Kinetic Studies of Site-Directed Mutational Isomalto-dextranase-Catalyzed Hydrolytic Reactions on a 27 MHz Quartz-Crystal Microbalance†

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ABSTRACT: A quartz-crystal microbalance (QCM) technique was applied to analyze effects of site-directed mutagenesis of a glycosidase (isomalto-dextranase) on the hydrolysis mechanism of the substrate binding (k_{on}, k_{off}, and K_d) and the catalytic process (k_{cat}), separately, by using a dextran-immobilized QCM in buffer solution. D266N, D198N, and D313N mutants, which are predicted as critical residues of the isomalto-dextranase hydrolytic activity, dramatically decreased the apparent enzyme activity. The D266N mutant, however, did not change the substrate binding ability (K_d), and the D198N and D313N mutants largely increased K_d values due to the increase of k_{off} and/or the decrease of k_{on} values, as well as the negatively small k_{cat} values. From these results, we estimate the reaction mechanism, in which Asp266 acts as only a general acid in the catalytic process, Asp198 acts as both nucleophile in the catalytic process and binding the substrate, and Asp313 acts as only the substrate binding.

In general, enzyme reactions have been kinetically studied by using a Michaelis−Menten equation (steady-state kinetics), in which the concentration of the enzyme−substrate (ES) complex was hypothesized to be nearly constant during the reaction, because it was relatively difficult to detect the concentration of the ES complex (*1*, *2*). The reaction rate was simply obtained from the initial rate of the product increase by various methods such as colorimetric and stopped-flow fluorescent methods (*3*−*7*). For analyses of the active center of enzymes, site-directed mutagenesis has been widely studied, and the kinetic effect of mutations has been again studied by using a Michaelis−Menten method from the product analysis. When the site-directed mutagenesis nearly diminished the enzyme activity and the product was hardly produced, it was difficult to distinguish whether mutations were effective to the substrate binding or the catalytic process since only the product was followed. If the formation and decay of the ES complex could be followed directly during the reaction, more precise and quantitative discussions on the site-directed mutagenesis are expected.

Isomalto-dextranase (1,6-α-D-glucan isomaltohydrolase, EC 3.2.1.94) is an *exo*-type enzyme that is capable to hydrolyze dextran and to release α-isomaltose units successively from the nonreducing ends of dextran chains via a retaining reaction mechanism (*8*, *9*). It is produced extracellularly by *Arthrobacter globiformis* T6 and *Actinomadura* sp. R-10 (*8*, *10*), and the cloning of the *imd* gene encoding this enzyme has been reported (*11*). It was classified as a member of a family comprising mainly eukaryotic α-galactosidases (*12*) and α-*N*-acetylgalactosaminidases (*13*), which is designated as a glycoside hydrolase (GH)[1] family 27 (*14*, *15*). The crystallization and preliminary X-ray diffraction analysis of isomalto-dextranase from *A. globiformis* T6 has been reported (*16*); however, the three-dimensional structure and reaction mechanisms of this enzyme are yet unknown. Multiple sequence alignments of a GH family 27 such as *A. globiformis* T6 isomalto-dextranase (IMD) (*11*), chicken α-*N*-acetylgalactosaminidase (NAG) (*13*), rice α-galactosidase (GALA) (*12*), and human α-galactosidase (GALH) (*17*) are shown in Figure 1. The letters with black background have been reported as identical active site residues, and three aspartic acids marked with red backgrounds and asterisks are thought to be critical residues of the enzyme activities. From the molecular modeling and the prediction of isomalto-dextranase structure from comparison of primary, secondary, and crystal structures of a GH family 27, we predict that three aspartic acids, Asp198, Asp266, and Asp313 (marked with red backgrounds and asterisks), are critical residues of the enzyme activity. Therefore, three kinds of site-directed mutants (D198N, D266N, and D313N) of isomalto-dextranase by substituting aspartic acids to asparagines are interest-

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[1] Abbreviations: QCM, quartz-crystal microbalance; GH, glycoside hydrolase; IMD, *Arthrobacter globiformis* T6 isomalto-dextranase; NAG, chicken α-*N*-acetylgalactosaminidase; GALA, rice α-galactosidase; GALH, human α-galactosidase.

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IMD    1 MATAVTARPG VPVTAAPPLR LASRNSVFTR SGAGPRYNNI YGYSFPH---
NAG    1                      LENGLART PPMGWLAWER FRCNVNCRED
GALR   1                      FENGLGRT PQMGWNSWNH FYCG------
GALH   1                  PGA RALDNGLART PTMGWLHWER FMCNLDCQEE

IMD   48 -NAPIPENEW KANIDWLAG- NFADFGYDIA CTDGWIEGSS RTTGNGYITS
NAG   29 PRQCISEMLF MEMADRIAED GWRELGYKYI NIDDCWAAKQ RDAE-GRLVP
GALR  23 ----INEQII RETADALVNT GLAKLGYQYV NIDDCWAEYS RDSQ-GNFVP
GALH  34 PDSCISEKLF MEMAELMVSE GWKDAGYEYL CIDDCWMAPQ RDSE-GRLQA

IMD   96 YNDSWQHDWA YWANYLAARK MKLGVYYNPL WVHRAAVEDA SKTVLGRPDV
NAG   78 DPERFPRGIK ALADYVHARG LKLGIY---- -----GDLGR -LTCGGYPGT
GALR  68 NRQTFPSGIK ALADYVHAKG LKLGIY---- -----SDAGS -QTCSNKMPG
GALH  83 DPQRFPHGIR QLANYVHSKG LKLGIY---- -----ADVGN -KTCAGFPGS

IMD  146 KIADLVVPGD FFARDIGGNQ LYWLDVTKSG AKEYVQGYVR YFKDLGVPYL
NAG  118 TL-------- ---------- ---------- --DRVEQDAQ TFAEWGVDML
GALR 108 SL-------- ---------- ---------- --DHEEQDVK TFASWGVDYL
GALH 123 FG-------- ---------- ---------- ---YYDIDAQ TFADWGVDLL

IMD  196 RIDFLSWYED GRDANIGQVN APHGRANYEL ALSWINEAAG EDMEVSLVMP
NAG  138 KLDGC----- --------YS SGKEQAQGYP QMARALNSTG RPIVYSCSWP
GALR 128 KYDNC----- --------ND AGRSVMERYT RMSNAMKTYG KNIFFSLC-E
GALH 142 KFDGC----- -------YCD SLENLADGYK HMSLALNRTG RSIVYSCEWP

IMD  246 ---HMFQD-- -GSAELANGD LVRNADADK GGWDRLSGMR QNWQDAWPNW
NAG  175 AYQGGLPPKV NYTLLGEICN LWRNYDDIQD -SWDSVLSIV DWFFTN-QDV
GALR 164 WGKEN----- PATWAGRMGN SWRTTGDIAD -NWGSMTSRA DENDQ-----
GALH 180 LYMWPFQKP- NYTEIRQYCN HWRNFADIDD -SWKSIKSIL DWTSFN-QER

IMD  290 ANPFCGFTGW SHRNGRGQLI LDGDFMRAST FA-SDEERKT MMNLMVAAGS
NAG  223 LQP----FAGP GHWN------ -DPDMLIIGN FGLSYEQSRS QMALWTIMAA
GALR 203 WAA----YAGP GGWN------ -DPDMLEVGN GGMSEAEYRS HFSIWALAKA
GALH 227 IVD----VAGP GGWN------ -DPDMLVIGN FGLSWNQQVT QMALWAIMAA
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FIGURE 1: Multiple sequence alignments of *A. globiformis* T6 isomalto-dextranase (IMD), chicken α-*N*-acetylgalactosaminidase (NAG), rice α-galactosidase (GALA), and human α-galactosidase (GALH). The letters with black background have been reported as identical active site residues, and three aspartic acids marked with red background and asterisks are thought to be critical residues of the enzyme activities.

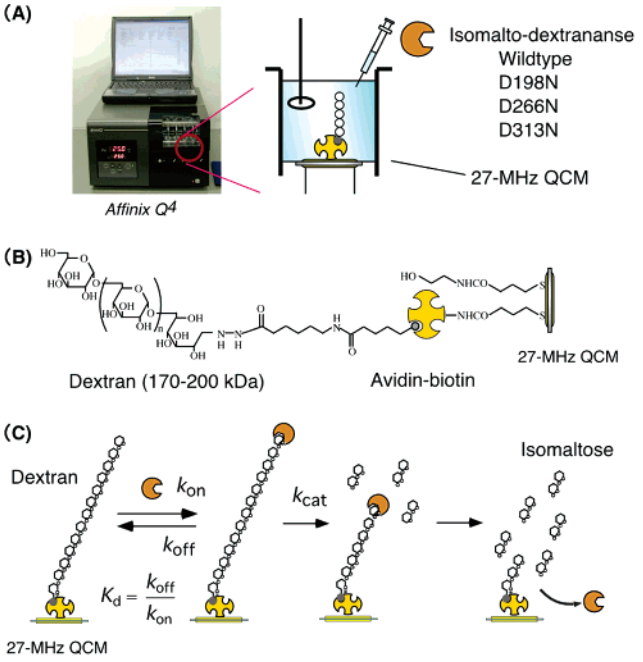

FIGURE 2: (A) Experimental setup (Affinix Q^4) of enzymatic hydrolyses on the dextran-immobilized 27 MHz quartz-crystal microbalance (QCM) catalyzed by the wild-type or site-directed mutagenic isomalto-dextranase in buffer solution: 20 mM acetate buffer, pH 5.2, and 150 mM NaCl at 25 °C. (B) Chemical structures of dextran immobilized on a QCM plate through an avidinbiotin linkage. (C) Reaction schemes and kinetic parameters obtained in this work.

ing whether these aspartic residues act as the binding site in addition to the catalytic site.

In this paper, we describe that a dextran-immobilized 27 MHz quartz-crystal microbalance (QCM) is a useful tool to detect whether the site-directed mutagenesis for isomalto-dextranase is effective to the substrate binding or the catalytic process. We prepared three kinds of mutant enzymes such as D198N, D266N, and D313N of isomalto-dextranase as well as a wild type. By using the substrate-immobilized QCM, we could follow directly and quantitatively each step of the hydrolysis (binding and dissociation of the enzyme and the catalytic process) from the ES complex formation as mass changes (see Figure 2) (*18*). The 27 MHz QCM is a very sensitive mass-measuring device in aqueous solution, and its resonance frequency is proved to decrease linearly with increasing mass on the QCM electrode at the nanogram level (*18*−*23*). We have previously reported that a 27 MHz QCM is a useful tool to directly and quantitatively detect various molecular interactions such as DNA−DNA hybridization (*20*), DNA−peptide interactions (*21*), glycolipid−protein interactions (*22*), enzyme reactions on DNA (*23*), and polysaccharides (*18*).

EXPERIMENTAL PROCEDURES

Preparation of Wild-Type and Mutated Isomalto-dextranases. The wild type and three mutants (D198N, D266N, and D313N) were produced from *Escherichia coli* BL21-(DE3) Rosetta cells carrying an expression plasmid pETG2Dsp-, according to the procedure of the previous paper (*24*). Oligonucleotide-directed mutagenesis was carried out using the plasmid pETG2Dsp- with the Quik-Change site-directed mutagenesis kit (Stratagene) for the construction of mutant isomalto-dextranases, D198N, D266N, and D313N, in which aspartic acid was replaced by asparagine.

Purification of Wild-Type and Mutant Enzymes. To denature the *E. coli* proteins, the crude extract was heat-treated at 55 °C for 30 min, and then the denatured host proteins were pelleted by centrifugation at 10000*g* for 20 min. The supernatant was loaded onto a phenyl-Toyopearl 650S column (Tosoh Co. Ltd., Tokyo, Japan; 2.2 × 20 cm) equilibrated with 20 mM Tris-HCl buffer (pH 7.5) containing 0.7 M $(NH_4)_2SO_4$. The enzyme was eluted with a linear 0.7−0 M $(NH_4)_2SO_4$ gradient in buffer. The isomalto-dextranase fractions were pooled, dialyzed against 20 mM Tris-HCl buffer (pH 7.5), and loaded onto a Q-Sepharose HP column (Amersham Biosciences Co.; 1.6 × 10 cm) equilibrated with same buffer. The enzyme was eluted with a linear 0−1 M NaCl gradient in buffer. The homogeneity of each isomalto-dextranase and its mutant was confirmed by SDS−PAGE (*25*).

Somogyi−Nelson Method (5, 6). Dextran saccharification activity of isomalto-dextranase in the bulk solution was measured by the conventional Somogyi−Nelson method. The reaction mixture of 25 μL of 0.1−5% (ca. 0.5−25 mM) dextran solution, 50 μL of acetate buffer (20 mM, pH 5.2), and 25 μL of the enzyme solution (15 μM) was incubated at 30 °C for an appropriate period. Reducing sugars formed per 1.0 mL of the mixture were determined by the colorimetric method of Somogyi−Nelson. One unit was defined as the amount of enzyme that released reducing sugars equivalent to 1.0 μmol of D-glucose from the substrate per minute under the assay conditions.

27 MHz QCM Setup and Its Calibration. An Affinix Q^4 was used as a QCM instrument (Initium Co. Ltd., Tokyo,

http://www.initium2000.com/) having four 500 μL cells equipped with a 27 MHz QCM plate (8 mm diameter of a quartz plate and an area of 4.9 mm^2 of Au electrode) at the bottom of the cell and the stirring bar with the temperature-controlling system (*18*, *20*−*23*). The Sauerbrey equation (*19*) was obtained for the AT-cut shear mode QCM:

$$\Delta F = -\frac{2F_0^{\,2}}{A\sqrt{\rho_q \mu_q}}\Delta m \qquad (1)$$

where ΔF is the measured frequency change (in hertz), F_0 the fundamental frequency of the quartz crystal prior to a mass change (27×10^6 Hz), Δm the mass change (g), A the electrode area (0.049 cm^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 performed such that a frequency decrease of 1 Hz corresponded to a mass increase of 0.62 ± 0.1 ng cm^{-2} on the QCM electrode, as described previously (*18*, *20*−*23*). The noise level of the 27 MHz QCM was ±1 Hz in buffer solutions at 25 °C, and the standard deviation of the frequency was ±2 Hz for 2 h in buffer solutions at 25 °C.

Preparation of Dextran-Immobilized QCM Plates. The biotinylated dextran (MW 170−200 kDa), in which the reducing ends were reacted with biotinamidocaproyl hydrazide, was anchored on NeutrAvidin-immobilized QCM according to previous papers (Figure 1) (*18*, *26*). The immobilized amount of the biotinylated dextran was maintained to be 150 ± 10 ng (ca. 0.8 pmol) cm^{-2}, which corresponds to ca. 10% coverage of the surface. This small coverage gives enough space for the binding of a large enzyme molecule.

Enzyme Reactions on Dextran-Immobilized QCM Plates. A dextran-immobilized QCM plate was soaked in 500 μL of 20 mM acetate buffer (pH 5.2) containing 150 mM NaCl and placed until the resonance frequency reached the steady state. Frequency changes responding to the addition of enzyme solution into the cell were followed with time. The solution was vigorously stirred to avoid any effect of slow diffusion of enzymes, and the stirring did not affect the stability and magnitude of frequency changes.

RESULTS AND DISCUSSION

The wild type and three mutants were produced and purified as described in Experimental Procedures. Three mutated isomalto-dextranases were found to have the markedly reduced level of the hydrolyzing activity for dextran, less than 0.01% of the wild type obtained by the conventional product analyses of the Somogyi−Nelson method (*5*, *6*) in the bulk solution (see Table 1). Therefore, it is difficult to discuss further enzyme activity by the conventional method to follow the product.

Figure 3A shows catalytic hydrolyses of dextran immobilized on a 27 MHz QCM plate catalyzed by different concentrations (7.5−45 nM, 3.8−23 pmol, in 0.5 mL) of the wild type isomalto-dextranase in the aqueous solution. The frequency simply increased (mass decreased) with the reaction time due to the hydrolysis of the dextran substrate on the QCM. When enzyme concentrations increased, initial slopes increased, and the frequency reached constant values [−30 ± 5 ng (ca. 0.2 pmol) cm^{-2} over the starting point] independent of the added enzyme concentrations. The amount corresponds to ca. 20% of the immobilized amount of the dextran substrate [150 ± 20 ng (ca. 0.8 pmol) cm^{-2}].

Table 1: Kinetic Parameters of Wild Type and Mutants of Isomalto-dextranase for Hydrolysis of Dextran Immobilized on the 27 MHz QCM[a] and Relative Enzyme Activity in Bulk Solution[b]

	k_{on}[a]/ 10^3 M^{-1} s^{-1}	k_{off}[a]/ 10^{-3} s^{-1}	K_d[a]/ 10^{-9} M	k_{cat}[a]/ s^{-1}	relative enzyme activity/%[b]
wild type			5.0	1 (2.5)[b]	100
D266N	78	0.4	5.1	∼0	0.011
D198N	167	39	234	∼0	0.003
D313N	29	15	518	∼0	0.002

[a] Obtained from the QCM method at 20 mM acetate buffer, pH 5.2, and 150 mM NaCl, 25 °C. [b] Obtained from the Somogyi−Nelson method in the bulk solution at 20 mM acetate buffer, pH 5.2, and 150 mM NaCl, 30 °C.

The dextran hydrolysis process of the wild-type enzyme is described as eq 2, where $[E]_0 \gg [S]_0$. The apparent hydrolysis rate constant (k_{app}) in the single turnover reaction is calculated from curve fittings of eqs 3−5, where $[E] = [E]_0$ and $[S] = [S]_0 - [ES]$.

$$E + S \underset{K_d}{\rightleftharpoons} ES \xrightarrow{k_{cat}} E + P \qquad (2)$$

$$k_{app} = \frac{d[P]}{dt} = k_{cat}[ES] \qquad (3)$$

$$[P] = [S]_0(1 - e^{\alpha k_{cat} t})\ \alpha = \frac{[E]_0}{[E]_0 + K_d} \qquad (4)$$

$$k_{app} = \frac{d[P]}{dt} = \alpha k_{cat} = \frac{[E]_0 k_{cat}}{[E]_0 + K_d} \qquad (5)$$

$$\frac{1}{k_{app}} = \frac{1}{k_{cat}} + \frac{K_d}{k_{cat}[E]_0} \qquad (6)$$

From the linear correlation between $1/k_{app}$ and different $1/[E]_0$ values of eq 6 (Figure 3B), the dissociation constant and catalytic hydrolysis rate constant could be obtained to be K_d = 5.0 nM and k_{cat} = 1.1 s^{-1}, respectively. The results are summarized in Table 1.

The catalytic activity of the wild-type enzyme was obtained to be $k_{cat} = 2.5$ s^{-1} by the conventional Somogyi−Nelson method in the condition of $[S]_0$ = 0.5−25 mM > $[E]_0 = 15$ μM. Although the reaction condition was different ($[E]_0$ = 3.8−23 pmol > $[S]_0$ = 0.8 pmol for the QCM method), both k_{cat} values showed good agreement each other.

Figure 4 shows frequency changes of the dextran-immobilized QCM, responding to additions of site-directed mutant enzymes (D198N, D266N, and D313N) as well as the wild type. When the wild type was employed, the hydrolysis (the mass decrease) was observed as already shown in Figure 3A. When mutant enzymes were employed, however, the frequency simply decreased (the mass increased), indicating that only the enzyme binding to the substrate is observed, but hardly observed the catalytic hydrolysis process. The binding ability seems to be in order of D266N ≫ D198N > D313N. The hydrolysis properties of these mutants were confirmed to be less than 0.01% of

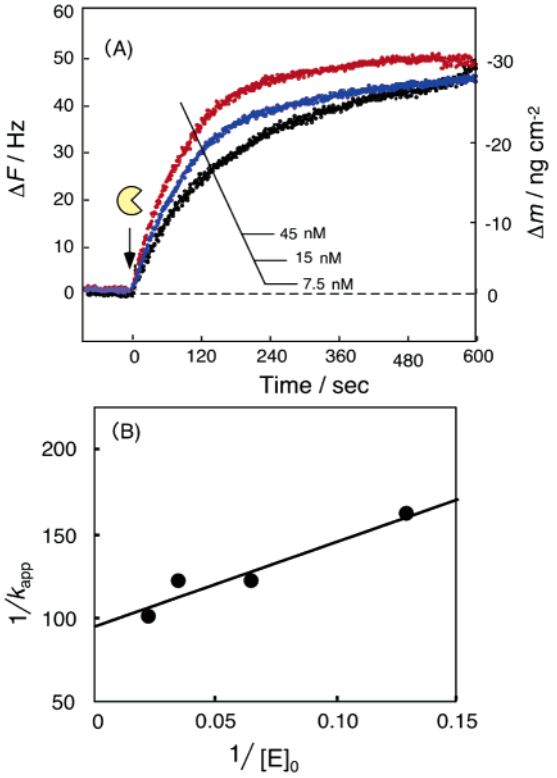

FIGURE 3: (A) Typical time courses of frequency increases (mass decreases) of the dextran-immobilized QCM, responding to the addition of different concentrations of the wild-type isomalto-dextranase. The immobilized amount of dextran (170−200 kDa) was 150 ± 10 ng cm^{-2} in 20 mM acetate buffer, pH 5.2, and 150 mM NaCl at 25 °C. (B) Linear reciprocal plots of k_{app} against the enzyme concentrations according to eq 6 in the text.

the wild type from the conventional Somogyi−Nelson method (*5*, *6*) in the bulk solution. Thus, frequency decreases in Figure 4 indicate the mass increases of the ES complex formation at time t on the QCM, which is given by eq 7.

$$\mathrm{E} + \mathrm{S} \underset{k_{off}}{\overset{k_{on}}{\rightleftharpoons}} \mathrm{ES} \tag{7}$$

$$\Delta m = [\mathrm{ES}] = [\mathrm{ES}]_{max}\{1 - \exp(-t/\tau)\} \tag{8}$$

$$\tau^{-1} = k_{on}[\mathrm{E}]_0 + k_{off} \tag{9}$$

As shown in Figure 5, the binding amount of the enzyme increased with increasing the enzyme concentration for all mutagenic enzymes. From linear correlation of τ^{-1} and $[\mathrm{E}]_0$ values of eq 9, the binding rate constant (k_{on}) and dissociation rate constant (k_{off}) could be obtained from the slope and intercept, respectively. The dissociation constant (K_d) could be obtained from the ratio of k_{off} to k_{on}. The kinetic results obtained for each mutant are summarized in Table 1.

The retaining enzymes as the GH family 27 members act via a double-displacement mechanism, wherein one of the catalytic carboxylate groups operates as a nucleophile to generate a glycosyl-enzyme intermediate. The second carboxylate group acts, in turn, as a general acid and general base catalyst to promote the formation and breakdown of the intermediate, respectively (*27*). Thus, in isomalto-dextranase, Asp198 exists as a catalytic nucleophile and Asp266 exists as a catalytic general acid and general base.

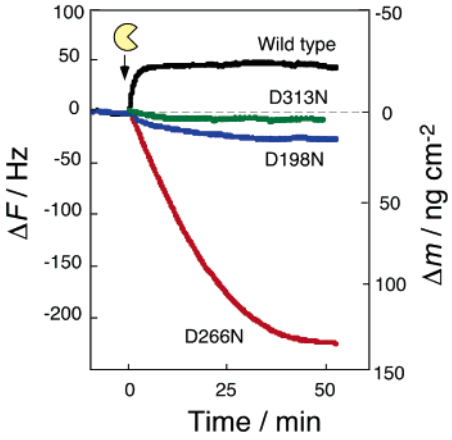

FIGURE 4: Time courses of frequency changes of the dextran-immobilized QCM, responding to the addition of the wild type and mutants of D198N, D266N, and D313N. The immobilized amount of dextran (170−200 kDa) was 150 ± 10 ng cm^{-2} in 20 mM acetate buffer, pH 5.2, and 150 mM NaCl at 25 °C.

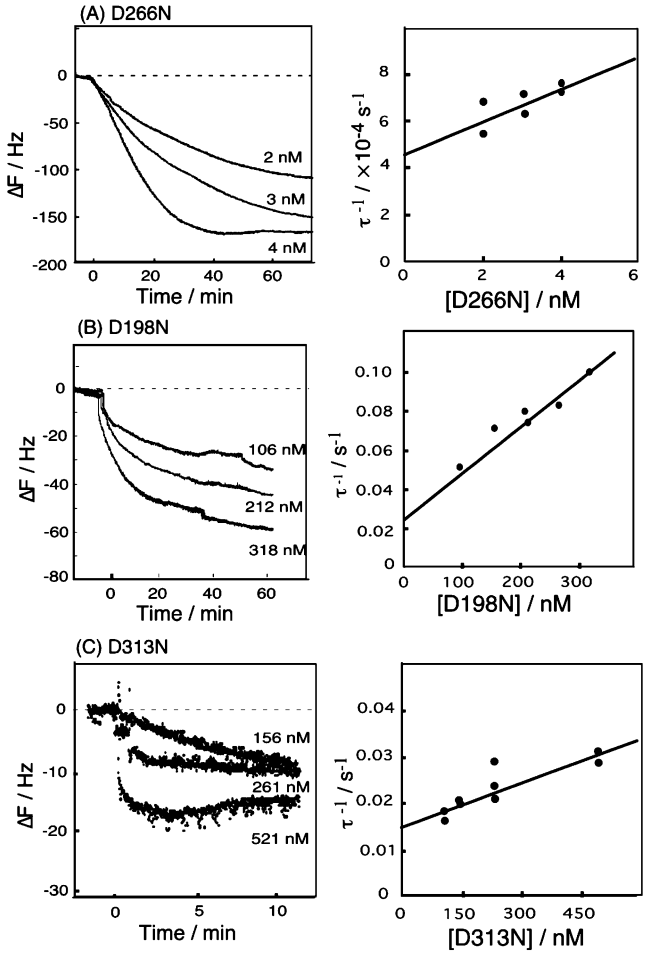

FIGURE 5: Typical time courses of frequency decrease (mass increase) of the dextran-immobilized QCM, responding to additions of (A) D266N, (B) D198N, and (C) D313N mutants, and each linear plot of the reciprocal relaxation time (τ^{-1}) against enzyme concentrations (eq 9). The immobilized amount of dextran (170−200 kDa) was 150 ± 10 ng cm^{-2} in 20 mM acetate buffer, pH 5.2, and 150 mM NaCl at 25 °C.

The role of Asp313, however, is not clear, although it is predicted to exist in the active site.

The substitution of the COOH to the $CONH_2$ group of D266N diminished the cleavage activity but not the binding activity (see Table 1). This means that the COOH residue

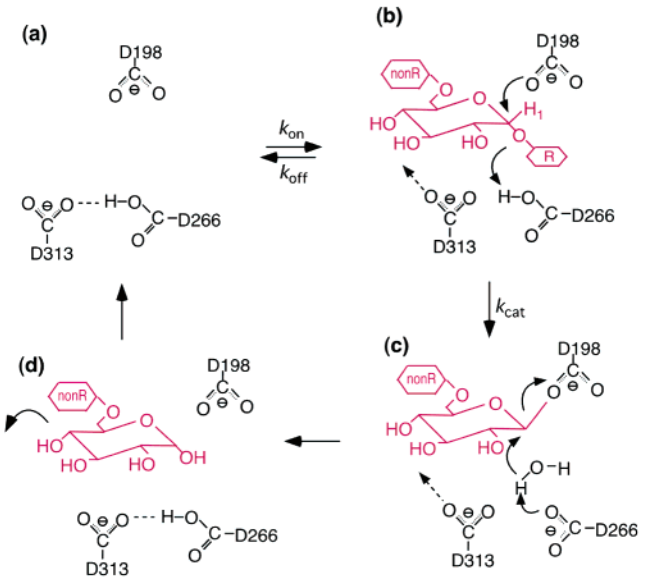

FIGURE 6: A catalytic model of retaining isomalto-dextranase based on both the predicted enzyme structure from a GH family 27 enzyme and our QCM kinetic results.

acts as a general acid to cleave the glucosidic linkage but does not interact with the substrate (see Figure 6b). The D198N mutant diminished the cleavage activity and increased the K_d value more than 40 times due to the large increase of k_{off} with the small increase of k_{on} values. This indicates that the carboxylate anion of Asp198 may interact with the $^{\delta+}C_1$ atom of the substrate as a nucleophile to generate an isomaltosyl-enzyme intermediate. The D313N mutant increased drastically the K_d value more than 100 times due to both the decrease of k_{on} and the increase of k_{off}, in addition to diminishing the cleavage activity. This indicates that Asp313 may strongly interact with the substrate binding probably through a hydrogen bond with the C_3−OH group to give a twisted and deformed conformation of the glucose ring at position −1 (*28*, *29*).

Recently, Tochihara and co-workers reported effects of site-directed mutagenesis of nine amino acids of isomalto-dextranase on the relative enzyme activity by the traditional Somogyi−Nelson method in the bulk solution (*30*). The substitution effect of D227A and D342A (corresponding to D198N and D313N in our study, respectively) agreed with our results, where the catalytic activity was nearly diminished. On the contrary, the substitution effect of D295A (corresponding to D226N) was different, in which D295A still retains ca. 45% activity, in contrast with the nearly diminishing activity of D226N. It is difficult to compare both results at present due to the different mutations. Moreover, Tochihara and co-workers obtained only the relative enzyme activity in percent (*30*), in contrast with our quantitative kinetic mutation studies.

From our kinetic results of mutants as well as the wild type by using the QCM system, we propose the following catalytic mechanism of isomalto-dextranase, as shown in Figure 6. (a) Before the binding of the substrate, the unionized Asp266 in the hydrophobic environment is interacting with Asp313 through a hydrogen bond. (b) The binding of the substrate to the enzyme disconnects this hydrogen bond, and Asp313 begins to bind to the C3−OH of the substrate and works to give a twisted and a deformed conformation of the glucose ring at position −1. In the glycosylation step, Asp266 provides general acid-catalyzed leaving group departure simultaneously with a nucleophilic attack by the carboxylate anion of Asp198 to form a isomaltosyl-enzyme intermediate. (c) In the deglycosylation step, Asp266 functions as a general base to activate the incoming nucleophile that hydrolyzes the isomaltosyl-enzyme intermediate. (d) Finally, the product (α-isomaltose) is released from the active site to revert to the starting point.

CONCLUSION

When the QCM method was applied to hydrolytic enzyme reactions, we could follow the enzyme binding process (ES complex forming process, k_{on}, k_{off}, and K_d) and the following catalytic hydrolysis process (k_{cat}) (*18*). In general, when kinetic parameters (s^{-1}) are the following relations, $k_{on}[E]_0 \ll k_{cat} \geq k_{off}$, the frequency simply increases, indicating an apparently catalytic process (mass decrease) as shown in the wild-type curve in Figure 4. In the case of $k_{on}[E]_0 \gg k_{cat} \geq k_{off}$, the frequency simply decreases, indicating apparently the enzyme binding process (mass increase), as shown in the D266N curve in Figure 4. On the contrary, in the case of $k_{on}[E]_0 \approx k_{cat} \geq k_{off}$, the frequency decreases at fast and then increases due to the competitive enzyme binding and the following catalytic process (*18*). Thus, various kinetic patterns can be analyzed from various patterns of frequency changes with time.

In the conventional Michaelis−Menten kinetics, only the hydrolysis product is usually followed. If the hydrolysis activity of the enzyme is completely diminished by the site-directed mutagenesis, it is difficult to predict whether the mutation affected the substrate binding and/or the cleavage process. When the QCM method is applied to mutation experiments, we could analyze the effect of mutations from the patterns of frequency changes and precise kinetic parameters such as k_{on}, k_{off}, K_d, and k_{cat} values. We believe that the combination of the QCM technique and the site-directed mutagenesis of enzymes can generally provide a new tool to analyze kinetically whether the functional group is involved in the substrate binding and/or the catalytic process, by following the ES complex formation.

REFERENCES

1. Segel, H. (1975) *Enzyme Kinetics*, John Wiley & Sons, New York.
2. Kuby, S. A., Ed. (1991) *A Study of Enzymes*, Vol. 1, CRC Press, Boca Raton, FL.
3. Papadopoulos, N. M., and Hess, W. C. (1960) Determination of Neuraminic (Sialic) Acid, Glucose and Fructose in Spinal Fluid, *Arch. Biochem. Biophys. 88*, 167−171.
4. Fox, J. D., and Robyt, J. F. (1991) Miniaturization of Three Carbohydrate Analyses Using a Microsample Plate Reader, *Anal. Biochem. 195*, 93−96.
5. Somogyi, M. (1952) Note on Sugar Determination, *J. Biol. Chem. 195*, 375−380.
6. Nelson, N. (1944) A Photometric Adaptation of the Somogyi Method for the Determination of Glucose, *J. Biol. Chem. 153*, 375−380.
7. Olsen, K., Svensson, B., and Christensen, U. (1992) Stopped-Flow Fluorescence and Steady-State Kinetic Studies of Ligand-Binding Reactions of Glucoamylase from *Aspergillus niger*, *Eur. J. Biochem. 209*, 777−784.
8. Sawai, T., Toriyama, K., and Yano, K. (1974) A Bacterial Dextranase Releasing Only Isomaltose from Dextrans, *J. Biochem. 75*, 105−112.

9. Okada, G., Takayanagi, T., Miyahara, S., and Sawai, T. (1988) An Isomalto-Dextranase Accompanied by Isopullulanase Activity from *Arthrobacter globiformis* T6, *Agric. Biol. Chem. 52*, 829−836.
10. Sawai, T., Ohara, S., Ichimi, Y., Okaji, S., Hisada, K., and Fukaya, N. (1981) Purification and Some Properties of the Isomaltodextranase of *Actinomadura* Strain R10 and Comparison with that of *Arthrobacter* globiformis T6, *Carbohydr. Res. 89*, 289−299.
11. Iwai, A., Ito, H., Mizuno, T., Mori, H., Matsui, H., Honma, M., Okada, G., and Chiba, S. (1994) Molecular Cloning and Expression of an Isomalto-Dextranase Gene from *Arthrobacter globiformis* T6, *J. Bacteriol. 176*, 7730−7734.
12. Fujimoto, Z., Kaneko, S., Momma, M., Kobayashi, H., and Mizuno, H. (2003) Crystal Structure of Rice α-Galactosidase Complexed with D-Galactose, *J. Biol. Chem. 278*, 20313−20318.
13. Garman, S. C., Hannick, L., Zhu, A., and Garboczi, D. N. (2002) The 1.9 Å Structure of α-*N*-Acetylgalactosaminidase: Molecular Basis of Glycosidase Deficiency Diseases, *Structure 10*, 425−434.
14. Henrissat, B. (1991) A Classification of Glycosyl Hydrolases based on Amino Acid Sequence Similarities, *Biochem. J. 280*, 309−316.
15. Henrissat, B., and Romeu, A. (1995) Families, Superfamilies and Subfamilies of Glycosyl Hydrolases, *Biochem. J. 311*, 350−351.
16. Akita, M., Mizuno, M., Tonozuka, T., Sakano, Y., Matsui, H., Hidaka, Y., Ito, S., and Horikoshi, K. (2004) Crystallization and Preliminary X-ray Study of Isomaltodextranase from *Arthrobacter globiformis*, *Acta Crystallogr. D60*, 572−573.
17. Garman, S. C., and Garboczi, D. N. (2004) The Molecular Defect Leading to Fabry Disease: Structure of Human α-Galactosidase, *J. Mol. Biol. 19*, 319−335.
18. (a) Nishino, H., Nihira, T., Mori, T., and Okahata, Y. (2004) Direct Monitoring of Enzymatic Glucan Hydrolyses on a 27-MHz Quartz-Crystal Microbalance, *J. Am. Chem. Soc. 126*, 2264−2265; (b) Nishino, H., Murakawa, A., Mori, T., and Okahata, Y. (2004) Kinetic Studies of Amylopectin Cleavage Reactions Catalyzed by Phosphorylase *b* using a 27 MHz Quartz Crystal Microbalance, *J. Am. Chem.* Soc. *126*, 14752−14757.
19. Sauerbrey, G. (1959) Verwendung von Schwingquarzen zur Wagung dunner Schichten und zur Microwagang, *Z. Phys. 155*, 206−222.
20. (a) Okahata, Y., Matsunobu, Y., Ijiro, K., Mukai, M., Murakami, A., and Makino, K. (1992) Hybridization of Nucleic Acids Immobilized on a Quartz-Crystal Microbalance, *J. Am. Chem. Soc. 114*, 8299−8300; (b) Okahata, Y., Kawase, M., Niikura, K., Ohtake, F., Furusawa, H., and Ebara, Y. (1998) Kinetic Measurements of DNA Hybridization on an Oligonucleotide-Immobilized 27 MHz Quartz-Crystal Microbalance, *Anal. Chem. 70*, 1288−1296.
21. (a) Okahata, Y., Niikura, K., Sugiura, Y., Sawada, M., and Morii, T. (1998) Kinetic Studies of Sequence-Specific Binding of GCN4-bZIP Peptides to DNA Stands immobilized on a 27 MHz Quartz-Crystal Microbalance, *Biochemistry 37*, 5666−5672; (b) Matsuno, H., Niikura, K., and Okahata, Y. (2001) Design and Characterization of Asparagine- and Lysine-Containing Alanine-Based Helical Peptides That Bind Selectively to A·T Base-Pairs of Oligonucleotides Immobilized on a 27 MHz Quartz Crystal Microbalance, *Biochemistry 40*, 3615−3622; (c) Furusawa, H., Kitamura, Y., Hagiwara, N., Tsurimoto, T., and Okahata, Y. (2002) Binding Kinetics of the Toroidal-Shaped PCNA to DNA Strands on a 27 MHz Quartz-Crystal Microbalance, *ChemPhysChem.*, 446−448.
22. (a) Ebara, Y., and Okahata, Y. (1994) A Kinetic Study of Concanavalin A Binding to Glycolipid Monolayers by Using a Quartz-Crystal Microbalance, *J. Am. Chem. Soc. 116*, 11209−11212; (b) Ebara, Y., Mizutani, K., and Okahata, Y. (2000) DNA Hybridization at the Air−Water Interface, *Langmuir 16*, 2416−2418.
23. (a) Niikura, K., Matsuno, H., and Okahata, Y. (1998) Direct Monitoring of DNA Polymerase Reactions on a Quartz-Crystal Microbalance, *J. Am. Chem. Soc. 120*, 8537−8538; (b) Matsuno, H., Niikura, K., and Okahata, Y. (2001) Direct Monitoring and Kinetic Studies of DNA Polymerase Reactions on a DNA-immobilized Quartz-Crystal Microbalance, *Chem. Eur. J. 7*, 3305−3312; (c) Matsuno, H., Furusawa, H., and Okahata, Y. (2005) Kinetic Studies of DNA Cleavage Reactions Catalyzed by an ATP-Dependent Deoxyribonuclease on a 27-MHz Quartz-Crystal Microbalance, *Biochemistry* (in press).
24. Tonozuka, T., Suzuki, S., Ikehara, Y., Mizuno, M., Kim, Y.-K., Nishikawa, A., and Sakano, Y. (2004) Heterologous Production and Characterization of *Arthrobacter globiformis* T6 Isomaltodextranase, *J. Appl. Glycosci. 51*, 27−32.
25. Laemmli, U. K. (1970) Cleavage of Structural Proteins during the Assembly of the Head of Bacteriophage T4, *Nature 227*, 680−685.
26. Shinohara, Y., Sota, H., Gotoh, M., Hasebe, M., Tosu, M., Nakao, J., Hasegawa, Y., and Shiga, M. (1996) Bifunctional Labeling Reagent for Oligosaccharides to Incorporate both Chromophore and Biotin Groups, *Anal. Chem. 68*, 2573−2579.
27. Hart, D. O., He, S., Chany, C. J., II, Withers, S. G., Sims, P. F. G., Sinnott, M. L., and Brumer, H., III (2000) Identification of Asp-130 as the Catalytic Nucleophile in the Main α-Galactosidase from *Phanerochaete chrysosporium*, a Family 27 Glycosyl Hydrolase, *Biochemistry 39*, 9826−9836.
28. Hasegawa, K., Kubota, M., and Matsuura, Y. (1999) Roles of Catalytic Residues in α-Amylases as Evidenced by the Structures of the Product-complexed Mutants of a Maltotetraose-forming Amylase, *Protein Eng. 12*, 819−824.
29. Kaneko, H., Kuriki, T., Handa, S., Takada, T., Takata, H., Yanase, M., Okada, S., Umeyama, H., and Shimada, J. (1998) How α- and β-Amylases Achieve their Perfect Stereo-Selectivity, *Res. Commun. Biochem. Cell Mol. Biol. 2*, 69−89.
30. Tochihara, T., Sasaki, K., Araki, O., Morimoto, N., Watanabe, K., Hatada, Y., Ito, S., Ito, H., and Matsui, H. (2004) Site-Directed Mutagenesis Establishes Aspartic Acids-227 and -342 as Essential for Enzyme Activity in an Isomalto-dextranase from *Arthrobacter globiformis*, *Biotechnol. Lett. 26*, 659−664.

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