JOURNAL OF BACTERIOLOGY, May 1975, p. 347-351
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Vol. 122, No. 2
Printed in U.S.A.

Insertion of a Minor Protein into the Outer Membrane of *Escherichia coli* During Inhibition of Lipid Synthesis

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Received for publication 20 January 1975

The antibiotic cerulenin, a specific inhibitor of fatty acid synthetase systems, was used to demonstrate that a minor protein component of the outer membrane of *Escherichia coli*, which serves as the receptor for the phage lambda, can be synthesized and inserted into the outer membrane during inhibition of lipid synthesis.

The envelope of *Escherichia coli* is composed of two membranes, the inner-most cytoplasmic membrane and the outer membrane which is exterior to the rigid peptidoglycan layer. Both membranes contain protein and phospholipid which are synthesized either in the soluble cytoplasm or at the cytoplasmic membrane itself. In addition the outer membrane contains lipopolysaccharide. Investigations of the assembly of the cytoplasmic membrane in glycerol auxotrophs have revealed that some proteins can be synthesized and incorporated into this membrane under conditions which do not permit the synthesis of lipids (5, 6, 7, 11). Assembly of the outer membrane requires a process which is not necessary for the assembly of the cytoplasmic membrane. Thus, the protein, lipid, and lipopolysaccharide components must be translocated from the site of their synthesis inside the cell to the final location exterior to the cytoplasmic membrane and the peptidoglycan. The mechanism of this translocation is unknown. In this paper I have asked if the insertion of newly synthesized protein into the outer membrane requires simultaneous lipid synthesis. The present data indicate that during the inhibition of lipid synthesis a minor protein of the outer membrane of *E. coli*, the receptor for phage lambda, is properly inserted as judged by its ability to function as a phage receptor. In these experiments, lipid synthesis was inhibited by the use of an antibiotic, cerulenin, which specifically blocks the activity of β-keto acyl thioester synthetase (3, 4).

MATERIALS AND METHODS

Chemicals. The antibiotic cerulenin was a generous gift of S. Ōmura. Cyclic adenosine 3',5'-monophosphate was purchased from Sigma Chemical Co. Sodium cholate was purchased from Schuchardt, Munich, Germany. Triton X-100 was obtained from Rohm and Haas. [^{32}P]phosphate was purchased from the Radiochemical Centre, Amersham, England. All other chemicals were the highest quality commercially available.

Bacterial strain. *E. coli* K-12 derivative HfrG6 (His$^-$) was used in all experiments. Cells were grown with shaking at 37 C in the low phosphate medium, M56LP (2): 10^{-1} M tris(hydroxymethyl)aminomethane (Tris)-hydrochloride, 10^{-2} M KCl, 1.5×10^{-2} M $(NH_4)_2SO_4$, 10^{-2} M $MgCl_2$, 3×10^{-4} M KH_2PO_4, pH 7.4, supplemented with histidine (0.1 mg/ml) and a carbon source as given in the experimental procedure.

Induction of receptor in presence and absence of cerulenin and labeling of phospholipids. The synthesis of receptor is repressed when cells are grown on glucose (catabolite repression) and this effect is reversed when cells are transferred to a medium containing cyclic adenosine 3',5'-monophosphate. In addition, the presence of maltose induces receptor synthesis. As described below, cells are transferred from repression conditions to conditions of derepression and induction, but for the sake of simplicity I shall refer to the transfer as induction.

Two 10-ml cultures in M56LP with glucose as the carbon source (2×10^{-2} M) were grown in parallel. When the cells reached an optical density of 0.6 (Beckman photometer: optical density of 1.0 represents approximately 5×10^8 cells/ml), they were rapidly centrifuged and transferred to 10 ml of fresh media, either (i) induction medium (M56LP containing 2×10^{-2} M maltose and 4×10^{-3} M cyclic adenosine 3',5'-monophosphate or (ii) induction-inhibition medium (M56LP containing maltose and cyclic adenosine 3',5'-monophosphate as above with the addition of cerulenin, 100 μg/ml final concentration). As a control, cells were transferred to M56LP containing glucose at the same concentration as the pretransfer medium. At the time of transfer, 50 μCi of [^{32}P]phosphate carrier free was added to each culture to follow phospholipid synthesis. At time intervals after transfer, samples were taken and analyzed for receptor activity and phospholipid synthesis as follows.

Phospholipid synthesis. Synthesis of phospholipid was followed as described by Ames (1). At 0, 5,

10, 15, and 20 min after transfer, 0.8-ml samples of the cultures were taken and 3 ml of methanol:chloroform (2:1) was added. After 10 min on ice, the extraction mixture was diluted with 1 ml of $CHCl_3$ followed by 1 ml of water. After all samples were taken, they were centrifuged and the $CHCl_3$ phase was removed by putting a pipette to the bottom of the tube. To count, 0.4 ml of the $CHCl_3$ phase evaporated to dryness in a scintillation vial and 10 ml of scintillation fluid (5 g of 2,5-diphenyloxazole and 0.1 g of *p*-bis[2-(4-methyl-5-phenyloxazolyl)]-benzene in 1 liter of toluene) was added. All points were done in duplicate.

Extraction of receptor. At 0, 10, and 20 min after transfer, 1.5-ml samples were taken and put on ice. Cells were pelleted by centrifugation, 3,000 × *g* for 20 min, and washed once with 10^{-2} M Tris buffer (pH 7.6), and the receptor was extracted as previously described (8); the washed cells were resuspended in 0.2 ml of 1% cholate, 2×10^{-3} M ethylenediaminetetraacetic acid, 10^{-2} M Tris buffer (pH 7.6). After a 15-min incubation at 37 C, the suspension was centrifuged at 12,000 × *g* for 40 min. The supernatant fraction contains the solubilized receptor. Each receptor fraction was diluted 100-fold with 10^{-2} M Tris buffer (pH 7.6) to assay.

Assay of the receptor activity. Presence of receptor was assayed as an inactivation of the phage lambda as previously described (8). One milliliter of a suspension containing between 3×10^3 and 6×10^3 plaque-forming units of phage λvh, a host range mutant of the virulent phage λv, in 10^{-2} M $MgSO_4$ was mixed with 0.5 ml of the cell fraction to be assayed diluted in 10^{-2} M Tris buffer (pH 7.6). The cell fraction was replaced by Tris buffer in the control mixture. Mixtures were incubated at 37 C and at times as indicated samples of 0.1 ml were taken and added to tubes containing 10^9 indicator bacteria (HfrG6) in 0.1 ml of 10^{-2} M $MgSO_4$, also at 37 C. After 5 additional min to allow adsorption, soft agar was added (2.5 ml) to each tube and the contents were plated.

Fractionation of the cell. To follow ^{32}P incorporation into the envelope (see Fig. 2) the cells were fractionated before CH_3OH:$CHCl_3$ extraction was carried out. Samples (0.8 ml) of ^{32}P-labeled cells were pelleted and the cells were resuspended in 10^{-2} M Tris buffer (pH 7.6) and broken by sonic treatment. The envelope was pelleted by centrifugation at 20,000 × *g* for 45 min, resuspended in 0.8 ml of 10^{-2} M Tris buffer (pH 7.6), and extracted with CH_3OH:$CHCl_3$ as described above.

In one experiment (see Fig. 3), the receptor was extracted from the outer membrane by differential solubilization of the envelope by the procedure of Schnaitman (9). Envelopes, isolated from an optical density of 5.0 of cells, were suspended in 4 ml of 2% Triton X-100, 10^{-2} M $MgCl_2$, and 10^{-2} M Tris buffer (pH 7.6) and incubated 15 min at room temperature (24 C). This procedure solubilizes the cytoplasmic membrane proteins but not the outer membrane which is pelleted by centrifugation for 60 min at 100,000 × *g*. The outer membrane was solubilized by resuspension in 2 ml of 2% Triton X-100, 5×10^{-3} M ethylenediaminetetraacetic acid, and 10^{-2} M Tris buffer, pH 7.6. The fractions were diluted 1:50 in 10^{-2} M Tris buffer (pH 7.6) and assayed as described above.

Kinetics of absorption. The technique has been previously described by Schwartz (10). Bacteria in exponential growth were resuspended in 10^{-2} M $MgSO_4$ to an optical density of 0.15. The bacteria were preincubated for several minutes at 30 C. At time zero, 0.1 ml of a phage suspension was added to 1.0 ml of bacteria to obtain 5×10^6 phage per ml of the final mixture. The mixture was incubated at 30 C, and, at time intervals, 0.1-ml samples were taken and diluted into 5 ml of 10^{-2} M $MgSO_4$ saturated with chloroform. The number of unabsorbed phage in each sample was then determined by plating dilutions on the indicator strain HfrG6.

RESULTS AND DISCUSSION

The receptor for the phage lambda, a minor protein component of the outer membrane, is synthesized under the control of the gene *lamB* located in a maltose operon (8). Thus, synthesis of this outer membrane protein is induced by maltose and inhibited by glucose through catabolite repression (10). The presence of the receptor is assayed as an inactivation of the phage lambda. The rate of this inactivation is proportional to the amount of receptor in the extract. Thus, the increase of receptor protein in the outer membrane after derepression and induction by maltose can be visualized as an increase in the rate of inactivation of the phage lambda.

An experiment was designed to ask if the receptor could be properly inserted in the outer membrane in the absence of lipid synthesis. Exponentially growing cells were transferred from conditions of catabolite repression (growth on glucose) to conditions of induction (growth on maltose in the presence of cyclic adenosine 3′,5′-monophosphate) in the presence and absence of the antibiotic cerulenin.

Figure 1a shows the receptor activity extracted from whole cells at 0, 10, and 20 min after the shift from repression to induction growth conditions. There is a 10-min lag before the increase in receptor can be detected. Between 10 and 20 min the receptor activity has increased fivefold as indicated by the increased rate of inactivation of the phage (Fig. 1a). The addition of cerulenin at the time of transfer has no effect on the amount of receptor activity which can be extracted from the cells.

Figure 1b shows that ^{32}P incorporation (counts per minute in the chloroform phase of a methanol:chloroform extract) in the same cultures was drastically inhibited by the addition of cerulenin. Between 10 and 20 min after induction, when the receptor increased fivefold,

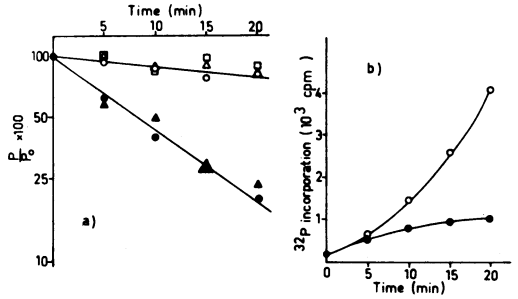

FIG. 1. *Effect of cerulenin. (a) Induction of lambda receptor activity in the presence and absence of cerulenin. Synthesis of the receptor was induced by transfer of bacteria from repression growth conditions to induction conditions as described in Materials and Methods. The receptor was extracted from whole cells with cholate/ethylenediaminetetraacetic acid, and the fractions were diluted and assayed for receptor activity by determining the kinetic of inactivation of phage λvh as described in Materials and Methods. The number of plaques, P, obtained at time, t, is plotted as percent of that (P_0) obtained at time t = 0. In the control, uninhibited culture receptor activity was assayed at times 0 (□), 10 (○), and 20 min (●) after transfer to induction conditions. A parallel culture received cerulenin at the time of transfer and receptor activity was assayed at 10 (△) and 20 min (▲) after transfer. (b) Phospholipid synthesis after transfer from repression to induction growth conditions in the presence and absence of cerulenin. At the time of transfer to induction conditions the cultures described in (a) received [^{32}P]phosphate to follow phospholipid synthesis. At 5-min intervals after transfer, samples were taken and [^{32}P]phosphate incorporation was followed in the $CHCl_3$ phase of a CH_3OH:$CHCl_3$ extract of whole cells from a control culture (○), i.e., no addition at time of transfer, and from an inhibited culture (●), i.e., cerulenin added at time of transfer.*

^{32}P was incorporated at only 9% of its uninhibited rate. (Incorporation of [^{14}C]leucine was not affected by addition of cerulenin during the course of the experiment [data not shown].)

The ^{32}P incorporation in Fig. 1b represents counts extracted from the whole cells. Figure 2 shows that this ^{32}P represents lipids which are associated with the envelope. The incorporation of ^{32}P into the chloroform fraction of extracts of whole cells and of the isolated envelopes shows the same time course. There is no lag in the initial appearance of ^{32}P in the envelope nor is there any lag in the inhibition of incorporation of ^{32}P into the envelope after addition of cerulenin. The data indicate that there is no large soluble pool of lipid and that incorporation of lipid into the envelope ceases on addition of cerulenin.

The receptor activity shown in Fig. 1a was extracted from whole cells. One could argue

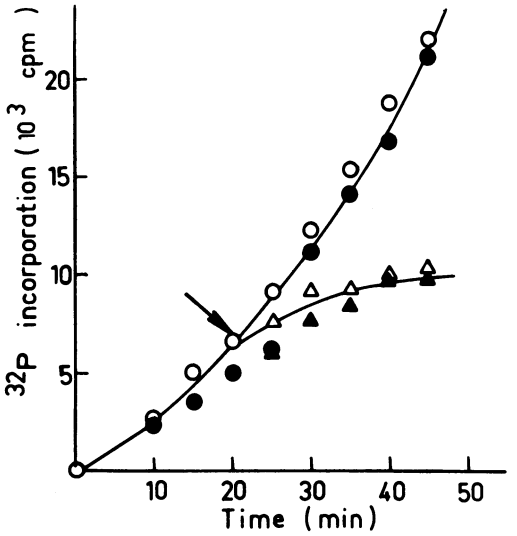

FIG. 2. *Phospholipid synthesis determined in whole cells and in isolated envelopes. [^{32}P]phosphate was added to an exponentially growing culture at time t = 0. At t = 20, the culture was divided in two fractions: one-half received cerulenin and the other half served as a control, i.e., no addition. [^{32}P]phosphate incorporation was followed by CH_3OH:$CHCl_3$ extraction of whole cells (control, ○; inhibited, △) and extraction of isolated envelopes (control, ●; inhibited, ▲).*

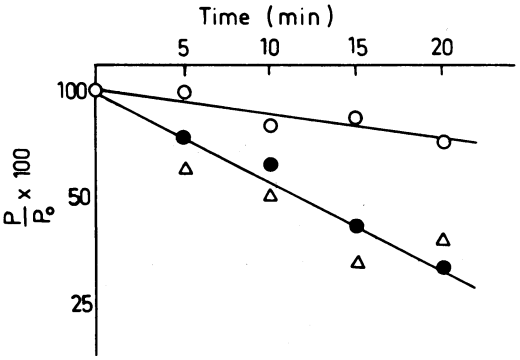

FIG. 3. *Induction of receptor activity assayed in situ. Two parallel cultures were induced for synthesis of the lambda receptor by transfer from repression growth conditions to induction conditions. One culture received no addition at the time of transfer, and the other received cerulenin. Fifteen minutes after induction receptor activity was assayed in situ as a kinetic of adsorption of phage to whole cells. The percentage of unadsorbed phage is plotted against the time of incubation of a mixture of whole cells and phage. The increase in receptor activity after induction is seen by comparing the rate of adsorption to cells grown in repression conditions (○) to that of induced cells. Induced in absence (●) and presence (△) of cerulenin.*

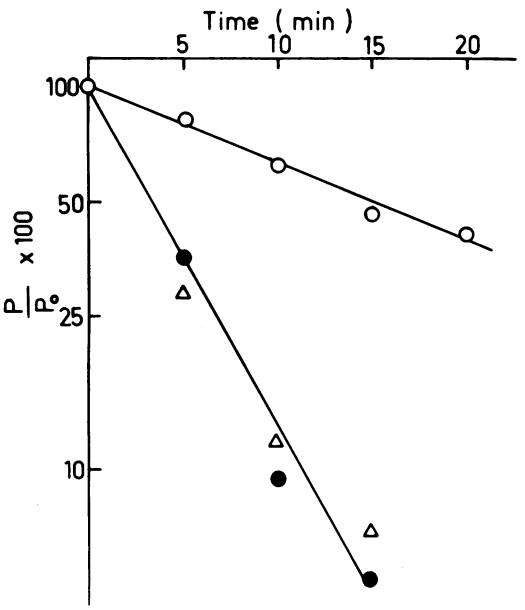

FIG. 4. *Receptor activity in the outer membrane fractions of cells induced in the presence and absence of cerulenin. Samples of the cells which were induced and assayed for in situ receptor activity (Fig. 3) were also fractionated and the outer membrane fractions were assayed for receptor by determination of the kinetic of inactivation of phage lambda. Outer membrane fraction of cells grown in repression conditions.* O; *outer membrane fractions 15 min after induction in the absence of cerulenin,* ●; *15 min after induction in the presence of cerulenin,* Δ. *The number of plaques at time t, is plotted as percent of that* (P_0) *obtained at time t = 0.*

that the receptor is synthesized in the presence of cerulenin, but not properly inserted and can still be extracted from whole cells in an active form. This possibility was eliminated by demonstrating that cerulenin does not affect the appearance of receptor sites on the cell surface, i.e., the outer membrane, and that all receptor activity in a cell extract is found in the outer membrane fraction.

Cells were induced for the lambda receptor in the presence or absence of cereulenin. The receptor was assayed in situ by following the kinetic of adsorption of phage to whole cells. Figure 3 shows that cerulenin does not affect the insertion of new, active receptor at sites available to phage on the outer membrane. Thus, the rate of adsorption of phage increases to the same extent after induction in the presence or absence of cerulenin.

Samples of the same cells were fractionated and the fractions were assayed for receptor activity. The only activity detected in both the control and in the inhibited culture was localized in the outer membrane; i.e., no activity was found in the soluble fraction nor in the cytoplasmic membrane (data not shown). The solubilized outer membrane from the control and the inhibited cultures show the same rate of inactivation of the phage (Fig. 4). Thus it is concluded that during inhibition of lipid synthesis the receptor protein is synthesized and translocated to its position in the outer membrane to the same extent as under normal growth conditions.

These data of course say nothing about the possibility of continuous redistribution of previously synthesized lipid between the two membranes. The translocation of the receptor protein might require simultaneous movement of lipid which continues after addition of cerulenin resulting in a depletion of lipid in the cytoplasmic membrane. This possibility has not been tested. The present experiments indicate that simultaneous lipid synthesis is not required for the translocation of a minor protein component to the outer membrane.

ACKNOWLEDGMENTS

This work was supported by the Swedish Cancer Society, the Natural Sciences Research Council and the Medical Research Council.

I would like to thank S. Ōmura for the generous gift of the antibiotic, cerulenin, and M. Schwartz for providing information concerning the conditions used to induce the lambda receptor. I am grateful to C. G. Kurland for advice and discussion and to G. L. Hazelbauer for critically reading the manuscript.

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