Supramolecular Holoenzymes: Activity Modulation of Endonuclease by the Use of Synthetic Bilayer Membranes as Regulatory Cofactors

Nobuo Kimizuka,* Atsushi Baba, and Toyoki Kunitake†

Department of Applied Chemistry
Faculty of Engineering, Kyushu University
Hakozaki 6-10-1, Higashi-ku, Fukuoka 812-8581, Japan

Received August 15, 2000

The artificial regulation of protein function has been a topic of active research.[1−4] Chemical modification has been used to introduce signal-responsive groups such as photofunctional groups[1−3] or affinity ligands[4] into proteins. However, such covalent modifications generally lower protein activity compared to corresponding endogenous proteins. In contrast to these synthetic approaches, the supramolecular regulation of protein activity is ubiquitously employed in biological systems. For example, protein kinase C, a key enzyme in intracellular signal transduction systems, is activated by diacylglycerol, anionic phosphatidylserine and Ca^{2+} ions on the intracellular surface of plasma membranes.[5] Such a messenger-induced activation of membrane-bound, dormant enzymes ensures their rapid response to extracellular signals and provides a new basis for the artificial manipulation of protein-regulation systems. In this study, we describe the control of the supramolecular activity of DNA-binding proteins by the use of bilayer membranes as regulatory cofactors.[6]

As a DNA-specific protein, the endonuclease HindIII (*Haemophilus influenzae* RdIII) was used. HindIII is a type II dimeric restriction enzyme containing a large number of cationic residues (35 lysines, 1 histidine and 10 arginines).[7] λ-DNA, dipalmitoyl phosphatidyl choline (DPPC) and Nitr-5 were used as received. Nitr-5 is a photoresponsive ligand that forms a stable Ca^{2+} complex, which releases Ca^{2+} ions upon illumination with 360-nm light.[8] Phosphate amphiphiles **1**−**3** were prepared in these laboratories[9] (see Chart 1). Amphiphile **1** and **2**, which form highly ordered bilayer membranes in water, contain L-glutamate and L-aspartate units, respectively.[9a,c]

Figure 1 shows the results of λ-DNA hydrolysis in the presence or absence of bilayer **1**. There are seven d(AAGCTT) sequences in λ-DNA and, of the eight fragments produced by the hydrolytic activity of HindIII, six fragments are visible in the gel electrophoresis in the absence of bilayer **1** (lane 1). In the absence of HindIII, hydrolysis was not observed for the mixtures of aqueous bilayer **1** and λ-DNA ([amphiphile]:[nucleotide] = 1:1 (lane 2) and 10:1 (lane 3)). All of the hydrolytic fragments were produced when HindIII was added to the premixed bilayer/DNA solutions and incubated at 37 °C (DNA + **1** ← HindIII, lanes 4 and 5). On the other hand, when HindIII was first incubated with bilayer **1**

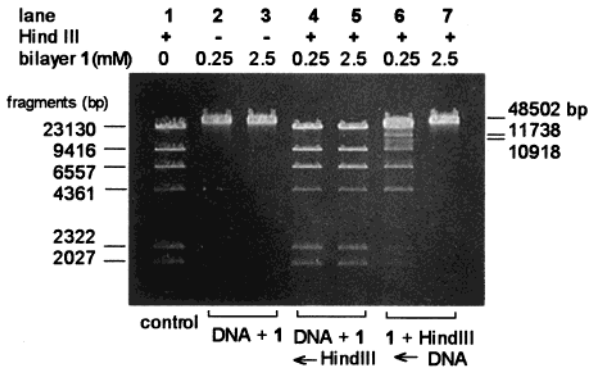
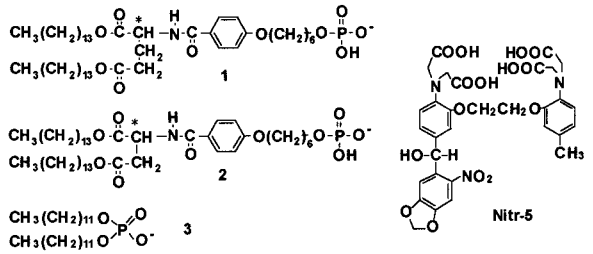


Figure 1. Electrophoretic analysis of the DNA-**1**−HindIII mixture. Each mixture contains 50 mM NaCl, 30 mM Tris-HCl (pH 7.9), 10 mM $MgCl_2$, 10 mM 2-mercaptoethanol, and λ-DNA (0.25 mM nucleotides). Reaction time was 1 h at 37 °C: lane 1, control; lanes 2 and 3, no HindIII; lanes 4 and 5; HindIII (1 μl, 10−12 units) was added to the mixture of bilayer **1** and λ-DNA; lane 6 and 7; λ-DNA was added to the mixture of bilayer **1** and HindIII.

Chart 1. Chemical Structures of Phosphate Amphiphile **1**−**3** and Nitr-5



prior to the addition of λ-DNA (**1** + HindIII ← DNA, lane 6), the two lower-molecular weight fragments (2322 and 2027 bp) disappeared, and were replaced by nondigested fragments of 11738 bp (= 2322 + 9416 bp) and 10918 bp (= 6557 + 4361 bp). A similar electrophoretic pattern was observed for the binary solutions of HindIII and λ-DNA (i.e., no bilayer), when the concentration of HindIII was lowered to 5% of that used in the experiment described above (data not shown). Therefore, these observations indicate that approximately 95% of HindIII in the solution is inactivated when it is bound to bilayer **1**, and that unbound species (∼5%) are responsible for the altered electrophoresis pattern. This result was further confirmed by employing larger amounts of bilayer **1** (lane 7, [amphiphile]:[nucleotide] = 10:1), where the hydrolytic fragments subsequently completely disappeared as a result of the shift in equilibrium toward the membrane-bound species. The binding of HindIII to bilayer **1** is based on electrostatic interactions, since the hydrolytic activity of HindIII was not influenced by the presence of zwitter ionic DPPC liposomes.

Interestingly, HindIII bound to bilayer **1** is not irreversibly denatured, as shown by temperature-dependence experiments (Figure 2). In the absence of bilayer **1**, HindIII hydrolyses λ-DNA in the temperature range of 25−60 °C, and normal scission fragments are obtained (lane 1, at 37 °C for example). On the other hand, in contrast to the altered electrophoretic pattern of **1** + HindIII ← DNA at 37 °C (lane 2. This is the same as lane 6 in Figure 1), incubation at the elevated temperatures of 45 and 55 °C lead to appearance of all the scission fragments (lanes 3 and 4). This observed recovery of hydrolytic activity clearly indicates that HindIII bound to bilayer **1** (at 37 °C) is in a dormant state, while it is activated at elevated temperatures as a result of its liberation from the bilayer surface.[10] On the other hand, when

† Present address: Frontier Research System, RIKEN, Wako, Saitama, 351-0198, Japan.

(1) Martinek, K.; Berezin, I. V. *Photochem. Photobiol.* **1979**, *29*, 637.
(2) (a) Willner, I.; Rubin, S. *Angew. Chem., Int. Ed. Engl.* **1996**, *35*, 367. (b) Hamachi, I.; Hiraoka, T.; Yamada, Y.; Shinkai, S. *Chem. Lett.*, **1998**, 537.
(3) (a) Westmark, P. R.; Kelly, J. P.; Smith, B. D. *J. Am. Chem. Soc.* **1993**, *115*, 3416. (b) Hohsaka, T.; Kawashima, K.; Sisido, M. *J. Am. Chem. Soc.* **1994**, *116*, 413.
(4) Hamachi, I.; Tajiri, Y.; Nagase, T.; Shinkai, S. *Chem. Eur. J.* **1997**, *3*, 1025.
(5) Mosior, M.; McLaughlin, S. *Biophys. J.* **1991**, *60*, 149.
(6) Parts of this study were presented at the 43rd SPSJ Annual Meeting, 1994, May, Nagoya, Japan.
(7) Old, R.; Murray, K.; Roizes, G. *J. Mol. Biol*, **1975**, *92*, 331.
(8) Tsien, R. Y.; Zucker, R. S. *Biophys. J.* **1986**, *50*, 843.
(9) (a) Nakashima, N.; Ando, R.; Fukushima, H. and Kunitake, T. *Chem. Lett.* **1985**, 1503. (b) Kunitake, T.; Okahata, Y. *Bull. Chem. Soc. Jpn.* **1978**, *51*, 1877. (c) Kim, J.-M.; Kunitake, T. *Chem. Lett.* **1989**, 959. (d) Baba, A. Masters Thesis, Faculty of Engineering, Kyushu University, 1994.
(10) It is possible that HindIII is temporarily denatured when it binds to the bilayer **1**.

10.1021/ja0030313 CCC: $20.00
Published on Web 02/03/2001

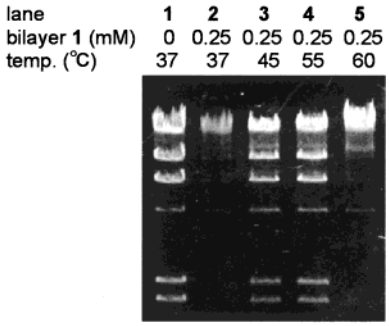


Figure 2. Effect of reaction temperature on endonuclease activity. All reaction mixtures contained HindIII (1 μl, 10−12 units). Lane 1, control (without bilayer **1**), lane 2, 37 °C; lane 3, 45 °C; lane 4, 55 °C; lane 5, 60 °C. Reaction time was 1 h.

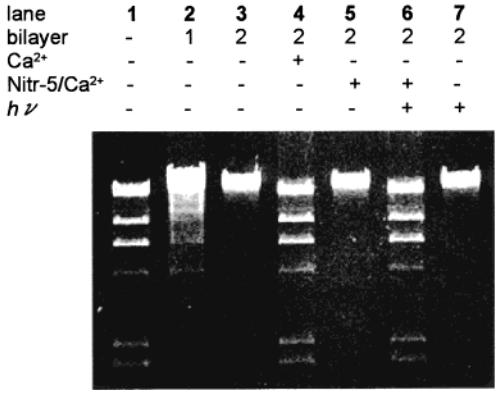


Figure 3. Effect of Ca^{2+} and photoirradiation of Nitr-5/Ca^{2+} on endonuclease activity. All reaction mixtures contained HindIII (1 μl, 10−12 units). Reaction time was 1 h at 37 °C, [nucleotide] = [amphiphile] = 0.25 mM: lane 1, control; lane 2, in the presence of bilayer **1**; lane 3, in the presence of bilayer **2**; lane 4, plus added Ca^{2+} ([Ca^{2+}] = 0.5 mM); lane 5, Nitr-5/Ca^{2+} was added ([Ca^{2+}] = 0.5 mM), without photoillumination; lane 6, Nitr-5/Ca^{2+} was photoilluminated; lane 6, photoillumination without Nitr-5/Ca^{2+}.

the incubation temperature of the aqueous mixture was further raised to 60 °C, the hydrolytic activity of HindIII was completely suppressed (lane 5). Given that HindIII in the absence of bilayer displayed normal hydrolytic activity at this temperature, it is likely that the HindIII was denatured by bilayer **1** in the liquid crystalline state.[11]

The affinity of HindIII to phosphate bilayers can be modified by replacing the L-glutamate unit of **1** with L-aspartate (amphiphile **2**). Figure 3 compares the result of λ-DNA hydrolysis in the presence of bilayers **1** and **2** ([amphiphile]:[nucleotide] = 1:1, 0.25 mM bilayer, 37 °C). The effects of Ca^{2+} ion-addition and photoillumination of the Nitr-5/Ca^{2+} complex for the **2** + HindIII ← DNA mixture are also shown. As described above (Figure 2), bilayer **1** restricted the activity of HindIII under this condition (Figure 3, lane 2.). In contrast, when HindIII was incubated with bilayer **2** at 37 °C, scission fragments were not produced (lane 3). Clearly, HindIII is more efficiently trapped by the aspartate-based bilayer **2**. The interaction between HindIII and bilayers is thus amenable to change, depending on the chemical structure of amphiphiles.

Interestingly, when Ca^{2+} ions were added to the above mixture, the normal scission pattern was regenerated (lane 4). It can be seen that the hydrolytic activity of HindIII, inhibited by bilayer **2**, is restored by the addition of Ca^{2+} ions. As Ca^{2+} ions strongly bind to phosphate bilayers,[9a] they must have replaced the membrane-bound HindIII. Such Ca^{2+}-induced activation was also observed for the mixture of **1** + HindIII ← DNA (data not shown). Liberation of the protein was specific to the presence of Ca^{2+} ions, since Mg^{2+} ions, which were abundantly present in the reaction mixture, exerted no influence on the membrane-bound HindIII.[12]

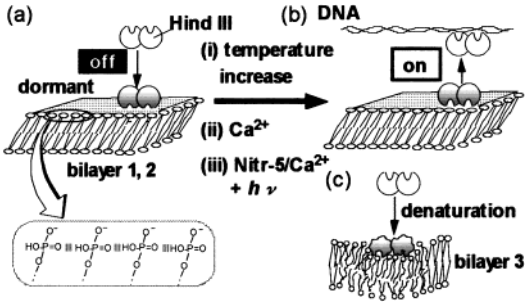


Figure 4. Schematic illustration of the supramolecular holoenzymatic system. (a) HindIII becomes dormant upon binding to bilayer **1**, **2** (off-state). (b) Triggered release of HindIII from the bilayer surface (on-state). (c) HindIII is irreversibly denatured upon binding to dialkyl phosphate bilayer **3**.

Unlike the glutamate- or aspartate-based phosphate bilayers, dialkyl phosphate bilayer **3** and sodium didodecyl sulfate (SDS) totally inhibited all enzymatic activity, regardless of the reaction temperature and addition of Ca^{2+} ions. Bilayer **3** is in the liquid crystalline state at 37 °C (T_c, 25 °C), and it can be easily deformed upon the binding of HindIII, leading to protein denaturation. Under the neutral pH employed in these experiments, bilayers **1** and **2** possess nondissociated P−OH groups, which can form hydrogen bonds with adjacent P═O groups either directly or via Tris molecules, thus making the bilayers intact upon binding of the protein.

The photoregulation of HindIII was also investigated (Figure 3). When λ-DNA was added to the ternary mixture of **2**, HindIIIand Nitr-5/Ca^{2+}, the enzymatic reaction did not proceed in the dark (lane 5). This confirms that Ca^{2+} is captured in the complex, and that HindIII remains in a dormant state on the bilayer surface. On the other hand, upon photoillumination of the above mixture, λ-DNA was digested and all of the hydrolytic fragments were produced (lane 6). Such activation was not observed for the photoilluminated mixture of **2** + HindIII ← DNA in the absence of Nitr-5/Ca^{2+} (lane 7). Therefore, photoreleased Ca^{2+} ions liberated HindIII from the bilayer surface, thus allowing the photoregulation of endonuclease activity to proceed.

A schematic illustration of the present supramolecular regulation system is shown in Figure 4. Phosphate bilayers formed from the glutamate, and aspartate amphiphiles serve as regulatory cofactors, and HindIII is bound to these bilayers as a dormant species (a). An increase in temperature (i) or the addition of Ca^{2+} ions (ii) liberates active HindIII from the bilayer surface, thus initiating the hydrolysis of DNA (b). Liberation of HindIII is also achieved by the photochemical release of Ca^{2+} ions (iii). On the other hand, HindIII is irreversibly denatured upon binding to the liquid crystalline bilayers of **1** (i.e., 60 °C) and **3** (c) or to SDS micelles.

In conclusion, the use of suitably designed phosphate bilayers as regulatory cofactors converted the restriction enzyme into a supramolecular holoenzyme, which requires thermal energy or Ca^{2+} ions for activation. This concept is simple and could be widely applicable to control functions of cationic proteins.

Acknowledgment. We are grateful to Professors M. Takagi and T. Ihara for their experimental assistance in the gel electrophoresis.

Supporting Information Available: Experimental details (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

JA0030313

(11) Aqueous bilayer **1** possesses a gel-to-liquid crystal phase transition temperature (T_c) of 59 °C.

(12) HindIII requires Mg^{2+} for its enzymatic activity. It is known that activity of type II restriction enzymes is relaxed or inhibited by other divalent ions.[13] In this system, Ca^{2+} ions are employed at a much lower concentration (0.5 mM), and such an undesirable effect can be eliminated.

(13) Hsu, M.; Berg, P. *Biochem.* **1978**, *17*, 131.