Effects of Changed Lipid Composition on Responses of Liposomes to Various Odorants: Possible Mechanism of Odor Discrimination†

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ABSTRACT: In a previous paper [Nomura, T., & Kurihara, K. (1987) *Biochemistry* (preceding paper in this issue)], we showed that azolectin liposomes are depolarized by various odorants and there is a good correlation between the responses in the liposomes and the frog or porcine olfactory responses. In this study, we examined effects of changed lipid composition on responses of liposomes to various odorants. The membrane potential changes in response to odorants were monitored with the fluorescent dye 3,3′-dipropylthiocarbocyanine iodide [diS-C_3(5)]. Egg phosphatidylcholine (PC) liposomes showed depolarizing responses to nine odorants among ten odorants tested. The magnitudes of depolarization by alcohols were similar to those in azolectin liposomes, but those by other odorants were much less than those in azolectin liposomes. Addition of sphingomyelin (SM) to PC led to an increase in the magnitude of depolarization by most odorants. Addition of phosphatidylethanolamine (PE) to PC (PE/PC = 0.25) led to depolarizing responses to four odorants among six odorants tested, and a further increase in PE content (PE/PC = 0.54) led to depolarizing responses only to two odorants. Addition of SM to the lipids of this composition of PC and PE [SM/(PC + PE) = 0.22] led to depolarizing responses to four odorants again. Liposomes made of a mixture of SM, PE, and PC exhibited depolarizing responses to four odorants tested, and addition of cholesterol to the lipids [cholesterol/(PC + PE + SM) = 0.05 and 0.11] led to depolarizing responses only to two and one odorant, respectively. Thus, changes in lipid composition of liposomes led to great changes in specificity of the responses to odorants. On the basis of the results obtained, a possible mechanism of odor discrimination in the olfactory system is discussed. Lipid composition of a receptor membrane of each olfactory cell is postulated to be different from cell to cell. Hence, each olfactory cell has a different sensitivity to various odorants. Response profiles in many olfactory cells in response to various odorants are transformed into a firing pattern among various olfactory axons, and the quality of odor is recognized in the brain.

The olfactory receptors sense chemical substances in external environments. In external environments, there are multifarious substances including alien substances for animals, and the olfactory receptors perceive and discriminate such multifarious substances. Such function of olfactory receptors is quite different from that of internal receptors, which perceive only certain specified chemicals such as hormones or neurotransmitter. A number of binding proteins for certain specified odorants were isolated (Pelosi et al., 1982; Pevsner et al., 1985; Price, 1981), but it is still unknown whether or not the proteins are receptor proteins (Pevsner et al., 1986; Price, 1984).

In olfactory receptors systems, there are a number of unique phenomena that are not seen in other receptors systems. For example, the threshold concentrations of general odorants are mainly determined by the partition coefficient between water and oil (Davies, 1971; Koyama & Kurihara, 1972). Ther are stereoisomers whose odors are different from each other, but the difference in odors of stereoisomers is not so remarkable in general odorants, while the stimulatory effect of pheromones depends greatly on the chirality of the molecules (Kurihara et al., 1986).

In a previous study (Nomura & Kurihara, 1987), we showed that liposomes made of azolectin are depolarized by various odorants and that concentrations of various odorants to depolarize azolectin liposomes are closely correlated to those to induce the frog and porcine olfactory responses. These results suggest that olfactory responses are induced by adsorption of odorants on the hydrophobic region of the lipid layer of receptor cells. The question arises how odorants are discriminated in the nonreceptor system such as the lipid layer. In this study, we examine the dependence of the responses to various odorants on lipid composition of liposomes. The results obtined show that the specificity of the responses to odorants greatly varies with lipid composition. On the basis of these results, a possible mechanism of odor discrimination is discussed.

Materials and Methods

Materials. Lipids of procine brain were extracted from fresh porcine brains according to Brigh and Dyer (1959) and stored as a 10% (w/v) solution in chloroform/methanol (2:1 v/v) at −20 °C until use. Egg phosphatidylcholine (PC)[1] was purchased from Nihon Seika Co. (Osaka). Egg phosphatidylethanolamine (PE) was extracted from fresh chicken egg yolks according to Lea et al. (1955). Sphingomyelin (SM) from chicken egg yolk was purchased from Sigma Chemical Co. (St. Louis, MO). The purity of PC, PE, and SM was checked by thin-layer chromatography on silica gel plates. Cholesterol was purchased from Wako Pure Chemicals (Tokyo) and recrystallized twice from methanol. The 10% (w/v) solutions

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[1] Abbreviations: PC, egg phosphatidylcholine; PE, egg phosphatidylethanolamine; SM, egg sphingomyelin; diS-C_3(5), 3,3′-dipropylthiocarbocyanine iodide; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid.

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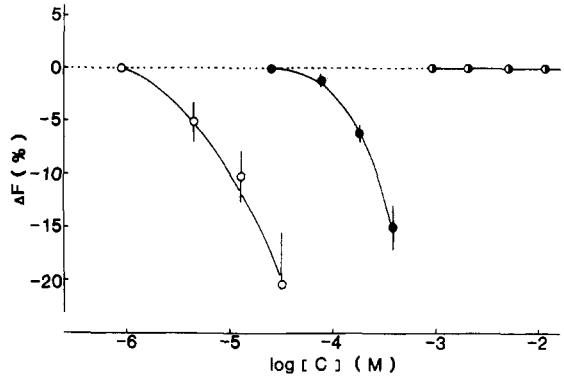

FIGURE 1: Responses of liposomes made of lipids extracted from porcine brain to *dl*-muscone (○), β-ionone (●), and *n*-amyl acetate (◑) as a function of odorant concentrations. Each point is the mean value of data obtained from at least four preparations.

of PC and PE in chloroform were stored at −20 °C until use. 3,3′-Dipropylthiocarbocyanine iodide [diS-C_3(5)] was purchased from Nihon Sensitive Dye Laboratories Co. (Okayama) and used as described previously (Nomura & Kurihara, 1987). Other chemicals used were of the best grade available.

Preparation of Liposomes. Liposomes were prepared essentially as described previously (Nomura & Kurihara, 1987). The dried lipid film in the flask was dispersed in 5 mM *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid (Hepes)–NaOH buffer (pH 7.3) containing 93 mM KCl and 7 mM NaCl. The lipid suspension was sonicated in a bath-type sonicator, followed by centrifugation of the sonicated suspension. The supernatant was used in this study. The phospholipid concentration was determined by measuring the phosphorus content (Allen, 1940).

Changes in the membrane potential of liposomes in response to odorants were monitored by measuring changes in the fluorescence intensity of diS-C_3(5). The fluorescence intensity changes were measured essentially as described previously (Nomura & Kurihara, 1987). The fluorescence intensity change ΔF is defined as

$$\Delta F = (F - F_0)/F_0 \times 100 \quad (1)$$

where F and F_0 represent the fluorescence intensity in the presence and the absence of odorants, respectively. The value of F_0 was unchanged for liposomes of different lipid composition used in this study. The final concentration of phospholipids was 50 μM. The temperature was maintained at 30 °C by circulating water through the cuvette holder of the photometer.

RESULTS

Figure 1 shows changes in the fluorescence intensity of diS-C_3(5) added to liposomes made of lipids extracted from porcine brain as a function of odorant concentrations. *n*-Amyl acetate does not affect the fluorescence intensity when its concentration is increased up to 40 mM. *dl*-Muscone and β-ionone decrease the fluorescence intensity, which indicates that these odorants hyperpolarize the liposomes. We refer to the membrane potential change where the potential at the inside of liposomes changes to a positive direction as depolarization and that to a negative direction as hyperpolarization. In olfactory systems, impulses of olfactory nerves are induced by depolarization of cells (Anderson & Ache, 1985; Suzuki, 1977; Trotier & MacLeod, 1983). Hence, it can be said that only the depolarizing response is an effective response. In the previous paper (Nomura & Kurihara, 1987), we showed that all the odorants tested depolarized azolectin liposomes. Thus, the responses of liposomes to odorants greatly depend on their

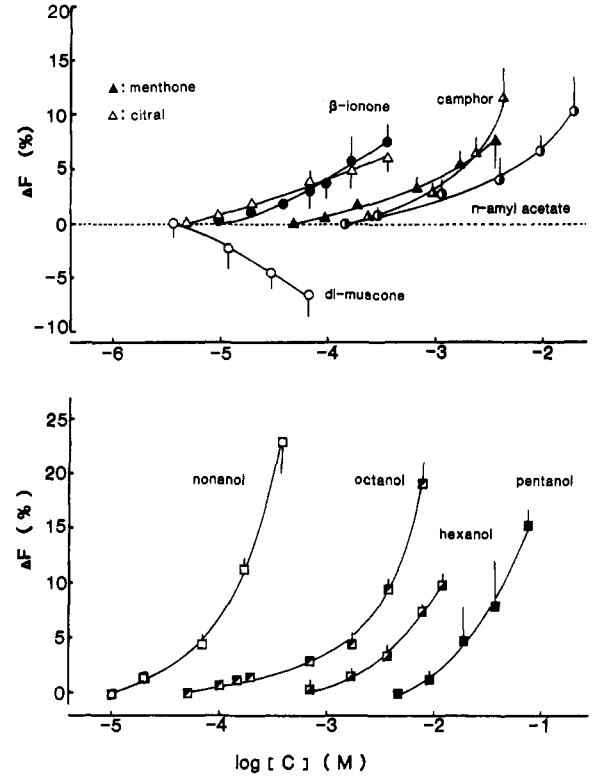

FIGURE 2: Responses of PC liposomes to various odorants as a function of odorant concentrations. Each point is the mean value of data obtained from at least four preparations.

lipid composition. In this study, we have examined systematically the dependence of the responses to various odorants on lipid composition of liposomes.

In a previous paper, Koyama et al. (1971) analyzed the chemical composition of the plasma membranes from bovine olfactory epithelium and found that the content of phospholipids is characteristically high and the phospholipids consist mainly of PC, PE, and SM. In the present study, we used the liposomes made of these phospholipids. Figure 2 shows the responses of PC liposomes to various odorants as a function of odorant concentrations. All the odorants except *dl*-muscone depolarize the liposomes. The magnitudes of depolarization of PC liposomes by β-ionone, citral, menthone, and *n*-amyl acetate are much less than those of azolectin liposomes, while those by alcohols are similar to those in azolectin liposomes. *dl*-Muscone led to a large depolarization in azolectin liposomes but induces hyperpolarizing response in PC liposomes.

Figure 3 shows effects of addition of SM to PC on responses of liposomes to nonanol, *n*-amyl acetate, β-ionone, citral, menthone, and *dl*-muscone as a function of odorant concentrations. The magnitude of depolarization by nonanol greatly increases with an increase in SM content. That by *n*-amyl acetate increases until SM/PC = 0.2 and decreases with a further increase in SM content. The magnitudes of depolarization by β-ionone, citral, and menthone increase with an increase in SM content with a lesser extent than that by nonanol and *n*-amyl acetate. *dl*-Muscone induces hyperpolarizing responses in liposomes containing SM as well as liposomes made of PC alone.

Figure 4 shows the effects of addition of PE to PC on responses of liposomes, nonanol, *n*-amyl acetate, β-ionone, menthone, citral, and *dl*-menthone as a function of odorant concentrations. *dl*-Muscone and citral induce large hyperpolarizing responses in liposomes containing PE. The magnitudes of depolarization by other odorants decrease with an increase of PE content above 0.25 (PE/PC ratio). The inset

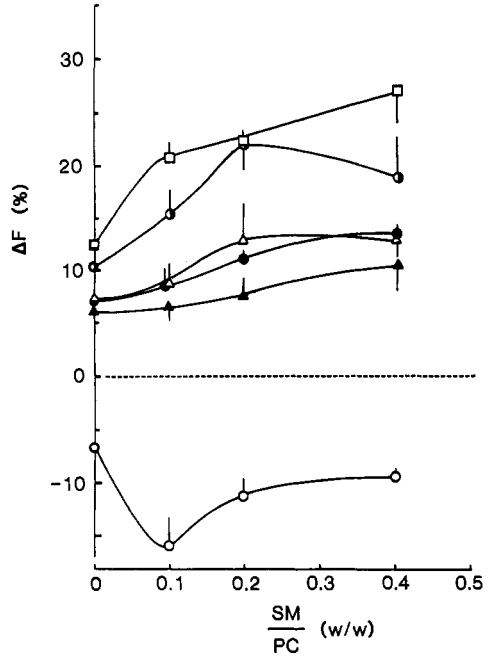

FIGURE 3: Effects of addition of SM to PC on responses of liposomes to nonanol (□), *n*-amyl acetate (◑), citral (△), β-ionone (●), menthone (▲), and *dl*-muscone (○) as a function of odorant concentrations. Each point is the mean value of data obtained from at least four preparations.

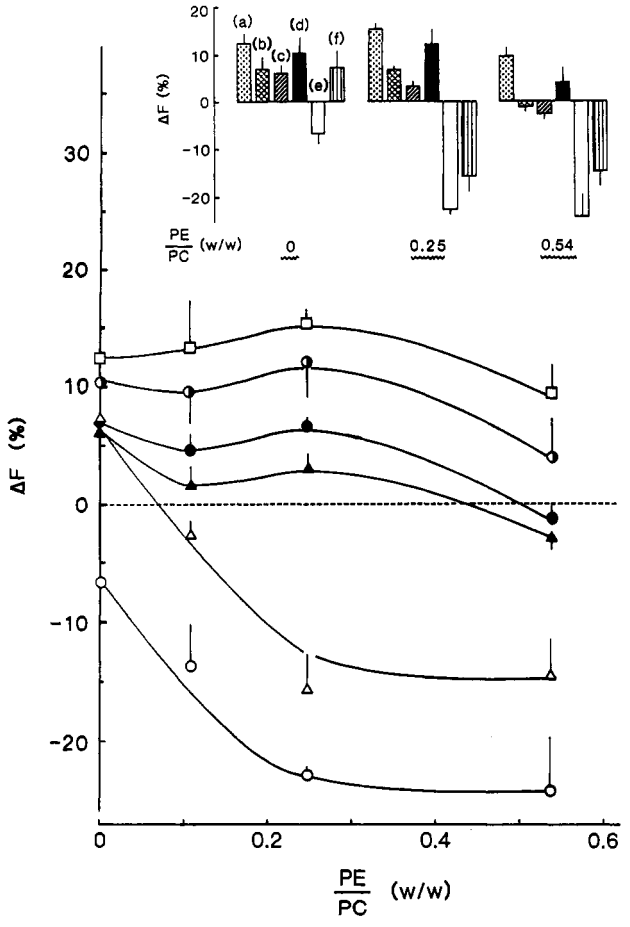

FIGURE 4: Effects of addition of PE to PC on responses of liposomes to nonanol (□), *n*-amyl acetate (◑), β-ionone (●), menthone (▲), citral (△), and *dl*-muscone (○) as a function of odorant concentrations. Each point is the mean value of data obtained from at least four preparations. The inset shows changes in response profiles of liposomes for nonanol (a), β-ionone (b), menthone (c), *n*-amyl acetate (d), *dl*-muscone (e), and citral (f).

shows the response profiles of PC liposomes containing PE in different contents. PC liposomes containing no PE exhibit

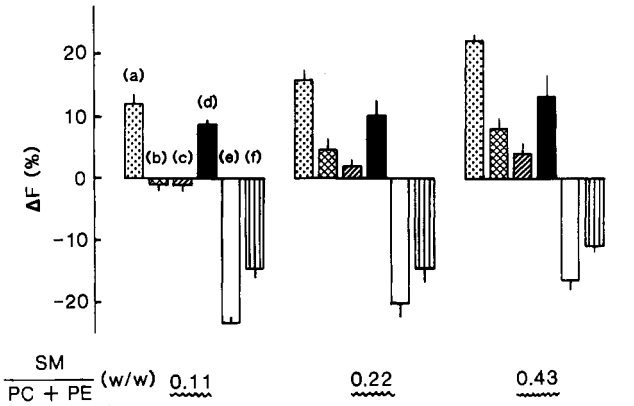

FIGURE 5: Effects of addition of SM to a mixture of PE and PC (PE/PC = 0.54, w/w) on response profiles of liposomes for nonanol (a), β-ionone (b), menthone (c), *n*-amyl acetate (d), *dl*-muscone (e), and citral (f). Each value is the mean value of data obtained from at least four preparations.

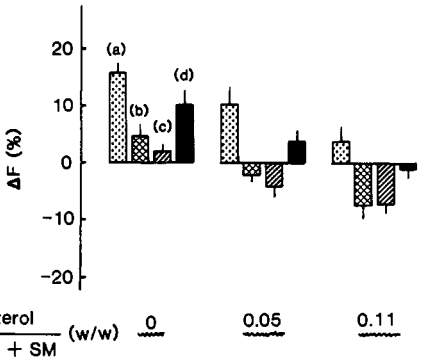

FIGURE 6: Effects of addition of cholesterol to a mixture of SM, PE, and PC [SM/(PC + PE) = 0.22, w/w] on response profiles of liposomes for nonanol (a), β-ionone (b), menthone (c), and *n*-amyl acetate (d). Each value is the mean value of data obtained from at least four preparations.

depolarizing responses to five odorants. PC liposomes containing PE (PE/PC = 0.25 and 0.54) exhibit depolarizing responses only to four and two odorants, respectively. Thus, addition of PE to PC leads to great changes in response specificity of liposomes to odorants.

Figure 5 shows effects of addition of SM to a mixture of PE and PC (PE/PC = 0.54) on the response profiles of liposomes to various odorants. While liposomes containing no SM exhibit depolarizing response only to two odorants (nonanol and *n*-amyl acetate) as shown in the inset of Figure 3, liposomes containing SM [SM/(PC + PE) = 0.22] exhibit depolarizing responses to four odorants (nonanol, β-ionone, menthone, and *n*-amyl acetate). A further increase in SM content leads to larger depolarizing responses to these odorants.

Figure 6 shows effects of addition of cholesterol to a mixture of SM, PE, and PC [SM/(PC + PE) = 0.22] on the response profiles of liposomes to various odorants. While liposomes containing no cholesterol exhibit depolarizing responses to four odorants (nonanol, β-ionone, menthone, and *n*-amyl acetate), addition of cholesterol [cholesterol/(PC + PE + SM) = 0.05] leads to hyperpolarizing responses to β-ionone and menthone. A further increase in cholesterol content [cholesterol/(PC + PE + SM) = 0.11] leads to a hyperpolarizing response to *n*-amyl acetate. That is, the liposomes exhibit a depolarizing response only to nonanol.

Discussion

In the previous paper (Nomura & Kurihara, 1987), we showed that azolectin liposomes respond to all the odorants examined. The present results demonstrate that changes in

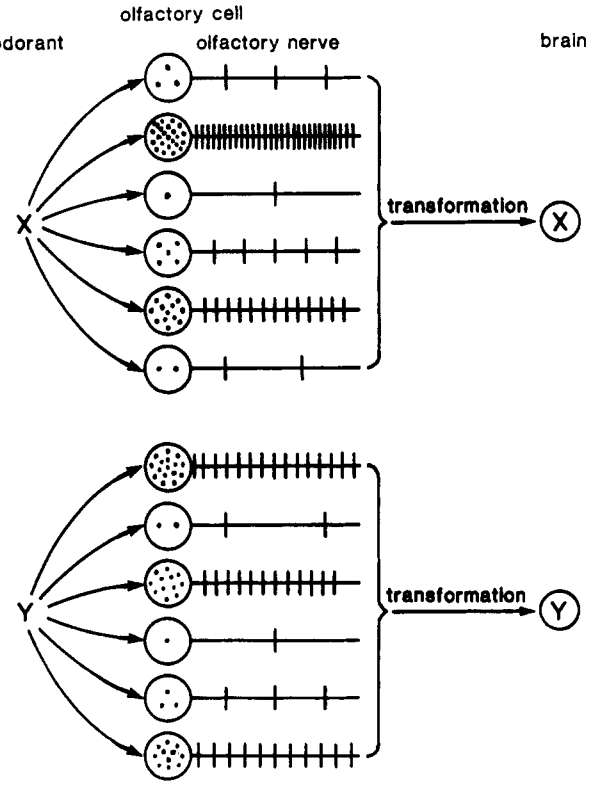

FIGURE 7: Schematic diagram illustrating mechanism of odor discrimination. Lipid composition of a receptor membrane of each olfactory cell is postulated to be different from cell to cell. The magnitude of depolarization, which is expressed as the number of dots in the figure, in many olfactory cells in response to an odorant "X" or "Y" is different from cell to cell. The magnitude of depolarization is converted to the number of impulses. The response profiles at the cell level for "X" and "Y" are transformed into the firing pattern among various olfactory axons, and the qualities of the odors "X" and "Y" are recognized in the brain.

lipid composition of liposomes greatly affect the magnitude and the direction (depolarization or hyperpolarization) of membrane potential changes in response to odorants. We can prepare liposomes that respond only to certain species of odorants using lipids of proper composition. Among various odorants, *dl*-muscone induced only hyperpolarizing responses in all the liposomes tested in this study, while it induced a large depolarizing response in azolectin liposomes. The hyperpolarization by *dl*-muscone was also observed electrically with the planar lipid bilayers made of a mixture of SM and PC or PE and PC (data not shown). The fact that *dl*-muscone hyperpolarizes most liposomes may be related to the following fact: there are animals that do not respond to *dl*-muscone (e.g., *dl*-muscone induced no response in the frog olfactory system; K. Kurihara et al., unpublished results).

As described in the previous paper (Nomura & Kurihara, 1987), the membrane potential changes in response to odorants are brought about by changes in the phase boundary potential. That is, the adsorption of odorants on the lipid membrane will induce a conformational change of the membrane, which alters the orientation of the fixed charges and dipoles within the membrane and in turn leads to changes in the phase boundary potential. Direction of the membrane potential changes (depolarization or hyperpolarization) in response to odorants depends on the direction of the changes in orientation of the fixed charges and dipoles. The present results demonstrate that the direction of the membrane potential changes of liposomes in response to an odorant depends on their lipid composition. This suggests that the direction of changes in orientation of the fixed charges and dipoles induced by adsorption of an odorant depends on the lipid composition of membranes.

Gesteland et al. (1982) examined sensitivity of a single olfactory cell of the rat to various odorants during maturation. The immature olfactory cell in the rat fetus responds nonselectively to nearly all of 14 odorants tested, and the mature cell responds only to about half of the odorants. That is, the immature olfactory cell has a large variety of receptor sites, some of which are lost when it matures. It is well-known that a single mature cell responds to many species of odorants, but the specificity of each cell to odorants is different from cell to cell (Sicard, 1985; Sicard & Holley, 1984). Such characteristics of each olfactory cell are a base for odor discrimination as discussed later. Azolectin liposomes mimic the function of the immature olfactory cell since the liposomes respond to many odorants. Liposomes, which do not exhibit depolarizing response to certain odorants, prepared from lipids of proper composition mimic the function of the mature olfactory cell.

On the basis of the present results, we propose the following mechanism of odor discrimination, which is illustrated in Figure 7. Lipid composition of a receptor membrane of each olfactory cell is postulated to be different from cell to cell. Hence, each olfactory cell has different sensitivity to various odorants. The magnitude of depolarization in many olfactory cells in response to an odorant is different from cell to cell. The relative magnitudes of the depolarization in many cells in response to an odorant are characteristics of the species of the odorant. The response profile at the cell level is transformed into a firing pattern among various olfactory axons, and the quality of the odor is recognized in the brain. In the above mechanism, only lipids are considered to be receptor sites for odorants, but the hydrophobic region of proteins may also be receptors sites for certain odorants although the proteins do not always imply those that bind only to specific odorant. Variation in combinations of lipids and proteins provides many different adsorption sites for odorants.

The present results support the mechanism of nonreceptor-mediated odor discrimination for general odorants. However, the above mechanism cannot be applicable to the reception of pheromones since their stimulatory effect depends greatly on the chirality of the molecules and a pheromone stimulates only its target animal. Probably specific receptor proteins are involved in the reception of pheromones.

Registry No. *dl*-Muscone, 956-82-1; β-ionone, 79-77-6; menthone, 89-80-5; citral, 5392-40-5; camphor, 76-22-2; *n*-amyl acetate, 628-63-7; nonanol, 143-08-8; octanol, 111-87-5; hexanol, 111-27-3; pentanol, 71-41-0; cholesterol, 57-88-5.

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Time-Resolved Tryptophan Fluorescence Anisotropy Investigation of Bacteriophage M13 Coat Protein in Micelles and Mixed Bilayers†

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ABSTRACT: Coat protein of bacteriophage M13 is examined in micelles and vesicles by time-resolved tryptophan fluorescence and anisotropy decay measurements and circular dichroism experiments. Circular dichroism indicates that the coat protein has α-helix (60%) and β-structure (28%) in 700 mM sodium dodecyl sulfate micelles and predominantly β-structure (94%) in mixed dimyristoylphosphatidylcholine/dimyristoylphosphatidic acid (80/20 w/w) small unilamellar vesicles. The fluorescence decay at 344 nm of the single tryptophan in the coat protein after excitation at 295 or 300 nm is a triple exponential. In the micelles the anisotropy decay is a double exponential. A short, temperature-independent correlation time of 0.5 ± 0.2 ns reflects a rapid depolarization process within the coat protein. The overall rotation of the coat protein–detergent complex is observed in the decay as a longer correlation time of 9.8 ± 0.5 ns (at 20 °C) and has a temperature dependence that satisfies the Stokes–Einstein relation. In vesicles at all lipid to protein molar ratios in the range from 20 to 410, the calculated order parameter is constant with a value of 0.7 ± 0.1 from 10 to 40 °C, although the lipids undergo the gel to liquid-crystalline phase transition. The longer correlation time decreases gradually on increasing temperature. This effect probably arises from an increasing segmental mobility within the coat protein. The results are consistent with a model in which the coat protein has a β-structure and the tryptophan indole rings do not experience the motion of the lipids in the bilayer because of protein–protein aggregation.

The interaction of membrane proteins with lipids has received considerable attention in the last decade. The interest in understanding this interaction arises from the notion that lipid composition and dynamics in membranes affect the properties of its proteins. A variety of biophysical techniques has already been applied to membrane proteins reconstituted in model membranes [for a survey, see Watts and De Pont (1985)]. Model membranes can easily be prepared and are free of the complexity of the natural membrane. In particular, time-resolved fluorescence anisotropy measurements [for a review, see Cundall and Dale (1983) and Beechem and Brand (1985)] and nuclear magnetic resonance (NMR)[1] provide complementary information about motion and order of lipids in bilayer systems [for a comparison, see Wolber and Hudson (1982) and Devaux and Seigneuret (1985)].

The objective of our research is to study the infection mechanism of nonenveloped viruses, like bacteriophage M13 (Hemminga, 1987) and plant viruses (Hemminga et al., 1985), at a molecular level. A suitable system to study the interaction of viral coat proteins with membranes is the M13–*Escherichia coli* system.

The major (gene 8 product) M13 coat protein is present in the long rodlike virus particle in numerous copies and functions as protection for its single-strand DNA. The virus enters *E. coli* by leaving the coat proteins in the cytoplasmic membrane (Marvin & Wachtel, 1975). After infection and DNA duplication, the newly synthesized procoats, a precursor form of the coat protein with a typical bacterial amino-terminal leader

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[1] Abbreviations: NMR, nuclear magnetic resonance; CD, circular dichroism; DMPC, 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine; DMPA, 1,2-dimyristoyl-*sn*-glycero-3-phosphatidic acid; SUVs, small unilamellar vesicles; SDS, sodium dodecyl sulfate; L/P ratio, phospholipid to coat protein molar ratio; cmc, critical micelle concentration; OD_{280nm}, optical density at 280 nm; S, order parameter; $r(0)$, initial anisotropy; $r(\infty)$, residual anisotropy; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; EDTA, ethylenediaminetetraacetic acid.

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