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Kinetics of association processes of D-fructose dehydrogenase onto liposome surfaces

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Summary

Association processes of *D*-fructose dehydrogenase (FDH) onto surfaces of liposomes which were composed of *N*-(5-dimethylamino-1-naphthalenesulfonyl)-*L*-α-dimyristoylphosphatidylethanolamide and *L*-α-dimyristoylphosphatidylcholine or *L*-α-dimyristoylphosphatidylglycerol (1:9) were investigated by the fluorescence stopped-flow technique. The association processes were divided into two relaxation processes: the faster process whose apparent rate constant monotonously increased with the concentration of FDH, and the slower process whose rate constant showed a saturation behavior. Taking the number of binding sites on the liposome surface into consideration, the corrected association rate constant of the faster process was 4.4 % of the theoretical value for a binary collision, probably due to a disadvantageous surface-searching and dehydration processes on the liposome and protein surfaces. The Arrhenius plots of the rate constants both for the faster and slower steps showed a discontinuous change around the gel to liquid-crystal phase transition temperature of the liposomes. Strong influences of deformability of liposomes, and state of hydrating water molecules around polar heads, on the rate of association processes were suggested.

Introduction

Biomembranes can be seen as a highly complicated molecular assembly composed of a fluid lipid bilayer in which various membrane components, such as enzymes, receptors and many other proteins and peptides are embedded or attached to (1). Not only in the process of the construction of their structures but also in the process to fulfill their function (transportation of information, substances and energy, for example) interaction between a protein and lipids or other proteins in biomembrane plays an important role. To reveal a mechanism of protein binding to biomembrane surfaces, liposomes which are composed of natural lipids or synthetic lipid analogues and reconstituted membrane proteins have been used as biomembrane mimetics (2), because they have simple and well-defined structures and compositions, and therefore simplification of the forces and interactions which drive the association process in living bodies is possible. We have been studying mutual recognition processes between complementary ligands on polymerized liposomes, and revealed the contribution of various interactions which drive

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these processes (3-6).

D-Fructose dehydrogenase (FDH) catalyzes the oxidation of *D*-fructose to 5-keto-*D*-fructose in biomembranes (7). Furthermore, the enzyme only catalyzes the oxidation of *D*-fructose in the presence of redox acceptors (ferricyanide and hexacyanoferrate (III), for example) (8). By making use of this property, the enzyme can be used as an analytical reagent for a microdetermination of *D*-fructose (9). Moreover, kinetic studies on the interaction between FDH and D-fructose or ferricyanide have been reported (10). As for associations between FDH and cell membranes or cell model membranes (liposomes, for example), a very slow association step between FDH and anionic liposomes (association rate constant, 4.3×10^{-4} s^{-1}) was reported (11). However, this rate constant is too small as a rate for the binding step between proteins and liposomes (1.25×10^{2} s^{-1}, for the binding step between myelin and anionic liposomes (12)). Therefore, detailed kinetic studies on the association between FDH and biomembranes have to be done. In this regard, we have examined here kinetics of association processes between FDH and liposomes as models of cell membrane.

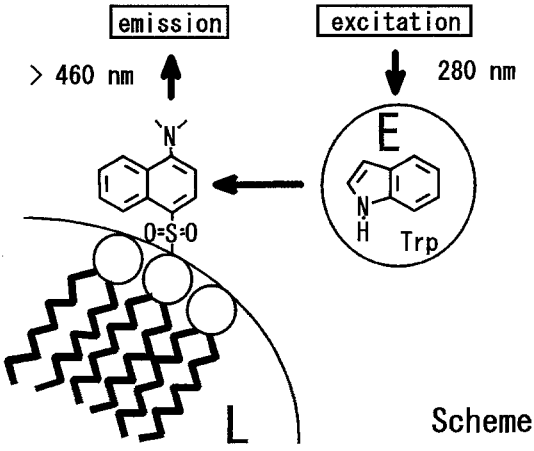
Experimental

Materials

D-Fructose dehydrogenase (from *Gluconobacter sp.*, M = 140000, FDH) was purchased from Toyobo Co., Ltd. Osaka, Japan. *L*-α-Dimyristoylphosphatidylcholine (DMPC), *L*-α-dimyristoylphosphatidyglycerol (DMPG), *L*-α-dipalmitoylphosphatidyl-ethanolamine (DPPE) and L-α-dipalmitoylphosphatidylcholine (DPPC) were purchased from Sigma, St. Louis, Mo., and used as supplied. *N*-(5-Dimethylamino-1-naphthalene-sulufonyl)-*L*-α-dipalmitoylphosphatidylethanol amide (Dan-DPPE) was prepared by the method of Pusey et al. (13). Other reagents were commercially available. Deionized water was distilled just prior to use for preparation of sample solutions and dispersions.

Preparation of solution of D-fructose dehydrogenase

FDH (50 mg) was dissolved in a McIlvaine buffer (consisted of citric acid and disodium hydrogenphosphate, 20 ml, 100 mM, pH 4.7 or 5.8), and the solution was



Scheme I

dialyzed against the buffer for 24 hs at 4 °C. Protein content was determined by the method of Lowry et al. (14), with cytochrome C as standard.

Preparation of liposomes

Lipids were dissolved into chloroform in a small round-bottomed flask. After evaporation of the solvent, the thin lipid film formed was dispersed into the McIlvaine buffer using a vortex mixer for 1 min at 40 °C, and an ultrasonifier (Bransonic 42, 240 W, Branson) for 3 min at 40 °C. To remove aggregates, the liposome suspension was finally passed through a membrane filter (pore size 0.22 mm, SLGV025LS, Millipore, Bedford, UK). Using a dynamic light scattering method (ELS-800, Otsuka Electronics, Hirakata, Japan; light source, He-Ne laser 6328 Å), hydrodynamic diameters of the liposomes were estimated.

Association processes

Association processes of FDH with liposomes was followed by the increase in fluorescence using a stopped-flow apparatus (RA-401, Otsuka Electronics) (excitation: 280 nm, emission: >460 nm), due to energy transfer from tryptophan residues of the enzyme to the dansyl groups introduced in the liposomes. The fluorescence was allowed to enter the detector using a cut-off filter (Y46, Hoya, Tokyo).

Determination of number of binding sites on liposome surface

Mixtures of various amounts of FDH and liposomes incubated for 20 min were ultracentrifuged (100,000 rpm for 15 min at 4 °C) and subsequently the concentration of FDH in the supernatant was determined by the Lowry method. An initial linear portion of the curve in the plot of the amount of FDH bound to the liposome versus the concentration of FDH added was extrapolated to the saturation point to obtain the total number of binding sites (N) on the liposome (2).

Results and discussion

Two-step association

By mixing the liposome suspension with an excess amount of FDH using the stopped-flow apparatus, the fluorescence intensity increased in a double exponential

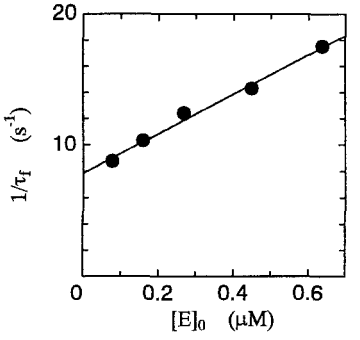


Figure 1(a) Plots of $1/\tau_f$ vs. $[E]_0$ for the DMPG-liposome system at pH 5.8 and 20 °C

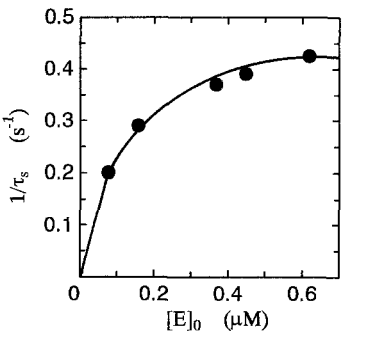


Figure 1(b) Plots of $1/\tau_s$ vs. $[E]_0$ for the DMPG-liposome system at pH 5.8 and 20 °C

manner (relaxation times, τ_f and τ_s). FDH contains some tryptophan residues although a number of the tryptophan residues is unknown (11). When the FDH molecule approaches to the liposome surface, the Trp residues in the enzyme molecule which are excited at 280 nm make a Förster style energy transfer to the dansyl groups, which induces the increase in fluorescence (Scheme I). In this system, two relaxation processes were observed and reciprocal of the relaxation time for the faster process increased with the concentration of FDH (Figure 1 (a)), whereas that for the slower process increased with the concentration of the enzyme and leveled off (Figure 1 (b)).

Similar association behaviors (the apparent rate constant for the faster process increased with the concentration of the enzyme, and that for the slower process increased with the concentration of the enzyme and leveled off) were also observed in many other association processes such as cytochrome c to liposome (15), a monoclonal antibody to haptens (16) and avidin to biotin (5) on liposome surfaces. If we assume that the association process observed here proceeds via a consecutive reaction between E and L (a fast binding step followed by a slow rearrangement step, Scheme II), reciprocals of relaxation times, $1/\tau_f$ and $1/\tau_s$, can be derived as equations (1) and (2).

$$E + L \underset{k_{-1}}{\overset{k_1}{\Leftrightarrow}} EL' \underset{k_{-2}}{\overset{k_2}{\Leftrightarrow}} EL'' \qquad [I\,I]$$

$$1/\tau_f = k_1([E] + [L]) + k_{-1} \qquad (1)$$

$$1/\tau_s = k_2([E] + [L])/[K^{-1} + ([E] + [L])] + k_{-2} \qquad (2)$$

where E and L are an enzyme (FDH in this case) and a ligand (liposomes), respectively. EL' and EL" are complexes of E and L. K^{-1} is equal to k_{-1}/k_1. According to this reaction scheme, when the initial concentration of FDH ($[E]_0$) is much higher than that of ligand ($[L]_0$), $1/\tau_f$ linearly increases with $[E]_0$ and $1/\tau_s$ is expected to show a saturation behavior. The experimental results are not inconsistent with expected concentration dependences.

From the slope in the plots of k_{obs} ($=1/\tau_f$) vs. initial concentration of FDH under the conditions $[E]_0 >> [L]_0$ (Figure 1 (a)), a second-order association rate constant, k_1, could be obtained as 2.6×10^7 $M^{-1}s^{-1}$ for the DMPG-Dan-DPPE (9:1) liposome (defined as DMPG-liposome, hereafter) at pH 5.8 (100 mM McIlvaine buffer) and 33 °C. The saturated value in Figure 1 (b) is corresponding to $k_2 + k_{-2}$. Since the Y-intercept in Figure 1 (b) which corresponds to k_{-2} is nearly zero, the saturated value is almost equal to k_2. The slower step might correspond to an rearrangement of the situation on the liposome surface (will be discussed latter). The considerably smaller rate constant (4.3×10^{-4} s^{-1} at pH 4.5 and 25 °C) reported by Kheirolomoom et al. (11) might correspond to the slow conformation change of FDH to attain the stablest contact with the lipid molecules surrounding the enzyme.

Estimation of true association rate constants

Since the liposomes can associate with a large number of FDH molecules due to a difference in size between the liposome (average diameter 700 Å obtained by the dynamic light scattering) and the enzyme (diameter 40 Å (17)), the association rate constant, k_1, estimated is not a true second-order association rate constant between one

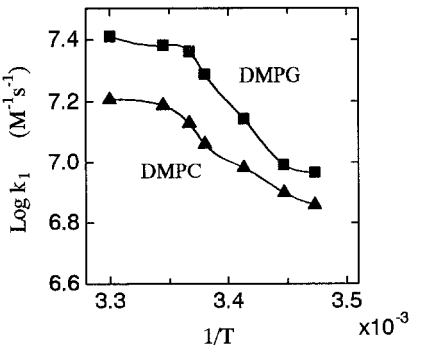


Figure 2 Arrenius plots of k_1 for the DMPC-liposome and the DMPG-liposome at pH 5.8.

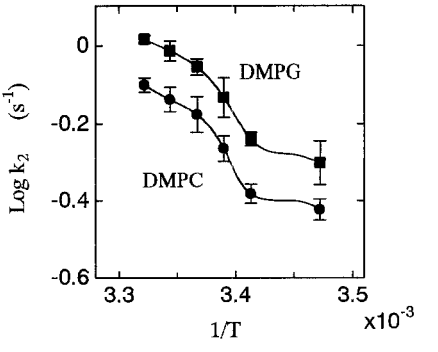


Figure 3 Arrhenius plots of k_2 for the DMPC-liposome and the DMPG-liposome at pH 5.8.

FDH molecule and one liposome. Equation (3) was used to estimate the true association rate constant, k_{corr}, in the liposomal system:

$$k_1 = k_{corr} / N \qquad (3)$$

where k_1 is the apparent association rate constant (2.6 x 10^7 $M^{-1}s^{-1}$ at 33 °C for DMPG-liposome from a slope of Figure 1 (a)), and N is the number of binding sites on the liposome surface. This equation can be applied to the diffusion-controlled association process between a mono-dentate ligand and a multi-dentate ligand (number of binding site, N) (14).

Assuming that the liposome (average diameter, 700 Å) was consisted of a single bilayer, and the occupied area per one lipid molecule was 58 $Å^2$ at the liquid crystal phase (18), the maximum number of FDH molecules (diameter 40 Å) which can be bound to the liposome theoretically was estimated to be 1,200. The N value which was experimentally estimated by ultracentrifugation method was 67, and this value is about 5.6 % of the maximum number of FDH which can be bound to the liposome. Using the equation (3) and the number of binding sites on the liposome (N = 67), we could estimate the true association rate constant, k_{corr}, as 1.7 x 10^9 $M^{-1}s^{-1}$ at 33 °C.

By a simple collision theory by Smoluchowski (19), the rate constant for a diffusion-controlled binary association was derived as equation (4):

$$k = 4\pi N_A(D_a+D_b)R_{ab}/1000 \qquad (4)$$

where N_A, D_a, R_{ab} are the Avogadro's number, the diffusion coefficient of the particle "a", and the closest distance between the centers of particles "a" and "b", respectively. By using the equation (4) we could evaluate the rate constant, k_{theor}, for the association of two spheres with the diameters of which are 40 Å (same as FDH) and 700 Å (same as liposome) at 33 °C in water as 3.9 x 10^{10} $M^{-1}s^{-1}$, which is about 23 times larger than the experimental result (k_{corr} = 1.7 x 10^9 $M^{-1}s^{-1}$).

Some examples of estimated collision efficiencies are 30 % for the association of blood clotting factor V to anionic liposomes (2), and 10 % for a binding of prothrombin to the same liposomes (20). As for a binding of IgE (approximated to be a sphere of radius 45 Å) to rat basophilic leukemia cell (radius 6 μm), k_{corr} was reported to be 3.0 x 10^{10} $M^{-1}s^{-1}$, and was much lower (1.2 %) than the theoretical value (2.5 x 10^{12} $M^{-1}s^{-1}$), too (21). For these association processes, all association rate constants were smaller than those of theoretical values.

In this study, k_{corr} value was about 4.4 % of the theoretical value. One of the reasons for the small k_{corr} value might be the necessity of energy that FDH may have to remove water molecules from the liposome to bind to the liposome surface (22). It was reported that the number of water molecules forming hydration layers around a head group of lipid molecules in aqueous suspensions are 11-16 per lipid molecules (23). We have been indicating that water layers around colloidal particles play an important role to determine the association rate between colloidal particles (24-26). Therefore, the association rate constant obtained in this experiment was not inconsistent with those for the systems previously examined.

Binding process

The Arrhenius plots of k_1 for the binding processes of FDH to DMPC-liposome or DMPG-liposomes at pH 5.8 are shown in Figure 2. At about 23 °C, there was a discontinuous region in both of the lines in Figure 2. The transition temperatures (Tm) of DMPC and DMPG were reported to be 24 °C (18). Therefore, the discontinuous regions in Figure 4 seem to correspond to the transition of the lipid bilayers from gel to liquid-crystal state, which demonstrates that the rate of binding process is strongly affected by the state of lipid bilayers. In the binding process of cytochrome c to negatively charged liposome surfaces, the phase transition of the liposomes also has significant influence on the reaction rate (15).

There are several differences in their structures and physical properties between lipid bilayers in gel phase and those in liquid-crystal phase. Examples included are density of lateral packing and the rate of diffusion of lipid molecules, and distribution of water molecules around polar heads. As discussed above the observed binding rate is much slower than the theoretical value for simple collision, which means energy consuming step exist in the process. The binding process is expected to include dehydration of liposome surface and rearrangement of lipid molecules in the layered structure, and both of the elemental steps are likely to be influenced by the phase of liposome.

When the temperature of a liposome suspension is raised above Tm, the number of water molecules around a head group of lipid molecules may increase as measured by NMR spectroscopy (27) and as simulated by the molecular dynamics (28), and hydrogen bonds between water molecules are perturbed as revealed by Raman spectroscopy (29). With the increase in the hydration number, the hydration force per one water molecule to the head group of the lipid molecule might decrease, which makes it easy to remove water molecules from the surface. The effective collision of FDH to the liposome surface would, therefore, be promoted, and consequently, the rate constant drastically increased around the discontinuous region.

Figure 2 also shows that the binding rate constant in the DMPG-liposome was larger than in the DMPC-liposome at pH 5.8. At pH 5.8, the DMPG-liposome has a negative charge whereas the DMPC-liposome has a slightly negative charge (corresponding to the presence of Dan-DPPE. DMPC is almost electrically neutral). Since FDH has a negative charge at pH 5.8 (pI = 5 (30)), FDH may feel stronger repulsive force from the DMPG-liposome than from the DMPC-liposome. Therefore electrostatic interaction between FDH and liposome seems to have a minor role to determine the binding rate. One of possible explanations for the experimental results is the difference in the looseness of the

packing of the lipid molecules due to difference in the electrostatic interaction between polar groups.

DMPG molecules have a net negative charge and repulse each other in liposome, whereas, DMPC is a zwitter ion and attracts each other. Therefore, the packing of the lipid molecules in the DMPG-liposome system is looser (the occupied area per one DMPG is 70 $Å^2$ in liquid-crystal phase (31)) than that in the DMPC-liposome (58 $Å^2$ in liquid-crystal phase (17)). FDH, accordingly, can not easily bind to the liposome surface due to the rigidity of the liposome surface and the hydrating water layer in the DMPC-liposome system.

This explanation is also supported the fact that the binding rates for the DMPG-liposome at pH 5.8 were larger than the rates at pH 4.7 (data not shown). Since degree of dissociation of phosphate groups of the lipid molecules at pH 5.8 is larger than at pH 4.7 in solution (pKa of phosphate groups is 3.5 (32)), repulsion between phospholipid molecules at pH 5.8 is larger than at pH 4.7. Consequently, the packing between lipid molecules at pH 5.8 may be comparatively looser than at pH 4.7. Because of the looseness of the liposome surface, FDH may easily bind to the liposome surface. After all, the rate constants for the binding step is closely connected with the looseness of the liposome surfaces.

Slower process

Figures 3 shows the Arrhenius plots of k_2 for the slower process (k_2). This figure shows that the curvatures observed in the slower process are similar to those for the binding process. These results may indicate that the difference in the packing of lipid molecules is also important for the slower step. Moreover, the rate constants for the DMPG-liposome system were larger than those for DMPC-liposome system again. It is difficult to describe this step in molecular term at this moment. However, since the protein molecules are incorporated in the bilayered structure of liposome at the final state, it is possible to guess that the slower step might correspond to a penetration of FDH into the lipid bilayer or rearrangement of the situation of the system on the liposome surface, for example, a minor conformational change in FDH or a desolvation in the micro-interface between the enzyme and the bilayer on the liposome, induced by the collision of the enzyme to the lipid molecules on the liposome surface. In either case, it is highly probable that lateral density and mobility of lipid molecules have significant effects on the reaction rate.

Conclusion

(1) Association processes of *D*-fructose dehydrogenase onto the surface of liposomes consist of the binding step whose apparent rate constant increases with the concentration of the enzyme, and consecutively the slower step whose apparent rate constant increases with the concentration of the enzyme and levels off.

(2) The rates of the association steps are affected by the rigidity of the liposome surface and the deformability of the liposomes.

Acknowledgment

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