Direct Monitoring of Both Phosphorolysis and Elongation of Amylopectin Catalyzed by Phosphorylase on a 27-MHz Quartz-crystal Microbalance

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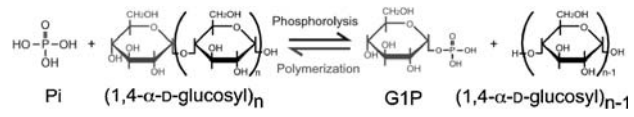
All kinetic parameters such as the enzyme binding process and the catalytic processes (phosphorolysis and polymerization) by phosphorylase from *Potato* could be obtained using an amylopectin-immobilized 27-MHz quartz-crystal microbalance (QCM).

Glycogen phosphorylase from *Potato* (EC 2.4.1.1) catalyzed the phosphorolysis of α-1,4-glucosidic linkages from the non-reducing ends of glucan such as amylopectin to produce a glucose 1-phosphate (G1P) in the presence of excess phosphoric acid (Pi) in vivo (Scheme 1).[1] Phosphorylase has been also known to catalyze the polymerization of α-1,4-glucosidic linkages of amylopectin in the presence of excess G1P in vitro,[2] in addition to the in vivo phosphorolysis. Enzyme reactions have been generally studied using a steady-state kinetics, in which the concentration of the enzyme–substrate (ES) complex is hypothesized to be nearly constant during the reaction, because it was difficult to detect the concentration of the ES complex.[3] The reaction rate was simply obtained from the initial rate of the product increase. In the case of phosphorylase, the phosphorolysis and polymerization reactions of amylopectin have been studied using the affinity gel-electrophoresis[4] and the enzymatic colorimetry to follow the production of G1P (phosphorolysis) or the production of Pi (polymerization) in the bulk solution.[5] If the formation and decay of the ES complex can be followed directly during the reaction, then more accurate kinetic parameters might be obtained.

In this paper, we describe that the amylopectin-immobilized 27-MHz quartz-crystal microbalance (QCM) is a useful tool to detect directly and quantitatively the each step of binding (k_{on}) and dissociation (k_{off}) of the enzyme to the substrate, catalytic phosphorolysis or polymerization rate constants (k_{cat}), and Michaelis constants for Pi ($K_{m,Pi}$) or G1P ($K_{m,G1P}$), from the ES complex formation and decomposition catalyzed by phosphorylase.

Glycogen phosphorylase (from *Potato*, EC 2.4.1.1, MW: 104 kDa) was isolated from Danshaku-potato from Hokkaido and purified as a single spot by an anion-exchange chromatography according to previous paper.[6] Amylopectin was prepared from glutinous rice (average MW: 100 ± 50 kDa), whose reducing end was biotinylated with biotinamidecaproylhydrazide, as previously described.[7,8] AFFINIX Q4 was used as a QCM instrument (Initium Co., Ltd, Tokyo: http://www.initium2000.com).[7,8] The 27-MHz QCM was calibrated to change frequency by -1 Hz, responding to the mass increase of 0.18 ng cm^{-2} on the electrode for amylopectin. The factor of 0.18 ng cm^{-2} per -1 Hz was also applied for phosphorylase binding to amylopectin on the 27-MHz QCM.[9] The biotinylated amylopectin was anchored on a streptavidin-immobilized QCM plate according to previous paper.[7,8] The immobilized amount of amylopectin was maintained at 110 ± 10 ng cm^{-2} (ca. 1.1 ± 0.1 pmol cm^{-2}), and corresponds to approximately 50% coverage of the Au surface (4.9 mm^2).



Scheme 1.

Figure 1 shows typical frequency changes as a function of time of the amylopectin-immobilized QCM, responding to the addition of phosphorylase and then phosphoric acid (Pi) or glucose 1-phosphate (G1P). The first step (A) showing frequency decrease (mass increase) indicates the enzyme binding to the

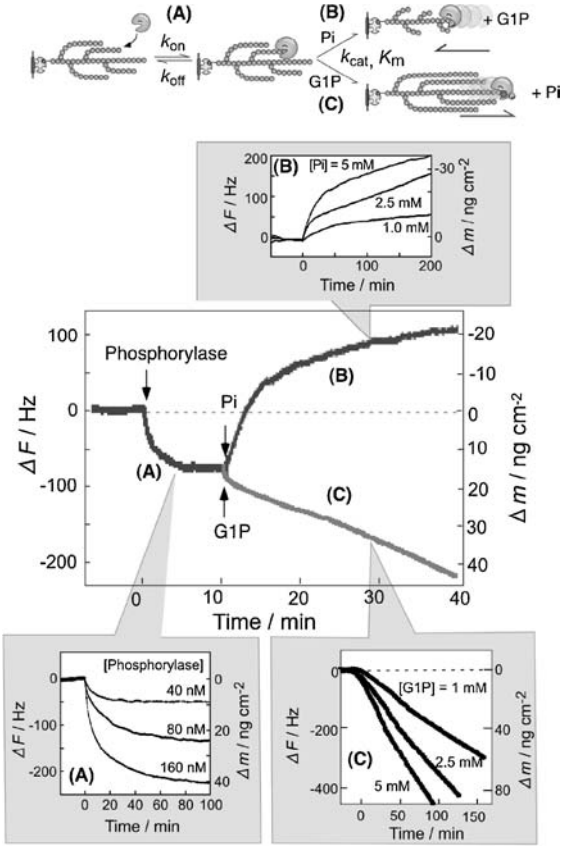


Figure 1. Typical time courses of frequency changes of the amylopectin-immobilized QCM, responding to additions of (A) phosphorylase (40–160 nM), (B) phosphoric acid (Pi, 1–5 mM), and (C) glucose 1-phosphate (G1P, 1–5 mM). [Amylopectin] = 0.11 nM on the QCM, pH 6.8, 50 mM citrate buffer, 100 mM NaCl, 25 °C.

Table 1. Kinetic parameters for phosphorolysis and polymerization of amylopectin on the 27-MHz QCM catalyzed by phosphorylase and Pi and G1P[a]

Reactions	Binding process			Catalytic process		
	$k_{on}/10^3\ M^{-1}\ s^{-1}$	$k_{off}/10^{-3}\ s^{-1}$	$K_d/\mu M$	k_{cat}/s^{-1}	K_m/mM	$k_{cat}/K_m\ (M^{-1}\ s^{-1})$
Phosphorylation	12	3.7	0.32	3.0	$K_{m,pi} = 2.1\ (1.9)$[b]	1400
Polymerization				2.3	$K_{m,G1P} = 2.5\ (1.9)$[b]	900

[a][Amylopectin] = 0.11 nM on the QCM, pH 6.8, 50 mM citrate buffer, 100 mM NaCl, 25 °C. Each measurement was carried out 3–5 times and the obtained parameters contain ±10% experimental errors. [b]Obtained in the bulk solution (Ref. 5).

substrate (the ES complex formation). After the frequency decrease reached equilibrium, when the excess Pi was injected, the frequency increase (mass decrease) was observed that indicates phosphorolysis of amylopectin on the QCM (step B). On the contrary, when the excess G1P was injected, the mass increase was observed showing the elongation of the amylopectin on the QCM (step C).

The binding of phosphorylase to the amylopectin on the QCM (step A) is described by eq 1. We followed the formation of *ES* complex over time as mass changes on the QCM. At time t, the mass of *ES* is given by eqs 2 and 3, where E and S indicate phosphorylase and amylopectin, respectively, and $[E]_0 > [S]_0$. Relaxation time (τ) associated with enzyme binding is calculated from curve fittings of the QCM frequency decrease in step (A). When the enzyme binding experiments were carried out at different enzyme concentrations (40–160 nM, Figure 1A), the linear correlation of $1/\tau$ and $[E]_0$ gave the binding (k_{on}) and dissociation (k_{off}) rate constants as a slope and an intercept, respectively, according to eq 4. The linear correlation (eq 4) is shown in a Supporting Information Figure S1.[10] The results are summarized in Table 1.

$$E + S \underset{k_{off}}{\overset{k_{on}}{\rightleftarrows}} ES \tag{1}$$

$$[ES]_t = [ES]_{max}\{1 - \exp(-t/\tau)\} \tag{2}$$

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

$$1/\tau = k_{on}[E]_0 + k_{off} \tag{4}$$

In step (B), both the phosphorolysis rate and amount increased with increasing Pi concentrations (1–5 mM, Figure 1B). The initial phosphorolysis rate (v_0) showed the saturation behavior with increasing Pi concentrations (data is shown in a supplementary Figure S2A).[10] This is simply expressed by the Michaelis–Menten equation (eqs 5 and 6), where *ES′* is the ternary complex of amylopectin–phosphorylase–Pi, and P_1 and P_2 are the phosphorolyzed amylopectin and G1P, respectively. K_m is the Michaelis constant for Pi of the ES complex, and k_{cat} is the phosphorolysis rate constant. From the linear correlation of Lineweaver–Burk plot (eq 7, data is shown in a supplementary Figure S2B),[10] $K_{m,Pi} = 2.1$ mM and $k_{cat} = 3.0\ s^{-1}$ were obtained from the slope and intercept, respectively. The obtained results are summarized in Table 1.

$$ES + Pi \underset{}{\overset{K_m}{\rightleftarrows}} ES' \xrightarrow{k_{cat}} E + P_1 + P_2 \tag{5}$$

$$v_0 = \frac{v_{max}[Pi]_0}{K_m + [Pi]_0} \tag{6}$$

$$\frac{1}{v_0} = \frac{K_m}{v_{max}} \cdot \frac{1}{[Pi]_0} + \frac{1}{v_{max}} \tag{7}$$

In step C, by the addition of G1P, the mass increase due to the elongation of amylopectin chains on the QCM was observed, and both the initial rate (v_0) and the polymerization amount increased with increasing the G1P concentrations (1–5 mM, Figure 1C). The polymerization rate constant (k_{cat}) and the K_m value for G1P were obtained according to eqs 5–7 (Data are shown in a Supporting Information Figure S3),[10] as well as the phosphorolysis. The results are summarized in Table 1. The K_m values for Pi and G1P obtained from the bulk reactions ($K_{m,Pi} = 1.9$ and $K_{m,G1P} = 1.9$)[5] were well consistent with those obtained by the QCM method ($K_{m,Pi} = 2.1$ and $K_{m,G1P} = 2.5$).

The K_d value of phosphorylase from *Potato* for the linear glucan of amylose was obtained to be 0.90 μM as the similar manner (data not shown), which was relatively consistent with $K_d = 0.32$ μM for the branched amylopectin. Thus, the binding ability of phosphorylase to α-1,4 glucan was little dependent whether it is branched or linear. The catalytic (phosphorylation and polymerization) rate constants ($k_{cat} = 2.3$–$3.0\ s^{-1}$) were very large compared with the decomposition rate constant ($k_{off} = 0.0037\ s^{-1}$) of the ES complex. The apparent first-order rate constant of the enzyme binding step is calculated to be $k_{on}[E]_0 = 12000\ M^{-1}\ s^{-1} \times 100\ nM = 0.0012\ s^{-1}$. Thus, phosphorylase slowly binds to amylopectin and proceeds to phosphorolysis or polymerization without releasing from the substrate ($k_{cat} \gg k_{on}[E]_0$ or k_{off}).

In conclusion, this is the first example to obtain all kinetic parameters (k_{on}, k_{off}, K_d, k_{cat}, and K_m) of the phosphorolysis and polymerization catalyzed by phosphorylase. It is important to grasp all kinetic parameters on the one device, in order to know precisely enzyme reactions. We believe that the QCM system is a useful method of detection for in situ enzyme reactions without any substrate labeling.

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10 Supporting Information is available electronically on the CSJ-Journal web site: http://www.csj.jp/journals/chem-lett/index.html.

Published on the web (Advance View) January 27, 2007; doi:10.1246/cl.2007.312