A Kinetic Study of Protein Binding to Ecabet Sodium Using Quartz-Crystal Microbalance

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To define the mechanism of the protection by ecabet sodium of the gastric mucosa, the characteristics of protein binding of this drug were investigated using a quartz-crystal microbalance (QCM) method. The binding rate constants (k_b) and the binding amounts (Δm) were obtained from time courses of the frequency decrease (mass increase) of the QCM. The binding constants to proteins of two ecabet analogues (G1, ecabet type and G2, non-ionic ecabet type) were dependent on the pH, leading to large k_b values at the acidic region. Furthermore, the k_b values of G1 with the addition of bovine serum albumin (BSA) and bovine serum fibrinogen (BSF) at the acid region were larger than those of G2. The difference in k_b values between G1 and G2 for porcine gastric mucin (PGM) is hardly discernible. Ecabet seems to be more heavily distributed in the ulcerous areas than in the intact mucosa, judging from the large binding constants of this drug to BSA and BSF compared with those to PGM. It is suggested that ecabet is bound to proteins by hydrophobic interaction, moreover, the electrostatic interaction between this drug and proteins (BSA and BSF) occurs at acidic pH region. On account of these interactions, ecabet sodium seems to have a more protective effect on an ulcer at intraluminal acidity than sucralfate. Finally, QCM was found to be a useful technique for detecting quantitatively the time course of binding proteins with drug.

Key words ecabet; quartz-crystal microbalance; protein binding

Ecabet sodium (Fig. 1A), synthesized from abietic acid, is widely used as an antiulcer drug.¹⁾ Ecabet is found to be more heavily distributed in the ulcerous areas than in the intact mucosa.^{2,3)} It has been shown that this property was due to the binding of the drug to serum and mucus proteins exuded from necrotic tissues.^{3,4)}

Studies on the binding behavior of proteins with drugs have been reported using various techniques: equilibrium dialysis,⁵⁾ ultrafiltration,⁶⁾ fluorescence reflection,⁷⁾ NMR,⁸⁾ frontal HPLC⁹⁾ and capillary electrophoresis.¹⁰⁾ These methods have future potential in the observation of protein bindings, however, they also pose some difficulties for *in situ* measurements.

In this study, the quartz-crystal microbalance (QCM) method, an easy *in situ* technique, was employed for detection of interactions between ecabet and proteins. To investigate the effect of ionic groups (SO_3^- , COO^-) in ecabet sodium on the protein binding, two analogues were synthesized and immobilized on electrodes of the quartz-crystal oscillator. The QCM method is known to provide sensitive mass measuring because its resonance frequency decreases with the increase of mass on the electrode at the nanogram level.^{11–15)} Binding amounts (Δm) and binding rate constants (k_b) of proteins can be determined quantitatively from the time courses of the frequency decrease (mass increase) of the oscillator.

Experimental

Materials Two ecabet analogues, G1 and G2, were synthesized and employed to investigate the protein binding of ecabet sodium. The structures of G1 and G2 are shown in Fig. 1B. G1, 7-(5-mercaptopentyloxy)-12-sulfodehydroabietic acid, containing thiol and methylene group as a spacer was prepared from ecabet. G2, methyl-7-(5-mercaptopentyloxy)dehydroabietate, containing thiol and methylene group as a spacer was prepared from abietic acid.

Bovine serum albumin (BSA, Mw=69000) was obtained from Nacalai Tesque (Kyoto, Japan). Bovine serum fibrinogen (BSF, Mw=340000) was obtained from Seikagaku Kogyo (Tokyo, Japan). Porcine gastric mucin

(PGM, Mw $\geq$ 1200000¹⁶⁾) was clear without a third repetition from Sigma and was purified through a gel filtration column (BIORAD, Bio-gel A).

The other chemicals used were of reagent grade from Wako Pure Chemical Industries (Osaka, Japan). Water used was purified with a Milli Q system.

Apparatus The quartz-crystal oscillator employed is a commercially available 9-MHz, AT-cut quartz with Au electrode on both sides (area 0.196 cm²). The frequency of the vibrating quartz was measured by a frequency counter, QCA 917 model (Seiko EG & G, Tokyo) attached to a microcomputer system (DELL, optiplex model). The QCM system used is shown in Fig. 2.

The following equation is valid for the AT-cut shear mode QCM.¹⁷⁾

Fig. 1. Chemical Structures of (A) Ecabet Sodium and (B) Its Two Analogues

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Fig. 2. Apparatus for the Frequency Measurements of the Quartz-Crystal Microbalance

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

where ΔF is the measured frequency shift (Hz), F_0 is the parent frequency of the QCM (8.9×10^6 Hz), Δm is the mass change (ng), A is the electrode area (0.196 cm^2), ρ_q is the density of quartz ($2.65 \text{ g} \cdot \text{cm}^{-3}$), and μ_q is the shear modulus ($2.95 \times 10^{11} \text{ dyn} \cdot \text{cm}^{-2}$). According to Eq. 1, calibration showed that a frequency decrease of 1 Hz corresponded to mass increase of 0.91 ng on the electrode of the oscillator. Therefore, Eq. 1 can be expressed simply as

$$\Delta F = -0.91 \Delta m \quad (2)$$

Preparation of Modified Au Electrode The Au electrodes on the quartz were washed successively with methanol and water, then immersed in a 5 mM aqueous solution of G1 (or methanol solution of G2) at room temperature for 24 h. Each ecabet analogue was modified on the Au electrode by a chemical bond of Au and S.¹⁸⁾ Finally, these Au electrodes modified were washed with water.

Measurement One side of the piezoelectric crystal was covered with a Teflon case to avoid contact with the ionic buffer solution. The other side of the piezoelectric crystal plate was immersed in buffer solution with stirring. When the frequency was stabilized in the buffer solution, the frequency change of the crystal responding to the addition of protein to the solution was followed with time at 37 °C. Buffer solutions used for measurements of protein binding were as follows: HCl-KCl buffer (Clark-Lubs buffer) solution for pH 1.2. Phosphate-citrate buffer (Mellvain buffer) for pH 3.0, 4.8, 5.5, 7.0 and 7.4. The ionic strength ($\mu=0.2$) was adjusted with KCl.

Results and Discussion

Modification of Ecabet Analogues on the Au Electrode

The amounts of modified ecabet analogues can be calculated from the frequency decrease according to Eq. 2. The calculated amounts of modified G1 and G2 are summarized in Table 1. About 40 ng of ecabet analogues were immobilized on the Au electrode.

Investigation by X-ray crystallography has shown ecabet sodium to have a molecular size of $7.1 \times 7.1 \times 12.7 \text{ \AA}$.¹⁹⁾ If both G1 and G2 are closely packed, the modified amounts of these analogues also can be estimated from the molecular size. These estimated values per electrode surface (0.392 cm^2) are 50 ng and 40 ng for G1 and G2, respectively. Accordingly, the modification ratios of G1 and G2 on the elec-

Table 1. Modification of G1 or G2 on the Au Electrode

	Δm (ng)	Modification ratio (%)
G1	33	66
G2	38	95

Fig. 3. Typical Time Courses of Frequency Changes of QCM with the Addition of BSA ($10 \mu\text{g/ml}$)

The modified electrode was used (a) G1 and (b) G2 at pH 3.0, (c) G1 and (d) G2 at pH 4.8. Temperature, 37 °C; ionic strength, $\mu=0.2$.

trode were calculated, and are summarized in Table 1. The modified amount of G1 was smaller than that of G2, probably because of the intermolecular electrostatic repulsion due to two anion groups (COO^- , SO_3^-) of G1.

Amounts of Protein Binding Figure 3 shows typical time courses of frequency changes (ΔF) of the piezoelectric crystal on the analogues responding to the addition of BSA ($10 \mu\text{g/ml}$, $1.45 \times 10^{-7} \text{ M}$) into buffer solution. When G1 modified Au electrode was used at pH 3.0, the frequency of the quartz-crystal oscillator decreased (mass increased) gradually responding to the addition of BSA and saturated at $-\Delta F = 79 \text{ Hz}$ ($\Delta m = 87 \text{ ng}$ according to Eq. 2) within 60 min (curve a). When the piezoelectric crystal plate was moved upward to the air phase and dried in air, the binding amount (Δm) of BSA calculated from the ΔF in the air phase before and after the deposition was 90 ng. This value was consistent with the saturated Δm (Δm_{max}) value (87 ng) obtained directly from the time course of ΔF in buffer solution. When G2 modified Au electrode was used at pH 3.0, the time course of ΔF (curve b) showed a similar pattern as in G1, although Δm values were different between G1 and G2. When G1 or G2 modified Au electrode was used at pH 4.8, the two Δm of BSA were almost the same, as shown in curves c and d in Fig. 3. In the same way, time courses of ΔF at other pHs (pH 1.2, 7.0 and 7.4) were investigated and Δm values were calculated from Eq. 2. The Δm_{max} values are shown in Fig. 4A. The Δm_{max} values of BSA for G1 and G2 were almost the same except for pH 3.0, and maximum values were obtained at its pI 4.8. Protein molecules were folded compactly at their pI and little intermolecular electrostatic repulsion of BSA was caused owing to the apparent electric charge of zero. The results obtained from QCM method were consistent with those obtained by other methods.^{20,21)} The Δm_{max} value of BSA at pH 3.0, 7.0 and 7.4 was reduced. The inter-

Fig. 4. Saturated Binding Amounts of Three Different Proteins to G1 and G2 under Various pH Buffers

(A) BSA, (B) BSF and (C) PGM. $\circ$, G1; $\bullet$, G2; $\times$, unmodified electrode. Protein concentration, $10 \mu\text{g/ml}$; temperature, 37°C ; ionic strength, $\mu=0.2$.

molecular electrostatic repulsion of BSA was due to the apparent electric charge. The reduction in Δm_{max} value of BSA for G1 at pH 3.0 was smaller than that for G2. The electrostatic interaction between a cationic residue (*e.g.*, Lys, Arg) of BSA and an anion group (SO_3^-) of G1 seems to contribute to Δm in G1. Protein binding to ecabet seems to be specific, as shown in Fig. 4, because binding amounts of proteins to G1 and G2 on the electrode were large compared with an unmodified electrode.

In the same way, time courses of ΔF of the QCM on the ecabet analogues were measured for the addition of BSF ($10 \mu\text{g/ml}$, $2.94 \times 10^{-8} \text{ M}$) and PGM ($10 \mu\text{g/ml}$). The results of Δm_{max} values of these proteins are shown in Figs. 4B and 4C; the value at pI 5.5 of BSF for G2 was the maximum. The reason for this result seems the same as that explaining BSA binding at pI. For G1, however, the Δm_{max} value at pH 4.8 was the maximum, and the reason for this is uncertainly. In the case of PGM, the Δm_{max} values for both G1 and G2 increased with a decrease of pH values and the difference in the pH dependence was hardly discernible.

Kinetic Analysis of Protein Binding The binding between protein and G1 (or G2) is described by Eq. 3,

Fig. 5. Linear Correlation between $\ln(1 - \Delta F / \Delta F_0)$ and Time at pH 3.0

$\circ$, G1; $\bullet$, G2. Concentration of BSA, $10 \mu\text{g/ml}$; temperature, 37°C ; ionic strength, $\mu=0.2$.

$$\text{protein} + \text{G1 (or G2)} \xrightleftharpoons[k_d]{k_b} \text{protein-G1 (or G2)} \quad (3)$$

where k_b and k_d are binding rate constant and dissociation rate constant, respectively. The binding ratios of protein to G1 or G2 are given by the ratios of the saturated frequency change (ΔF_0) to frequency changes (ΔF) at each measurement time. If the dissociation is assumed to be slow and protein concentration is in greater excess than that of analogues, the binding reaction can be regarded as pseudo 1st order reaction²²⁾ and the binding rate (V_a) can be determined according to Eq. 4,

$$V_a = -\frac{d(1 - \Delta F / \Delta F_0)}{dt} = k_b C_0 (1 - \Delta F / \Delta F_0) \quad (4)$$

where C_0 is concentration of protein. Equation 4 can be expressed as Eq. 5 through the integration of Eq. 4,

$$\ln(1 - \Delta F / \Delta F_0) = -k_b C_0 t \quad (5)$$

The binding rate constant (k_b) of protein to G1 (or G2) can be obtained from the slope of Eq. 5. The plotting results between $\ln(1 - \Delta F / \Delta F_0)$ and t in the addition of BSA at pH 3.0 are shown in Fig. 5; linear correlations with $r^2=0.9957$ were observed for both G1 and G2. Then, the k_b values at six different pHs (pH 1.2, 3.0, 4.8, 5.5, 7.0 and 7.4) were obtained from the time courses of the binding process using Eq. 5, and these values are summarized in Table 2. It was found that the k_b values of G1 and G2 increased with a decrease of pH values. It is known that conformational change of BSA occurs and hydrophobic domain is exposed at the acidic pH region.^{23–26)} The main interaction between hydrophobic domain of BSA and hydrophobic terpene framework of ecabet thus occurs at the acidic buffers, leading to the large k_b values. However, at pH 1.2, binding interaction could not be explained only by the hydrophobicity since the binding site of BSA at pH 1.2 may be out of shape. Other interactions, such as an ionic one, may also be involved in the binding of ecabet to BSA.⁴⁾

The k_b value of G1 at pH 3.0 was larger than that of G2. These values were 2.4×10^{-2} and $1.9 \times 10^{-2} \text{ m}^3 \cdot \text{kg}^{-1} \cdot \text{s}^{-1}$, namely, 1.6×10^4 and $1.3 \times 10^4 \text{ M}^{-1} \cdot \text{s}^{-1}$ for G1 and G2, respectively. This can be interpreted both by the electrostatic and the hydrophobic interaction between BSA (Lys, Arg) and

Table 2. Binding Rate Constants (k_b) of BSA, BSF and PGM to G1 or G2^{a)}

pH	k_b ($10^{-4} \text{ m}^3 \cdot \text{kg}^{-1} \cdot \text{s}^{-1}$)					
	BSA		BSF		PGM	
	G1	G2	G1	G2	G1	G2
1.2	196±13.0	251±27.9	179±11.2	181±10.6	21±2.6	18±2.5
3.0	238±4.2	192±18.6	98±5.5	79±3.5	16±2.5	18±2.5
4.8	72±6.5	79±4.2	44±4.0	32±4.5	18±0.6	18±1.5
5.5	—	—	30±2.5	15±1.7	16±1.5	15±0.6
7.0	20±2.9	42±5.6	—	—	—	—
7.4	36±6.9	63±6.6	67±3.2	86±9.1	9±1.5	12±1.5

a) Values are expressed as means±S.D. ($n=3$).

G1 (SO_3^-). The k_b values of G1 and G2 at pI 4.8 were almost the same. That values of G1 at the alkaline region was smaller than that of G2. This can be interpreted as being due to the large electrostatic repulsion between G1 (SO_3^- , COO^-) and BSA (anion residue).

When BSF was used, the k_b values of G1 and G2 at pH 1.2 were maximum ($1.8 \times 10^{-2} \text{ m}^3 \cdot \text{kg}^{-1} \cdot \text{s}^{-1}$, namely, $6.2 \times 10^4 \text{ M}^{-1} \cdot \text{s}^{-1}$), and at pI 5.5 were the smallest of all k_b values. The k_b value of G1 at the acid region was larger than that of G2, and is believed to be the electrostatic interaction due to SO_3^- group of G1.

When PGM was used, the k_b values for G1 and G2 slightly increased with a decrease of pH values; however, the difference of the values between G1 and G2 was hardly discernible. The ionic groups of ecabet probably were not influenced for PGM binding. PGM is a mixture of various proteins and has a molecular weight distribution, therefore, the k_b values for PGM were expressed in $\text{M}^{-1} \cdot \text{s}^{-1}$.

In conclusion, it is believed that ecabet is more heavily distributed to the ulcerous areas than to the intact mucosa, judging from the large binding constants of this drug to BSA and BSF compared with those to PGM. These results agreed well with those reported by Ito *et al.*⁴⁾ It is suggested that ecabet is bound to proteins by hydrophobic interaction, and that the electrostatic interaction between this drug and proteins (BSA and BSF) occurs at the acidic pH region. Because of these interactions, ecabet sodium seems to have more of a protective effect on an ulcer at intraluminal acidity than dose sucralfate.²⁷⁾ Finally, QCM was found to be a useful technique for detecting quantitatively the time course of binding proteins with a drug.

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