Biochimica et Biophysica Acta, 729 (1983) 161–166
Elsevier Biomedical Press

BBA 71579

ELECTRON SPIN RESONANCE PROBING OF LIPOPOLYSACCHARIDE DOMAINS IN THE OUTER MEMBRANE OF *ESCHERICHIA COLI*

RICHARD T. COUGHLIN [a], A. HAUG [b] and ESTELLE J. McGROARTY [a,c,*]

[a] *Department of Biophysics,* [b] *MSU-DOE Plant Research Laboratory, and* [c] *Department of Biochemistry, Michigan State University, East Lansing, MI 48824 (U.S.A.)*

(Received September 20th, 1982)
(Revised manuscript received December 15th, 1982)

Key words: ESR; Lipopolysaccharide domain; (E. coli membrane)

(1) Scatchard analysis of the binding of the cationic electron spin resonance probe 4-(dodecyldimethylammonium)-1-oxyl-2,2,6,6-tetramethylpiperdine bromide to lipopolysaccharide and to phospholipid indicated that this probe has a 5 fold greater affinity for anionic lipopolysaccharide than for phospholipid. In the intact outer membrane this cationic probe likely associates with the lipopolysaccharide-containing outer monolayer. (2) The temperature dependence of the mobility of the cationic spin probe in the outer membrane indicates that a structural transition occurs at 9°C in the outer monolayer. (3) A similar 9°C transition was detected in the outer membrane using the spin probe 5-doxyl stearate. This anionic probe has been shown to preferentially partition into the phospholipid enriched domains of the outer membrane. (4) A porin-lipopolysaccharide-peptidoglycan complex probed with the cationic probe also was shown to undergo a thermally induced structural change at 9°C. (5) Purified lipopolysaccharide, however, was shown to have structural transitions at 20°C and 40°C. (6) It is proposed that a structural rearrangement of the intact outer membrane occurs at approx. 9°C in both the lipopolysaccharide and phospholipid domains of the outer membrane. Furthermore, this structural transition appears to be highly dependent on lipid-protein interactions. A second thermotropic transition that occurs in the outer membrane at approx. 40 to 42°C appears to result mainly from changes in the lipopolysaccharide domain structure.

Introduction

The outer membrane of gram negative bacteria is a highly asymmetric structure. The outer monolayer is composed of anionic lipopolysaccharide and protein, whereas the inner monolayer contains phospholipid and protein. Moreover, phospholipids and lipopolysaccharide isolated from *Escherichia coli* upon reconstitution are reportedly unable to coexist in a single phase [1]. Within the intact outer membrane of *Salmonella*, spin labeled fatty acids have been shown to preferentially partition into the phospholipid domains of the inner monolayer [2] perhaps due to the greater fluidity of these domains compared to that of the lipopolysaccharide domains, and to charge repulsion between the anionic head groups of the fatty acid probe and lipopolysaccharide. Analysis of extracted phospholipid from the outer membrane of 37°C grown *E. coli* indicated that the phospholipid undergoes a structural transition at approx. 4 and

* To whom correspondence should be addressed.

Abbreviations: Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; CAT_{12}, 4-(dodecyldimethylammonium)-1-oxyl-2,2,6,6-tetramethylpiperidine bromide; SDS, sodium dodecyl sulfate; ESR, electron spin resonance.

24°C (Ref. 3 and unpublished data). Results reported here demonstrate that the structural transition of purified lipopolysaccharide begins at 20°C and ends at 40°C. We also found that the cationic electron spin resonance (ESR) probe 4-(dodecyldimethylammonium)-1-oxyl-2,2,6,6-tetramethyl bromide (CAT_{12}) preferentially partitions into lipopolysaccharide compared to phospholipid domains. Applying this cationic probe and the acyl chain probe 5-doxyl stearate we have evidence to suggest that both lipid domains located on either side of the outer membrane undergo a cooperative temperature dependent rearrangement. The onset of this rearrangement at 9°C in both lipid domains of the intact membrane appears to be determined mainly by protein-lipid interactions. In contrast, the end of the thermotropic phase change in the intact outer membrane at around 42°C may be determined by rearrangements within the lipopolysaccharide domains.

Materials and Methods

Cell growth and membrane isolation

Culture of *E. coli* strain W1485 F^- were grown at 37°C in M9 minimal medium supplemented with 0.4% glucose (final concentration). Cultures were harvested, and the cytoplasmic and outer membranes were isolated and characterized as described previously [4]. The degree of purity of the separated membranes equalled or exceeded that previously described [4].

Lipid isolation

Lipopolysaccharide was extracted from whole cells with aqueous phenol [5]. The extracted lipopolysaccharide was sedimented at $78000 \times g$ for 60 min and washed twice with double distilled water. The resuspended sample was dialyzed extensively against double distilled water yielding the native lipopolysaccharide product. Electrodialysis of native lipopolysaccharide resulted in an acidic sample (pH approx. 4) which was neutralized with NaOH to pH 7. Phospholipid was extracted from isolated outer membranes with chloroform/methanol (2 : 1, v/v) as previously indicated [6].

Porin-lipopolysaccharide-peptidoglycan isolation

A porin-lipopolysaccharide-peptidoglycan complex was isolated from cells grown and harvested as described above. Cells were washed in 10 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (Hepes), pH 7.5, and lysed using a French pressure cell at 12000 lb/inch^2. Total membranes were treated with 20 μg/ml deoxyribonuclease I (Sigma Chemical Co.), pelleted and washed twice with double distilled water. The porin-lipopolysaccharide-peptidoglycan complex was extracted with 1% sodium dodecyl sulfate (SDS). The protein composition of the complex was characterized on SDS polyacrylamide slab gels [7].

Assays

Succinate dehydrogenase activity was determined by the method of Osborn et al. [8] to quantitate cytoplasmic membrane levels. Lipopolysaccharide levels were quantitated by analysis of 3-deoxy-D-mannooctulosonic acid content [9]. Protein concentration was assayed using the procedure of Lowry et al. [10]. Peptidoglycan levels were quantitated by measuring the muramic acid content of acid hydrolyzed peptidoglycan [11].

Spin labeling

The fatty acid spin probe 5-doxyl stearate was dissolved as a 30 mM solution in absolute ethanol. An aliquot of the probe was dried onto the bottom of a clean test tube under a stream of N_2, and the sample was added at room temperature with mild vortexing. The spin label comprised less than 0.1% of the lipid weight. The CAT_{12} was always less than 0.3% of that of the lipid acyl chains present in the sample. The partitioning of CAT_{12} into lipid was calculated as previously described [12]. All electron spin resonance experiments were carried out as described earlier using a Varian Century Line electron spin resonance spectrometer X-band, model E112 [13]. The temperature dependence of the order parameter, S, and the hyperfine splitting parameter, $2T_{\parallel}$, was analyzed in terms of linear components using a linear regression program developed for transition point analysis [14]. Discontinuities in plots of the temperature dependence of $2T_{\parallel}$ and S are interpreted to reflect structural rearrangements of the lipid domains in which the probe is bound [15].

Results

Membrane spin labeling

Two electron spin resonance probes, CAT_{12} and 5-doxyl stearate (Fig. 1), were used to monitor intact outer membranes, isolated lipopolysaccharide and porin-lipopolysaccharide-peptidoglycan complexes. Negatively charged 5-doxyl stearate has been shown to partition into those lipid domains which contain lower levels of anionic groups. The position of the nitroxide group allows 5-doxyl stearate to probe acyl chain mobility. In contrast, the positively charged CAT_{12} is thought to partition into more anionic lipid domains, and the position of the nitroxide within the head group allows CAT_{12} to probe the motion within the lipid head group region. ESR spectra of CAT_{12} in outer membranes indicate that the spin label movement in the headgroup region is sensitive to temperature (Fig. 2). At temperatures above 9°C broadening of the low field peak suggests that CAT_{12} partitions into more than one environment. Fig. 3 shows the temperature dependence of $2T_{\parallel}$ reported by CAT_{12} and 5-doxyl stearate bound to outer and cytoplasmic membranes. Above 30°C line broadening of the CAT_{12} signal in the outer membrane was too great to allow resolution of $2T_{\parallel}$. At low temperatures, the CAT_{12} in the outer membrane appears to experience a slightly more rigid environment than does 5-doxyl stearate. In contrast, the CAT_{12} probe in the cytoplasmic membrane appears to reside in a similar or slightly more fluid environment than 5-doxyl stearate.

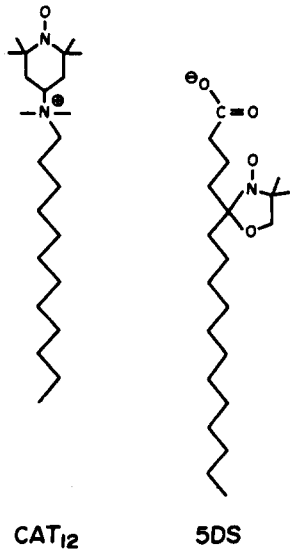

Fig. 1. The chemical structure of the two electron spin resonance probes CAT_{12} and 5-doxyl stearate (5DS).

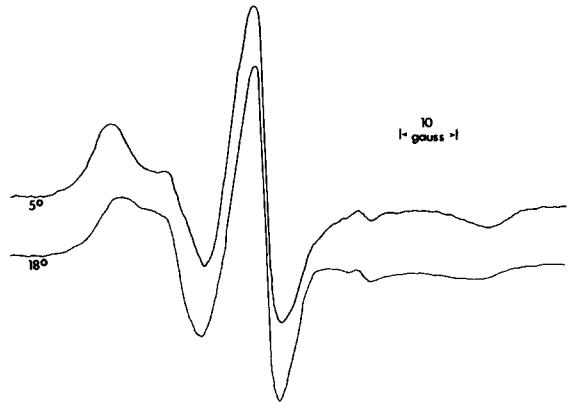

Fig. 2. Electron spin resonance spectra of CAT_{12} bound to intact outer membranes from cells grown at 37°C, in 10 mM Hepes, pH 7.5. The membrane concentration was approx. 20 mg/ml protein and probe was added at a 1:300 (probe:lipid acyl chain) dilution. The spectra were recorded at 5 and 18°C.

Lipid spin labeling

The partitioning of CAT_{12} into purified phos-

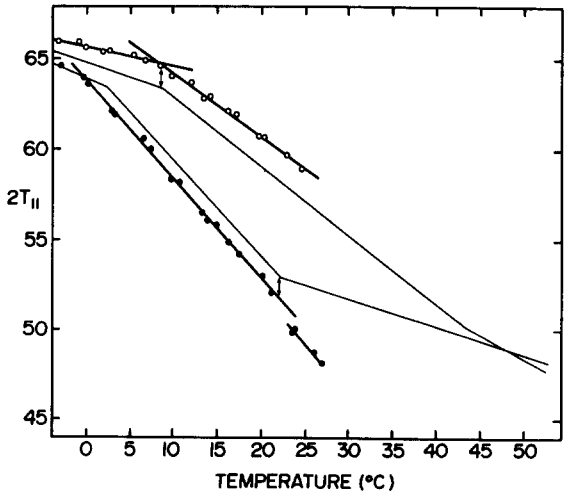

Fig. 3. The temperature dependence of the hyperfine splitting parameter, $2T_{\parallel}$. of CAT_{12} bound to outer membranes (○) and cytoplasmic membranes (●) from *E. coli* W1485F⁻ grown at 37°C. Results of 5-doxyl stearate (solid line) probed outer membranes (top) and cytoplasmic membranes (bottom) are included for reference (Ref. 4).

pholipid and lipopolysaccharide was analyzed at 37°C as a function of probe concentration in order to assess the preferential association of CAT_{12} with specific domains in the outer membrane. From Scatchard analysis of CAT_{12} binding (Fig. 4) it can be concluded that this cationic probe has approx. 5-fold greater binding affinity for lipopolysaccharide than for phospholipid. The binding of this probe to both types of lipid appears to be non-cooperative and at a single type of site. Similar Scatchard analysis using 5-doxyl stearate is not possible because of micelle formation at high probe concentrations.

Plots of the temperature dependence of the spectral parameters, $2T_{\parallel}$ and S, for 5-doxyl stearate and CAT_{12} bound to purified lipopolysaccharide, indicate discontinuities at 20°C and 40°C (Fig. 5). These results suggest that structural transitions occur at these temperatures which can be detected within both the headgroup and the acyl chain regions. Comparison of ESR spectra of CAT_{12} bound to isolated lipopolysaccharide and intact outer membrane measured at 37°C indicates that

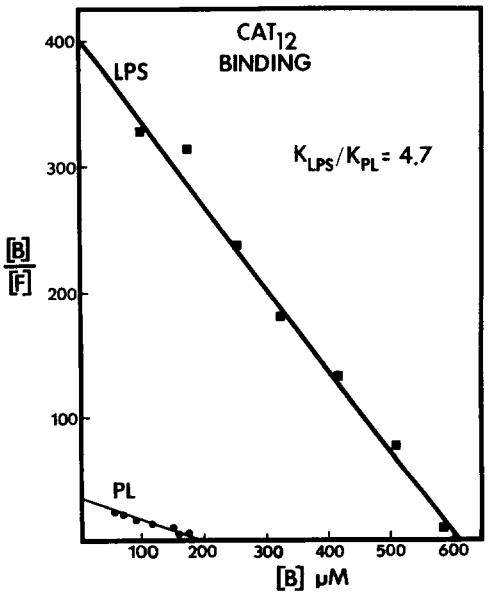

Fig. 4. Scatchard analysis of CAT_{12} binding to electrodialyzed lipopolysaccharide (■, LPS) and to phospholipid (●, PL) from the outer membrane of *E. coli* W1485F⁻ grown at 37°C. CAT_{12} partitioning was measured at 37°C on samples of lipopolysaccharide and phospholipid at 500 μM concentration as a function of probe concentration.

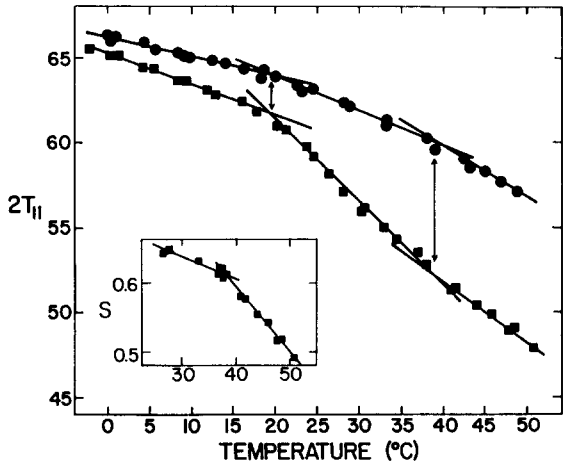

Fig. 5. The temperature dependence of the hyperfine splitting parameter, $2T_{\parallel}$, and the order parameter, S, (Insert) of CAT_{12} (●) and 5-doxyl stearate (■) bound to electrodialyzed lipopolysaccharide.

the headgroup region in the pure lipopolysaccharide is more rigid than that of lipopolysaccharide in the intact membrane (data not shown).

Porin-lipopolysaccharide-mucopeptide labeling

To assess whether lipopolysaccharide tightly associated with protein was similar in its fluid prop-

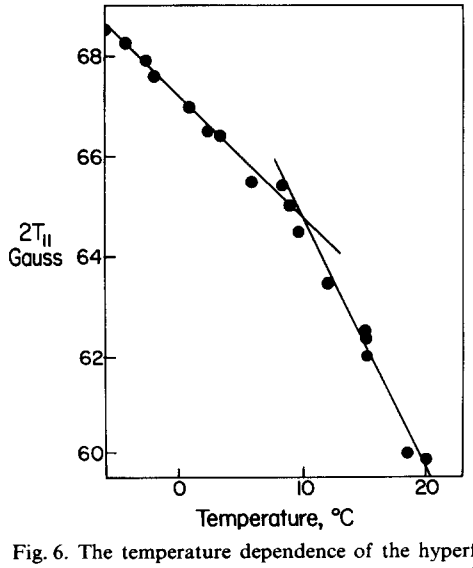

Fig. 6. The temperature dependence of the hyperfine splitting parameter, $2T_{\parallel}$ of CAT_{12} bound to porin-lipopolysaccharide-peptidoglycan complexes isolated from *E. coli* W1485F⁻ grown at 37°C.

erties to that in the intact outer membrane or to the isolated lipopolysaccharide, a porin-lipopolysaccharide-mucopeptide complex was isolated and probed with CAT_{12}. The porin-lipopolysaccharide-mucopeptide complex was shown to contain approx. 70% protein, 15% lipopolysaccharide and between 5 and 15% peptidoglycan by weight. SDS polyacrylamide gels indicated that the protein was comprised of approximately equal amounts of porins 1a and 1b with only minor contamination from other membrane proteins (data not shown). These complexes were probed with CAT_{12}, and the plots of the temperature dependence of $2T_{\parallel}$ indicated a discontinuity at 9°C (Fig. 6). The mobility of the probe within this complex was similar to that of the probe bound to intact outer membranes when compared at temperatures above 9°C. Below 9°C, the head group mobility detected with CAT_{12} in the isolated porin-lipopolysaccharide-mucopeptide complex was comparable to that of isolated lipopolysaccharide but less than that of the intact outer membrane.

Discussion

Thermotropic structural rearrangements have been shown to occur in the lipid domains of biological membranes, and these transitions usually occur over a broad temperature range [15]. These changes often affect membrane enzyme activities and other membrane functions [16]. It is also known that the presence of membrane proteins and the composition of the lipid's acyl chains dramatically affect the temperature of the membrane's thermotropic rearrangement of the lipid components and the lipid acyl chain mobility. In this study we set out to determine whether the structure of pure lipopolysaccharide was different from that of the glycolipid associated with protein in the intact outer membrane and in isolated protein-lipopolysaccharide-mucopeptide complexes. Previously we had shown that 5-doxyl stearate labeled outer membranes undergo thermotropic structural transitions at temperatures different from those of the extracted phospholipids (Ref. 3 and unpublished data). Outer membranes from cells grown at 37°C labeled with 5-doxyl stearate appear to undergo a transition beginning at 9°C and ending at 42°C whereas the phospholipid extracted from the same membrane undergoes a structural transition between 4 and 24°C (Ref. 3 and unpublished data). Since 5-doxyl stearate is reported to probe phospholipid domains of the outer membrane [2], this probe was of limited use in evaluating the importance of other outer membrane components in modulating the transition temperature.

We report here the use of a cationic electron spin resonance probe, CAT_{12}, to analyze the structure of the outer membrane. We have shown that CAT_{12} preferentially partitions into lipopolysaccharide more readily than into phospholipids, and in the intact outer membrane this probe likely partitions with the same specificity. Using this probe to analyze the structure of lipopolysaccharide in the intact membrane we have found that lipopolysaccharide associated with protein in the intact membrane and in the isolated porin-lipopolysaccharide-peptidoglycan complex has a thermotropic transition beginning at 9°C. In contrast, extracted lipopolysaccharide probed with either CAT_{12} or 5-doxyl stearate undergoes a thermotropic transition starting at 20°C and ending at 40°C. Since the lipopolysaccharide domains in the intact outer membrane, probed with CAT_{12}, appear more fluid compared with pure lipopolysaccharide, it is proposed that proteins when associated with lipopolysaccharide disorder the head group region. Such disordering of the lipopolysaccharide may also cause the decrease in the temperature of the beginning of the thermal transition. Similar decreases in the thermal transition of phospholipids have been reported when the lipid is mixed with membrane proteins [17]. The end of the thermal transition in the intact outer membrane can also be detected using the anionic probe 5-doxyl stearate. The end of the transition in the intact membrane detected with 5-doxyl stearate (42°C) is very similar to the end of the transition of the isolated lipopolysaccharide (approx. 40°C). Thus it would seem that temperature dependent structural rearrangements which occur in the lipopolysaccharide domains of the outer monolayer affect the structure of the phospholipid domains of the inner monolayer. Since in the intact outer membrane both 5-doxyl stearate and CAT_{12} detect a 9°C structural transition, both lipid domains

may be coupled in their structural rearrangements, perhaps through the structure or packing of transmembrane proteins.

In the porin-lipopolysaccharide-peptidoglycan complexes, CAT_{12} detected a more rigid environment below 9°C than was detected with the same probe in the intact outer membrane. The domains being probed in these two structures are likely to be similar in composition. However, in the porin-lipopolysaccharide-peptidoglycan complex the lipopolysaccharide present is probably tightly bound to the protein. We have shown that this porin-lipopolysaccharide complex is enriched in divalent cations compared to the intact outer membrane (unpublished data), and these cations may be involved in tightly binding lipopolysaccharide to the porin proteins.

The cationic probe CAT_{12} detected a slightly more rigid environment in the outer membrane than did 5-doxyl stearate (Fig. 3). These differences are more difficult to interpret due to differences in the location of the probes within the membrane. First, the nitroxide free radical in the CAT_{12} probe is located in the headgroup region and is exposed to a polar aqueous environment. In contrast, the nitroxide free radical in 5-doxyl stearate is located five carbons into the acyl chain of the probe and is likely probing the mobility of the lipid acyl chains at this level. Secondly, as previously suggested, these two probes are probing different lipid domains within the outer membrane which may have dramatically different head group and/or acyl chain mobility. However, even though 5-doxyl stearate and CAT_{12} locate in different environments, both reported a similar structural transition beginning at 9°C. These results suggest the involvement of transmembrane proteins in coupling structural changes between the two monolayers.

Acknowledgements

This work was supported in part by a grant from Research Corporation, by BRSG Grant 2-SO RR07049-15 awarded by the Biomedical Research Support Grant Program, Division of Research Resources, National Institutes of Health, and by United States-DOE contract DE-AC02-76-ER-1338.

References

1 Takeuchi, Y. and Nikaido, H. (1981) Biochemistry 20, 523–529
2 Nikaido, H., Takeuchi, Y., Ohnishi, S.-I. and Nakae, T. (1977) Biochim. Biophys. Acta 465, 152–164
3 Janoff, A.S., Gupte, S. and McGroarty, E.J. (1980) Biochim. Biophys. Acta 598, 641–644
4 Janoff, A.S., Haug, A. and McGroarty, E.J. (1979) Biochim. Biophys. Acta 555, 56–66
5 Galanos, C., Lüderitz, O. and Westphal, O. (1969) Eur. J. Biochem. 9, 245–249
6 Folch, J., Lees, M. and Stanley, G.H.S. (1957) J. Biol. Chem. 226, 497–509
7 Ames, G.F.-L. (1974) J. Biol. Chem. 249, 634–644
8 Osborn, M.H., Gander, J.E., Parisi, E. and Carson, J. (1972) J. Biochem. 247, 3962–3972
9 Dröge, W., Lehmann, V., Lüderitz, O. and Westphal, O. (1970) Eur. J. Biochem. 14, 175–184
10 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randal, R.J. (1951) J. Biol. Chem. 193, 265–275
11 Hadzija, O. (1974) Anal. Biochem. 60, 512–517
12 Melhorn, R.J. and Packer, L. (1979) Methods Enzymol. 56, 515–526
13 Janoff, A.S., Coughlin, R.T., Racine, F.M., McGroarty, E.J. and Vary, J.C. (1979) Biochem. Biophys. Res. Commun. 89, 565–570
14 Brunder, D.G., Coughlin, R.T. and McGroarty, E.J. (1981) Comput. Biol. Med. 11, 9–15
15 Steim, J.M., Edner, O.J. and Bargoot, F.G. (1968) Science 162, 909
16 Linden, C.D., Wright, K.L., McConnell, H.M. and Fox, C.F. (1973) Proc. Natl. Acad. Sci. U.S.A. 70, 2271–2275
17 Boggs, J.M., Stamp, D. and Mascarello, M.A. (1981) Biochemistry 20, 6066–6072