The Molecular Organization of Lipids in the Membrane of
Escherichia coli: Phase Transitions*
(x-ray diffraction/auxotroph/thermotropic phase transitions/Arrhenius plots)

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ABSTRACT X-ray diffraction techniques have been used to investigate the physical state of the lipids in the membrane of an unsaturated fatty acid auxotroph of Escherichia coli. Thermo- tropic phase transitions have been detected in membranes prepared from cells grown on various fatty acid supplements. Below the transition temperature, the X-ray diffraction pattern features a sharp ring at 420 pm (4.2 A) due to the close hexagonal packing of the apolar groups of the lipids; above the transition temperature the lipids are in a less organized liquid-crystalline state that gives rise to a diffuse band centered at a Bragg spacing of 460 pm (4.6 A). This transition occurs at or below the temperature at which the cells were grown. Disparities between this transition temperature and the temperature of discontinuities in the Arrhenius plots for proline transport and sucinic dehydrogenase activity lead us to conclude that the distribution of lipids within the membrane is heterogeneous.

The importance of the apolar portion of the membrane lipids in the structural and functional properties of biological membranes has recently been emphasized by investigations with Mycoplasma laidlawii (1, 2) and with mutant strains of Escherichia coli that cannot synthesize unsaturated fatty acids (3-10). From the detection of thermotropic phase transitions in the lipids of these membranes (1, 2, 11) and related functional studies (4-10), it would appear that the physical state of the membrane lipids is an important factor in determining certain characteristics such as permeability and enzyme activity. A previous investigation on correlated structural and functional properties (5) used monolayer studies of an isolated lipid fraction to characterize the physical state of the membrane lipids. However, there are indications (12, 13) that the physical state of lipid extracts of membranes is quite different from that of the lipid component of intact membranes. For this reason we have studied the physical state of the lipids within the membrane using X-ray diffraction techniques.

MATERIALS AND METHODS

Bacterial Strain and Growth Conditions. Strain O147-1, an unsaturated fatty acid auxotroph derived from E. coli K12, Strain CR8 (4) was used throughout these studies. Cells were grown at 37°C in 1 liter of medium contained in a 2 liter flask under conditions reported earlier, except that the carbon source was 2% glycerol and 2% amino-acid mixture.

Preparation of Membranes. Cells were harvested at the logarithmic phase of growth, washed at 4°C once with 0.01 M Tris-HCl (pH 8.0) containing 1% Triton X-100 and once with the same buffer without Triton. Spheroplasts were prepared essentially according to the procedure of Repaske (14). The cells were suspended in a solution of 20% sucrose in 0.03 M Tris-HCl (pH 8.0) at 25°C, at an optical density of 0.5-0.9 units at 600 nm. EDTA and lysozyme were added at a final concentration of 0.54 μmol and 41 μg/ml, respectively. Incubation was performed at room temperature for 30 min with mild stirring by a magnetic stirrer. Membranes were then prepared from the spheroplasts and washed according to the method of Kaback (15, 16), except that the homogenized spheroplast suspension was diluted 30- to 50-fold into the lysing buffer instead of a 200-fold dilution. The crude membranes were finally homogenized in a solution of 20% sucrose in 0.03 M Tris-HCl (pH 7.6) containing 10 mM MgSO4 and layered on top of a discontinuous sucrose density gradient of 50-60% sucrose in the same buffer, and centrifuged for 2 hr at 68,000 X g in a SW 27 rotor. Membranes from cells grown on unsaturated fatty acids separated in two bands above the 58% solution, while membranes containing Bromo fatty acids banded above the 56% sucrose solution. A barely visible pellet was observed at the bottom of the tubes that consisted mainly of whole cells and unbroken spheroplasts. The membranes were collected and subjected to a second purification by sucrose density gradient centrifugation.

Membranes were routinely examined by the electron microscope after fixation with glutaraldehyde and osmium tetroxide. The preparations consisted essentially of single and double membrane-bounded vesicles similar to those described by Kaback for strains of E. coli K12, or W (17). The membranes displayed a clear "unit membrane" structure about 7 nm (70 Å) in width; there was little evidence of contamination from nonmembranous material. Protein was measured by the method of Lowry et al. (18) after the membranes were dissolved in 1 N NaOH.

Lipid Extraction and Fatty Acid Analysis. Extraction of lipids and isolation of the phospholipid fraction were described (4, 8). The total lipid extract of the whole cells was partitioned between chloroform and 0.5 M Na2CO3 to remove the residual unsaturated fatty acid carried over from the growth medium. The chloroform layer was washed with distilled water. After evaporation to near dryness, the lipid solution was further purified by successive rounds of addition and evaporation of benzene. Finally, the total lipids were taken up in chloroform and separated from the precipitated impurities by filtration through fine-pore sintered glass. The

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fatty acid composition of the phospholipids and total lipids were determined as described (4, 8). 12-Bromostearic acid was synthesized from 12-hydroxystearic acid (19). The details of its analysis will be published elsewhere.

Proline Uptake. Uptake of proline by the membranes was assayed according to the procedure of Kaback (17).

Succinic Dehydrogenase Activity. Succinic dehydrogenase (EC 1.3.99.1) activity of the membranes was measured according to the method of Guiditta and Singer (20), with 2,6-dichlorophenol indophenol as an electron acceptor. The temperature of the cuvette compartment of a Gilford model 2000 was controlled with the use of a Haake thermostat. The temperature of the reaction mixture was continuously monitored with a thermistor probe.

X-ray Diffraction. For X-ray diffraction, membranes were pelleted from distilled water at 150,000 × g for 30 min and a sample was either sealed in a thin-glass capillary tube, or placed across the gap of a plastic specimen holder, and mounted in a thermally controlled specimen chamber fitted with thin Melinex windows. In the case of specimens on plastic holders, the humidity of the atmosphere surrounding the sample was controlled by a stream of moist nitrogen as described (21). Samples of membrane lipid extracts were mixed with water (about 1:1), sealed in a 1-mm glass capillary tube, and allowed to equilibrate for 24 hr. The specimen chamber was mounted on a wide-angle diffraction camera in which the X-ray beam was collimated by a system of pinholes, and the diffraction patterns were recorded on an Ilford Industrial G X-ray film over 6 hr by the use of nickel-filtered Cu Ka radiation.

RESULTS

X-ray diffraction

Thermal phase transitions were observed in all the membrane preparations studied. The transition was characterized in the wide-angle diffraction pattern by a gradual change from a diffuse band centered at a Bragg spacing of 460 pm (4.6 Å) to a sharp band at 420 pm (4.2 Å) as the temperature was lowered (Fig. 1). Microdensitometer traces and visual inspection of the diffraction patterns showed that this change took place over a temperature range of about 10°C. The temperature ranges, together with the approximate mid-point temperature for this transition for membranes from cells grown on five different fatty acids are shown in Table 1. From this table, it can be seen that the fatty acid used to support growth of the cells greatly affects the transition temperature. However, in considering these effects, the fatty acid composition of the membrane phospholipids must be taken into account and these are listed for the same membrane preparations in Table 2. Introduction of a fatty acid having a more "fluid" or highly expanded character (e.g., linolenic or myristoleic acid) in place of the oleic acid supplement might have been expected to have decreased the transition temperature, but due to the compensatory lower incorporation of these acids (from 52 to 30%) the transition temperature was raised (from 24 to 41°C). In the case of a trans-un satu rated fatty acid (e.g., elaidic acid), the more condensed character of this lipid over the normal cis acid,

Table 1. Effect of supplemental fatty acid on transition temperatures as determined by X-ray diffraction

<table>
<thead>
<tr>
<th>Fatty acid added to growth medium</th>
<th>Transition temperature (°C)*</th>
<th>Membrane</th>
<th>Lipid extract</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oleate</td>
<td>19–20 (24)</td>
<td>6–14 (10)</td>
<td></td>
</tr>
<tr>
<td>Linolenate</td>
<td>36–46 (41)</td>
<td>21–31 (28)</td>
<td></td>
</tr>
<tr>
<td>Myristoleate</td>
<td>36–46 (41)</td>
<td>—</td>
<td></td>
</tr>
<tr>
<td>Elaidate</td>
<td>30–40 (35)</td>
<td>—</td>
<td></td>
</tr>
<tr>
<td>12-Bromostearate</td>
<td>19–20 (24)</td>
<td>—</td>
<td></td>
</tr>
</tbody>
</table>

* The values given are the range of temperatures during which transition occurs. The values in parentheses represent the midpoint transition temperature.
and its increased incorporation into the membrane phospholipids, results in an increase in the transition temperature. In all of these instances, the transition temperatures were at or below the temperature at which the cells were grown.

In addition to the above mentioned diffraction bands a diffuse band at about 1.05 nm (10.5 Å) was observed as well as the very broad diffraction band due to water at 340 pm (3.4 Å). Both these bands were unaffected over the temperature range (0-46°C) that was used in this study.

FIG. 5. Arrhenius plots for succinic dehydrogenase activity. Each reaction mixture (1.0 ml) contained 0.02 M succinate-0.01 M KCN-0.05 M potassium phosphate (pH 7.6), and 60 µM 2,6-dichlorophenol indophenol. The reaction was started by addition of 10-20 µl of membrane suspension [3.5 mg of protein per ml of 0.1 M potassium phosphate (pH 6.6) containing 0.01 M MgSO4]. Membranes were prepared from cells supplemented with olate (●), elaidate (■), and linolenate (▲).

Similar phase transitions were observed in hydrated samples of a total lipid extract from cells grown on oleic or linolenic acids (Fig. 2). The temperature ranges for these transitions (shown in Table 1) were different for the two supplemental fatty acids, and were also substantially below the transition ranges for intact membranes grown on the same fatty acid supplements. This difference was not due to oxidation of unsaturated fatty acids after exposure to X-rays, since analyses of the lipids before and after X-ray exposure did not show such oxidation. The absence of the diffuse 1.05 nm (10.5 Å) diffraction band in the diffraction pattern from the isolated lipids indicated that this band in the pattern from membrane samples was due to some component that is not a lipid.

In membrane samples that had been partially oriented, the sharp 420 pm (4.2 Å) band showed an enhanced intensity in the equatorial direction (Fig. 3) that was perpendicular to the plane of orientation of the membranes. These membranes were oriented by removal of free intermembranous water but the presence of the weak 340 pm (3.4 Å) band due to water indicated that the membranes were still hydrated.

**Proline uptake**

The effect of temperature and fatty acid supplement on the initial rate of proline uptake by the membranes is shown in Fig. 4. The temperature at which discontinuities in the Arrhenius plots were observed are in decreasing order from elaidic (26°C)-to oleic (19°C)-to linolenic acid (14°C), indicating a sensitivity of the proline transport process to the degree of unsaturation in the supplemental fatty acids. The slow rate of uptake below the transition temperature was not due to leakiness of the membrane, since in control experiments (data not shown), vesicles preloaded with [14C]-proline at temperatures above transition did not lose their radioactivity when incubated at temperatures below transi-
tion. As noted by Wilson and Fox (10) for both the glucoside and galactoside transport systems, the slopes of the Arrhenius plots above the transition temperatures are similar for different fatty acid supplements, but vary below these temperatures. The data indicate that the lipid sites in the membrane that are oriented generally in the plane of the membrane (4.6 A) 460 pm recently (also in PHAGE Transitions in E. coli Membranes 3183

Succinic dehydrogenase activity

The succinic dehydrogenase activity of the membranes also exhibited discontinuities in the Arrhenius plots, which are unique for each type of membrane (Fig. 5) but, unlike the proline uptake, the order at which the discontinuities were observed does not follow the order in the degree of unsaturation in the supplemental fatty acid (oleate 11°C, linolenate 23°C, and elaidate 22°C). Furthermore, the increase in slopes below the transition temperatures is not as pronounced as in the case of proline uptake (Fig. 4). Thus, it appears that the succinic dehydrogenase activity of the membrane is not as sensitive to its lipid environment as is the proline uptake, and that it could function with either fully saturated or highly unsaturated lipids. As in the case of transport, the slope of the Arrhenius plots are comparable above, but vary below the transition temperatures. It should be noted here that the effect of lipid composition on the succinic dehydrogenase activity at various temperatures could be the resultant of several processes that are required for the expression of this activity such as substrate or dye penetration into the membrane or the actual lipid–enzyme interactions. In any case, the response of this membrane activity to temperature and lipid composition is quite different from that of proline uptake and can indicate an association with lipids that are structurally distinguishable from those associated with the site involved in transport of proline.

DISCUSSION

The similarity between the broad 460-pm (4.6 Å) diffraction band that is generally obtained from biological membranes and that obtained from liquid paraffins (22) indicates that the apolar part of the membrane lipids is in a liquid (or expanded) state. Comparison with the sharp 420-pm (4.2 Å) band from the hexagonal phase of long-chain paraffins (23) and also with certain phospholipid-water mixtures (24) indicates that the sharp band is due to the close hexagonal packing of the hydrocarbon chains. On this basis, the gradual thermal transition observed in the diffraction patterns of these membranes has been attributed to a phase change in the fatty acyl chains from the closely packed (condensed) state to a more fluid (expanded) state. Such a phase change has previously been detected in the membrane of Mycoplasma laidlawii by the use of differential scanning calorimetry (1, 11) and X-ray diffraction techniques (2).

An orientation, perpendicular to the low-angle diffraction, in the intensity of the broad 460-pm (4.6 Å) band has been observed in diffraction patterns from myelin (25) and more recently in patterns from erythrocyte ghosts (26) and disc membranes of retinal-rod outer segments (27). Although the 460-pm (4.6 Å) band may contain contributions from membrane components other than lipids, such an orientation (also seen in these studies in the 420-pm (4.2 Å) band) indicates that the hydrocarbon chains of the lipids are generally oriented perpendicular to the plane of the membrane. Whether this is due to a monolayer or bilayer of lipid cannot be uniquely determined from the present results, but the structural feature of the unit membrane seen in electron micrographs of these preparations indicates that the E. coli membrane contains a lipid bilayer. Current work using low-angle diffraction techniques on membranes from cells grown on bromostearic acid (which provides a heavy atom label in the membrane) will enable us to give a more detailed description of the structural characteristics of the E. coli membrane. Recently, Engelmann (28) studied the low-angle X-ray diffraction of mycoplasma membranes and concluded that a bilayer is the predominant lipid structure.

The difference in the transition temperature detected between membranes and lipid extracts (Table 1) may be due to many factors; one such factor could be the immobilizing effect of the nonlipid components of the membrane (i.e., proteins) on the physical state of the lipids within the membrane. A previous analysis of wide-angle diffraction patterns of membranes and their lipid extracts (29) has also indicated such an effect. More recently, Rottom et al. (13) showed that the freedom of motion of spin labels in the native Mycoplasma membrane was less than that in dispersed mycoplasmal membrane-lipids, indicating the influence of membrane proteins on the physical state of membrane lipids. In any case, these results emphasize the need for the measurement of the physical state of the lipids while within the membrane and in association with other membrane components in order to draw meaningful conclusions about the relationship between that physical state and functional properties of the membrane.

Discontinuities in the Arrhenius plots of several membrane-associated functions have been interpreted in terms of a phase change in the membrane lipids (5, 7, 10, 30). That is, the increase in activation energy below the discontinuity is a reflection of the closer packing of the hydrocarbon chains of the membrane lipids. The discontinuity observed in the Arrhenius plot of either proline uptake or succinic dehydrogenase activity is unique for each membrane.

The temperatures at which physical changes in membrane lipids were detected by X-ray diffraction do not coincide with temperatures at which breaks in the Arrhenius plots of proline uptake or succinic dehydrogenase activity were observed. One of two major conclusions can be drawn from this dis-
parity. (a) Either that the lipid environment associated with these functions is identical, due to the random distribution of lipids in the membrane, and that the uniqueness of the discontinuities in the Arrhenius plots is due mainly to differences in lipid–protein interactions; (b) or that the disparity observed is due mainly to a heterogeneity in the molecular organization of the membrane. That is, within a given membrane, a specific class of lipids having a physical character determined by its apolar as well as polar groups, is associated with a specific function. While we leave the former hypothesis (a) open and subject to further investigation, we present some observations compatible with the latter (b).

The temperature of discontinuities in the Arrhenius plots of proline uptake and succinic dehydrogenase activity are all below the transition temperatures observed by X-ray diffraction. This indicates that, while the majority of the membrane lipids are in a condensed state, those associated with these two functional properties are in a more fluid state. The broad temperature range of the transition detected by X-ray diffraction could then be due to the overlapping of several phase transitions of different membrane lipid components.

If we consider only the apolar groups, the possible lipid types available in membranes from cells grown on three different fatty acid supplements are shown schematically in Fig. 6. This presentation was based on the data shown in Table 2 and Silbert’s observations on the positional distribution of fatty acids in the molecular types of phospholipids in unsaturated fatty acid auxotrophs of E. coli (6). In the case of elaidate-enriched membranes, the fluid character of the different lipid components is more similar to each other than is the case for linolate-enriched membranes, because the physical properties of trans- acids are close to those of saturated acids (31); oleate membranes have intermediate properties. Also, the fact that only 30% of the fatty acids of the lipids of linolate-enriched membranes are unsaturated tends to provide a wider spectrum in the fluidity of the different lipid components than is encountered with elaidate and oleate-enriched membranes (Fig. 6).

The more homogeneous nature of the lipid components in elaidate-enriched membranes is reflected by the observation that the transition temperatures for proline transport and succinic dehydrogenase activity are closer to each other and to the lipid phase transition temperature than is the case for membranes containing oleic or linolic acids. While the order of the transition temperatures for proline uptake follows the order in the degree of fluidity of the fatty acid supplement, this is not the case for succinic dehydrogenase activity. This could indicate a more stringent requirement of proline transport for the more fluid lipid components of a membrane. A similar sensitivity of sugar transport to the fatty acid supplement has been observed in other fatty acid auxotrophs of E. coli (5, 7, 10) and Wilson and Fox suggested that sugar transport sites may be associated with the most mobile or liquid sites on the membrane. However, the observation that the order of transition temperatures for succinic dehydrogenase activity does not follow the order in the degree of fluidity of the fatty acid supplements is indicative of a lesser sensitivity of this activity to its lipid environment.

Similar disparities in the discontinuities of Arrhenius plots for respiration and sugar transport have been observed by Overath et al. (5) and these may also be interpreted in terms of a heterogeneous distribution of lipid within the membrane. The results presented here together with other observations (10, 32–34), concerning the effects of lipids on the functional properties of membranes, are consistent with there being a heterogeneity in the distribution of lipids within the membrane and that in a given membrane specific lipids are required for the expression of optimal activity of specific membrane functions.

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