Photoaffinity Labeling of Fatty Acid-binding Proteins Involved in Long Chain Fatty Acid Transport in *Escherichia coli*

Dev Mangroo and Gerhard E. Gerber†

*From the Department of Biochemistry, McMaster University, Hamilton, Ontario L8N 3Z5, Canada*

(Received for publication, February 18, 1992)

The photoreactive fatty acid 11-m-diazirinophenoxyl-[11-3H]undecanoate was shown to be taken up specifically by the fatty acid transport system expressed in *Escherichia coli* grown on oleate. This photoreactive fatty acid analogue was therefore used to identify proteins involved in fatty acid uptake in *E. coli*. The *fadL* protein was labeled by the probe, confirmed to be exclusively in the outer membrane and to exhibit the heat modifiable behavior typical of outer membrane proteins. The apparent pI of the incompletely denatured form of the protein having the mobility of a 33-kDa protein was 4.6 while that of the fully denatured form was consistent with the calculated value of 5.2. The denaturation was reversible depending upon the protein to detergent ratios. The photoreactive fatty acid partitions into the outer membrane, resulting in extensive photolabeling of the lipid; a high affinity fatty acid-binding site is not apparent in total membranes labeled using free fatty acids due to this large binding capacity of the outer membrane. However, when the free fatty acid concentration was controlled by supplying it as a bovine serum albumin complex, the *fadL* protein exhibited saturable high affinity fatty acid binding, having an apparent $K_d$ for the probe of 63 nM. The methods described very readily identify fatty acid-binding proteins: the fact that even when the sensitivity was increased 500-fold, no evidence was found for the presence of a fatty acid-binding protein in the inner membrane is consistent with the proposal that fatty acid permeation across the plasma membrane is not protein mediated but occurs by a simple diffusive mechanism.

*Escherichia coli* can utilize long chain fatty acids as the sole carbon source due to the coordinated expression of enzymes involved in fatty acid uptake and metabolism (1–3). A combination of genetic and biochemical studies has shown that fatty acid uptake in *E. coli* involves at least two proteins (1–8). The first is a 43-kDa protein encoded by the *fadL* gene which has been shown to be necessary for fatty acid permeation of the outer membrane (4–7, 9, 10). The mechanism by which this protein facilitates the transmembrane movement of fatty acid is not understood. Although binding analysis using *fadL* mutants led to the proposal that this protein binds fatty acid (10, 11), no direct evidence which supports this conclusion has been reported. The second protein involved in fatty acid uptake is fatty acyl-CoA synthetase which is encoded by the *fadD* gene (1–7, 9, 10). This enzyme is essentially cytosolic and is the first enzyme of the β-oxidation pathway. At present it is not known whether one of the above proteins or another protein which may be required for fatty acid translocation across the inner membrane is involved in the saturable, rate-limiting step in fatty acid uptake.

Protein-mediated translocation of many compounds across the plasma membrane of *E. coli* is rate limiting in their uptake and is coupled to the electrochemical potential (12, 13). Fatty acid uptake has been reported to be inhibited when the membrane potential was abolished by uncouplers and to increase as the magnitude of proton gradient was increased; this led others to propose that translocation of fatty acid across the inner membrane may be rate limiting and involve a proton-fatty acid cotransporter (14). However, we have recently shown that the rate-limiting step is not directly coupled to or affected by the magnitude of the electrochemical potential (15). It is therefore unclear whether fatty acid permeation of the inner membrane involves a protein or occurs by passive diffusion. In mammalian cells it is also not known which of these two mechanisms is responsible for fatty acid movement across the plasma membrane since evidence for both has been reported (16–21).

Photoreactive fatty acid analogues have the potential for identifying proteins involved in the transmembrane movement of fatty acids and their metabolism. The diazirinophenoxyl (DAP) fatty acid analogues were shown previously to be used as long chain fatty acids by rat liver microsomal enzymes for the synthesis of fatty acyl-CoA and phospholipids, for phospholipid synthesis and fatty acid acylation of membrane proteins in L-cells (22, 23), and for phospholipid synthesis in *E. coli* (37, 38). The diazirine is stable under physiological conditions and chemically unreactive prior to photolytic activation. The carbene generated by photolysis of the diazirine at 365 nm has a short half-life and is capable of inserting into C–H and C–C bonds. Unlike other chemical cross-linking reagents, the chemical reactivity of the carbene does not rely on the presence of hydroxyl, carboxylic, amino or thiol functional groups (24) and therefore would result in the covalent attachment of the fatty acid even to the hydrophobic amino acid residues expected to make up the fatty acid-binding site. The fact that these photoreactive fatty acids are recognized as long chain fatty acids by a variety of metabolic enzymes indicates that they should label proteins involved in fatty acid uptake. We have therefore used the DAP fatty acid to determine whether a protein may be required for fatty acid permeation of the inner membrane and whether the *fadL* protein binds fatty acid.

The abbreviations used are: DAP, m-diazirinophenoxyl; BSA, bovine serum albumin; MSH, mercaptoethanol; 2-D PAGE, two dimensional polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate.

* This work was supported by the Medical Research Council of Canada Grant MA6488 (to G. E. G.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† To whom correspondence should be addressed. Tel.: 416-525-1709; Ext. 2454; Fax: 416-522-9033.
We report that the photoreactive fatty acid is recognized by the long chain fatty acid transport system in *E. coli* and is capable of identifying membrane-bound fatty acid-binding proteins. The *fad*L outer membrane protein was directly shown to bind fatty acid saturably and with high affinity using this photoaffinity labeling approach. The fact that a fatty acid-binding protein could not be detected in the inner membrane suggests that fatty acid permeation of the plasma membrane of *E. coli* occurs by passive diffusion.

**MATERIALS AND METHODS**

The ML308 (*Δ* zhu*) and RS3338 (fadR*) strains of *E. coli* were obtained from the American Type Culture Collection and from the Genetics Stock Center, Yale University, New Haven, CT, respectively. All bacterial strains were stored in Luria-Bertani medium containing 15% glycerol (w/v) at −20 °C (25). DNase I, RNase A, lysozyme, thiamine, Brij 58, pl marker proteins (3.6-6.6), and BSA were purchased from Sigma. Prestained molecular weight markers were obtained from Amersham Corp. and 11-DAP-[11-3H]undecanoic acid (3.75-15 Ci/mmol) was synthesized as described (26, 27). The tissue and gel solubilizer, Solvable, was purchased from Du Pont-New England Nuclear.

**Cell Growth—** Aliquots of cells grown initially in Luria-Bertani medium at 37 °C with shaking, were diluted into M9 minimal medium containing 0.4% BSA pre-equilibrated to pH 6.5, at a protein concentration of 5-10 mg/ml, were frozen in water and incubated at 25 °C with stirring for 30 min. Water (19.5 ml) was added to the frozen aliquot of the incubation mixture and the incubation continued for 5 min.

Fatty Acid-BSA Method of Labeling—11-DAP-[11-3H]undecanoic acid ([11-3H]undecanoic acid) was synthesized as described (26) and incorporated into the lipids of mesophiles and hornflies (26, 27). The tissue and gel solubilizer, Solvable, was purchased from Du Pont-New England Nuclear.

**RESULTS**

**Fatty Acid-Binding Proteins**

The photolyzed sample (20 µg of protein) was added to 10 µl of 25 mM Tris-HCl, pH 6.8, containing 2.5 mM MgCl₂ and 0.53% SDS (w/v) (A) or 1.2% SDS (w/v) (B) and boiled for 5 min. A solution (26 µl) containing 17.5% MSH (v/v), 7% amphoteries (3.5/10.4/6.6/8, 1:2:2) and 4.8% Nonidet P-40 (w/v) was added to preparations A and B, respectively. Urea (18 mg) was added to 17.5 µl (5 µg of protein) of each preparation and analyzed by 2-dimensional (2-D) PAGE as described below.

**Isoelectric Focusing—** An aliquot (40 µl) of the photolyzed sample (20 µg of protein) was added to 10 µl of 250 mM Tris-HCl, pH 6.8, containing 2.5 mM MgCl₂ and 0.53% SDS (w/v) (A) or 1.2% SDS (w/v) (B) and boiled for 5 min. A solution (26 µl) containing 17.5% MSH (v/v), 7% amphoteries (3.5/10.4/6.6/8, 1:2:2) and 4.8% Nonidet P-40 (w/v) was added to preparations A and B, respectively. Urea (18 mg) was added to 17.5 µl (5 µg of protein) of each preparation and analyzed by 2-dimensional (2-D) PAGE as described below.

**2-Dimensional Polyacrylamide Gel Electrophoresis—** Electrophoresis was performed as described by Amies and Nikaido (31). A Bio-Rad Mini Protein 2 apparatus. Electrofocusing points of protein were determined by comparison to proteins of known pl values.

**Preparation of Antiserum to Synthetic Peptide of the *fad*L Protein**—A COOH-terminal peptide (acycteinylated-INEGPQFQESGK-NH₂) of the *fad*L protein was synthesized and coupled to keyhole limpet hemocyanin using m-maleimidobenzoic acid-N-hydroxysuccinimide ester (Multiple Peptide Systems, San Diego, CA). Antiserum to the coupled peptide was raised in rabbits (9) and screened by Western immunoblotting as described (34) except the blots were treated with 1% BSA. Bound antibodies were detected with [125I]-protein A followed by autoradiography (34).

**Preparation of Whole Cells for SDS-PAGE Analysis**—The photoaffinity labeled proteins were dissolved in 10 mM Tris-HCl, pH 8.0, 15 µl of a solution containing 100 µg/ml lysozyme, and 1.5 mM sodium EDTA, pH 7.8, was added and the suspension incubated at 25 °C for 30 min. The incubation mixture was diluted with 15 µl of a solution containing 0.2 M magnesium sulfate and 600 mM potassium chloride buffered at 37 °C in 1% sodium dodecyl sulfate. The sample was boiled for 5 min and analyzed by SDS-PAGE as described (33).

**Miscellaneous—** Protein was quantitated as described by Lowry et al. (35) using BSA as the standard. Fluorography was performed as described by Bonner and Laskey (36). Silver staining of proteins were performed as described by the supplier (Bio-Rad). Electrophoretic transfer of proteins onto nitrocellulose was performed as specified by the supplier (Bio-Rad). Silver staining of proteins were performed as described by the supplier (Bio-Rad).

**RESULTS**

**Labeling of Total Lysate at 37 °C**—The photolysis of lysates from both fatty acid and glucose grown cells incubated with 11-DAP-[11-3H]-undecanoic acid results in labeling of several proteins that have an affinity for fatty acids (Fig. 1); the absence of labeling of the 70-, 55-, 43-, 40-, 37- and 18-kDa proteins (arrows) in the lysate of glucose grown cells shows that the expression of these proteins is induced by fatty acid and repressed by glucose. The levels of labeling of proteins in
the lysate of glucose grown cells are insensitive to the temperature or time of preincubation with the photoreactive probe. The extent of labeling of the proteins in the lysate from cells grown in oleate also remained constant at 0 °C as the incubation was increased from 1 to 5 min. However, at 37 °C the intensities of labeling of the 43-, 37-, 35-, and 18-kDa proteins (arrows) at 1 min were higher than those observed at 0 °C. As the incubation at 37 °C was increased to 5 min, in addition to an increase in the intensity of labeling of the above proteins, three other proteins having molecular masses of 70, 55, and 18 kDa were intensely labeled. The fact that these proteins were not significantly labeled at 0 °C, as well as the latency of labeling at 37 °C, suggests that these proteins are labeled by the photoreactive fatty acid that had undergone metabolism. The radioactive material at the bottom of the gel is related to photolabeling of the lipids.

**Uptake of 11-DAP-[11-3H]undecanoate**—Cells grown on oleate were capable of taking up the probe while glucose grown cells were not (Fig. 2). The internalization of 11-DAP-[11-3H]undecanoate (10 μM) at 37 °C resulted in minor labeling of the 35- and 37-kDa proteins (Fig. 3A, arrows). The extent of labeling of these proteins remained essentially constant as the incubation was continued for 8 min. The fact that absolutely no labeling was observed for the proteins labeled in the cell lysate (Fig. 1) indicates that these cells totally exclude the probe. Photolysis of oleate grown cells after a brief preincubation with 11-DAP-[11-3H]undecanoate resulted in significant labeling of the 70-, 55-, 43-, 40-kDa proteins and minor labeling of the 35- and 18-kDa proteins (Fig. 3B, arrows). The extent of labeling of these proteins is dramatically reduced with increasing preincubation times beyond 1 min, indicating that the photoreactive probe is being depleted.

**Labeling of Total Membranes from ML308 and fadR fadL Cells**—The membranes were labeled with a high concentration of 11-DAP-[11-3H]undecanoate (10 μM) and analyzed by 2-D PAGE. Several proteins having molecular masses of 75, 37 (inner arrows), and 33 kDa (box) were heavily labeled by the probe in total membranes prepared from oleate grown ML308 cells (Fig. 4A). The 75- and 37-kDa proteins (inner arrows) were also labeled in total membranes from ML308 grown on glucose (Fig. 4B) and fadR fadL cells (Fig. 4C) while the 33-kDa protein was not detected. A shorter exposure of
the gel of Fig. 4A shows that the 33-kDa protein was labeled to the same extent as the 75- and 37-kDa proteins (data not shown). However, as detected by silver staining, the 75- and 37-kDa proteins are major membrane proteins (Fig. 4, D and E, inner arrows), whereas the 33-kDa protein is a relatively minor one (Fig. 4D, box).

Specific Labeling and 2-D Analysis of the Labeled Membrane Solubilized at Different SDS to Protein Ratios—Membranes from oleate grown ML308 cells were labeled with a low concentration of 11-DAP-[11-3H]undecanoate (200 nM) using the fatty acid-BSA method and solubilized at 100 °C using an SDS to protein ratio of 2.6 or 6 prior to analysis. When the sample was solubilized at an SDS to protein ratio of 2.6 a labeled protein with an apparent molecular mass of 33 kDa and a pI of 4.6 was observed (Fig. 5A, box). However, solubilization of the sample using an SDS to protein ratio of 6 resulted in this protein now migrating as a 43-kDa protein with a pI of 5.2 (Fig. 5B, box). Analysis of total fadR fadL membranes solubilized at an SDS to protein ratio of 6 shows that no proteins were labeled by the probe. The labeled 66-kDa proteins present in the membranes from fadR fadL and oleate grown ML308 cells are due to residual BSA that is not removed during washing of the photolyzed membranes. The silver-stained protein patterns also showed that the increase in the SDS to protein ratio from 2.6 to 6 resulted in a shift of the apparent molecular mass of the 33-kDa protein (Fig. 5D, box) to 43 kDa (Fig. 5E, box).

Immunoblot Analysis of Membranes from fadR fadL Mutant and ML308 Grown on Oleate—Total membranes from both types of cells were solubilized at 100 °C using an SDS to protein ratio of 6 and subjected to 2-D PAGE. The separated membranes were probed with a polyclonal antiserum to a synthetic peptide derived from the COOH-terminal sequence of the fadL protein. A 43-kDa protein having a pI of 5.2 was recognized by the antiserum in the membranes prepared from oleate grown cells (Fig. 6A), but not in the membranes of the fadL mutant (Fig. 6B). The antisera also cross-reacted with the 37- and 25-kDa proteins present in both membranes but to a much lower extent. These results, together with those of Fig. 5, clearly show that the 43-kDa protein is the fadL protein whose pI is 5.2 when the protein is denatured.
Saturability of Labeling of the fadL Protein—Total membranes were labeled at the various probe concentrations which were controlled by varying the 11-DAP-[11-3H]undecanoate to BSA ratio and analyzed by SDS-PAGE. The protein band corresponding to the fadL protein was excised and its radiolabel was quantitated by liquid scintillation counting. As the free concentration of the photoreactive fatty acid was increased from 50 to 1400 nM, the fadL protein was labeled saturably (Fig. 7). From a Lineweaver-Burk analysis of the data from Fig. 4, it was determined that the K_s for 11-DAP-[11-3H]undecanoate was 63 nM.

Labeling of Inner and Outer Membranes—Inner and outer membranes were separated from a total membrane preparation by sucrose density centrifugation as described (29). The isolated inner and outer membranes were labeled separately with a high, non-limiting concentration (20 μM) of 11-DAP-[11-3H]undecanoate under equilibrium conditions. The method was modified in order to increase the sensitivity by at least 500-fold (the specific activity of the probe was increased to 15 Ci/mmol; the probe concentration was increased to 20 μM; the analysis of purified membranes allowed an increase in the amount of relevant protein on the gel; the exposure time was increased to 15 days). This increased sensitivity is confirmed by the observed increase in the level of labeling of the fadL protein in the outer membrane (compare Fig. 8D, inner arrows to Fig. 4A, box). The labeled membranes were solubilized at 100 °C using an SDS to protein ratio of 2.6 and subjected to 2-D PAGE. Silver staining showed that several outer membrane proteins (Fig. 8B) are present in the inner membrane fraction (Fig. 8A) in small quantities; these include the 75-kDa proteins (outer arrow) and the 37-kDa proteins (inner arrow). Furthermore, the outer membrane is contaminated with a small amount of the inner membrane, indicated by the presence of a small amount of a major inner membrane protein having a molecular mass greater than 100 kDa (Fig. 8B, inner arrow). These results clearly show that the separation method is efficient and that the resulting inner and outer membrane fractions are essentially pure. In the outer membrane (Fig. 8D), several proteins including those of 75 kDa (outer arrow), 37 kDa (outer arrow), and the fadL protein, distributed between the incompletely and completely denatured forms (33 and 43 kDa, inner arrows, respectively) are labeled while the only labeled proteins (75, 37, and 33 kDa) observed in the inner membrane (Fig. 8C), are those derived from the small percentage of outer membrane contamination.

DISCUSSION

Photoreactive fatty acid analogues have the potential for identifying proteins involved in the transmembrane movement of long chain fatty acids and their metabolism. These analogues have been shown to be recognized as long chain fatty acids by a variety of mammalian (22, 23, 26) and E. coli enzymes (37, 38). The photoreactive fatty acid 11-DAP-[11-3H]undecanoate labeled several proteins in the lysate from E. coli grown on oleate or glucose (Fig. 1) and was taken up by oleate grown but not by glucose grown cells (Fig. 2; also compare Fig. 3 with Fig. 1). These results show that the probe was being taken up specifically via the transport system expressed in oleate grown cells and is capable of labeling proteins having an affinity for fatty acids. The absence of uptake of the probe and photolabeling of cytoplasmic proteins in glucose grown cells shows that there is no passive permeation of the probe in the absence of the transport system. It has been shown in vivo that the photoreactive fatty acids are incorporated into E. coli membrane lipids (37, 38). The latency of labeling of the 70-55- and 18-kDa proteins in the lysate of oleate grown cells at 37 °C (Fig. 1, arrows) indicates that these proteins are not being labeled by the photoreactive probe but by one of its metabolites; the absence of labeling after a 2-min incubation of oleate grown cells with a low concentration of 11-DAP-[11-3H]undecanoate (10 μM) at 37 °C is consistent with the interpretation that the probe is being metabolized. The fact that the photoreactive fatty acid is recognized as a long chain fatty acid by the E. coli transport system and metabolic enzymes clearly showed that this probe is appropriate for labeling proteins involved in this process.

The fadL protein has been shown to be necessary for fatty acid permeation of the outer membrane (4-7, 9, 10, 39) of E. coli. However, the mechanism by which this protein facilitates the transmembrane movement of fatty acids is not understood. It was presumed that the fadL protein binds fatty acids based on the following observations: fadD fadL cells bind four to seven times more fatty acid than fadD fadL cells (10,
of silver staining of the 75- and 37-kDa proteins is low (compare Fig. 4, A with D, box) which shows that the probe was recognized by the transport protein. The saturability of labeling of the fadL protein was determined using the fatty acid-BSA method. As shown in Fig. 7, the labeling of the fadL protein was saturable with a $K_d$ of 63 nM. These results clearly show for the first time that the fadL protein has a high affinity for fatty acids and that this binding is saturable. This also shows that the photoreactive fatty acid analogue can identify a membrane-bound fatty acid-binding protein. The saturability of labeling of the fadL protein demonstrates that this protein has a substrate-binding site. The lamB and Tsx proteins, required for permeation of maltose and nucleoside, respectively, across the outer membrane of E. coli, have been shown to have substrate binding sites (43, 44) and to function as substrate-specific diffusion channels (44, 45).

Although the precise mechanism of the fadL protein in facilitating fatty acid permeation across the outer membrane is not known, it is possible that the fadL protein functions similarly.

The mechanism of fatty acid permeation of the inner membrane of E. coli is not known. Although genetic evidence suggests that acyl-CoA synthetase is involved in the process of fatty acid permeation, it has been suggested that fatty acid translocation across the inner membrane may involve a protein since it was shown that fatty acid uptake was inhibited when the membrane potential was abolished by uncouplers (14). The fact that the probe was recognized by the transport system as a long chain fatty acid suggests that an inner membrane fatty acid-binding protein required for permeation would become labeled by the probe. However, labeling of pure inner membrane with a nonlimiting concentration of 11-DAP-[11-3H]undecanoate under equilibrium conditions and using methods having 500-fold increased sensitivity resulted in no protein unique to the inner membrane being labeled other than those proteins (75 and 37 kDa) derived from the small percentage of outer membrane contaminant (Fig. 6C).

The approach described using a photoreactive fatty acid analogue is easily capable of identifying membrane-bound fatty acid-binding proteins; the fact that even the high sensitivity version of this method was unable to detect any fatty acid-binding protein in purified inner membranes strongly suggests the presence of such a site, the saturability of labeling of the fadL protein was high suggesting that this protein has a high affinity for fatty acids. The nonspecific labeling of the major proteins suggests that some proportion of labeling of the fadL protein is also due to non-specific labeling under these conditions.

The specific labeling of high affinity fatty acid-binding sites requires the use of very low concentrations of fatty acids. At these concentrations, high affinity sites in membranes are usually not apparent due to the fatty acid interacting with the large number of low affinity sites available (membranes, glassware, etc.). In order to saturate these low affinity sites at low free fatty acid concentrations, the fatty acid is routinely presented as a BSA complex (16-21) which serves as a reservoir for free fatty acid. The free fatty acid concentration can be determined as described (30). Using this method of labeling the fadL protein was intensely labeled (Fig. 5A, box) while very little labeling of the major proteins (e.g. 37 kDa) was observed in isolated membranes prepared from oleate grown ML308 (Fig. 5A) and fadR fadL (Fig. 5C); this indicates that the fadL protein was labeled specifically under these conditions and supports our conclusion that the major proteins (75 and 37 kDa) are labeled nonspecifically (Fig. 4, inner arrows).

The fact that the fadL protein was labeled specifically at low concentrations of the photoreactive probe suggests that the protein has a fatty acid-binding site. In order to confirm the presence of such a site, the saturability of labeling of the fadL protein was determined using the fatty acid-BSA method. As shown in Fig. 7, the labeling of the fadL protein was saturable with a $K_d$ of 63 nM. These results clearly show for the first time that the fadL protein has a high affinity for fatty acids and that this binding is saturable. This also shows that the photoreactive fatty acid analogue can identify a membrane-bound fatty acid-binding protein. The saturability of labeling of the fadL protein demonstrates that this protein has a substrate-binding site. The lamB and Tsx proteins, required for permeation of maltose and nucleoside, respectively, across the outer membrane of E. coli, have been shown to have substrate binding sites (43, 44) and to function as substrate-specific diffusion channels (44, 45). Although the precise mechanism of the fadL protein in facilitating fatty acid permeation across the outer membrane is not known, it is possible that the fadL protein functions similarly.

The observation that the fadL protein was labeled by 11-DAP-[11-3H]undecanoate suggested that the protein is capable of binding fatty acids. However, in addition to the fadL protein being labeled by the probe, other proteins having molecular masses of 75 and 37 kDa are also labeled in total membranes (Fig. 4A, inner arrow) as well as in pure outer membrane (Fig. 8D, outer arrows) prepared from oleate grown cells. The 75- and 37-kDa proteins are present in large amounts in comparison to the fadL protein (Fig. 4D, inner arrows); the ratio of the intensity of labeling to the intensity of silver staining of the 75- and 37-kDa proteins is low (compare Fig. 4, A with D, inner arrows) as would be expected of proteins having a low affinity for fatty acids. Labeling of these proteins is therefore nonspecific being due to the photoreactive fatty acid partitioning into the membrane and interacting with the hydrophobic domains of the protein. In contrast, the ratio of the intensity of labeling to the intensity of silver staining of the fadL protein (compare Fig. 4, A with D, box) is high suggesting that this protein has a high affinity for fatty acids. The nonspecific labeling of the major proteins suggests that some proportion of labeling of the fadL protein is also due to non-specific labeling under these conditions.

The specific labeling of high affinity fatty acid-binding sites requires the use of very low concentrations (nanomolar) of the fatty acid. At these concentrations, high affinity sites in membranes are usually not apparent due to the fatty acid interacting with the large number of low affinity sites available (membranes, glassware, etc.). In order to saturate these low affinity sites at low free fatty acid concentrations, the fatty acid is routinely presented as a BSA complex (16-21) which serves as a reservoir for free fatty acid. The free fatty acid concentration can be determined as described (30). Using this method of labeling the fadL protein was intensely labeled (Fig. 5A, box) while very little labeling of the major proteins (e.g. 37 kDa) was observed in isolated membranes prepared from oleate grown ML308 (Fig. 5A) and fadR fadL (Fig. 5C); this indicates that the fadL protein was labeled specifically under these conditions and supports our conclusion that the major proteins (75 and 37 kDa) are labeled nonspecifically (Fig. 4, inner arrows).
suggests that fatty acid permeation of the plasma membrane of *E. coli* does not involve such a protein. The process is thus likely to be one of non-protein-mediated passive diffusion of the fatty acid across the plasma membrane followed by conversion of the fatty acid to its non-permeant CoA form by acyl-CoA synthetase.

**Acknowledgments**—We thank William Nuttley for his help with the antibody preparation and Andy Leung for his excellent technical assistance.

**REFERENCES**

1. Klein, K., Steinberg, R., Flethen, B., and Overath, P. (1971) *Eur. J. Biochem.* 9, 442–450
2. Overath, P., Raufuss, E. M., Stoffel, W., and Ecker, W. (1967) *Biochem. Biophys. Res. Commun.* **29**, 24–33
3. Overath, P., Pauli, G., and Schaier, H. U. (1969) *Eur. J. Biochem.* **7**, 559–574
4. Maloy, S. R., Ginsburg, C. L., Simons, R. W., and Nunn, W. D. (1981) *J. Biol. Chem.* **256**, 3735–3742
5. Ginsburg, C. L., Black, P. N., and Nunn, W. D. (1984) *J. Biol. Chem.* **259**, 8437–8443
6. Nunn, W. D., and Simons, R. W. (1978) *Proc. Natl. Acad. Sci. U. S. A.* **75**, 3377–3381
7. Nunn, W. D., Simons, R. W., Egan, P. A., and Maloy, S. R. (1979) *J. Biol. Chem.* **254**, 9130–9134
8. Freeman, F. E., and Bennet, W. (1975) *Arch. Biochem. Biophys.* **159**, 434–443
9. Black, P. N., Said, B., Ghosn, C. R., Besch, J. R., and Nunn, W. D. (1987) *J. Biol. Chem.* **262**, 1412–1419
10. Nunn, W. D., Colburn, R. W., and Black, P. N. (1986) *J. Biol. Chem.* **261**, 167–171
11. Kumar, G. B., and Black, P. N. (1991) *J. Biol. Chem.* **266**, 1348–1353
12. Kaback, H. R. (1971) *Methods Enzymol.* **22**, 99–120
13. Ramos, S., and Kaback, H. R. (1977) *Biochemistry* **16**, 854–858
14. Kameda, K., Suzuki, L. K., and Imai, Y. (1985) *Biochim. Biophys. Acta* **123**, 227–234
15. Mangroo, D., and Gerber, G. E. (1991) *FASEB J.* **5**, 6002
16. Stremler, W., Strohmeyer, G., and Berk, P. D. (1986) *Proc. Natl. Acad. Sci. U. S. A.* **83**, 3584–3588
17. Stremler, W., Strohmeyer, G., Borehard, F., Kochwa, S., and Berk, P. D. (1986) *Proc. Natl. Acad. Sci. U. S. A.* **82**, 4–8
18. Berk, P. D., Wade, H., Horiyo, Y., Potter, B. J., Sorrentino, D. Zhow, S.-L., Isola, L. M., Stump, D., Kang, C.-L., and Thang, S. (1990) *Proc. Natl. Acad. Sci. U. S. A.* **87**, 3484–3488
19. Noy, N., and Zakim, D. (1980) *Biochemistry* **19**, 3521–3525
20. Cooper, R. B., Noy, N., and Zakim, D. (1989) *J. Lipid Res.* **30**, 1719–1726
21. Storch, J., and Kleinfield, D. M. (1986) *Biochemistry* **25**, 1717–1726
22. Capone, J., Leblanc, P., Gerber, G. E., and Ghosh, H. P. (1983) *J. Biol. Chem.* **258**, 1395–1398
23. Leblanc, P., and Gerber, G. E. (1983) *Can. J. Biochem. Cell Biol.* **62**, 375–378
24. Bailey, H. (1983) *Photoaffinity Labeling in Biochemistry and Molecular Biology* (Work, T. S., and Burdon, R. H., eds) pp. 1–188, Elsevier, New York
25. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
26. Leblanc, P., Capone, J., and Gerber, G. E. (1982) *J. Biol. Chem.* **257**, 14586–14589
27. Leblanc, P., and Gerber, G. E. (1984) *Can. J. Chem.* **62**, 1767–1771
28. Witholt, B., Boekhout, M., Brock, M., Kingma, J., Heerikhuizen, H. Van., and Ley, L. (1976) *Anal. Biochem.* **74**, 160–170
29. Osborn, M. J., Gander, J. E., Parisi, E., and Carson, J. (1972) *J. Biol. Chem.* **247**, 3962–3972
30. Spector, A. A., John, K., and Fletcher, J. E. (1969) *J. Lipid Res.* **10**, 56–69
31. Ames, G. F.-L., and Nikaido, K. (1976) *Biochemistry* **15**, 616–623
32. O’Farrell, P. H. (1975) *J. Biol. Chem.* **250**, 4007–4021
33. Laemmli, U. K. (1970) *Nature* **227**, 680–685
34. Burnette, W. N. (1981) *Anal. Biochem.* **112**, 195–203
35. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
36. Borner, W. M., and Laskey, R. A. (1974) *Eur. J. Biochem.* **46**, 83–88
37. Greenberg, G. R., Chakrabarti, P., and Khoura, H. G. (1976) *Proc. Natl. Acad. Sci. U. S. A.* **73**, 266–275
38. Olson, W., Schaechtert, M., and Khoura, H. G. (1979) *J. Bacteriol.* **137**, 1443–1446
39. Nunn, W. D. (1986) *Microbiol. Rev.* **50**, 179–192
40. Black, P. N. (1990) *Biochim. Biophys. Acta* **1046**, 97–105
41. Black, P. N. (1991) *J. Bacteriol.* **173**, 435–442
42. Eisele, J.-U., and Rosenbusch, J. P. (1990) *J. Biol. Chem.* **265**, 10217–10220
43. Luckey, M., and Nikaido, H. (1980) *Biochim. Biophys. Res. Commun.* **93**, 166–171
44. Maier, C., Bremer, E., Schmidt, A., and Benz, R. (1988) *J. Biol. Chem.* **263**, 2485–2490
45. Luckey, M., and Nikaido, H. (1980) *Proc. Natl. Acad. Sci. U. S. A.* **77**, 167–171