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SHORT COMMUNICATION

Selectivity in substrate–enzyme complexation studied by surface forces measurement

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Abstract The selectivity of substrate in substrate–enzyme complexation of heptaprenyl diphosphate synthase was directly investigated using colloidal probe atomic force microscopy (AFM). This enzyme is composed of two dissociable subunits, which exhibits a catalytic activity only when they are associated together in the presence of a cofactor, Mg^{2+}, and a substrate, farnesyl diphosphate (FPP). We have recently succeeded to directly demonstrate a specific interaction involved in this enzyme reaction and obtain new insights into the molecular mechanism of the reaction using the approach based on the colloidal probe AFM. The AFM measurement showed the adhesive force between the subunits only in the presence of both Mg^{2+} and FPP. In this study, we studied the substrate selectivity in the complexation by monitoring the adhesive force. The substrates studied are pyrophosphate (PPi), isopentenyl diphosphate (IPP), geranyl diphosphate (GPP), farnesyl monophosphate (FP), and farnesyl geranyl diphosphate (FGPP). No adhesion was observed in the case of PPi, IPP, and GPP. On the other hand, the significant adhesion was observed for phosphate derivatives, which bear prenyl units longer than three. This is in good agreement with the selectivity of the substrates by this enzyme, which catalyzes the condensation reaction of four IPP molecules with FPP to give heptaprenyl (C_{35}) diphosphates. Our study showed a useful methodology for examining the elemental processes of biological reactions.

Keywords Surface force measurement · Heptaprenyl diphosphate synthase · Substrate-enzyme complexation · Molecular mechanism · Colloidal probe AFM

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Introduction

The elucidation of specific interactions involved in biological reactions is essential in biological science. Especially, the interactions between protein molecules have attracted increasing attention in the current biological science. It has become a new target after many genomes and protein structures have become known.

The interactions of proteins and other biomolecules have been actively studied using both the conventional and novel nano-scale measurements. They include ultracentrifugation [1], surface plasmon resonance (SPR) [2], and a quartz-crystal microbalance (QCM) [3]. Among them, the atomic force microscopy (AFM) [4] and surface forces measurement (SFA) [5] occupy a unique position because they can directly monitor interaction forces by employing a spring balance. However, the protein systems studied so far are rather limited to relative simple ones such as the interactions between antigen-antibody [6, 7] or protein unfolding [8–10]. It is important to further develop the potential of this measurement for studying biological systems. Using the approach based on the colloidal probe AFM, we have recently succeeded to directly demonstrate specific interaction involved in the recognitions of DNA by a protein and in an enzyme reaction and obtain new insights into their molecular mechanisms [11–13].

Prenytransferases (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* [14–17], which forms the HepPP with a chain length of C_{35}, is composed of two nonidentical protein subunits, neither of which has a catalytic activity alone. These subunits have been assumed to associate in the presence of a substrate, allylic substrate (*E*,*E*)-farnesyl diphosphate (FPP), and a cofactor, Mg^{2+}, to form a catalytically active complex, which represents an intermediate state during the catalysis (Fig. 1a) [18]. This catalytically active complex has been characterized by the gel filtration and cross-linking experiments [18]. However, there was no direct evidence to support the assumption that the two subunits would associate to form a transient dimer by specific interactions between them. Recently, we have directly demonstrated a specific interaction involved in this enzyme reaction using the colloidal probe AFM. Two subunits of proteins were immobilized on two opposing surfaces respectively, and the reaction intermediates were formed by bringing them together by AFM to the contact in the presence of both Mg^{2+} and FPP. AFM was used to manipulate each elementary step (collision and separation) of the reaction and measure the interactions. This study has also shown that AFM is a powerful tool for investigating elementary steps involved in the biological reactions.

In this study, we evaluated the substrate selectivity in the enzyme reaction employing a colloidal probe AFM. The interactions between subunits I and II were studied in solutions containing substrate analogues, which bear various chain lengths or the monophosphate group instead of the diphosphate. Substrate analogues studied are pyrophosphate (PPi; C_0), IPP(C_5), geranyl diphosphate (GPP; C_{10}), farnesyl monophosphate (FP; C_{15}), and fanresylgeranyl diphosphate (FGPP; C_{25}), and shown in Fig. 2 together with the original substrate, FPP (C_{15}).

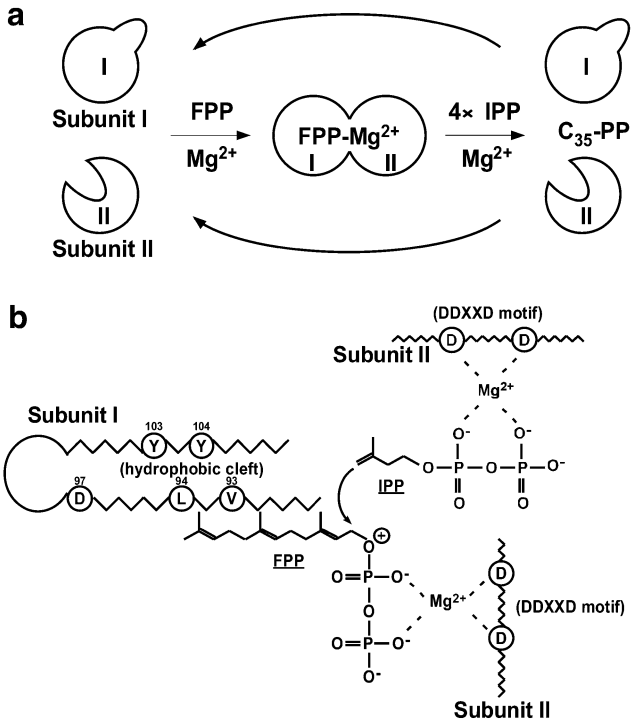

Fig. 1 **a**) A hypothetical mechanism of the catalytically active complex of HepPP synthase of *B. subtilis* [18]. **b**) A hypothetical scheme for the binding of FPP and IPP between the two subunits of *B. subtilis* HepPP synthase [19, 20]

Experiments

Materials The two subunits (subunits I and II, containing 251 and 323 amino acid residues, respectively) of HepPP synthase of *B. subtilis* are overproduced in *Escherichia coli* cells and purified separately as previously described [18]. These subunits are modified with six histidines (polyhistidine) at their N-terminals. PPi was purchased from Wako. IPP, GPP, (*E*,*E*)-FPP, (*E*,*E*)-FP, and (*E*,*E*)-FGPP were obtained by the same procedures as in previous studies [21–23]. The preparation of *N*-(8-(1, 2-di(octadecyloxy) propanoxy)- 3,6-dioxaoctyl)iminodiacetic acid (DSIDA) was previously described [24]. Dioctadecyl dimethyl ammonium bromide (DODA) was purchased from Sogo Pharmaceutical and used as received. Tris(hydroxymethyl) aminomethane (Tris), NaCl, and $MgCl_2$ were purchased from Nacalai Tesqu. All other reagents were of analytical grade. The water was purified by a Nonapure II and distilled twice by an FI-stream 48D glass still system (Barnstead).

Preparation of protein modified surfaces The glass surfaces were modified with DODA and DSIDA-Cu^{2+} monolayers by

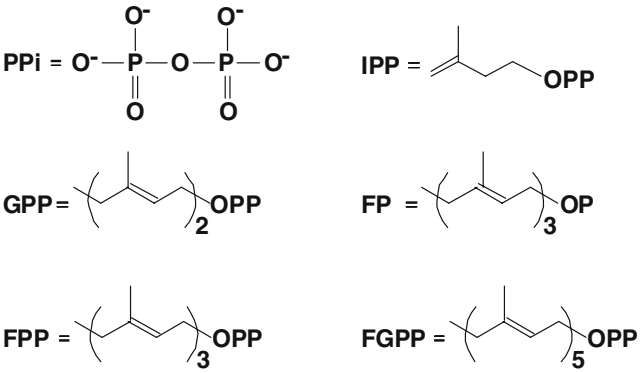

Fig. 2 Structures of substrate and its analogues

the Langmuir–Blodgett deposition at 20.0±0.1 °C using a computer-controlled film balance system (NL-BIO20-MWC, Nippon Laser and Electronics). The sample surfaces were prepared by following a previously described procedure [13, 25]. The surface was rendered hydrophobic by depositing DODA monolayer at a surface pressure of 35 mN/m, in the up-stroke mode, at a rate of 3.0 mm/min. The DSIDA-Cu^{2+} monolayer was transferred to the hydrophobic glass surface at a surface pressure of 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, $(8\pm1)\times10^{-8}$ M, for both I and II, which showed the saturated adsorption on the DSIDA-Cu^{2+} monolayer determined by QCM in a 0.1 mM Tris–HCl buffer containing 1.0 mM NaCl (data are not shown). 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.

Surface forces measurement 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 [26] using an AFM (Seiko II, SPI3700-SPA300). The measurements were carried out basically similar to previous studies [25, 27]. A colloidal glass sphere (Polyscience, 10–20 μm radius) was attached to the top of a cantilever (Olympus, RC-800PS-1) with epoxy resin (Shell, Epikote1004). The individual spring constant of the cantilever was determined by measuring the resonance frequency of the cantilever before and after adding the mass (glass sphere) at the end of the cantilever [28]. Interactions between the sphere and a glass substrate (Matsunami, microcover glass) were measured in a homemade closed AFM fluid cell. The obtained forces were normalized by the radius (R) of the sphere using the Derjaguin approximation [29],

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

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

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 and 1.0 mM $MgCl_2$ by varying the 15 μM substrate [PPi, IPP, GPP, (*E*,*E*)-FPP, (*E*,*E*)-FP, or (*E*,*E*)-FGPP] (Fig. 3). The concentration of the buffer was chosen to be 1.0 mM to check the isoelectric point of the proteins to avoid non-specific interaction mainly due to the hydrophobic and/or the van der Waals attraction. The measurement was performed at the pH at which the proteins are electrically charged.

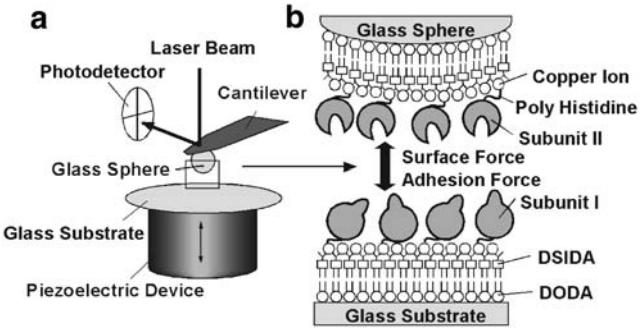

Fig. 3 Schematic drawings of experimental set-up: surface forces measurement system employing a colloidal probe atomic force microscope (**a**) and sample surfaces (**b**). Surface force, F, is determined as the product of the spring constant of the cantilever, k, and cantilever deflection, ΔD, which is obtained from the direction of the laser light reflected on the back of the cantilever

Results and discussion

Original substrate: farnesyl diphosphate (FPP; C_{15})

We have demonstrated a specific interaction between subunits I and II of HepPP synthase using the colloidal probe AFM [13]. The major results are summarized here. For FPP, that is, an original substrate, adhesive force is observed *upon separation* in the presence of both Mg^{2+} and FPP; however, only the repulsive force is observed *upon approach*. The average value of the adhesive forces was 0.21±0.06 mN/m (Fig. 4). One may note that the jump-out distance observed here is much larger than the zero-contact due to the elongation of the protein complexes. The slightly larger repulsion may be also due to the elongation and/or the hysteresis of the piezo drive. Under this condition, these forces were reproducible after repeating compression. Only repulsion was observed if either FPP or Mg^{2+} was absent in the solution, indicating that all of subunits I and II, FPP, and Mg^{2+} are necessary for the enzyme–substrate complexation. We used the "apparent adhesive force" because we did not count the elastic force appeared in the force profile before the jump-out. If the elastic force of protein is considered, the actual adhesive force could be larger. However, it is not so simple to include this deformation; therefore, we used the apparent value.

It should be interesting to investigate how AFM could differentiate the substrate specificity. Therefore, we studied substrate analogues, which had various number of prenyl units or the monophosphate group instead of the diphosphate.

Substrate analogues exhibiting no adhesive force

Pyrophosphate (C_0)

Figure 5 shows the interaction forces between subunits I and II upon approach in the 0.1 mM Tris buffer solution

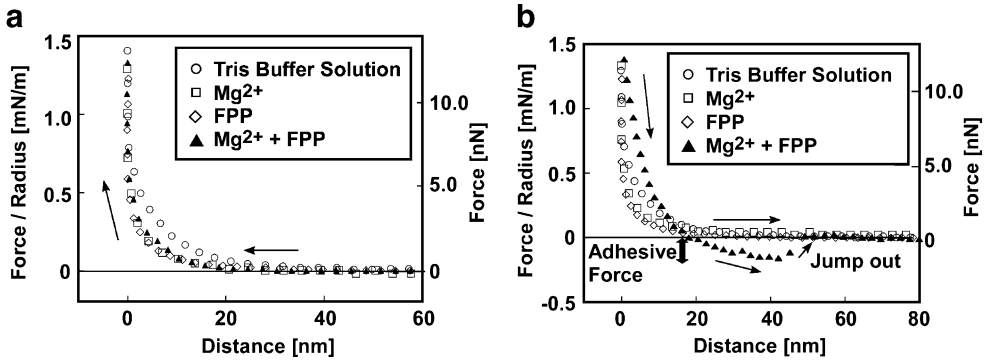

Fig. 4 Force profiles of interactions between subunits I and II surfaces **a** upon approach and **b** upon separation under various condition at pH= 8.3. *Open circle* 0.1 mM Tris buffer solution containing 1.0 mM NaCl; *open square* 0.1 mM Tris buffer solution containing 1.0 mM NaCl and 0.1 mM Mg^{2+}; *filled diamond* 0.1 mM Tris buffer solution containing 1.0 mM NaCl and 15 μM FPP; *filled triangle* 0.1 mM Tris buffer solution containing 1.0 mM NaCl, 0.1 mM Mg^{2+} and 15 μM FPP

containing only 1.0 mM NaCl or containing 1.0 mM NaCl, 1.0 mM Mg^{2+}, and 15 μM PPi, which has no hydrocarbon chain, at pH=8.3. Only repulsive force was observed upon approach in the Tris buffer solution in both the absence and presence of Mg^{2+} and PPi, indicating that there was no interaction between subunits I and II. Similarly, upon separation, only repulsive force was observed even in the presence of Mg^{2+} and PPi. There was no adhesive force upon separation. Force curves were completely reversible both on approach and on separation. These results indicate that the enzyme–substrate complex was not formed when PPi was used as a substrate, and the prenyl chain was necessary.

Isopentenyl diphosphate (C_5) and geranyl diphosphate (C_{10})

The interaction forces between subunits I and II were similarly studied in the 0.1 mM Tris buffer solutions containing only 1.0 mM NaCl or containing 1.0 mM NaCl, 1.0 mM Mg^{2+}, and 15 μM IPP or GPP. Only repulsive forces were observed upon approach and separation for both IPP and GPP; therefore, the enzyme–substrate complex was not formed for substrate analogues bearing five or ten carbon chains.

Substrate analogues exhibiting adhesive force

Farnesyl monophosphate (C_{15})

Figure 6 shows the interaction forces between subunits I and II in the 0.1 mM Tris buffer solution containing only 1.0 mM NaCl or containing 1.0 mM NaCl, 1.0 mM Mg^{2+}, and 15 μM FP in which one phosphate group of FPP is lacking. In the presence of Mg^{2+} and FP, the repulsive force was observed upon approach and adhesive forces upon separation. The average adhesive force was 0.23±0.05 mN/m and practically the same as FPP. Under this condition, these

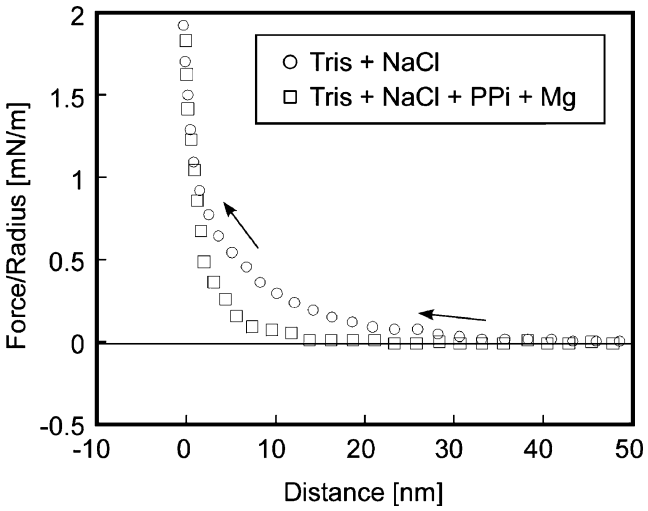

Fig. 5 Force profiles of interactions between subunit I and subunit II surfaces in the 0.1 mM Tris buffer solution containing 1.0 mM NaCl upon approach. Absence (*open circle*) and presence (*open square*) of 1.0 mM Mg^{2+} and 15 μM PPi

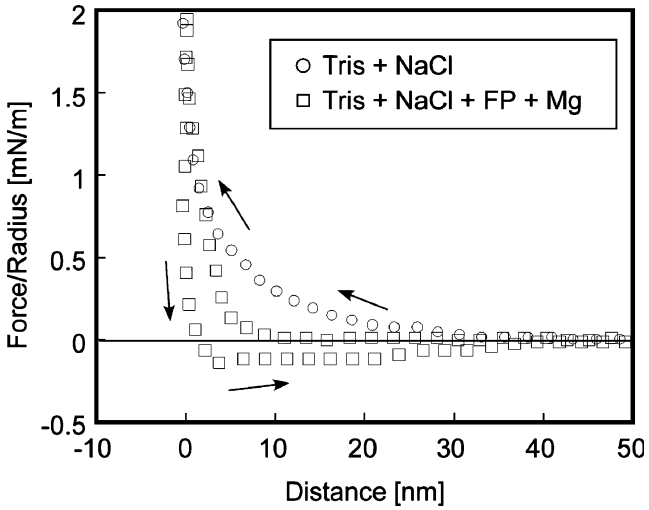

Fig. 6 Force profiles of interactions between subunits I and II surfaces in the 0.1 mM Tris buffer solution containing 1.0 mM NaCl, 1.0 mM Mg^{2+}, and 15 μM FP. *Open circle* 0.1 mM Tris buffer solution containing 1.0 mM NaCl; *open square* 0.1 mM Tris buffer solution containing 1.0 mM NaCl and 1.0 mM Mg^{2+} and 15 μM FP

forces were reproducible after repeating compression. The biochemical study and the X-ray crystal structure analysis have shown that the diphosphate group is necessary for interaction between the substrate and the subunit because the diphosphate group binds to the aspartic acid residues of subunit II through Mg^{2+} [30–32]. However, the adhesive force observed in the presence of the monophosphate group indicated that the enzyme–substrate complex could be formed employing only the monophosphate group. It has been shown that FP has a protective effect similarly to FPP in the presence of Mg^{2+} [22]. Therefore, FP seems to bind to the FPP binding sites of subunits I and II, although a precise mechanism how FP could be involved in the enzyme–substrate complex formation has not been clearly understood so far. Two oxygen atoms of the monophosphate group were probably associated with subunit II through Mg^{2+}, and the prenyl tail of FPP binds to a hydrophobic pocket of subunit I.

Fanresylgeranyl diphosphate (C_{25})

Figure 7 shows the interaction forces between subunits I and II in the 0.1 mM Tris buffer solution containing only 1.0 mM NaCl or containing 1.0 mM NaCl, 1.0 mM Mg^{2+}, and 15 μM FGPP, which has 25 carbons. The weak repulsive force was observed upon approach similarly to other substrate analogues. However, a significant adhesive force was observed upon separation, although the trace of the adhesive force was somewhat different from those for FPP. This peculiar adhesion force profile could be related to the elongation of protein because the similar profiles were reported for unfolding of proteins [8–10]. The same force profiles were always observed at the first measurement for different positions on the substrate; however, the force profiles after the second compression were different. It was likely that the proteins could not fold back to the original state after they were significantly elongated. The recent X-ray crystal structure analysis revealed that the hydrocarbon group of the substrate bound to the hydrophobic pocket of subunit I, possibly by hydrophobic interaction. In the case of FPP, we have indeed determined that the observed adhesion force should correspond to the interaction force between subunit I and FPP [13]. For this mechanism, the binding of substrate to subunit I should become stronger when the number of carbons of substrates increases. Owing to stronger adhesion than FPP expected for FGPP, it should become difficult to separate FGPP and subunit I, resulting in the elongation of proteins. The average adhesive force obtained in the presence of FGPP was 0.22±0.07 mN/m.

The selectivity of substrate in substrate–enzyme complexation of heptaprenyl diphosphate synthase

As seen in this study, no adhesive force was observed in the absence of substrate and for PPi, IPP, and GPP. On the other hand, some adhesive force was observed in the presence of FP, FPP, or FGPP. Therefore, we could conclude that more than 15 carbons are required for complexation of subunits I and II. The strength of adhesion forces observed was almost the same as summarized in Fig. 8. The selectivity observed in this study well agreed with the function of this enzyme, which catalyzes the polymerization reaction of FPP with four IPP. During the catalytic reaction, the complex of C_{25} FGPP with subunits I and II should be formed by Mg^{2+} ion bridging and the hydrophobic interaction. It will be interesting to test how HepPP interacts with subunits. However, unfortunately, enough amount of HepPP was not available for our experiments. It is also interesting that the monophosphate is enough to form the substrate–enzyme

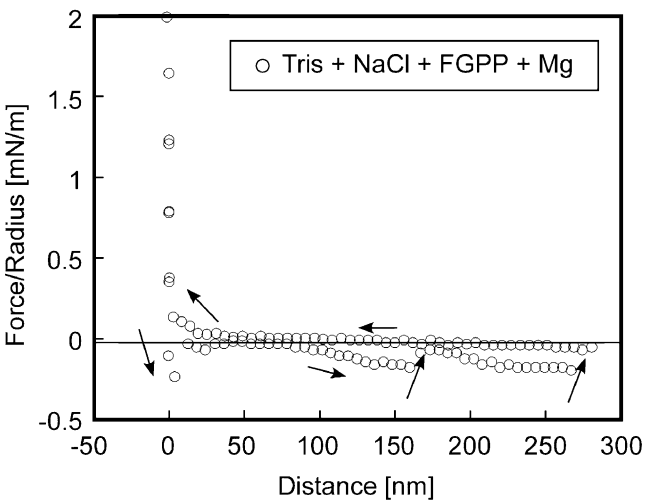

Fig. 7 Force profiles of interactions between subunits I and II surface in the 0.1 mM Tris buffer solution containing 1.0 mM NaCl, 1.0 mM Mg^{2+}, and 15 μM FGPP

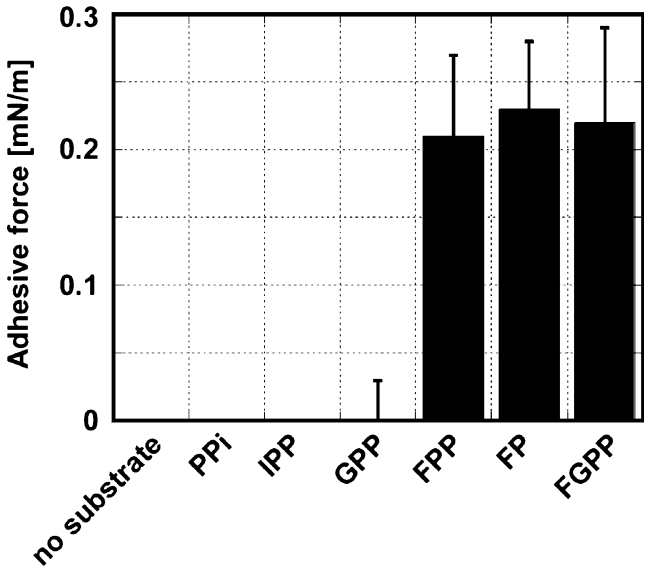

Fig. 8 Histogram of adhesion force for various substrate

complexes, addressing a question for a future study about the role of the diphosphate group.

Conclusion

In this study, the selectivity of substrate in substrate–enzyme complexation of HepPP synthase was directly investigated using colloidal probe AFM. Adhesion force between subunits I and II was observed only in the presence of the substrate containing more than 15 carbons, which is reasonable for the enzymatic function of HePP synthase. The adhesion measurement showed that the monophosphate is sufficient for the complexation. This result suggests that there is an as yet possible unidentified role of the phosphate group. The approach used in this study is useful for investigating the elementary steps of enzyme reaction.

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