmuir monolayer (480−600 ng cm^{-2}, ca. 7 × 10^{-12} mol cm^{-2}). Biotinylated DNA strands (20−21 bp) were immobilized on the avidin-bound QCM surface (95 ± 5 ng cm^{-2}, ca. 7 × 10^{-12} mol cm^{-2}) by immersing the QCM for about 30 min into the aqueous solution of DNA. The biotinylated DNA strand can be calculated to bind to one of four binding sites of an avidin molecule. The immobilized amount of DNA strands on the QCM could be controlled by the immersing period.

One-to-One Binding of the bZIP Dimer to the CRE Site. Figure 3 shows typical time courses of frequency decrease, $-\Delta F$ (mass increase, Δm) of the DNA duplex-immobilized 27-MHz QCM, responding to the addition of bZIP or ss-bZIP in the aqueous solution of 20 °C, pH 7.5, 20 mM Tris-HCl, 2 mM EDTA, 10 mM $MgCl_2$, and 0.2 M KCl. In the case of a CRE-immobilized QCM being employed, when a large excess of bZIP was injected in the solution (0.2 μM), the frequency gradually decreased (mass increased) in time and saturated at $-\Delta F$ = 160 ± 10 Hz (Δm = 95 ± 5 ng cm^{-2}) within 10 min (curve a). After the QCM plate was picked up to the air phase at the saturation and dried in air, the binding amount of bZIP was calculated to be Δm = 90 ± 10 ng cm^{-2} from the frequency decrease in the air phase before and after the bZIP binding. This value was consistent with the in-situ binding amount of curve a in the aqueous solution. The molecular weight of a CRE (21-bp) duplex was accidentally almost twice that of bZIP, 13 800 and 6 650, respectively. Thus, the binding of about 95 ng cm^{-2} (14 × 10^{-12} mol cm^{-2}) of bZIP to about 95 ng cm^{-2} (6.8 × 10^{-12} mol cm^{-2}) of the CRE duplex means that two bZIP molecules (i.e., a dimer) bind to one CRE site of a 21-bp DNA strand.

When a CRE-random (41 bp) was immobilized on a QCM, in which a random 20-bp sequence was linked to the end of the CRE site, a similar frequency decrease was observed (curve b). Thus, the additional random sequence added to the CRE site did not affect bZIP binding to the CRE site. Thus, a sliding mechanism, in which bZIP weakly binds to the random region and moves to the specific CRE site, does

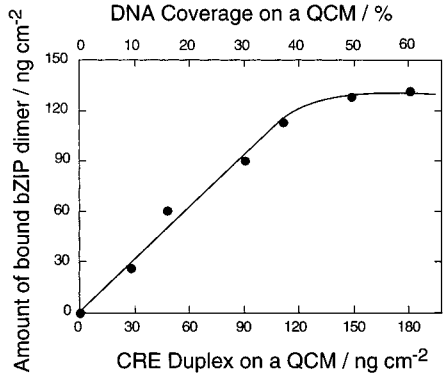

FIGURE 4: Effect of the immobilized amount of CRE duplex on the QCM on the binding amount of bZIP dimer (20 °C, pH 7.5, 20 mM Tris-HCl, 2 mM EDTA, 10 mM $MgCl_2$, 0.2 M KCl).

not seem to be important. Additionally the adjacent random sequence does not have a negative effect such as steric hindrance.

When the random 20-bp duplex was immobilized alone on the QCM, bZIP hardly bound (curve c). When an excess amount (0.4 μM) of CRE was present in the solution before bZIP was added, the binding of bZIP to the CRE-immobilized QCM was inhibited. This is apparently due to the CRE-bZIP formation in the solution that blocks bZIP from binding to CRE on a QCM. This result clearly indicates that bZIP binding to the CRE site is sequence-specific.

When ss-bZIP was added to the solution instead of bZIP, essentially no binding to the CRE site was observed (curve d). This indicates that the leucine-zipper region is important for sequence-specific binding to the CRE site. Dimerization constants of a leucine-zipper region of bZIP without DNA have been reported to be 10^{7-8} M^{-1} (*20*−*22*). Since our experiments were carried out in the bZIP concentration range $10^{-7}-10^{-6}$ M (mainly 10^{-6} M), bZIP is expected to exist as a dimer when it binds to the CRE site. We expected that ss-bZIP could mimic the bZIP dimer, however, not only binding affinity is decreased but also sequence specificity was largely decreased. Kim et. al. also observed from a gel mobility shift assay that ss-bZIP does not have specific binding affinity to DNA at 20 °C (*10*).

Effect of the Immobilized Amount of CRE Strands. The amount of CRE (21 bp) immobilized on the avidin-bound QCM could be controlled. The amount of bZIP bound as a function of the amount of CRE immobilized on the QCM is shown in Figure 4. When the immobilized amount of CRE was increased, the binding amount of bZIP increased linearly with a slope of about 1 as a weight ratio. Since the molecular weights of a CRE duplex and a bZIP dimer are almost the same (13 800 and 13 300, respectively), the slope indicates that one bZIP dimer binds to one CRE duplex. When the immobilized amount of CRE was larger than 100 ng cm^{-2} (ca. 40% area coverage of the QCM electrode), the binding amount of bZIP dimer reached a constant value. This indicates that a bZIP dimer can bind to a CRE duplex with a 1:1 molar ratio when the duplex coverage is less than 40% of the QCM electrode. Above this coverage the bZIP dimer has difficulty binding to the densely packed DNA strands on a QCM. The following experiments were carried out by using the QCM immobilized with 95 ± 5 ng cm^{-2} of DNA duplex (ca. 30% area coverage) to avoid these steric hindrances.

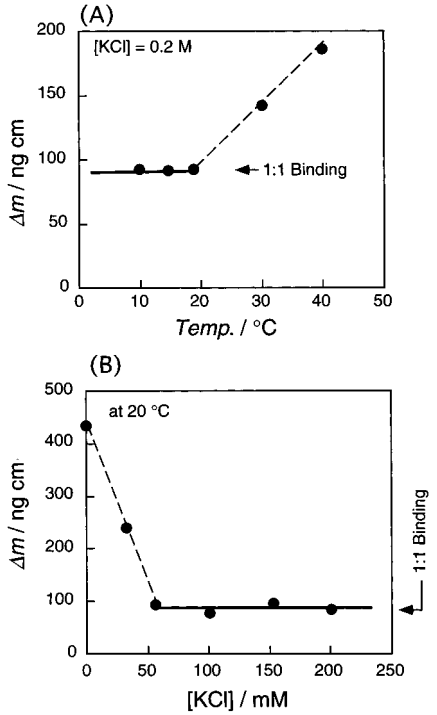

FIGURE 5: Effect of (A) temperature and (B) ionic strength on the bZIP dimer binding to the CRE duplex on the QCM (pH 7.5, 20 mM Tris-HCl, 2 mM EDTA, 10 mM $MgCl_2$).

The Effect of Temperatures and Salt Concentrations on 1:1 Binding. Figure 5A shows the effect of temperature on the binding of the bZIP dimer to the CRE duplex (95 ± 5 ng immobilized) on the QCM. Below 20 °C, 90 ± 10 ng cm^{-2} of bZIP bound to the CRE duplex, which means one bZIP dimer bound to one duplex (1:1 binding). With increasing temperatures above 20 °C, the amount of bZIP bound increased. This implies that nonspecific binding of bZIP occurs at higher temperatures possibly due to strong hydrophobic interactions.

As shown in Figure 5B, when the ionic concentration was kept above 50 mM KCl, 1:1 binding could be observed. At concentrations of KCl below 50 mM, a lot of nonspecific binding was also observed. This indicates that bZIP tends to bind nonspecifically due to electrostatic interactions at low ionic strength conditions. The QCM technique can provide quantitative confirmation of specific 1:1 binding as a mass change.

The Effect of DNA Sequences on bZIP Binding. The following kinetic experiments were carried out in the presence of 0.2 M KCl and at 20 °C, which shows 1:1 binding. The CRE site is a well-known recognition sequence ($^{5'}$ATGACGTCAT$^{3'}$ shown in bold in Figure 1) for a bZIP dimer (*2*−*8*). To investigate the sequence specificity of bZIP, various oligonucleotides were examined as binding sites (see Figure 1). CREhalf (21 bp) contains half of the CRE site ($^{5'}$ATGAC$^{3'}$), with the other half of the sequence replaced by five ATs. CE (21 bp) contains a recognition site of other bZIP proteins, in which four base pairs ($^{5'}$AT*TG*CG*CA*AT$^{3'}$ shown in italics) are different from the CRE sequence. CECR (21 bp) contains half of the CE site ($^{5'}$ATTGC$^{3'}$) and half of the CRE site ($^{5'}$GTCAT$^{3'}$).

Figure 6A shows typical time courses of bZIP binding to CRE, CREhalf, CECR, CE, and random DNAs on the QCM. Data for CRE and random DNA have already been shown

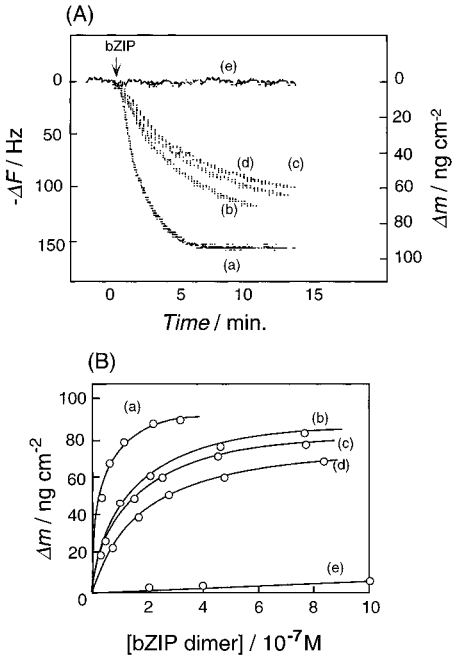

FIGURE 6: (A) Time courses of bZIP binding to the 27-MHz QCM immobilized with (a) CRE (21 bp), (b) CREhalf (21 bp), (c) CECR (21 bp), (d) CE (21 bp), and (e) random 20 bp (20 °C, pH 7.5, 20 mM Tris-HCl, 2 mM EDTA, 10 mM $MgCl_2$, 0.2 M KCl, [bZIP] = 0.2 μM). (B) Saturation binding behavior against bZIP dimer concentrations.

in Figure 3. Apparent binding rates to CREhalf, CECR, and CE sites that contain a half-binding site of CRE decreased to about half in comparison to the binding to a full CRE site. The final binding amounts were closed to that of the CRE site.

When the injected concentration of bZIP was changed in the solution, the binding amount (Δm) increased as the bZIP concentration increased. When Δm was plotted against [bZIP dimer], simple saturation curves containing one component were obtained as shown in Figure 6B. These saturation binding curves were displayed as reciprocal plots, eq 1, between $[\text{bZIP dimer}]_0/\Delta m$ and $[\text{bZIP dimer}]_0$, where

$$\frac{[\text{bZIP dimer}]_0}{\Delta m} = \frac{[\text{bZIP dimer}]_0}{\Delta m_{\max}} + \frac{1}{\Delta m_{\max} K_a} \quad (1)$$

$[\text{bZIP dimer}]_0$ indicates the initial concentration of bZIP as a dimer.

Simple straight lines were obtained according to eq 1 (data not shown). Association constants (K_a) and the maximum binding amount ($\Delta m_{\max}$) of dimer bZIP to DNAs were calculated from the slope and intercept of the linear relationship indicated by eq 1. Results are summarized in Table 1.

The binding of bZIP to DNA containing a CRE, CECR, or CE site was also examined by using a conventional gel mobility shift assay (*5*−*12*). A typical result for the binding of bZIP to ^{32}P-end-labeled CRE (21 bp) is shown in Figure 7. Increasing the bZIP concentration resulted in the gradual appearance of a band with a lower electrophoretic mobility (the complex of dimer bZIP and CRE). The association constant (K_a) was estimated according to eq 1, and the results are summarized in Table 1.

K_a values obtained from the QCM method were in good agreement with those obtained from the conventional gel mobility shift assay. When half of the DNA sequence of the CRE site was changed to nonspecific ATs (CREhalf), or to other specific sequences (CECR), or when the entire CRE site was changed to the CE site (a recognition site of other bZIP proteins), K_a values decreased to about half [(10−18) × 10^6 M^{-1}] of the value obtained with the CRE site (31 × 10^6 M^{-1}). K_a values of bZIP to a random sequence and of ss-bZIP to the CRE site were less than 1 × 10^6 M^{-1}.

Table 1: Maximum Binding Amount ($\Delta m_{\max}$), Association Constants (K_a), and Binding and Dissociation Rate Constants (k_1 and k_{-1}) between bZIP Dimer or ss-bZIP and DNAs Obtained by QCM Measurements,[a] As Well As K_a Values Obtained by Gel Mobility Shift Assay (in Parentheses)

DNA sequences	peptides	$\Delta m_{\max}$/ng cm^{-2}	$K_a/10^6$ M^{-1}	$k_1/10^2$ M^{-1} s^{-1}	$k_{-1}/10^{-4}$ s^{-1}
CRE	bZIP	95	31,[a] 31,[b] (22)[c]	630	21
CREhalf	bZIP	90	10[a]		
CECR	bZIP	88	18,[a] 18,[b] (18)[c]	750	42
CE	bZIP	85	15,[a] 15,[b] (13)[c]	680	46
random	bZIP		<1[a]		
CRE	ss-bZIP		<1[a]		

[a] Obtained from the saturation method using eq 2. [b] Obtained from the curve fitting method using eqs 3−5. [c] Obtained from gel mobility shift assay.

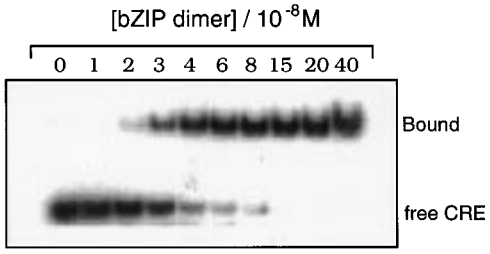

FIGURE 7: Gel mobility shift assay of ^{32}P-end-labeled CRE (21 bp) after incubation with bZIP (20 mM Tris-HCl, pH 7.5, 0.2 M KCl, 10 mM $MgCl_2$, 1 mM EDTA, 20 °C, [^{32}P-end-labeled CRE 21 bp] = 1 nM, [bZIP] = 0−0.4 μM).

The maximum binding amounts ($\Delta m_{\max}$) of bZIP to all DNA sequences were almost the same within experimental errors (see Table 1). Thus, when the CRE site changed to a half-recognition site or a slightly different site, the binding affinity decreased, but the bZIP dimer could bind up to the 1:1 ratio. The QCM method has a future in obtaining the absolute binding amount relative to the conventional gel mobility method.

Circular Dichroism (CD) Spectra Changes. Since the basic region of bZIP contains many positively charged Lys or Arg residues as shown in Figure 2, the basic region forms a random coil conformation in the absence of DNA. When bZIP binds to the specific CRE site, the basic region is known to form an α-helical structure due to the neutralization of basic amino acids by anionic phosphates in DNA chains (*23*). This phenomenon has been observed by circular dichroism spectrum changes. Figure 8A shows difference CD spectra showing the conformational changes of bZIP in the absence and presence of CRE, CECR, and CE (21 bp) DNA. When DNA was added to the solution, the magnitude of the θ_{223} values increased due to the formation of α-helical structures of the basic region of bZIP.

Saturation behaviors of θ_{223} values with increasing DNA concentrations were observed as shown in Figure 8B. Since reliability of θ_{223} values was relatively low and DNA could

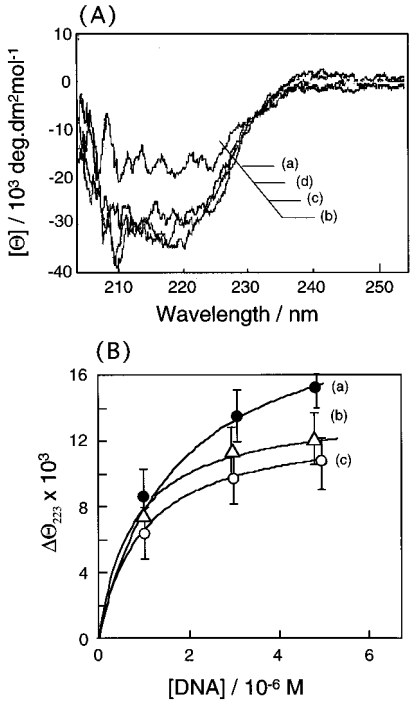

FIGURE 8: (A) CD spectra of bZIP (a) in the absence of DNA, and in the presence of (b) CRE, (c) CECR, and (d) CE 21 bp DNAs. CD spectra of the bZIP−DNA complex were subtracted by those of only DNAs (20 mM Tris-HCl, pH 7.5, 0.2 M KCl, 10 mM $MgCl_2$, 1 mM EDTA, 20 °C, [bZIP] = 2 μM, [DNA] = 0−6 μM). (B) Saturation behaviors of θ_{223} values against DNA concentrations.

not be added in excess of bZIP in difference CD spectrum measurements, K_a values could not be obtained precisely. However, K_a values were estimated to be 10^7 M^{-1} and the binding affinity of the bZIP dimer to DNA sites was on the order CRE > CREhalf = CE. This is fairly consistent with the results obtained by the QCM method as well as the gel mobility shift assay.

Binding and Dissociation Rate Constants. Detailed binding kinetics of the bZIP dimer to DNAs can be calculated from the time courses of frequency decreases (mass increases) of Figures 3 and 6. The binding between the bZIP dimer and DNAs on the QCM is described by eq 2

$$[\text{bZIP dimer}] + [\text{DNA}] \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} [\text{bZIP dimer}-\text{DNA}] \quad (2)$$

The amount of bZIP dimer-DNA complex formed at time t after injection is given by eqs 3−5

$$[\text{bZIP dimer}-\text{DNA}]_t = [\text{bZIP dimer}-\text{DNA}]_\infty (1 - e^{-1/\tau t}) \quad (3)$$

$$\Delta m_t = \Delta m_{max} (1 - e^{-1/\tau t}) \quad (4)$$

$$\tau^{-1} = k_1 [\text{bZIP dimer}]_0 + k_{-1} \quad (5)$$

Figure 9 shows linear correlations of the reciprocal of the relaxation time of the binding (τ^{-1}) against various bZIP dimer concentrations (eq 5). The binding and dissociation rate constants (k_1 and k_{-1}) could be obtained from the slope and intercept of Figure 9, respectively. K_a values could also be obtained from the ratio of k_1 and k_{-1}. The obtained kinetic parameters are summarized in Table 1. The K_a values

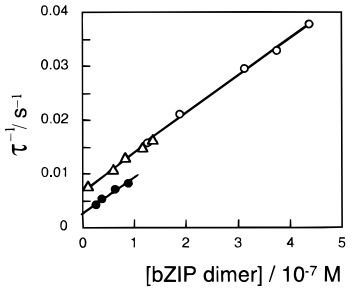

FIGURE 9: Linear reciprocal plots of relaxation time (τ) against [bZIP dimer] according to eq 5: (●) CRE, (△) CECR, and (○) CE 21-bp DNAs.

obtained from the curve fitting method according to eqs 3−5 were fairly consistent with those obtained from a saturation method (see eq 1 and Figure 6). Binding rate constants (k_1) were almost the same for all DNA sequences, but the dissociation rate constant (k_{-1}) for the CRE site is half of those for the CECR and CE sites, which is reflected in the two times larger K_a value for the CRE site than those of the CECR and CE sites. Thus, the sequence selectivity of bZIP is mainly determined by the dissociation process, but not the binding process. In the sequence selective binding experiments of NFI proteins (MW = 4.5×10^4) (*24*) to the 30-bp DNA strand containing a $^{5'}TGGN_{6-7}GCCAA^{3'}$ sequence by using the same 27-MHz QCM system, the selective binding showed the smaller k_{-1} values compared to the nonspecific binding of NFI to the random sequences (*25*).

CONCLUSION

The 27-MHz QCM is highly sensitive and quantitative to detect in situ sequence-selective peptide binding to DNA strands in the aqueous buffer solution without using any DNA labeling in ionic aqueous solutions. From time courses of frequency changes, kinetic parameters such as the binding amount (Δm), the association constants (K_a), and the binding and dissociation rate constants (k_1 and k_{-1}) could be obtained. The association constants obtained by QCM measurements were consistent with those obtained by the conventional gel mobility shift assay and CD spectrum changes. We believe that the highly sensitive 27-MHz QCM technique will provide a new tool for in situ kinetic studies of the sequence-specific binding of peptides and/or proteins to DNA strands such as genetic regulatory proteins to promoter and enhancer regions of transcribed genes in aqueous solutions.

EXPERIMENTAL SECTION

Materials. Oligonucleotides were automatically synthesized using a DNA synthesizer (Pharmacia Biotech, AB) and commercially available amidites. 5′-Biotinylated oligonucleotides were prepared using biotinylating amidite (Biodite, Pharmacia Biotech, AB). Synthesized DNA sequences are shown in Figure 1 as CRE (21 bp), CRE-random (41 bp), CREhalf (21 bp), CE (21 bp), CECR (21 bp), and random sequence DNA (20 bp). All of the resultant oligonucleotides were purified with anion-exchange chromatography, and their concentrations were determined by an optical density measurement taken at 260 nm.

The bZIP 56-mer peptide was prepared from a single stepwise manual solid-phase peptide synthesis using Fmoc

(9-fluorenylmethoxycarbonyl) amino acids and PyBOP in the presence of 1-hydroxybenzotriazole (*26*). The coupling reaction was performed with 1.12 g of Fmoc-PAL-PEG-PS resin (0.18 mmol/g) and Fmoc amino acid (0.5 mmol) in the presence of PyBOP (0.5 mmol), *N*-methylmorphorin (0.75 mmol), and 1-hydroxybenzotriazole (0.5 mmol) in anhydrous DMF for 45 min. Completion of the coupling was monitored by the Kaiser test (*27*), and the coupling reaction was repeated until completion. Removal of the Fmoc group was performed by treatment with 20% piperidine−DMF. The amino termini were acetylated with acetylimidazole, and the peptides were cleaved from the resin with a cleavage mixture containing bromotrimethylsilane (1.35 mL), thioanisole (1.2 mL), 1,2-ethanedithiol (0.6 mL), and *m*-cresol (0.2 mL) in trifluoroacetic acid (7.48 mL), then desalted by Sephadex G-10 chromatography in 5% acetic acid. Purification was done by reversed-phase HPLC with an Ultron VX-Peptide column (Sinwa Chemical Industry; 20 × 250 mm) and linear gradient of acetonitrile−water with 0.2% trifluoroacetic acid (8−30% acetonitrile in 50 min; flow rate, 5 mL/min). MS (electrospray; 50% acetonitrile, 0.05% formic acid) calculated for [M^+], 6651.69; found, 6652.76.

Synthesis of the disulfide dimer of the basic region of bZIP (23-mer) was carried out similarly (*11*). An aqueous solution (0.2 M Tris-HCl, pH 8.5, 800 μL) of the 23 mer peptide having a Cys residue at the C end (15 nmol) was kept at 0 °C for 5 h under aerobic conditions. The solution was allowed to warm to room temperature and was quenched with the addition of acetic acid. Purification of the crude product by Sephadex G-10 (5% acetic acid as an eluent) and reversed-phase HPLC on a Ultron VX-Peptide column (Sinwa Chemical Industry; 20 × 250 mm) with 0.2% trifluoroacetic acid−acetonitrile as an eluent yielded the disulfide-bonded dimer.

Avidin was obtained from Calzyme Co., and other chemicals were purchased from Tokyo Kasei Co. and Sigma Co. and used without further purification.

A 27-MHz QCM and It's Calibration. A 27-MHz, AT-cut QCM is commercially available from Showa Crystals Co., Chiba, 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) (*18, 19*). One side of the quartz crystal was sealed with a rubber casing, maintaining it in an air environment to avoid contact with the ionic aqueous solution, while the other is exposed to aqueous buffer solution (*14, 16*). A cased 27-MHz QCM was connected to an oscillation circuit designed to drive the quartz in aqueous solution. The frequency changes were followed by a universal frequency counter (Hewlett-Packard Co., Ltd., Tokyo, model 53131A) attached to a microcomputer system (Macintosh Power Book 170, Apple, Co.) (*14, 16*).

The following Sauerbrey's equation (eq 6) has been

$$\Delta F = -\frac{2F_0^{\,2}}{A\sqrt{\rho_q \mu_q}}\Delta m \qquad (6)$$

obtained for the AT-cut shear mode QCM (*17*), 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}).

Calibration of the 27-MHz QCM was carried out analogously to the calibration of our conventional 9-MHz QCM (*14, 16*). When a respective amount of polymer solution was cast or LB film of lipid monolayers was deposited on the bare Au electrode side of the QCM plate, a linear relationship was observed between the deposited amount of mass and the frequency decrease of the QCM, independent of the method 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 (*18, 19*). Thus, the sensitivity for the mass change of a 27-MHz QCM was increased by about 10 times, in comparison to our conventional 9-MHz QCM (Δm = 6.5 ng cm^{-2}) (*14, 16*). The noise level of the 27-MHz QCM was ±5 Hz in buffer solution at 20 °C, and the standard deviation of the frequency was ±5 Hz for 12 h in buffer solution at 20 °C. These values were the same level as the conventional 9-MHz QCM (*14, 16*).

Immobilization of DNA on a 27-MHz QCM. Immobilization of a biotinylated DNA to an avidin-bound QCM is schematically shown in Figure 1. The cleaned bare Au electrode side of the QCM plate was soaked into the aqueous solution (3 mL) of 3,3′-dithiodipropionic acid (1 mM) at room temperature, and the frequency decrease was saturated at about 100−150 Hz (mass increase of 60−90 ng cm^{-2}) after 20 min. This means 3,3′-dithiodipropionic acid (area per molecule: 0.4 nm^2) covered roughly as a Langmuir monolayer on the Au electrode (4.9 mm^2). Before drying, the carboxylic acid on the QCM was reacted with *N*-hydroxysuccinimide in the presence of water-soluble carbodiimide [1-ethyl-3-(3-dimethylaminopropyl)carbodiimide] in the aqueous solution. The frequency decrease was roughly equilibrated at 50−100 Hz after 30 min. The QCM having the activated carboxyl groups was immersed in 1 mL of the aqueous buffer solution (pH 7.9, 10 mM Tris-HCl, 0.2 M NaCl) of avidin (10 μg, M*w* = 68 000). The frequency decrease reached equilibrium at 800−1000 Hz (480−600 ng cm^{-2}) for 1 h. This means that avidin (section area: 80 nm^2) bound as a monolayer on the electrode (25−30 ng on 4.9 mm^2). Avidin was not able to be removed from the electrode after rinsing with aqueous solution several times. The QCM was immersed into the aqueous solution (1 mL) of ethanolamine (1 M) for 30 min to deactivate the carboxyl group as β-hydroxyethylamide.

As a control experiment, when a bare QCM was soaked into the aqueous buffer solution of avidin (10 μg in 1 mL), a simple adsorption of avidin was hardly observed in this concentration. When the concentration of avidin was increased to 30−100 μg in 1 mL, however, nonspecific adsorption was observed.

The avidin-bound QCM was immersed into 1 mL of the aqueous buffer solution (pH 7.9, 10 mM Tris-HCl, 0.2 M NaCl) of biotinylated DNA (20−21 bp, 1 μM) at 25 °C. After 30 min, when the frequency decreased about 150 ± 10 Hz (mass increase of 95 ± 5 ng cm^{-2}) in about 30 min., the QCM was picked up to control the immobilization amount. The biotinylated DNA strand is calculated to bind to one of four binding sites of an avidin molecule. The immobilized amount of biotin-DNA was controlled by the immersing time in the DNA solution.

Binding of bZIP Peptides to DNA on a QCM. A duplex DNA-immobilized QCM was soaked into 8 mL of aqueous

solution (pH 7.5, 20 mM Tris-HCl, 2 mM EDTA, 10 mM $MgCl_2$, 0.2 M KCl) at the respective temperature (usually at 20 °C), and the resonance frequency of the QCM was defined as the 0 position after equilibrium. The stability and the drift of the 27-MHz QCM frequency in the solution were ±5 Hz for 12 h at 20 °C. The frequency change of the QCM responding to the addition of 10−100 μL of aqueous solution of bZIP was recorded with time. The solution was stirred to avoid any effect of the diffusion of peptides, and the stirring did not affect the stability and the amount of frequency changes.

Gel Mobility Shift Assay. Binding between bZIP and a DNA duplex was also studied by a conventional gel mobility shift assay as described previously (*8*, *11*, *12*). Aqueous solutions of bZIP and ^{32}P-end-labeled DNA duplex (21 bp) were incubated for 30 min (pH 7.5, 20 mM Tris-HCl, 1 mM EDTA, 10 mM $MgCl_2$, 0.2 M KCl, 6% sucrose) and applied to poly(acrylamide) gel at 20 °C (20 mM Tris-HCl, 20 mM phosphate, 0.1 mM EDTA). The mobility was analyzed by autoradiography. The concentration of bZIP was determined by amino acid analysis (Waters AccQ-Tag Chemistry Package, Millipore, α-aminobutylic acid as a standard).

Circular Dichroism (CD) Spectra. The spectra were observed by a J-600 spectropolarimeter (Nippon Bunkou Co., Tokyo) at 20 °C using a quartz cell (2-mm cell length, pH 7.5, 20 mM Tris-HCl, 2 mM EDTA, 10 mM $MgCl_2$, 0.2 M KCl). At first the CD spectrum of DNA only was observed, and after incubation with bZIP for 10 min., the spectrum of bZIP-DNA was observed. The CD differential spectrum of bZIP was obtained by subtracting the DNA spectrum from the bZIP-DNA spectrum.

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