purpose. Very high-quality x-ray observations with synchrotron radiation and Raman observations free from the effect of the surrounding diamond anvils are the important next step.

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Monolayer Crystallization of Flagellar L-P Rings by Sequential Addition and Depletion of Lipid

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The L-P ring complex is thought to be a molecular bushing that supports flagellar motor rotation at about 10,000 revolutions per minute with presumably very little friction. Structural studies of this complex have been limited because only very small amount of samples are available. Therefore devising an efficient method of crystallization was essential. The addition of a phospholipid and its subsequent slow depletion by phospholipase A_2 have been used to successfully grow well-ordered monolayer crystals that extend up to about 10 micrometers. The interaction of the L-P ring complex with lipid membranes was also visualized during this process.

BACTERIA SWIM BY ROTATING ONE OR more flagellar filaments driven by rotary motors at the base of each flagellum. The hook–basal body (HBB) complex is a part of the flagellar motor that can be easily detached from the cell membrane and consists of four ring structures and a rod connected to a hook (Fig. 1) (*1*). The L-P ring complex is composed of the outer two of the four rings and is thought to be a bushing that supports the flagellar motor, bearing its rapid rotation in both the clockwise and counterclockwise direction. Its structure, particularly at its inner surface, is interesting from a microtribiological aspect. The outermost ring, which sits in the outer membrane, is designated the L ring and is composed of 27-kD protein subunits; the other ring, which is thought to be associated with the peptidoglycan layer, is called the P ring and consists of 38-kD protein subunits (*2*). Although the overall shape of the L-P ring complex has been revealed by cylindrically averaged image reconstruction of the basal body from electron micrographs with the use of single particle averaging (*3*), more detailed structure, including organization of the subunits in the ring complex, remains to be elucidated. Three-dimensional image reconstruction from electron micrographs is especially useful for analysis of such intrinsic membrane proteins because of their potential tendency to form monolayer crystals. However, this method has so far been applied only to proteins available in large amounts, which are necessary for screening conditions to obtain well-ordered crystals large enough for analysis. A large-scale preparation of the L-P ring complexes has been hindered by the fact that each bacterium has only about ten of them. Therefore, it was essential to devise an efficient method of crystallization.

In order to maximize the limited amount of the sample available, the procedure for the purification of the L-P ring complex was improved (*4*) from the one developed by Aizawa *et al.* (*2*) in two respects. First, a high concentration of urea was used instead of acid degradation for more reproducible partial degradation of HBB complex. Second, contaminants such as outer membrane components and DNA fragments were removed by enzymatic digestion. In Fig. 2A, axial views are shown of the purified L-P ring complex, and Fig. 2B shows lateral views, which have been called "staples" (*2*). The open side of this staple has been assigned to the L ring and the closed side to the P ring (*3, 5*).

After the removal of urea by centrifugation and resuspension, it was impossible to keep the L-P ring complexes dispersed even in the presence of 2% Triton X-100. Various kinds and concentrations of other detergents, ionic and non-ionic, gave no improvement. However, when a small amount of a phospholipid, dimyristoylphosphatidylcholine (DMPC), was added to the L-P ring aggregates, there appeared small patches of ring arrays apparently embedded in phospholipid membrane sheets or vesicles (Fig. 2C). The rings were packed closely in a well-ordered hexagonal lattice. Besides these patches, there were also single L-P rings with flaps or vesicles attached (Fig. 2D). Comparison between Fig. 2B and Fig. 2D shows that these extra structures, which are presumably lipid membranes, are attached to the L ring portion of the complex. This observation shows directly that the L ring has a high affinity for phospholipid and that the L-P ring complex can be effectively incorporated and concentrated in membranes through this property.

Overnight incubation at 30° to 37°C following the addition of the phospholipid favored the growth of well-ordered arrays of submicrometer size. Longer incubation, however, induced the formation of random aggregates of L-P ring complexes rather than larger arrays. In order to find better crystallization conditions, the effect of the ratio of Triton X-100 and DMPC on array formation was examined (Fig. 3, A to C). When the detergent:phospholipid ratio was high, the resulting arrays were planar, well ordered, and tightly packed, but also contained large cracks that separated ordered areas and limiting their size (Fig. 3A). When the ratio was low, there were large continuous vesicular membranes in which L-P ring complexes were embedded and arrayed loosely (Fig. 3C). Since no ratio we tried was entirely satisfactory, we used a strategy of first incubating the above mixture at a high DMPC concentration to facilitate incorporation of the L-P ring complexes into large and continuous proteomembranes and then slowly depleting the DMPC to pack the L-P rings into well-ordered arrays. We hypothesized that

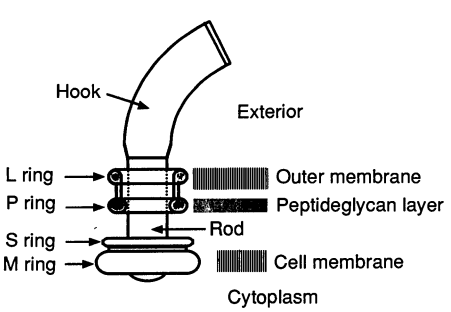

Fig. 1. Schematic diagram of the HBB complex of *Salmonella typhimurium*, showing the configurations and the locations of four rings: L, P, S, and M. The cross section of the L-P ring complex is hatched.

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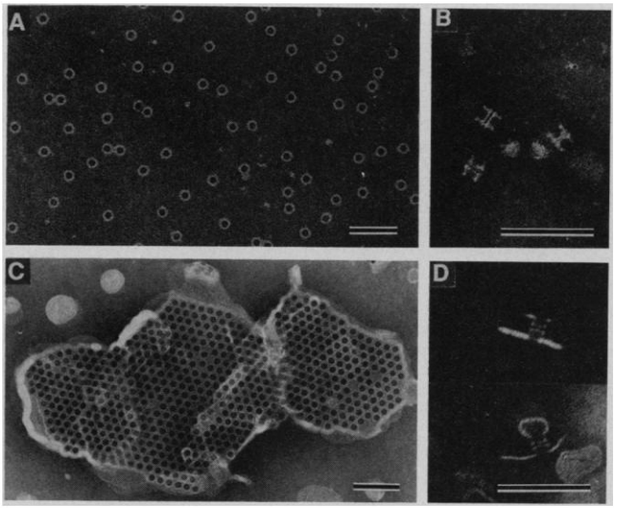

Fig. 2. Electron micrographs of the L-P ring complex negatively stained with 2% phosphotungstic acid (pH 7.0). (**A**) Axial and (**B**) lateral views of purified L-P ring complexes in the presence of 7.5 M urea; bipolar dimers of the L-P ring complex are shown in lateral views. (**C**) Regular array of the L-P ring complexes induced by an addition of DMPC to a final concentration of 0.5%. (**D**) The L-P ring complexes with membranous flaps or vesicles attached to the L ring portion, which coexist with the regular array shown in (C). Scale bars: 0.1 μm.

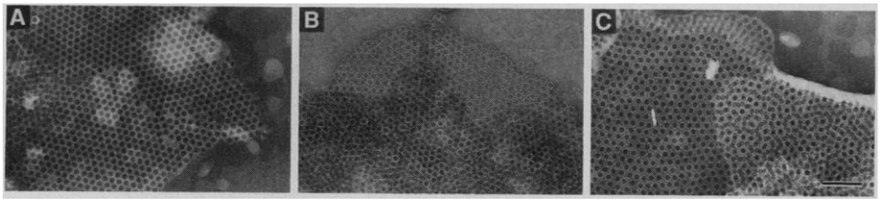

Fig. 3. Effects of the ratio of Triton X-100 (T) and DMPC (D) on the formation of L-P ring complex arrays. The detergent and phospholipid were added to the L-P ring solution to final concentrations as follows: (**A**) 2% (w/v) T and 0.5% (w/v) D; (**B**) 0.5% T and 0.5% D; and (**C**) 0.5% T and 1% D. The solution was then incubated at 30°C for 1 hour. Specimens for electron microscopy were prepared by the mercury-surface spread method developed by Yoshimura *et al.* (*11*) as described in (*12*). Scale bar: 0.1 μm.

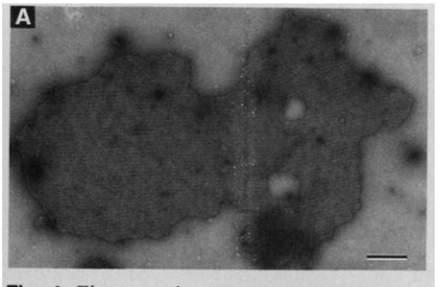

Fig. 4. Electron micrographs of a large monolayer crystal of the L-P ring complex at (**A**) low and at (**B**) high magnification. The inset in (B) is a computed Fourier transform. Scale bar: 1 μm in (A) and 0.1 μm in (B). Monolayer crystallization was performed as described in (*13*).

the second process should be slow enough to minimize perturbations to the preformed arrays. Mild phospholipase A_2 digestion (*6*) was used rather than dialysis because the amount of the sample was very small.

In Fig. 4, an example is shown of the monolayer crystal obtained by this method. The L-P rings are arrayed in a well-ordered hexagonal lattice over the whole patch of several micrometers without a distinct lattice dislocation. Although single or multiple vacancies are found, they do not disturb the order of the array. The inset of Fig. 4B is a computed Fourier transform, in which the diffraction spots are visible up to the sixth order (the ninth order at a signal-to-noise ratio of 2), showing the degree of crystallinity. This result suggests that the rotational disorder is relatively small if it exists. The inner and outer surfaces of the L-P ring complex are very smooth in appearance and their diameters are about 13 and 20 nm, respectively. Most of the L-P ring complexes in the array show an angular periodicity but it does not continue clearly around the entire circumference. We estimate the rotational symmetry of the L-P ring from such features to be around 20, which is close to the number of subunits in the L ring (28.3 ± 5.0) and P ring (24.1 ± 4.3) estimated biochemically (*7*).

The previous report on the L-P ring structure by Stallmeyer *et al.* (*3*) was based on electron micrographs of a dispersed sample. They have estimated the inner and outer diameters to be 10 and 24 nm, respectively. This thicker appearance than that in our observation may have been caused by flattening of individual particles upon drying. The outer boundary was particularly obscure and variable, suggesting some residual material adhering to the L ring as well. Their tentative estimation of the rotational symmetry was 12, only about one half of the biochemically estimated stoichiometry. Thus the fine structure of the L-P ring seems to be better preserved in the crystalline array.

The L-P ring complex has been demonstrated to have remarkable stability against chemical treatments. This feature is shared by bacterial porins, which are major proteins of the outer membrane (*8*). From secondary structure predictions based on amino acid sequences predicted from DNA, both the L and P ring proteins have high β-structure contents (*9*), which is also characteristic to porins (*10*), although no consensus sequences have been found between the L-P ring proteins and the porins.

The method of monolayer crystallization described here, involving incorporation of a protein complex into a lipid membrane and slow condensation in the membrane by enzymatic depletion of the lipid, should be useful in studies of other membrane proteins, particularly to those with low solubility in detergent solutions or those that are available only in limited quantities.

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12. After the addition of sucrose to a final concentration of 10%, 5 μl of the sample solution was spread on a clean liquid mercury surface kept at 30°C. The spread sample was transferred to a carbon-coated grid by pressing the grid against the mercury surface. For these samples, this method was effective for spreading the lipid membrane and resulted in planar sheets, but it did not facilitate the growth of the arrays in the membrane.
13. The L-P ring solution (10 μl) was mixed with an equal volume of 4% (w/v) DMPC dissolved in TEC solution (*4*) in a 0.5-ml Eppendorf tube. The tube was tightly sealed with Parafilm and the solution was incubated overnight at 37°C in a humid chamber to minimize the evaporation of the liquid. Then 2 μl of a solution containing phospholipase A_2 (50 U/ml) and 10 mM $CaCl_2$ was added and the resulting solution was incubated overnight at room temperature. Monolayer crystals were adsorbed onto a carbon-coated grid for electron microscopy.
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Dopamine Activation of an Orphan of the Steroid Receptor Superfamily

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The chicken ovalbumin upstream promoter transcription factor (COUP-TF) is a member of the steroid receptor superfamily and participates in the regulation of several genes. While a number of functions have been ascribed to COUP-TF, no ligand or activator molecule has been identified, and thus it is classified as one of a group of orphan receptors. Activation of COUP-TF by physiological concentrations of the neurotransmitter dopamine was observed in transient transfection assays. Treatment of transfected cells with the dopamine receptor agonist α-ergocryptine also activated COUP-dependent expression of a reporter gene. COUP-TF that contained a deletion in the COOH-terminal domain was not activated by these compounds. These observations suggest that dopamine may be a physiological activator of COUP-TF.

THE COUP (CHICKEN OVALBUMIN upstream promoter) transcription factor is a member of the steroid-thyroid hormone receptor superfamily (*1*). COUP-TF participates in the regulation of the chicken ovalbumin gene (*2*), the rat insulin II gene (*3*), the proopiomelanocortin (POMC) gene (*4*) and the apo–very low density lipoprotein II (apo VLDLII) gene (*5*). A *Drosophila* protein related to COUP-TF termed Seven Up (*6*) regulates retinal cell differentiation and shares 93% sequence identity with human COUP-TF in the COOH-terminal or putative ligand binding domain. Because an activator ligand for COUP-TF has not been identified, it is classified as one of a list of orphan receptors in search of either a ligand or an activator. Dopamine is the predominant catecholamine in the retina (*7*), yet the function of dopamine in this tissue is unclear. Dopamine action is mediated by its interaction with D1 and D2 G protein–coupled receptors that stimulate and inhibit adenylyl cyclase, respectively (*8*). Here we report the activation of human COUP-TF by dopamine and a dopamine receptor agonist α-ergocryptine.

To search for potential COUP-TF ligands we constructed a chimeric COUP receptor that contained the NH_2- and COOH-terminal regions of human COUP-TF and the DNA binding domain of the chicken progesterone receptor (cPR) (Fig. 1A). This strategy was implemented to prevent competition from endogenous COUP-TF in transfected cells. Transient transfection assays were conducted in a PR-negative monkey kidney cell line (CV1) transfected with the chimeric COUP construct and a reporter plasmid (PRETKCAT) (*9*); the reporter contained two copies of a progesterone response element (PRE) located upstream of the herpes simplex virus thymidine kinase promoter linked to a chloramphenicol acetyl transferase (CAT) gene. Candidate compounds were tested for their ability to activate COUP-dependent CAT gene expression.

Because of the possible function of COUP-TF in human retinal development, dopamine and related compounds were tested. Concentrations of dopamine as low as 3 μM stimulated CAT gene activity five- to tenfold (Fig. 2A). Dopamine (100 μM) did not stimulate CAT activity in cells transfected with the parent expression vector P91023 (B), which lacked COUP-TF coding sequences. This demonstrates that dopamine stimulation of transcription required COUP-TF and did not result from a general increase in transcriptional activity. The dopamine receptor agonist α-ergocryptine also stimulated COUP-dependent CAT gene expression at concentrations as low as 300 nM. Concentrations of α-ergocryptine greater than 8.5 μM inhibited the response. More than 150 other candidate compounds did not activate gene expression in this assay system (*10, 11*). Of the other catecholamines tested, only L-dopa elicited a weak transcriptional response (*10, 12*). Activation of COUP-TF therefore appears to be relatively specific for dopamine.

Another chimeric COUP-TF (COUPΔ, Fig. 1B), which contained a deletion within the COOH-terminal domain, was tested in this assay system. Translation in vitro of RNA derived from this mutant COUP cDNA produced a stable protein of the correct size, which did not bind to a PRE oligonucleotide in gel retardation assays (*10*). This mutant protein is not capable of transcriptional activation. The inability of dopamine and α-ergocryptine to stimulate CAT gene expression in cells transfected with this mutant COUP-TF (Fig. 2B) demonstrates that an intact COUP-TF capable of transactivation is essential for the dopamine response to be observed. This result argues against the hypothesis that the effect on transcription is brought about by dopamine-mediated stimulation of transcription factors unrelated to COUP-TF.

We performed immunoblot analysis to

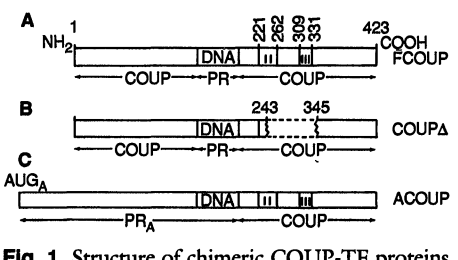

Fig. 1. Structure of chimeric COUP-TF proteins (*22*). (**A**) The chimeric COUP-TF (FCOUP) with the NH_2- and COOH-terminal domains of human COUP-TF fused to the DNA binding domain of cPR. The boundaries of domains II and III, which are conserved among the steroid receptor family (*1*), are indicated by amino acid number. (**B**) The COOH-terminal deletion mutant of (A) (COUPΔ). (**C**) The PR_A-COUP chimera (ACOUP) with the NH_2-terminal and DNA binding domains of cPR_A fused to the COOH-terminal domain of COUP-TF.

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