Lipid and protein segregation in *Escherichia coli* membrane: Morphological and structural study of different cytoplasmic membrane fractions

(fatty acid auxotroph/freeze-fracture electron microscopy/high-angle x-ray diffraction)

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**ABSTRACT** Lipid and protein segregations can be induced in *E. coli* cytoplasmic membranes by conformational transitions of their lipid hydrocarbon chains from a disordered to an ordered state. For *E. coli* strain K 1059 (an unsaturated fatty acid auxotroph) supplemented with linolenic acid, the segregation leads to large areas of membrane surfaces having distinctly different morphological characteristics (smooth compared with strongly particulated fracture faces, as visualized by freeze-fracture electron microscopy). The different regions are physically separated by osmotic lysis of spheroplasts at temperatures below those of the order-disorder transition of the lipid hydrocarbon chains. The analysis of the different cytoplasmic membrane fractions provides a direct demonstration and allows a direct analysis of the segregation. As compared to the nonfractionated membranes, the membrane regions corresponding to the smooth fracture surfaces are poor in proteins, rich in lipids, and enriched in saturated fatty acids, while the membrane regions corresponding to the strongly particulated fracture surfaces are rich in proteins, poor in lipids, and enriched in unsaturated fatty acids. Quantitative information about the extent of these segregations is obtained from high-angle x-ray diffraction of the different membrane fractions and of the corresponding total lipid extracts.

Segregation of lipids and proteins in biological membranes can be induced by conformational transitions of the hydrocarbon chains of the membrane lipids from a disordered to an ordered state (1–4). Lipid segregation is the consequence of membrane lipid heterogeneity, which may lead to a lateral lipid phase separation during the disorder-to-order transition (5, 6). As a result of the phase separation, two types of membrane domains with different lipid composition are observed: domains enriched with lipids containing disordered hydrocarbon chains and domains enriched with lipids having ordered hydrocarbon chains. Since hydrophobic membrane proteins deep embedded in the hydrophobic core of the membrane (integral proteins (7)) cannot be accommodated easily in the ordered domains, lipid segregation will induce segregation of proteins, and the hydrophobic proteins will migrate preferentially into domains enriched with lipids containing disordered hydrocarbon chains.

Protein segregation can be visualized best by means of electron microscopy utilizing freeze-fracture techniques (1–3). Membranes frozen from temperatures above the order-disorder transition of the hydrocarbon chains exhibit a random distribution of intramembrane particles which presumably represent integral membrane proteins or complexes of these proteins with lipids. When frozen from temperatures below the transition, membrane fracture faces usually display a mixture of smooth surfaces devoid of particles and surfaces containing aggregated particles. In certain special cases, lipid segregation can also be visualized by freeze-fracture electron microscopy (2, 3).

We have demonstrated previously (3) that cytoplasmic membrane vesicles of *Escherichia coli* containing specific unsaturated fatty acids exhibit relatively large areas (i.e., several thousand Å in diameter) of smooth and particulated surfaces when frozen from temperatures below the order-disorder transition of the hydrocarbon chains. Since the two domains are radically different, discrete fractions of cytoplasmic membrane vesicles can be isolated that exhibit different relative amounts of smooth and heavily particulated surfaces (8, 9). In a preliminary report (8) we described the chemical and morphological characteristics of these different membrane fractions isolated by isopycnic centrifugation. Here, we extend the study to the analysis of their structural characteristics as determined by high-angle x-ray diffraction. Taking into account chemical, morphological, and structural information about the different fractions, we discuss the nature of the lipid and the protein segregation induced by the transition of the hydrocarbon chains of the lipids from a disordered to an ordered conformation.

**MATERIALS AND METHODS**

The techniques used in this study have been described previously (3, 8, 10). Further details are given in the text and figure legends.

**RESULTS**

Preparation and Chemical Characterization of Cytoplasmic Membrane Fractions. *E. coli* K 1059, an unsaturated fatty acid auxotroph, was grown on a medium supplemented with linolenic acid (cis-Δ9,12,15C18). The formation of spheroplasts from whole cells followed the procedure of Kaback (11), with slight modifications (10). Cytoplasmic membrane vesicles were obtained by osmotic lysis of the spheroplast suspension either at a temperature just above (46°C) or just below (4°C) the temperature range for the order-disorder transition of the membrane lipid hydrocarbon chains. After purification (11), the cytoplasmic membranes were isolated on a continuous sucrose gradient (30–70% wt/vol). When lysis is carried out at 4°C, three distinct membrane fractions are obtained: a light fraction (fraction I, density: 1.11), an intermediate fraction (fraction II, density: 1.17) and a heavy fraction (fraction III, density: 1.23). When lysis is performed at 46°C, only one fraction is recovered (fraction II′, density: 1.17). We have reported previously that the protein profiles as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis are quite different for the three fractions, I, II, and III. It has been shown in particular that some bands are missing in the light fraction I (8). However, the relative proportions of the phospholipid species are similar in the three fractions (data not shown). Some other chemical characteristics of the different fractions are summarized in Table 1.

**Morphological Characterization of Cytoplasmic Membrane Fractions.** Fig. 1 presents freeze-fracture electron mi-
Table 1. Characteristics of cytoplasmic membrane fractions

<table>
<thead>
<tr>
<th>Property</th>
<th>Fraction I</th>
<th>Fraction II</th>
<th>Fraction III</th>
<th>Fraction II'</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spheroplasts osmotically lysed at</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4°</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Buoyant density</td>
<td>1.11 (1.08)*</td>
<td>1.17</td>
<td>1.23</td>
<td>1.17</td>
</tr>
<tr>
<td>Phospholipids (mg/mg of protein)</td>
<td>2.23 (4.5)*</td>
<td>0.66</td>
<td>0.54</td>
<td>0.66</td>
</tr>
<tr>
<td>Lipopolysaccharide (mg/mg of protein)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fatty acid composition (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C14:0</td>
<td>6</td>
<td>5</td>
<td>3</td>
<td>5</td>
</tr>
<tr>
<td>C16:0</td>
<td>67</td>
<td>59</td>
<td>55</td>
<td>60</td>
</tr>
<tr>
<td>C18:2</td>
<td>25</td>
<td>36</td>
<td>41</td>
<td>35</td>
</tr>
<tr>
<td>Unknown</td>
<td>2</td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Saturated</td>
<td>73 (77)*</td>
<td>64</td>
<td>58</td>
<td>65</td>
</tr>
<tr>
<td>Unsaturated</td>
<td>25 (20)*</td>
<td>36</td>
<td>41</td>
<td>35</td>
</tr>
</tbody>
</table>

E. coli K 1059 was grown as described (3) with linolenic acid as the exogenous unsaturated fatty acid. Membranes were prepared from whole cells as described (refs. 8 and 11, and see text). Proteins, lipids, fatty acid, and lipopolysaccharide were determined and characterized as described in ref. 8.

* These values are corrected for contamination by membranes displaying a normal concentration of particles on the fracture faces (i.e., similar to the nonfractionated membrane II'). See text for explanation.

† Pure outer membrane is characterized by a lipopolysaccharide content of 1–1.2 mg/mg of protein (10). Thus, fraction III appears to be contaminated by some 15–20% with outer membrane.

crographs of the cytoplasmic membrane fractions. As described previously (3), the fracture faces of cytoplasmic membranes isolated from spheroplasts osmotically lysed at 46° (fraction II') display a random distribution of particles when rapidly frozen from 46° (Fig. 1A) and a mixture of smooth and particulated surfaces when frozen from 0° (Fig. 1B). The fracture faces of the cytoplasmic membrane fractions isolated from spheroplasts osmotically lysed at 4° are markedly different for the three fractions I, II, and III (8). Fraction I (frozen from 46°) displays a mixture of different types of membrane vesicles (Fig. 1C). A statistical analysis performed on over 200 vesicles indicates that it consists in mass of 70% large membrane vesicles (4000–6000 Å in diameter) exhibiting fracture faces devoid of particles or displaying some individual scattered particles, and 30% smaller membrane vesicles (1000–3000 Å in diameter) displaying fracture faces with a concentration of particles similar to that of the nonfractionated membranes (fraction II'). Fraction II (frozen from 46°) is very similar to the nonfractionated vesicles (Fig. 1D). It is however contaminated by fraction I, as indicated by the presence of a small percentage of vesicles (10% in mass) exhibiting fracture faces devoid of particles or with only scattered particles. Finally, fraction III (frozen from 46°) displays mainly membranes with highly particulated fracture faces (Fig. 1E).

Structural Characterization of Cytoplasmic Membrane Fractions. High-angle x-ray diffraction of the different membrane fractions and of the corresponding total lipid extracts from the fractions has been carried out as described (ref. 3, and legend to Fig. 2). At sufficiently high temperature, the high-angle x-ray diffraction spectra of all preparations display a broad band centered at around 4.5 Å which is characteristic of disordered hydrocarbon chains. As the temperature decreases, a sharp reflection centered at 4.2 Å appears superimposed on the broad band, indicating that some of the hydrocarbon chains have become ordered. The intensity of this reflection increases with decreasing temperature and reaches a constant value below a certain temperature (Fig. 2).

The ratios of ordered/total hydrocarbon chains for the various membrane fractions and total lipid extracts are shown in Figs. 3 and 4 as a function of temperature. The ratios are determined from the integrated intensity of the 4.2 Å reflection (ref. 3, and legend to Fig. 3).

A comparison of the order-disorder transitions for a given membrane and the corresponding total lipid extract shows that they are qualitatively similar. However, quantitatively the amount of hydrocarbon chains taking part in the transition may be smaller for the membrane than for the corresponding total lipid extract. This indicates that the membrane proteins may produce some degree of disordering of the lipid hydrocarbon chains.

The high-angle x-ray diffraction spectra of fractions II and II' are similar (see Fig. 3 and ref. 3). Each displays two order-disorder transitions centered at 10° and at 35°, respectively. In contrast, the spectrum of fraction I displays a single transition centered at 35°, while the spectrum of fraction III displays a single transition centered at 18°.

DISCUSSION

The segregation of lipids and proteins within different areas of the bacterial membrane leads to large areas of membrane surfaces having distinctly different morphological characteristics (smooth compared with strongly particulated fracture faces). Spheroplasts lysed at 4° give rise to a heterogeneous population of cytoplasmic membrane vesicles having distinct chemical, morphological, and structural characteristics (fractions I, II, and III). The same spheroplasts lysed at 46° give rise to only one cytoplasmic membrane fraction (fraction II'). These results clearly indicate that segregation of lipids and proteins within different areas of the membrane must exist in the spheroplasts at temperatures below those of the order-disorder transition of the hydrocarbon chains. It has also been reported that E. coli cells (13) display fracture faces with both large smooth surfaces and surfaces with aggregated particles at these temperatures. Moreover, the isolation of discrete cytoplasmic membrane fractions from spheroplasts lysed at 4° indicates that the spheroplast membrane must be disrupted in a manner that is dependent to some extent upon the nonrandom distribution of smooth and particulated domains. Since mainly three discrete fractions are isolated, the disruption is not statistical, and most
FIG. 1. Freeze-fracture electron microscopy. The membranes were treated as described in ref. 8. The replicas were obtained with a Balzer BAF 3000 freeze-etch unit and examined with a Philips EM 301 electron microscope. (A and B) Nonfractionated membranes II' isolated from
probably takes place preferentially along the lines of demarcation between smooth and particulated areas. These lines of demarcation presumably represent regions of contact between lipids having disordered and lipids having ordered hydrocarbon chains (see below). These regions are most likely to be areas in which there is weak interactions. This contention is substantiated by permeability studies across lipid liposomes. It has been shown that the liposomes are particularly permeable to solutes at a temperature corresponding to that of the mid-order-disorder transition of the hydrocarbon chains, i.e., at a temperature where domains of lipids with ordered and domains of lipids with disordered hydrocarbon chains coexist.

Cytoplasmic membrane fraction I, which displays (when frozen from 46°) predominantly fracture surfaces with practically no particles or with some scattered particles, clearly originates from membrane regions of the spheroplasts displaying, at low temperature, the smooth fracture surfaces. Cytoplasmic membrane fraction III, which displays (when frozen from 46°) fracture surfaces with heavily aggregated particles, must originate from membrane regions of the spheroplasts displaying heavily particulated surfaces at low temperature. Fraction II is quantitatively the predominant fraction and has the same characteristics as the sole cytoplasmic membrane fraction obtained from spheroplasts lyed at 46° (fraction II'), where no segregation occurs. This fraction probably originates from individual spheroplasts, which give rise to single membrane vesicles upon lysis at 4° (i.e., one spheroplast lyzes to form one membrane vesicle) 15.

A similar type of physical separation can be achieved by subjecting the spheroplasts, as described by van Heerikhuizen et al. (9), to high pressure (French press) rather than osmotic lysis at 4°. The advantage of osmotic lysis is that it leads to relatively large and apparently intact cytoplasmic membrane vesicles that are potentially able to catalyze active transport.

FIG. 2. High-angle x-ray diffraction of cytoplasmic membranes as a function of temperature. A Philips PW 10.10 was used as the x-ray source. The beam was linearly focused by a monochromator. The diffracted x-ray beam was detected with a linear position, sensitive, proportional counter (12). Other experimental conditions and the preparation of the membranes and the lipid extracts for the x-ray study are described in ref. 3.

FIG. 3. Disorder-order transitions of the hydrocarbon chains of lipids for different membrane fractions. (▼) Fraction I; (●) fraction II; (●) fraction III. The amount of ordered hydrocarbon chains in the various samples was determined quantitatively by comparison of the integrated intensity of their reflection at 4.2 Å to that of a standard consisting of elaidic acid (trans-Δ9, C<sub>18.1</sub>) mixed with 5% water. At 0° practically 100% of the hydrocarbon chains of the standard lipid are in an ordered state (10).

Until recently, the existence of lipid-protein segregation during the transition from the disordered to the ordered conformation of lipid hydrocarbon chains has been inferred mainly from the appearance of fracture faces with different morphological characteristics. This segregation is now experimentally confirmed, and the chemical analysis of different fractions provides a direct demonstration of this phenomenon. As compared to the composition of the nonfractionated membranes (fraction II'), the membrane regions corresponding to the smooth fracture surfaces are poor in proteins and rich in lipids. Moreover, the lipids in these regions are enriched in saturated fatty acids (fraction I). The membrane regions corresponding to the fracture surfaces displaying aggregated particles (fraction III) are poor in lipids and enriched in proteins. Moreover, the lipids in these regions are enriched in unsaturated fatty acids. Therefore, two types of segregation exist: segregation of lipids from the proteins and segregation of different lipid classes.

Valuable additional quantitative information regarding these segregations is obtained from the high-angle x-ray diffraction data. The origin and significance of the two order-disorder transitions observed for the nonfractionated membranes (fraction II and II') have been discussed (3). Briefly, because of the simple fatty acid composition of the membrane lipids (mainly C<sub>16:0</sub> synthesized by the cell and C<sub>18:3</sub> provided during growth), three types of lipids exist: totally saturated lipids having two saturated fatty acid chains, totally unsaturated lipids having two unsaturated fatty acid chains, and mixed lipids having one saturated and one unsaturated fatty acid chain. Due to the very fluid character of the linolenic fatty acid chains, the totally unsaturated lipids are not expected to take part in order-disorder transitions at temperatures above 0°. Thus, the existence of two order-disorder transitions above that temperature has been taken as an indication of at least partial segre-

spheroplasts osmotically lyed at 46°. (A) Membranes frozen from 46°. (B) Membranes frozen from 0°. X 75,000. (C–E) Membrane fractions I, II, and III, respectively, isolated from spheroplasts lyed at 4° and frozen from 46°. (C and D) X27,300; (E) X75,000. For all figures, the shadowing is from bottom to top.
taking part in the transition in the membrane as compared to the corresponding total lipid extract is the result of the contamination of the membrane displaying fracture faces devoid of particles by membranes displaying fracture faces with a higher concentration of particles. Taking this into account, the amount of hydrocarbon chains taking part in the transition in a membrane displaying only smooth fracture faces should be similar to that of the corresponding total lipid extract. It can thus be concluded that the proteins introducing the largest perturbations are those visualized as particles on the fracture surfaces and thus corresponding to integral hydrophobic proteins. They perturb the transitions inasmuch as they interact and disorder the lipids in their immediate environment.

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Fig. 4. Disorder-order transitions of the hydrocarbon chains of total lipid extracted from different membrane fractions. (△) Fraction I; (●) fraction II; (●) fraction III. See legend to Fig. 3 for explanation.