# Imprinted Chiral Molecular Recognition in Dipeptide Crystals immobilized on a Quartz-crystal Microbalance

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Enantioselective binding and dissociation processes of D- or L-AcLeuOEt into (*R*)-phenylglycyl-(*R*)-phenylglycine crystals, whose layered cavity had been imprinted with the same guest molecules, were studied by using a quartz-crystal microbalance in aqueous solution.

Induced-fit molecular recognition between proteins and their substrates has been the focus of much attention in recent years. We report here chiral molecular recognition of amino acid derivatives by crystals of the dipeptide (*R*)-phenylglycyl-(*R*)-phenylglycine, as a model of host proteins, whose cavity had been imprinted with the same guest molecule. The enantioselective imprinting and molecular recognition processes were followed quantitatively as a mass change by using a dipeptide-deposited quartz-crystal microbalance (QCM). QCMs are known to provide very sensitive mass-measuring devices because their resonance frequency decreases with increases of the mass on the QCM in a nanogram level (see Fig. 1).[1–4]

(*R*)-Phenylglycyl-(*R*)-phenylglycine (*R*,*R*) was prepared by the method repeated previously; X-ray crystallography of a single crystal had confirmed that it formed a layered structure owing to the strong intermolecular interactions between the terminal $CO_2^-$ and $NH_3^+$ groups.[5] A chloroform dispersion of the (*R*,*R*)-dipeptide was cast as a film on both sides of an Au electrode (area: 16 mm$^2$) on a QCM plate (9 MHz; AT cut). The dipeptide film was physically stable and non-swelled on the QCM in hot water (20–80 °C) and even in organic solvents such as benzene and methanol, as confirmed by the small frequency (mass) changes of the QCM during soaking. The QCM was connected to a handmade oscillator designed to drive the quartz at the resonance frequency in aqueous solution.[2] The frequency changes were followed by a universal frequency counter (Iwatsu Co., Tokyo, Model SC 7201) attached to a microcomputer system (NEC Co., Tokyo, Model PC 9801). Calibration showed that a frequency decrease of 1 Hz corresponded to a mass increase of 1.05 ng on the QCM electrode (16 mm$^2$) both in aqueous solution and in air (experimental error: ± 5 Hz).[2]

When the QCM deposited with the (*R*,*R*)-dipeptide (3.0 µg, 10.6 nmol) was soaked in an aqueous solution (100 µmol in 10 ml) of *N*-acetyl-*O*-ethyl-L-leucine (L-AcLeuOEt) and the temperature increased gradually from 20 to 80 °C, the frequency of the QCM decreased (mass increased) with increasing temperature ($\Delta F$ = −762 Hz; $\Delta m$ = 800 ng; 4.1 nmol), and the frequency did not revert to its original value after the temperature was decreased to room temperature. The (*R*,*R*)-dipeptide (10.6 nmol) was calculated to include L-AcLeuOEt (4.1 nmol) as a *ca.* 2:1 complex in the cavity. Changes in frequency due to the temperature change (viscosity and density changes in water) were allowed for from measurements on a bare QCM under the same conditions. When the dipeptide-deposited QCM was soaked in hot water without guest molecules, the corrected frequency hardly changed. The viscoelasticity of the crystal on the QCM was confirmed to be constant during the experiments by measuring

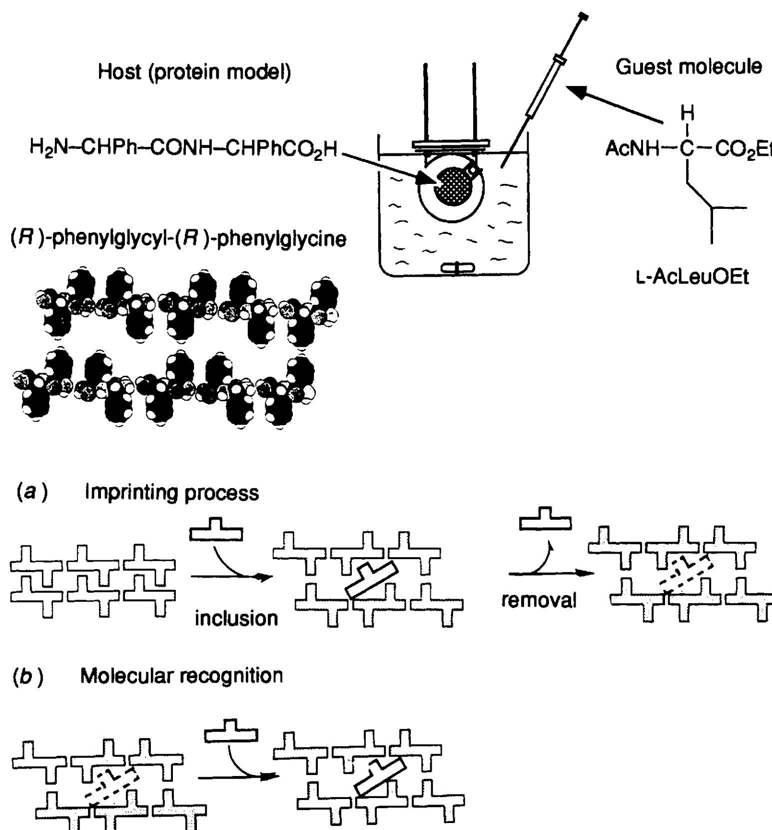


**Fig. 1** A schematic illustration of imprinting and molecular recognition processes of (*R*)-phenylglycyl-(*R*)-phenylglycine aggregates followed by a QCM

**Table 1** Kinetic parameters of binding ($k_1$) and dissociation ($k_{-1}$) of L- and D-AcLeuOEt into the (*R*,*R*) dipeptide aggregates on the QCM at 25 °C

| Dipeptide host | Guest | $k_1$/dm$^3$ mol$^{-1}$ s$^{-1}$ | $k_{-1}$/10$^{-3}$ s$^{-1}$ | $K_a$ (= $k_1/k_{-1}$)/dm$^3$ mol$^{-1}$ |
|---|---|---|---|---|
| Non-imprinted | L-AcLeuOEt | 8.7 | 2.6 | 340 |
| | D-AcLeuOEt | 7.2 | 2.2 | 330 |
| Imprinted with L-AcLeuOEt | L-AcLeuOEt | 0.011 | 0.00113 | 9700 |
| | D-AcLeuOEt | 0.017 | 0.14 | 86 |

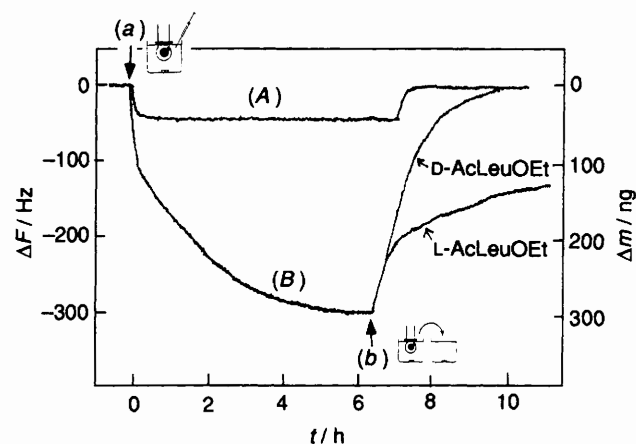


**Fig. 2** Frequency changes of the (*R*,*R*) dipeptide-deposited QCM responding to the addition [at (*a*)] of L- and D-AcLeuOEt in water (100 μmol in 5 ml) at 25 °C. At (*b*), the QCM was moved to fresh water at 25 °C. (*A*): Non-imprinted (*R*,*R*)-dipeptide aged in hot water without guest molecules. (*B*): (*R*,*R*)-Dipeptide imprinted with L-AcLeuOEt in hot water, in which binding and dissociation processes involve two steps: the fast step near the surface, followed by slow step inside the crystal.

resonance resistance (*R* values) by an impedance analyser (Yokogawa Hewlett-Packard, Co., model 4192A). These findings indicate that L-AcLeuOEt is included as a guest molecule in the cavity of the (*R*,*R*)-dipeptide during aging processes in the hot water; it is stable in the cavity and not removed at room temp. (see Fig. 1).

Powder X-ray diffraction indicated that the (*R*,*R*)-dipeptide on the QCM plate exists as a layered structure showing a regular long spacing of 7.0 and 7.8 Å.[6] After the inclusion of L-AcLeuOEt in the dipeptide aggregates, the layer spacing increased to 12.4 Å. This indicates that the dipeptide aggregates incorporate the substrates in the layer cavities by expanding the layer spacing. When the dipeptide aggregates included with L-AcLeuOEt in the cavity on the QCM were washed with methanol, the frequency reverted to the original value (mass) of the host molecule itself, indicating the complete removal of guest molecules from the cavity. However, the layer spacing of the dipeptide aggregates remained 12.4 Å, and did not revert to the original 7.0 and 7.8 Å. This suggests that the dipeptide aggregates memorize (are imprinted with) the guest molecules in the layered cavity after removal of the substrates, as shown schematically in Fig. 1.

The (*R*,*R*) crystal can incorporate the D-isomer (D-AcLeuOEt) in the cavity during the aging process with expansion of the layer spacing from 7.8 to 12.7 Å; however, the layer spacing reverted to its original value after removing the guest by washing with methanol.

Fig. 2 shows typical time courses of frequency changes in an aqueous solution of the QCM immobilized with the (*R*,*R*)-dipeptide whose cavity had been imprinted with L-AcLeuOEt, when ethanolic solutions of L- and D-AcLeuOEt were injected (1900 ng; 100 μmol in 10 ml) as guest molecules at 25 °C [curve (*B*)]. Both L- and D-AcLeuOEt led to gradual frequency decreases (mass increases) with time, showing binding into the dipeptide layers on the QCM ($\Delta m$ = 300 ng; 1.55 nmol): calculated extent *ca.* 40% (mol/mol) of the imprinted cavity. When the QCM was moved to fresh water after reaching equilibrium at the arrow (*b*) in Fig. 2, the frequency for D-isomer reverted rapidly to the original value (the mass of the host itself) indicating complete removal of guest molecules. In contrast, the removal rate for the L-isomers was very slow. In the case of the non-imprinted dipeptide crystals that had been aged in hot water without guest molecules, both L- and D-AcLeuOEt were hardly adsorbed at all in the host layers on the QCM, and both binding and dissociation rates were very fast [curve (*A*)].

The binding rate constant ($k_1$) was obtained from the time-course of the frequency decrease (mass increase) after the injection of guest molecules at the arrow (*a*), and the dissociation rate constant ($k_{-1}$) was obtained from the frequency increase (mass decrease) after removal of the QCM to fresh water at the arrow (*b*) in Fig. 2. The results are summarized in Table 1.

In the case of the non-imprinted dipeptide, association constants ($K_a$ = $k_1/k_1$) were nearly equal and low for both L- and D-AcLeuOEt molecules, whereas, in the case of the dipeptide imprinted with L-AcLeuOEt, the $K_a$ value for L-AcLeuOEt was 110 times larger than that from D-AcLeuOEt. The $K_a$ of D-isomer for the imprinted dipeptide decreased significantly compared with the value for the non-imprinted dipeptide decrease significantly compared with the value for the non-imprinted dipeptide aggregates. The large difference of $K$ values between L- and D-isomers for the imprinted dipeptide was a result of the dissociation rate constant ($k_{-1}$), but not the binding rate constant ($k_1$). Thus, the molecular recognition for the L- and D-isomers in the cavity of the dipeptide imprinted with L-isomers was a result of the dissociation process, but not the binding process. It is noteworthy that the dissociation process plays a more important role than the binding process in selective molecular recognition; equilibrium *binding* constants ($K$) have been discussed mainly in conventional studies by using spectral techniques.

In summary, dipeptide layered aggregates show chiral binding for amino acid derivatives *via* the imprinting (induced-fit) process; QCM systems are useful for determining kinetics for molecular recognition processes by obtaining both binding and dissociation rate constants from time courses of frequency (mass) changes.

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