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INFRARED SPECTRA OF OUTER AND CYTOPLASMIC MEMBRANES OF *ESCHERICHIA COLI*

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SUMMARY

Outer and cytoplasmic membranes of *Escherichia coli* were prepared by a method based on isopycnic centrifugation on a sucrose gradient. The infrared spectra of solid films of these membranes were studied. The cytoplasmic membrane had an amide I band at 1657 cm^{-1} and an amide II band at 1548 cm^{-1}. The outer membrane had a broad amide I band at 1631–1657 cm^{-1} and an amide II band at 1548 cm^{-1} with a shoulder at 1520–1530 cm^{-1}. Upon deuteration, the amide I band of the cytoplasmic membrane shifted to 1648 cm^{-1}, whereas the band at 1631 cm^{-1} of the outer membrane remained unchanged. After extraction of lipids with chloroform and methanol, the infrared spectra in the amide I and amide II regions of both membranes remained unchanged. Although the outer membrane specifically contained lipopolysaccharide, this could not account for the difference in the infrared spectra of outer and cytoplasmic membranes. It is concluded that a large portion of proteins in the outer membrane is a β-structured polypeptide, while this conformation is found less, if at all in the cytoplasmic membrane.

INTRODUCTION

The cell envelope of *Escherichia coli* contains two membranous components, the outer membrane and the cytoplasmic membrane. They have been separated from each other [1–3], and structural and functional differences between these components have been studied [1–10].

Recent evidence indicates that infrared spectroscopy is a useful technique for studying the conformation of proteins of cellular membranes and the interrelationships between membrane proteins and lipids [11–17]. In the present study, infrared spectra of the outer membrane from E. coli was compared with those of the cytoplasmic membrane. The outer membrane was found to differ significantly from the cytoplasmic membrane in the conformation of its proteins.

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METHODS

Bacterial strain

E. coli YA21 was used, a leucine-auxotroph mutant derived from *E. coli* K12 (met$^-$, F$^-$, λ^-) used in the previous study [2].

Preparation of outer and cytoplasmic membranes

Outer and cytoplasmic membranes were prepared principally according to the method described previously [1] with some modifications (Mizushima, S. and Yamada, H., unpublished). Details of the procedure will be published elswhere. Briefly, cells were grown in a modified Fraser and Jerrels' medium [18] in which the amount of glycerol and polypeptone was one third of that of the original composition. For the preparation of spheroplasts, ice-cold reagents were slowly added to 35.5 ml of the washed cell suspension (1 g dry weight) in ice–water in the following order: 20 ml of 0.1 M Tris–HCl buffer (pH 8.0, at 24 C), 18 ml of 2 M sucrose, 3.5 ml of 1% Na-EDTA (pH 7.0) and 3.5 ml of 0.5% lysozyme. The mixture was warmed to 30 C within 2–3 min and kept at this temperature for 60 min. A part of the outer membrane was released from spheroplasts during the incubation. The released membrane was separated from spheroplasts by centrifugation at 15 000 rev./min for 10 min, sedimented by high-speed centrifugation (38 000 rev./min for 45 min) and purified by centrifugation on a sucrose gradient (outer membrane I). A membrane fraction was also prepared from the spheroplasts and separated into a cytoplasmic and an outer membrane fraction by sucrose density gradient centrifugation (cytoplasmic membrane and outer membrane II). Membranes were recovered by centrifugation (38000 rev./min, 20 min), washed three times with water and suspended in water to make the protein concentration 10–15 mg/ml. For ^{2}H labeling, membranes were washed three times with ^2H_2O.

Infrared spectra

A Hitachi Model EPI-G3 or Jasco Model IR-G infrared spectrophotometer was used. A standard polystyrene film (Hitachi Ltd., Tokyo) was used for the calibration of absorption peak frequencies.

For the preparation of solid films, a preparation (20–40 μl, 0.3–0.6 mg protein) was spread on a KRS-5 plate into rectangular shape (10 mm × 20 mm) and was dried in air at room temperature. In case of the ^{2}H-labeled preparations, solid films were prepared in vacuo inside the moisture control cells equipped with KRS-5 plates. All measurements on ^{2}H-labeled preparations were carried out in the absence of ^2H_2O vapour because ^2H_2O vapour (32% saturation; with $CaCl_2$ saturated with ^2H_2O) did not make any difference in the spectra. Infrared spectra in ^2H_2O solution were measured with a KRS-5 liquid absorption cell (path length 0.05 mm).

Other methods

Lipopolysaccharide was prepared from the cell envelope of the same strain according to the method of Osborn [19]. Purified murein of *E. coli* was a gift from Dr Yanai.

The amount of lipopolysaccharide in outer membrane preparations was

estimated from their 2-keto-3-deoxyoctonic acid content which was determined as described by Osborn et al. [20]. The purified lipopolysaccharide was used as reference material. The amount of murein in outer membrane preparations was estimated from their diaminopimelic acid content which was determined by means of an amino acid analyser (Japan Electron Optics Lab., JLC-5AH). Samples were hydrolysed with 6 M HCl at 110 °C for 20 h. The purified murein was used as reference material.

RESULTS

Infrared spectra of outer and cytoplasmic membranes

Fig. 1 shows the amide I and amide II regions of the infrared spectra of dried films cast from aqueous suspensions of outer and cytoplasmic membranes. The amide I band of the cytoplasmic membrane showed a peak centered at 1657 cm^{-1}, indicating an α-helical and/or random-structured polypeptide. The lack of absorption around 1631 cm^{-1} indicates that there is little β-structured polypeptide in the cytoplasmic membrane. On the other hand, the amide I band of outer membrane showed a broad peak at 1631–1657 cm^{-1} covering the region associated with β-structured polypeptides as well as an α-helical and/or random-coiled structure.

Another difference between the two preparations was observed in the amide II band. The amide II band of the outer membrane showed a peak at 1548 cm^{-1} with a shoulder at about 1520–1530 cm^{-1} where the amide II band of β-structured polypeptides should be expected [21,22]. The shoulder was hardly visible in the spectrum of the cytoplasmic membrane. Outer membrane II separated from cytoplasmic membrane by centrifugation on a sucrose gradient showed essentially the same spectrum as that of outer membrane I (Fig. 2).

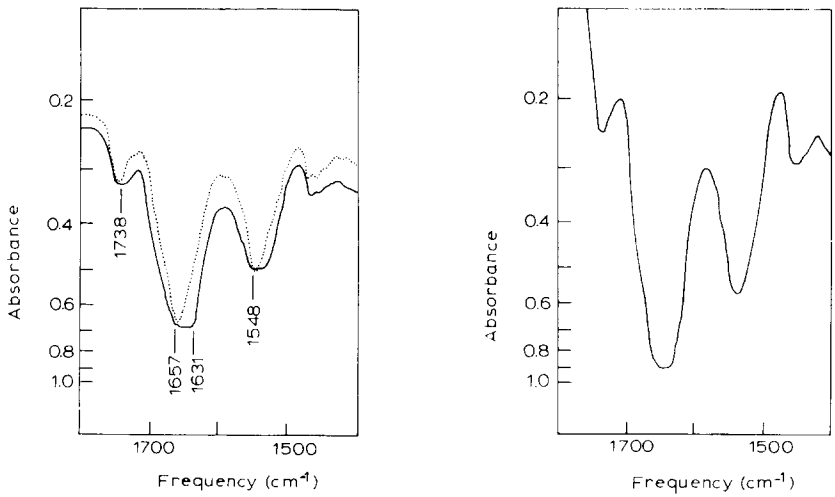

Fig. 1. Infrared spectra of a solid film of outer and cytoplasmic membranes. ——, outer membrane I; – – – –, cytoplasmic membrane. Hitachi EPI-G3 infrared spectrophotometer was used.

Fig. 2. Infrared spectrum of a solid film of outer membrane II. Hitachi EPI-G3 infrared spectrophotometer was used.

As shown in Fig. 3, the extraction of lipids from dried films with chloroform methanol (2:1, v/v) did not alter the spectra of the amide I and amide II regions. Judging from the disappearance of the band at 1740 cm^{-1} due to C=O stretching vibration of fatty acid esters [14], the removal of lipids from the cytoplasmic membrane seemed complete. A small peak in the spectrum of the lipid-depleted outer membrane at 1740 cm^{-1} must be due to fatty acid esters in lipopolysaccharides.

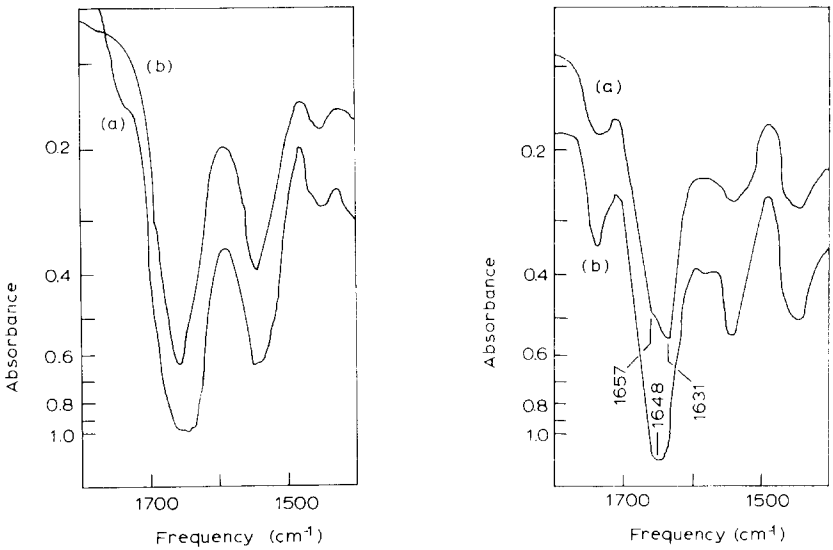

Fig. 3. Effect of lipid extraction on infrared spectrum of membranes. A solid film of membrane was kept overnight in chloroform methanol (2:1, v/v) and dried in vacuo. (a) lipid-depleted outer membrane I; (b) lipid-depleted cytoplasmic membrane. Hitachi EPI-G3 infrared spectrophotometer was used.

Fig. 4. Infrared spectra of a solid film of ^2H-labeled outer and cytoplasmic membranes. (a) outer membrane I; (b) cytoplasmic membrane. Hitachi EPI-G3 infrared spectrophotometer was used.

Infrared spectra of ^2H-labeled outer and cytoplasmic membranes

Fig. 4 shows infrared spectra of dried films cast from ^2H-labeled suspensions of membrane preparations. Upon ^2H labeling, the amide I band of the cytoplasmic membrane was shifted to 1648 cm^{-1}. This indicates that a large portion of the amide I band of cytoplasmic membrane was due to the random coil conformation, for random coil is the only conformation which readily changes the position of the amide I band upon ^2H labeling [22]. In the ^2H-labeled outer membrane, a peak at 1631 cm^{-1} is predominant in the amide I region, supporting the above-mentioned observation that β-structured polypeptides are significant in the outer membrane.

Fig. 5 shows infrared spectra of membranes washed and suspended in ^2H_2O. Spectra around the amide I region were identical to those obtained on dried films cast from ^2H_2O suspensions.

In all spectra examined so far, there was no resolved shoulder around 1685 cm^{-1}, characteristic of the antiparallel-chain pleated sheet [21].

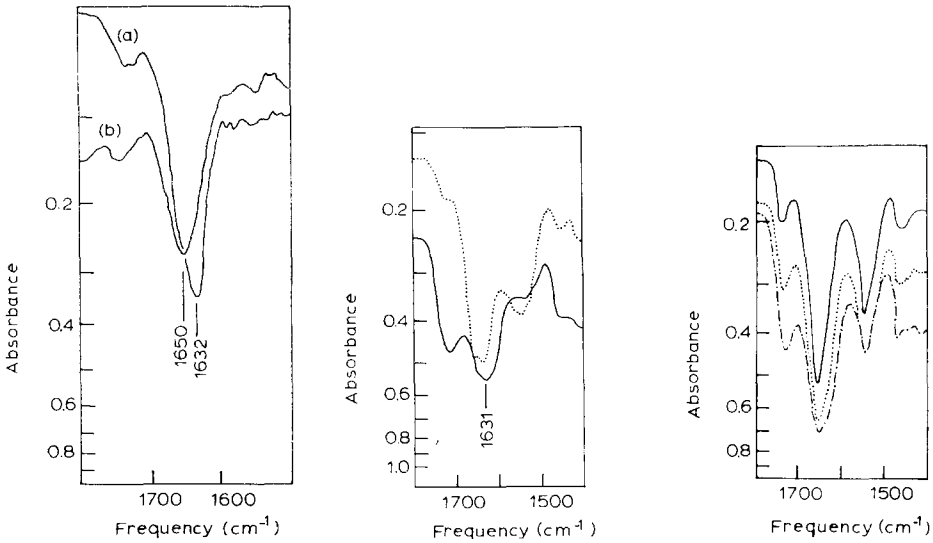

Fig. 5. Infrared spectra of outer and cytoplasmic membranes in ^2H_2O. Protein concentration of membrane preparation was about 30 mg/ml. (a) outer membrane I; (b) cytoplasmic membrane. Hitachi EPI-G3 infrared spectrophotometer was used.

Fig. 6. Infrared spectra of a solid film of lipopolysaccharide and murein. 1.6 mg of lipopolysaccharide in 800 μl of water or 0.35 mg of murein in 70 μl of water was cast over KRS-5 plate. - - -, lipopolysaccharide; – – – – –, murein. Jasco IR-G infrared spectrophotometer was used.

Fig. 7. Infrared spectra of a solid film cast from a mixture of cytoplasmic membrane and lipopolysacharide. Cytoplasmic membrane (0.32 mg protein) in 40 μl of water was mixed with various amounts of lipopolysaccharide (2 mg/ml) and cast over KRS-5 plate. Jasco IR-G infrared spectrophotometer was used. Amount of lipopolysaccharide per mg protein: ——, 0 mg; - - - - -, 0.63 mg; -·-·-, 3.12 mg.

Influence of other constituents of the membrane preparation on infrared spectra

Besides proteins and lipids, lipopolysaccharide and murein were thought to be constituents of the outer membrane. The outer membrane used for infrared spectroscopy contained 0.3–0.8 mg of lipopolysaccharide per mg protein while the amount of murein was only about 20 μg/mg protein.

The infrared spectrum of purified lipopolysaccharide is shown in Fig. 6. Although the spectrum was quite different from that of the outer membrane, it had an absorption band around 1630 cm^{-1}. The possibility that the presence of lipopolysaccharide could account for the difference in the spectra of outer and cytoplasmic membranes was then examined. Fig. 7 shows the infrared spectra of the cytoplasmic membrane mixed with different amounts of lipopolysaccharide. The amide I region of a sample in which the protein: lipopolysaccharide ratio was the same as that in the outer membrane was similar to that of the cytoplasmic membrane. Spectra of samples with a higher lipopolysaccharide content were different from that of the outer membrane (Fig. 7). Although the difference was not so significant as in the case of the amide I region, the amide II region of the cytoplasmic membrane with lipopolysaccharide was also different from that of the outer membrane (Fig. 7). The infrared spectrum of murein is shown in Fig. 6.

Although the amide I region of murein was very similar to that of the outer membrane, such a small amount of murein as found in the outer membrane preparation did not make any significant difference in the infrared spectrum of the membrane preparations.

DISCUSSION

Extensive studies have been carried out on the positions and shifts of amide I and II bands. It is now possible to relate small differences in band position to specific differences in the conformations of polypeptide chains [21]. The β-structured polypeptide chain shows amide I and amide II bands at 1628–1635 cm^{-1} and at 1521–1525 cm^{-1}, respectively, while the α-helical or random-coil polypeptide shows amide I and amide II bands at 1652–1657 cm^{-1} and 1545–1551 cm^{-1}, respectively. Although the random-coil polypeptide chain is known to have an amide I band slightly higher in frequency than that of the α-helical chain [21], quantitative analysis on the content of polypeptides of each conformation is hardly possible.

The infrared spectra of cellular membranes have been studied extensively. Spectra of erythrocyte ghosts [11,12], plasma membrane and endoplasmic reticulum of Ehrlich ascites carcinoma [14] showed no indication of the β-structured polypeptide chain in these membranes. Recently, however, the presence of β-conformation in the mitochondrial membrane [15] and rat adipocyte plasma membrane [17] has been shown by infrared spectroscopy.

In the present study, we have found some significant differences in infrared spectra of cytoplasmic and outer membranes both of which were derived from the same bacterial cells. Differences were found to be due to the presence of a significant amount of β-structured polypeptide chain in the outer membrane from the following observations: (1) The amide I band of the outer membrane showed a broad peak (1631–1657 cm^{-1}) covering the region due to β-structured polypeptide. (2) Upon ^{2}H labeling, the peak at 1631 cm^{-1} remained unchanged. (3) Lipid depletion of membrane preparations with chloroform and methanol did not change the spectra of amide I bands. (4) The presence of lipopolysaccharide, another main constituent of the outer membrane, could not account for the characteristic absorption around 1631 cm^{-1}. (5) The amount of murein, which showed absorption at 1630–1640 cm^{-1}, was insignificant in the outer membrane preparation studied in the present paper. A significant amount of murein was found in an outer membrane preparation prepared in a different way [20]. (6) In the spectrum of the outer membrane, a shoulder was observed at 1520–1530 cm^{-1} where the amide II band of β-structured polypeptide is localized.

Proteins of the outer membrane are supposed to be synthesized inside the cytoplasmic membrane. Therefore, these membrane proteins have to be excreted through the cytoplasmic membrane which is composed of many proteins. How do bacteria distinguish proteins which are to be excreted out of the cytoplasmic membrane and incorporated into the outer membrane, from those to be incorporated in the cytoplasmic membrane? It would be interesting, on this point, if some differences in tertiary structure were observed between outer membrane and cytoplasmic membrane proteins.

By the method described in this paper, about half of the outer membrane (outer membrane I) was always released from spheroplasts, while the other half remained on spheroplasts (outer membrane II). As far as infrared spectra are concerned, however, no difference was found between them.

Finally, our study indicates that infrared spectroscopy is a useful technique for a rapid distinction between outer and cytoplasmic membranes.

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