Direct Observation of Specific Interaction between Enzyme-substrate Complexes Using Colloidal Probe Atomic Force Microscopy

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The interactions between the enzyme-substrate complex of heptaprenyl diphosphate synthase were directly investigated using colloidal probe atomic force microscopy. This enzyme is composed of two dissociable subunits which exhibit a catalytic activity only when they are associated together in the presence of Mg^{2+} and farnesyl diphosphate (FPP). We observed the adhesive force between the subunits only in the presence of both Mg^{2+} (0.1 mM) and FPP (15 μM). This is the first direct demonstration of the specific interaction involved in the enzyme reaction.

Prenyl diphosphate synthases catalyze the sequential head-to-tail condensation of isopentenyl diphosphate (IPP) with allylic substrates to give linear prenyl diphosphates in the biosynthetic pathway of isoprenoid compounds. Heptaprenyl diphosphate (HepPP) synthase from *Bacillus subtilis*, which forms the HepPP with a chain length of C_{35}, is composed of two nonidentical protein subunits, neither of which has catalytic activity alone. These subunits have been assumed to associate in the presence of the allylic substrate (*E*,*E*)-farnesyl diphosphate (FPP) and Mg^{2+} (a cofactor) to form a catalytically active complex, which represents an intermediary state during the catalysis (Figure 1).[1] However, there has been no direct evidence to support the assumption that the two subunits would associate to form a transient dimer by some specific interactions between them.

The importance of the direct force measurement has rapidly increased in the current research on protein interactions. For example, using atomic force microscopy (AFM), Gaub et al.[2] measured the binding of biotin and streptavidin. Relatively strong interactions such as those between an antigen and antibody as well as the unfolding–folding process were mainly studied, because it is difficult to observe weaker interactions though they are common in most protein interactions. In order to study weak interactions, many protein molecules are needed to give sufficient responses. Moreover, it is necessary to immobilize these proteins in a fixed orientation to observe the interactions only ascribed to the specific interaction sites.

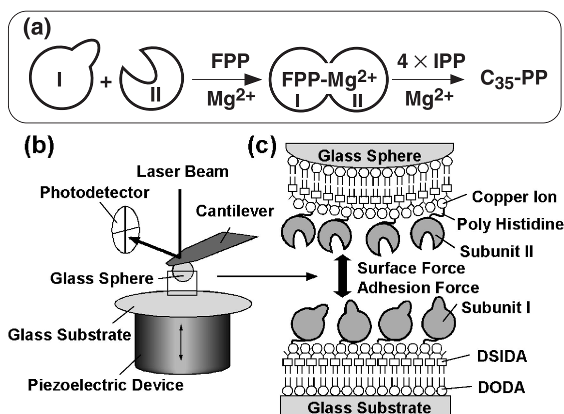

Figure 1. Hypothetical mechanism of the catalytically active complex of HepPP synthase of *B. subtilis*[1] (a), and schematic drawings of experimental set-up: surface forces measurement system employing the atomic force microscope (b), and sample surfaces (c).

Recently, we prepared the Langmuir–Blodgett monolayers of a chelate amphiphile which could bind poly(histidine) tagged proteins, and succeeded in the direct measurement of the interaction between these monolayers using the colloidal probe AFM.[3] It should be interesting to employ this approach for studying enzymatic processes. In this study, we immobilized subunits I and II of HepPP synthase on glass surfaces, and measured the interactions between them in a similar manner (Figure 1).

The subunits (subunit I or subunit II) of HepPP synthase of *B. subtilis* were overproduced in *E. coli* cells and purified as previously described.[1] These subunits were modified with six histidines [poly(histidine)] at the N-terminus. The preparation of *N*-[8-[1,2-di(octadecyloxy)propyloxy]-3,6-dihydroxyoctyl]iminodiacetic acid (DSIDA) has been previously described.[4] Dioctadecyl dimethyl ammonium bromide (DODA) was purchased from Sogo Pharmaceutical and used as received. All other reagents were of analytical grade.

The interaction force (*F*) between the protein modified glass sphere and plate was measured as a function of the surface separation distance (*D*) by the colloidal probe method[5] using an AFM (Seiko II, SPI3700-SPA300). The measurements were carried out basically similar to previous studies using colloidal glass spheres (Polyscience) and glass substrate (Matsunami, micro cover glass).[4,6] A colloidal glass sphere (10–20 μm radius) was attached to the top of a cantilever (Olympus, RC-800PS-1) with epoxy resin (Shell, Epikote1004). Interactions between the sphere and a glass substrate were measured in a home-made closed AFM fluid cell. The obtained forces were normalized by the radius (*R*) of the sphere using the Derjaguin approximation,[7]

$$F/R = 2\pi G_f. \qquad (1)$$

where G_f is the interaction free energy per unit area between two flat surfaces.

The glass surfaces were modified with proteins by the Langmuir–Blodgett deposition at $20.0 \pm 0.1\,^\circ C$ using a computer controlled film balance system (NL-BIO20-MWC, Nippon Laser & Electronics). A DODA solution, a DSIDA–Cu^{2+} solution and the hydrophobic glass modified with DODA were prepared as already reported.[3] The DSIDA–Cu^{2+} monolayer was transferred on the hydrophobic glass surface at a surface pressure of

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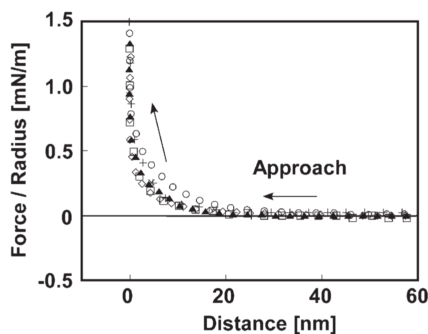

Figure 2. Force profiles of interactions between subunit I and subunit II upon approach under various conditions. ○, Tris buffer solution; □, Tris buffer solution containing Mg^{2+}; ◇, Tris buffer solution containing FPP; ×, Tris buffer solution containing IPP; ▲, Tris buffer solution containing Mg^{2+} and FPP; +, Tris buffer solution containing Mg^{2+} and IPP.

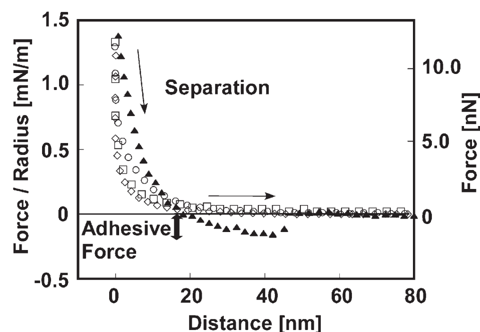

Figure 3. Force profiles of interactions between subunit I and subunit II upon separation under various conditions. ○, Tris buffer solution; □, Tris buffer solution containing Mg^{2+}; ◇, Tris buffer solution containing FPP; ▲, Tris buffer solution containing Mg^{2+} and FPP. Particle size, 15 µm radius.

40 mN/m from the protein solution subphase (1.0×10^{-7} M) (M $=$ mol dm^{-1}) in the down-stroke mode at a rate of 3.0 mm/min. The concentration of the proteins was chosen to be slightly higher than the concentration, $(8 \pm 1) \times 10^{-8}$ M, for both I and II, which showed the saturated adsorption on the DSIDA–Cu^{2+} monolayer. The adsorption process was determined by a quartz crystal microbalance in a 0.1 mM Tris–HCl buffer containing 1.0 mM NaCl. The transfer ratio of the protein bound DSIDA–Cu^{2+} monolayer on the hydrophobic glass was found to be 0.6 ± 0.1. The surface density of protein was 2.7×10^{-8} mol/m^2.

The interactions between the protein-modified surfaces were measured in 0.1 mM Tris–HCl buffer solution (pH = 8.3) containing 1.0 mM NaCl. The influence with and without 0.1 mM $MgCl_2$ (cofactor), 15 µm FPP (substrate), and 15 µm IPP (another substrate) were studied.

Figure 2 shows the interaction forces between subunits I and II *upon approach*. Only repulsive forces were observed under all the solution conditions. We compressed the layers till the force of 4.0 mN/m. Under this condition, these forces were reproducible after repeating compression. The isoelectric points of subunit I and II are known to be 5.1 and 5.2, respectively.[8] Therefore, each subunit must be negatively charged in the solution at pH = 8.3, giving rise to the repulsive double layer force. Indeed, the observed repulsive force was described by an exponential function as expected for the double layer force, and its decay length of 9.2 ± 0.4 nm was in good agreement with the Debye length of 9.2 nm calculated for the corresponding salt concentration of 1.1 mM.[7]

Figure 3 presents the surface forces between subunits I and II *upon separation* at pH = 8.3. In the solutions containing only Mg^{2+}, FPP or IPP, the interaction was always repulsive, and reversible both on approach and on separation. However, in the solution containing both Mg^{2+} and FPP, adhesive forces were observed upon separation though the repulsive force was observed upon approach. The intensity of the apparent adhesive force taken as shown in Figure 3 was 0.20 ± 0.06 mN/m. This value was constant for the piezo driving velocity of 70–720 nm/s. When FPP was replaced by IPP, the interaction was only repulsive again. One may note that the interactions between identical proteins (subunits I and I, or II and II) were always repulsive under all solution conditions (data not shown). This confirmed (1) that the proteins were charged; and (2) there was no specific and nonspecific interaction between the identical proteins. Therefore, the observed adhesive force could be attributed to the specific interaction between subunits I and II which were associated by Mg^{2+} and FPP. Our observation well agreed with the report that a catalytically active complex is formed only in the presence of FPP and Mg^{2+} (Figure 1).[1] Thus, it is likely that we could form the intermediate complex by bringing two subunits into contact by AFM, and detect the adhesive force which possibly bridged the subunits by FPP and Mg^{2+}.

In conclusion, by using the LB method, subunits I and II of HepPP synthase were immobilized on glass surfaces, and the interaction between them was directly measured using the colloidal probe AFM. It was possible, for the first time, to detect the interactions involved in the complex formation of enzyme subunits, a cofactor and a substrate (FPP in our case). This study demonstrated the potential of the force measurement for studying the elementary steps of biological interactions involved in complex biological reactions.

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