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Spin labeling of a cysteine residue of the *Escherichia coli* outer membrane lipoprotein in its membrane environment

(electron spin resonance/peptidoglycan/lipoprotein mutant)

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ABSTRACT **A method was developed to attach a spin label to a specific site on the structural lipoprotein of the *Escherichia coli* outer membrane *in situ*. This method takes advantage of the fact that the outer membrane of wild-type *E. coli* contains few residues reactive towards sulfhydryl reagents. A mutant *E. coli* strain has been isolated [Suzuki, H., Nishimura, Y., Iketani, H., Campisi, J., Hirashima, A., Inouye, M. & Hirota, Y. (1976) *J. Bacteriol.* 127, 1494–1501] in which the second position from the carboxy terminus of the lipoprotein is changed from arginine into a cysteine residue. The membrane fraction of this mutant was treated with *N*-(1-oxyl-2,2,5,5-tetramethylpyrrolidinyl)maleimide in the presence of EDTA and 2-mercaptoethanol. Spin label was found to be preferentially incorporated into the lipoprotein. The spectrum of the spin-labeled membrane shows two components, both arising from spin label at the same site near the carboxy terminus. The strongly immobilized component has a maximum hyperfine splitting value of 53 G, and the weakly immobilized component, 37 G. A fraction of the lipoprotein is covalently bound to the peptidoglycan layer through its carboxy-terminal lysine; the spectrum of the isolated bound form of the lipoprotein was identical to that of the free form. When the matrix protein, the other major outer membrane protein, was removed by mutation, the spectrum of the lipoprotein was altered, suggesting that these two proteins are closely associated.**

The outer membrane of *Escherichia coli* contains an unusual lipoprotein of molecular weight 7200 (1, 2). Salient features of this lipoprotein include its abundance (750,000 copies/cell), the covalent attachment of three fatty acid residues near the amino terminus, and the existence of the lipoprotein in two forms, one free and one covalently attached to the peptidoglycan layer through the carboxy-terminal lysine. The great stability of the protein permits its isolation in homogeneous form using harsh detergent solutions (3). Extensive studies have been carried out on the purified lipoprotein and the aggregates it forms *in vitro* (3–5). Recently a lipoprotein mutant (6) has been isolated in which the penultimate arginine residue is replaced by cysteine (7).

Here we describe a method to study the lipoprotein in its membrane environment. Using sulfhydryl-specific reagents and the lipoprotein mutant (6), we found conditions that permit the preferential labeling of the lipoprotein in a membrane fraction by virtue of its exposed sulfhydryl group. A spin label sulfhydryl reagent attached to this sulfhydryl group allowed us to study the environment of the lipoprotein and its interaction with peptidoglycan and the matrix protein, the other major protein of the outer membrane.

Spin labels have been used extensively to study membrane lipids (8) and soluble proteins (9). The acetylcholine receptor of postsynaptic membranes and the ADP carrier of mitochondrial membranes have been studied using spin label analogs that have high affinity for these proteins (10, 11). To our knowledge selective labeling of other membrane proteins with a covalently attached probe has not been reported. The wealth of biochemical and genetic technology available facilitated this project, but the general approach described here might be applicable to other membrane proteins as well.

MATERIALS AND METHODS

Bacterial Strains and Culture Medium. The following strains of *E. coli* K12 were used: K63-1 (F^+ *lpp-1* [previously assigned as *lpm* (6)] *his fadD gal str*); K63-2 (F^+ *lpp*$^+$ *his fadD gal str*). A mutant lacking matrix protein, K63-1-1, was isolated from *E. coli* K63-1 by selecting a TuI phage-resistant strain (12).

Membrane Preparation. The membrane fractions were prepared by differential centrifugation as previously described (13). The bound form of the lipoprotein was prepared as described by Braun and Sieglin (14).

Reaction of Iodoacetic Acid with the Membrane Fraction. The membrane fraction prepared from about 1.5×10^{10} cells was suspended in 2 ml of 0.1 M sodium phosphate buffer, pH 6.8, containing 1% (vol/vol) 2-mercaptoethanol and 1 mM Na_2EDTA. The suspension was incubated at 37° for 2 hr. After the incubation, the membrane suspension was centrifuged at 100,000 × *g* for 30 min. The membrane pellet was resuspended in 0.5 ml of 0.1 M sodium phosphate buffer, pH 6.8, and 10 μCi of iodo[^{3}H]acetic acid (700 Ci/g, New England Nuclear) was added to the suspension. The reaction mixture was incubated at 37° for another 2 hr and then the reaction was stopped by adding 10 μl of 2-mercaptoethanol and 1.5 ml of acetone. The precipitate thus formed was collected by centrifugation at 13,000 × *g* for 10 min and washed with 75% (vol/vol) acetone. The final membrane pellet was then solubilized in 0.2 ml of the solubilizing solution for 20 min at 70° and subjected to sodium dodecyl sulfate ($NaDodSO_4$)/polyacrylamide gel electrophoresis as previously described (13) except that 2-mercaptoethanol was omitted. Disc $NaDodSO_4$/gel electrophoresis was carried out with use of the fluorescent internal molecular weight standards (15). Slab $NaDodSO_4$/gel electrophoresis was carried out according to the method of Anderson *et al.* (16).

Reaction of the Spin Label with the Membrane Fraction. The membrane fraction prepared from 1×10^{11} cells was suspended in 4 ml of 0.1 M sodium phosphate buffer, pH 6.8, containing 1% 2-mercaptoethanol and 1 mM EDTA. The suspension was incubated at 37° for 2 hr. The membrane fraction was recovered from the suspension by centrifugation (100,000 × *g* for 30 min) and the pellet was washed twice with 0.1 M sodium phosphate buffer, pH 6.8. The final membrane pellet

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Abbreviations: $NaDodSO_4$, sodium dodecyl sulfate; ESR, electron spin resonance.

was resuspended in 4 ml of 0.1 M sodium phosphate buffer, pH 6.8, and 2 mg of *N*-(1-oxyl-2,2,5,5-tetramethylpyrrolidinyl)-maleimide (Syva, Palo Alto, CA) was added to the suspension. The reaction mixture was incubated at 0° for 2 hr with gentle shaking and then at 5° for another 10 hr (17). After the reaction, the membrane fraction was collected by centrifugation at 100,000 × *g* for 30 min and washed three times with 0.1 M sodium phosphate buffer, pH 6.8.

Electron Spin Resonance (ESR) Spectra. The membrane preparation was resuspended in 0.1 ml of 0.1 M sodium phosphate buffer, pH 6.8. The samples were then transferred to a 50-μl disposable pipette sealed at one end (8) and ESR spectra were obtained using a Varian E-104A spectrometer equipped with a temperature control accessory (Varian Associates, Palo Alto, CA).

RESULTS AND DISCUSSION

Incorporation of iodoacetic acid into the lipoprotein

E. coli K-63-1 carries a mutation (*lpp-1*) in the structural gene for the lipoprotein. This mutant lipoprotein has an extra cysteine residue, which permits dimer formation of the mutant lipoprotein in the membrane (6). Recently we found that this extra cysteine was derived from the arginine residue at the 57th position as a result of a single point mutation from CG^{U}_{C} (for arginine) to UG^{U}_{C} (for cysteine). Because the mutant lipoprotein has a free sulfhydryl group (SH) not present in the wild type, we treated a membrane fraction with SH reagents in the hope that we could incorporate label specifically into the lipoprotein. When iodo[^{3}H]acetic acid was added to the membrane fraction from *E. coli* K63-1 there was no detectable incorporation of iodoacetic acid into the membrane fraction (data not shown). Pretreatment of the membrane fraction with 2-mercaptoethanol in the presence of EDTA as described in *Materials and Methods* resulted in extensive incorporation of iodo[^{3}H]acetic acid into the membrane fraction. The radioactivity was mainly associated with lipoprotein as judged by NaDodSO$_4$/gel electrophoresis of the membrane fraction (Fig. 1 *upper*). About 80% of total radioactivity incorporated into the membrane fraction was recovered in the lipoprotein peak. No other proteins migrate to this region of the NaDodSO$_4$ gel (1). From the radioactivity, it was calculated that more than 70% of the mutant lipoprotein was labeled with iodo[^{3}H]acetic acid. The addition of EDTA during 2-mercaptoethanol treatment was found to be essential; in the absence of EDTA very low incorporation of iodo[^{3}H]acetic acid into the lipoprotein resulted (Fig. 1 *upper*). Treatment with EDTA removes 30–50% of the lipopolysaccharide from intact cells, rendering the cells permeable to some metabolites and drugs that are normally excluded (18). The bilayer structure of the outer membrane is retained after EDTA treatment and some structural domains are exposed, while others may be altered (19). Our results indicate that the membrane proteins of *E. coli* are not highly crosslinked by disulfide bridges, in contrast to the situation in animal cells (20). Thus, we expect that treatment with 2-mercaptoethanol has little effect on the structure of the membrane.

After solubilization of the membrane fraction from the mutant strain with NaDodSO$_4$, iodoacetic acid treatment labeled the mutant lipoprotein and also a second major protein component of apparent molecular weight 28,000 (Fig. 1 *lower*). This new peak is probably *ompA* protein, another major protein of the outer membrane which is known to contain two cysteine residues (21, 22). These cysteine residues in the *ompA* protein appear not to be reactive toward iodo[^{3}H]acetic acid unless the membrane fraction is solubilized in NaDodSO$_4$. From these

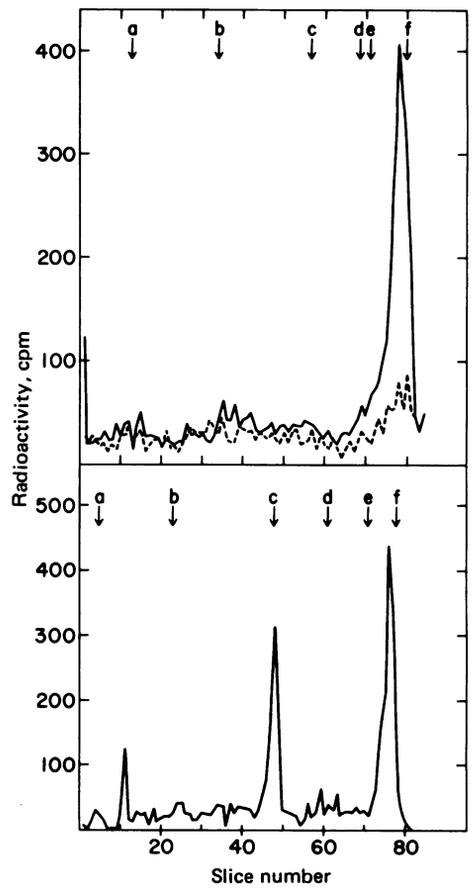

FIG. 1. NaDodSO$_4$/polyacrylamide gel electrophoresis of the membrane fractions treated with iodo[^{3}H]acetic acid. (*Upper*) The membrane fraction from *E. coli* K63-1. The membrane fractions were pretreated with 2-mercaptoethanol in the presence (solid line), and absence (broken line) of EDTA, followed by treatment with iodo[^{3}H]acetic acid. (*Lower*) The membrane fraction from an *lpp-1* mutant strain was solubilized in 1% NaDodSO$_4$ containing 1% 2-mercaptoethanol and then treated with iodo[^{14}C]acetic acid, as described previously (6). Arrows indicate the positions of the internal molecular weight standards: a, dimer, and b, monomer of 5-dimethylaminonaphthalene-1-sulfonyl (dansyl) bovine serum albumin; c, dimer and d, monomer of dansyl egg white lysozyme; e, cytochrome *c*; f, dansylinsulin (15).

results it appears that the mutant lipoprotein in its membrane environment can be modified specifically by virtue of its exposed SH group. Moreover, this procedure allows selective labeling of the lipoprotein because there are few SH groups exposed to the membrane fraction.

Specific spin labeling of the lipoprotein *in situ*

Using the method described above, we were able to incorporate a SH-specific spin label reagent, *N*-(1-oxyl-2,2,5,5-tetramethylpyrrolidinyl)maleimide, into the mutant lipoprotein *in situ*. When wild-type (K63-2) and mutant (K63-1) membrane fractions were treated with the maleimide spin label reagent as described in *Materials and Methods*, 3–5 times as much spin label was incorporated into the K63-1 membrane fraction (Fig. 2).

In order to confirm that the spin label was preferentially incorporated into the lipoprotein, the spin-labeled membrane fraction was subjected to NaDodSO$_4$/gel electrophoresis, the lipoprotein was extracted from the gel, and the spin label paramagnetism in each fraction was assayed by resonance spectrometry. About 70% of the total spin label incorporated was

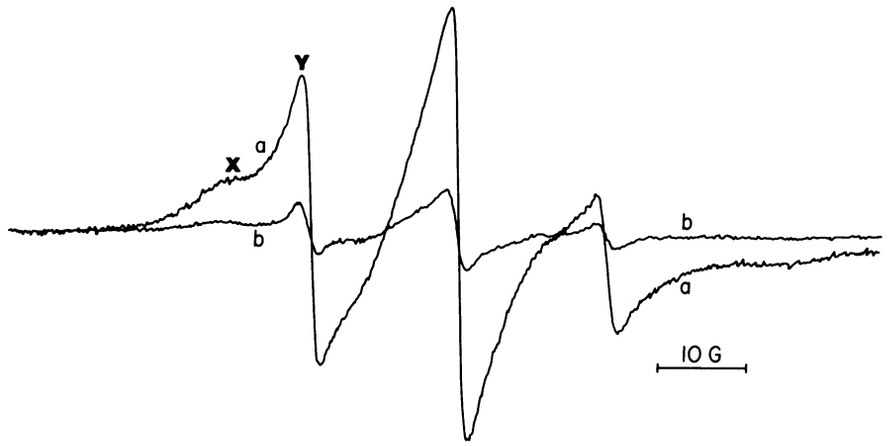

FIG. 2. ESR spectra of the membrane fractions from *E. coli* K63-1 and K63-2. Spin label was incorporated as described in *Materials and Methods*. a, *E. coli* K63-1; b, *E. coli* K63-2.

recovered in the lipoprotein fraction in the case of the mutant strain. In the case of the wild-type strain less than 35% of the total label incorporated was recovered in the lipoprotein fraction. These results, together with the requirement for 2-mercaptoethanol pretreatment, imply that spin label is preferentially incorporated into the cysteine residue at the 57th position of the mutant lipoprotein under these conditions. From these data we estimate that ~10% of the label on the mutant lipoprotein is at sites other than cysteine-57.

The ESR spectra of spin-labeled membrane preparations from the mutant (K63-1) and the wild type (K63-2) are shown in Fig. 2. The K63-1 membrane preparation, carrying spin label predominantly on the cysteine of the lipoprotein, reveals at least two spectral components. These two components are most clearly seen as two peaks (*X* and *Y*) in the low field region of the spectrum. The maximum hyperfine splitting values for these two spectral components are 57 G and 37 G, respectively (1 G = 10^{-4} T). This result requires that labels see at least two different environments, and that these two populations of spin label do not exchange environments rapidly compared to the reciprocal of the frequency separation of *X* and *Y* ($<10^{-7}$ sec) (23).

To test the possibility that the two spectral components might be spin label attached to free and bound forms of the lipoprotein, we separated free and bound forms from a spin-labeled membrane preparation. Free and bound forms of the lipoprotein can be separated by treatment with 2% $NaDodSO_4$, which dissociates the free form from the outer membrane (see *Materials and Methods*). Fig. 3 shows the ESR spectrum of spin-labeled lipoprotein after treatment with 2% $NaDodSO_4$ but before the free form has been removed by centrifugation. In the presence of 2% $NaDodSO_4$ the spin label shows a nearly homogeneous spectrum, quite similar to that of component *Y* in Fig. 2. The lipoprotein retains most of its secondary structure in 2% $NaDodSO_4$, as judged by circular dichroism measurements (5). After centrifugation to pellet the peptidoglycan with the bound form of the lipoprotein, approximately equal amounts of spin label paramagnetism were recovered in the supernatant (free form of the lipoprotein) and pellet fractions. This result requires that both the free and bound form of the lipoprotein are labeled. The ESR spectra of both fractions (data not shown) were identical to the spectrum of the sample before centrifugation (Fig. 3). We therefore cannot exclude the possibility that components *X* and *Y* are related to environmental differences for the free and bound forms, but it seems likely that the motion of the spin label is not influenced by covalent attachment of the carboxy terminus of the lipoprotein to the peptidoglycan layer.

The *N*-(1-oxy-2,2,5,5-tetramethylpyrrolidinyl)maleimide spin label presented two-component ESR spectra when attached to specific sulfhydryl residues of hemoglobin (23), troponin (24), and elongation factor Tu (25). For the case of hemoglobin the spin label assumes two isomeric states relative to the protein (23, 26). It appears from these results that the ESR spectrum of this spin label is not strongly influenced by the particular protein to which it is attached (17). Nevertheless this probe is very

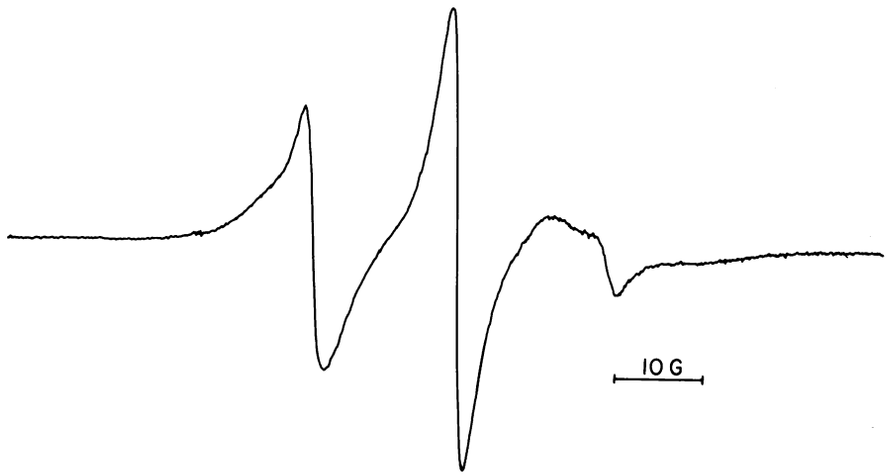

FIG. 3. ESR spectrum of the membrane fraction from *E. coli* K63-1 treated with 2% $NaDodSO_4$ at 100° for 2 min.

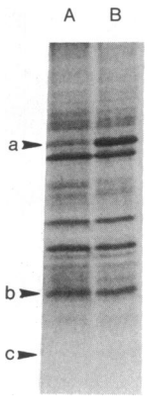

FIG. 4. $NaDodSO_4$/polyacrylamide gel electrophoresis of the membrane fraction derived from K63-1-1 and K63-1. The slab gel electrophoresis was carried out with 17.5% acrylamide. The gel was stained with Coomassie blue. A, K63-1-1; B, K63-1. Arrows a, b, and c indicate the positions of the matrix protein, lipoprotein dimer, and lipoprotein monomer, respectively.

sensitive to conformational changes in the protein (23–25) which are reported either as changes in the mobility of one spectral component or as a change in their ratio. It is, therefore, not surprising that the ESR spectrum of spin-labeled K63-2 membrane is nearly identical in shape to that of labeled K63-1 membranes (Fig. 2).

As a rough estimate, we expect the "extinction coefficient" (23) of component *X* to be roughly one-half that of component *Y*. We then estimate that nearly equal amounts of the spin label experience environments (or conformations) *X* and *Y*. If $NaDodSO_4$ serves to shift the equilibrium between the two hypothetical conformational states, isosbestic points should appear when the membrane preparation is treated with increasing $NaDodSO_4$ concentrations (23).

Interaction of lipoprotein and matrix protein

The other major protein of the outer membrane is the matrix protein of molecular weight 36,500 (27). A derivative (K63-1-1) that lacks the matrix protein was isolated from K63-1. The $NaDodSO_4$ gel pattern of the membrane fraction of K63-1-1 is shown in Fig. 4, where one can see a striking decrease of the matrix protein content. After spin labeling, the K63-1-1 membrane fraction gave an ESR spectrum similar to that for K63-1 (see Fig. 5), except that the ratio of spectral components *X*:*Y* was ~1:2 in K63-1-1 versus ~1:3 in K63-1. The spin label bound to lipoprotein is, therefore, more immobilized in the presence of matrix protein, suggesting that the matrix protein is located near the carboxy terminus of the lipoprotein in the outer membrane. In agreement with this result, DeMartini and Inouye (27) have found that the matrix protein adheres more tightly to the peptidoglycan layer in the presence of the lipoprotein.

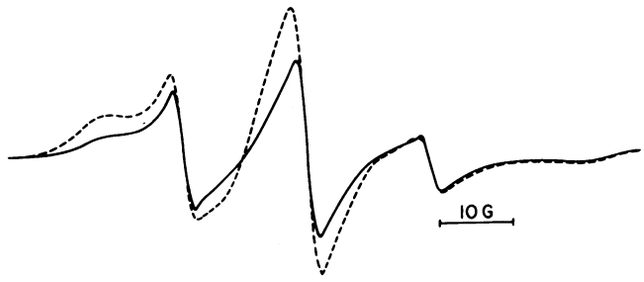

FIG. 5. ESR spectra of the membrane fractions from *E. coli*: K63-1 (solid line); K63-1-1 (broken line).

It should be possible to pursue these results, combining physical probes with suitable mutations. We have also obtained NMR signals from membrane samples containing ^{13}C or ^{19}F labels at specific sites in the lipoprotein (28). NMR probes located near the carboxy terminus reveal rapid molecular motion in this region of the molecule, in agreement with the ESR results presented here. Combining the spin label and NMR probes presents additional possibilities for mapping the lipoprotein *in situ*.

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