Iodination of *Escherichia coli* with Chloramidine T: Selective Labeling of the Outer Membrane Lipoprotein

ROBERT S. MUNFORD and EMIL C. GOTSCHLICH

The Rockefeller University, New York, New York 10021

Received for publication 14 January 1977

Iodination of *Escherichia coli* cells with chloramidine T preferentially labels the free and murein-bound forms of the outer membrane lipoprotein. Iodination for 15 s at 15°C labels the two forms of the lipoprotein almost exclusively, whereas iodination for 60 s at 25°C also labels the other major outer membrane proteins. Chloramine T iodination is a rapid, simple technique for labeling the outer membrane lipoprotein.

Although the outer membrane of *Escherichia coli* contains relatively few major proteins, little is known about their organization in the membrane. There is evidence that two of the proteins are exposed to the surface of the cell, since protein I (molecular weight, 38,000) and protein II* (molecular weight, 33,000) are receptors for phage (9, 21). Protein IV, the lipoprotein of molecular weight 8,000 first described by Braun and his colleagues (3), exists in two forms in the membrane. It is synthesized as a "free" form, and approximately one-third of the copies become covalently linked to murein during cell growth (13). Since the four major proteins can be cross-linked by dimethyl imidoesters, it has been argued that they are tightly associated within the membrane (7).

In studies intended to detect surface exposure of the major outer membrane proteins, we have found that it is possible to iodinate a tyrosine residue located on the inner surface of the membrane. This is the sole tyrosine of the lipoprotein, which in the murein-bound form is located three amino acid residues from the lipoprotein’s covalent linkage to murein. This paper describes experiments with chloramine T as the iodinating agent. Brief iodination with this agent labels the two forms of the lipoprotein almost exclusively, whereas longer iodination also labels the other major outer membrane proteins.

**MATERIALS AND METHODS**

Bacterial strains. *E. coli* K-12 was provided by Norton Zinder of The Rockefeller University. *E. coli* O6.K2:H1 and *E. coli* O6 antisera were obtained from the Center for Disease Control, Atlanta, Ga.

Serological identification of *E. coli* O6 was repeated for each subculture used for iodination experiments.

Radioisotopes and chemicals. Isotopes were obtained from New England Nuclear Corp., Boston, Mass. [131I]Iodo-NEN-033 in 0.01 N NaOH was used for labeling experiments within 1 week of preparation and diluted immediately prior to use. [3,5-3H]Tyrosine (69.4 Ci/mmol) and L-[U-14C]Arginine (279 mCi/mmol) were used. All chemicals were reagent grade and supplied by standard sources.

Media and growth conditions. *E. coli* organisms were maintained on minimal medium A agar plates (17) and grown at 37°C to log phase (1 × 10⁸ to 3 × 10⁹ organisms/ml) in liquid medium A containing 0.4% glucose and 20 μg of thiamine per ml. MgCl₂ was added to give the desired Mg²⁺ concentration (usually 5 mM). For experiments in which the effect of MgCl₂ concentration was studied, a liquid medium was used that contained 0.51% sodium β-glycerophosphate, 0.74% KCl, and 0.1% (NH₄)₂SO₄, pH 7.4, with glucose and thiamine in the above concentrations.

Preparation of cell envelopes. Cells were centrifuged and resuspended in distilled water. After sonic treatment (model W185, Heat Systems-Ultrasonics, Inc.) at 100 W for four or five 30-s pulses in an ice bath, low-speed centrifugation (8,000 × g for 10 min) to pellet unbroken cells, and ultracentrifugation (100,000 × g for 45 min), the pelleted cell envelopes were washed once in 0.01 M sodium phosphate buffer, pH 7.4, and frozen at −70°C (12). For lysozyme digestion, the envelopes were resuspended in 200 μl of sodium phosphate buffer and incubated with 100 μg of lysozyme (EC 3.2.1.17; Worthington Biochemicals Corp., Lysof OCC, Freehold, N.J.) per ml at 37°C for 60 min.

For sodium dodecyl sulfate-polycrylamide gel electrophoresis (SDS-PAGE), samples were suspended in an SDS mixture (2% SDS—10% glycerol—10 mM tris(hydroxymethyl)aminomethane (Tris)-hydrochloride (pH 7.5)) and heated at 100°C for 5 min or at 37°C for 2 h. Bovine serum albumin, ovalbumin, lysozyme, and insulin were treated with dansyl chloride and used along with cytochrome c as molecular weight markers (co electrophoresis with sam-
ple) for tube gels (11). Electrophoresis followed the method of Weber and Osborn (22). For most experiments, 11% polyacrylamide was used. For 5% polyacrylamide gels, 0.5% agarose was added. Gels were removed from the tubes, fluorescent bands were marked, and the gels were cut into 0.75-mm slices. For counting of $^{14}$C and $^3$H, gel slices were digested with 0.1 ml of 30% $\text{H}_2\text{O}_2$ at 55°C for several h, cooled to 4°C, and suspended in 3 ml of Aquasol (New England Nuclear Corp.). Counting efficiencies were 75 and 25% for $^{14}$C and $^3$H, respectively, using a Packard model 3330 liquid scintillation counter. $^{125}$I was counted in a Packard model 3022 gamma spectrometer.

Chloramine T iodination. Cells in the exponential phase of growth were pelleted and excess medium was carefully removed. The cells (approximately $4 \times 10^{10}$ colony-forming units) were resuspended in 200 $\mu$L of liquid medium A (omitting glucose and thiamine) and brought to the desired temperature. Chloramine T ($25 \mu$g in 5 $\mu$L) and $^{125}$I ($25$ to $100 \mu$Ci) were added while blending in a Vortex mixer. The reaction was terminated after the desired time interval by adding 125 $\mu$g of sodium metabisulfite and 2 mg of KI. Cells were washed three times with 5 ml of ice-cold medium A and solubilized immediately for SDS-PAGE or frozen until further use.

Intrinsic labeling. Cells were grown to early exponential phase in 100 ml of medium A supplemented with 10 $\mu$g of L-arginine and L-tyrosine per ml. $[^3]$H]arginine and $[^3]$H]tyrosine were added in the desired quantities, and cells were harvested after an additional generation of growth.

Immunoprecipitation. Purified murein-lipoprotein was isolated by the method of Braun and Sieglin (4). Amino acid analysis of this preparation (JEOL 6-AH analyzer programmed to separate glutamic acid and aspartic acid, and diaminopimelic acid in addition to the standard amino acids) was consistent with murein-lipoprotein without protein contamination. Antiserum was prepared by injecting a New Zealand red rabbit with 0.5 mg of this murein-lipoprotein in complete Freund adjuvant intrascapularly, followed by three injections of 0.5 mg in incomplete Freund adjuvant at intervals of 2 weeks. Immunoprecipitation of iodinated cell envelope proteins was performed as described by Halegoua et al. (6). Precollection serum from the same rabbit was used as a control.

Identification of the iodinated species. Iodinated cells were digested directly with chloroform-methanol (2:1), ethanol-ether (3:1), or 10% trichloroacetic acid. To determine the iodinated species in gel slices from peaks of $^{125}$I activity, gel slices were immediately placed in 200 $\mu$L of antiodiant solution (0.01% [wt/vol] butylated hydroxyanisole, 5 $\mu$L of alphatocopherol per ml, 1.5 mg of NaI per ml in 0.05 M Tris-hydrochloride [pH 8.8], counted, and then miniced and extracted several times with 200 $\mu$L of the same solution. A total of 250 $\mu$g of Pronase, grade B (Calbiochem, San Diego, Calif.), per ml was added (final volume, 1 to 2 ml), and the mixture was allowed to sit in the dark at room temperature for 48 h; an additional 250 $\mu$g of Pronase was added at 24 h. The digested proteins were then extracted with chloroform-methanol (2:1) or extracted into acidified n-butanol for thin-layer chromatography on silica gel, using butanol-acetone-1 N NH$_4$OH (1:4:1) (19). Portions were also treated with an equal volume of cold 20% trichloroacetic acid, and the supernatants were chromatographed in 1 M acetic acid on a G-25 Sephadex column (0.9 by 56 cm) previously shown to separate $^{125}$I, monoiodotyrosine, and diiodotyrosine (15). In control experiments, it was shown that there was no iodination of carrier L-tyrosine by $^{125}$I during the procedures used.

RESULTS

Incorporation of radioactive amino acids into cell envelope proteins during growth. To define the spectrum of major cell envelope proteins in the E. coli strains used, lysozyme-treated cell envelopes from E. coli K-12 cells grown for one doubling time in the presence of 50 $\mu$Ci of L-$[^3]$H]tyrosine and 25 $\mu$Ci of L-$[^3]$C]arginine were analyzed by SDS-PAGE after solubilization in the SDS mixture at two temperatures. After solubilization at 37°C, there were four major peaks, with approximate molecular weights of 57,000, 32,000, 17,500, and 9,000 (Fig. 1A). These molecular weights and the approximate ratios of arginine to tyrosine found in the peaks correspond to the published values for the four E. coli major outer membrane proteins (5, 10). If the envelopes were solubilized at 100°C, the two larger proteins both assumed an apparent molecular weight of 40,000 (Fig. 1B), again in agreement with previous data (14, 20). Identical results were obtained with E. coli O6 cell envelopes. To assure complete solubilization of proteins, iodinated cells were routinely solubilized in SDS at 100°C.

Chloramine T iodination. E. coli K-12, iodinated at room temperature for 60 s with chloramine T ($25 \mu$g) and $^{125}$I ($25 \mu$Ci), demonstrated an unexpected profile of $^{125}$I incorporation when whole cells were solubilized in SDS at 100°C and tested by SDS-PAGE. Greater than 50% of the counts were found in two peaks, one at the origin of the gel (Fig. 2, designated "B") and the other at an approximate molecular weight of 9,000 (Fig. 2, designated "F"). Similar results were found with E. coli O6. The nature of the iodinated material at the origin of the gel was studied further in K-12 cells that were iodinated for 15 s with 25 $\mu$g of chloramine T at 15°C; under these conditions, 68% of the total radioactivity on the gel appeared in peaks B (23%) and F (45%). SDS-PAGE profiles were compared for iodinated cells, cell envelopes, and lysozyme-digested cell envelopes (Fig. 2B to D). The profiles were similar for cells and cell envelopes (Fig. 2B and C). After lysozyme
iodination of *E. coli* with chloramine T

When cells iodinated for 60 s at 25°C were solubilized in SDS at 37°C for 60 min, peaks of radioactivity were found at approximately 57,000 and 32,000 daltons, corresponding to proteins I and II* as detected by intrinsic labeling with radioactive amino acids (Fig. 1).

Immunoprecipitation of murein-bound and free lipoprotein. Cells were labeled with chloramine T at room temperature, and cell envelopes were prepared. SDS-PAGE on the cell envelope preparation before (Fig. 3A) and after (Fig. 3B) solubilization in SDS and immunoprecipitation with antiserum to murein-lipoprotein indicated that only peaks B and F were retained in the precipitate. If cell envelopes were treated with lysozyme prior to immunoprecipitation, peak B was no longer present and radioactivity increased in the range of 9,000 to 18,000 daltons (Fig. 3C). Twelve percent of the 125I activity in the envelope preparation was precipitated by the antiserum, before and after lysozyme treatment.

Identification of the iodinated species. Chloroform-methanol (2:1) or ethanol-ether (3:1) treatment of iodinated whole cells extracted small amounts of 125I (5% or less). In contrast, 10 to 40% of the counts were solubilized if iodinated cells were precipitated with 10% trichloroacetic acid. With G-25 Sephadex chromatography, these counts appeared entirely in a peak corresponding to free 125I. When trichloroacetic acid-extracted cells were washed

![Fig. 1. SDS-PAGE profile of *E. coli* K-12 cell envelopes after lysozyme treatment. Organisms were grown in 100 ml of medium A for one doubling time after the addition of 50 μCi of [3H]tyrosine and 25 μCi of [14C]arginine. (A) Envelopes solubilized in SDS mixture at 37°C for 2 h. (B) Envelopes solubilized in SDS mixture at 100°C for 6 min. Dansylated molecular weight markers: a, bovine serum albumin (68,000); b, ovalbumin (47,000); c, lysozyme dimer (31,000); d, lysozyme (16,000); e, cytochrome c (12,800); f, insulin (6,500). ———, [3H]tyrosine; ———, [14C]arginine.](https://jib.asm.org/)
Further identification of the iodinated species was confined to peaks B and F, since iodination of the single tyrosine in these proteins was unexpected. After Pronase digestion, less than 5% of the counts was extractable into (2:1) chloroform-methanol (the system used to extract the lipid portion of Pronase-digested lipoprotein [8]), less than 10% was precipitable by 10% trichloroacetic acid, and more than 80% was extractable into acidified n-butanol. Further analysis found the $^{125}$I in moniodotyrosine or as free $^{125}$I (Table 1).

**DISCUSSION**

The selective iodination of the *E. coli* outer membrane lipoprotein found in this study was unexpected. Other membrane proteins are thought to be exposed to the cell surface (9, 21) and thus might have tyrosines accessible to iodination, whereas the sole tyrosine of the murein-bound lipoprotein is located on the inner surface of the membrane, three amino acid residues from the lipoprotein's covalent linkage to murein (4).

We first demonstrated that the free and murein-bound forms of the lipoprotein are iodinated when intact *E. coli* cells are exposed to chloramine T and $^{125}$I. Because of its attachment to murein, the murein-bound lipoprotein is not soluble in boiling SDS. This property was first used by Braun and Rehn to isolate the protein (3). When *E. coli* cell membranes are solubilized in SDS at 100°C, the spectrum of proteins found by SDS-PAGE includes material that does not enter the gel. After solubilization of iodinated cells, we observed this material (peak B) in 11, 7.5, and 5% polyacrylamide gels. After lysozyme digestion of the murein layer and solubilization in hot SDS, no material was found at the origin of the gel, and activity increased in the 9,000- to 18,000-dalton region (Fig. 2B to D). Since free lipoprotein migrates in such gels with an apparent molecular weight of 8,000 (peak F), the material of slightly higher molecular weight is thought to represent lipoprotein with attached murein fragments of heterogeneous length. This criterion was first used by Inouye et al. to demonstrate that murein-bound lipoprotein is formed from a free lipoprotein precursor (13). We obtained further evidence that the iodinated material in peak B is murein-lipoprotein by immunoprecipitation of SDS-solubilized envelopes with antiserum to murein-lipoprotein (Fig. 3). If envelopes were first treated with lysozyme, iodinated material was found in only the 8,000- to 18,000-dalton range of the gel (Fig. 3C). This observation indicated that the material in peak B was pre-

**Fig. 3. Immunoprecipitation of SDS-solubilized cell envelopes.** *E. coli* O6 cells were labeled with chloramine T for 60 s at room temperature, and cell envelopes were prepared as described in the text. SDS-PAGE profiles were determined for cell envelopes before (A) and after (B) immunoprecipitation of SDS-solubilized envelopes with antiserum to murein-lipoprotein and after lysozyme treatment of the cell envelopes prior to SDS solubilization and immunoprecipitation (C). Molecular weight markers were as described in the legend to Fig. 1.
cipitated by antibodies to murein (or lipoprotein) and that it was of appropriate molecular weight to be murein-lipoprotein. These results suggest strongly that free and murein-bound lipoprotein are iodinated.

It was also important to verify that the iodinated species was the lipoprotein’s tyrosine. We were concerned that the unsaturated lipids at the opposite end of the lipoprotein from the tyrosine might be iodinated and that during handling of the iodinated cells an active I species might transfer from the lipid to the tyrosine. Precautions to prevent this transfer were taken after the cells were iodinated and washed, but identical results were obtained. We therefore think that this possibility is an unlikely explanation for the appearance of the ^I in peaks B and F in monoiodotyrosine.

As demonstrated by immunoprecipitation with antiserum to murein-lipoprotein, the free lipoprotein is iodinated by the methods used. Since the orientation of the free lipoprotein in the outer membrane is unknown, it is possible that its carboxy terminal end might be exposed to the surface, thus allowing the tyrosine to be available for iodination (2), and any labeled murein-bound lipoprotein could result from attachment of iodinated free lipoprotein to murein. This possibility seems unlikely, since murein-bound lipoprotein was iodinated when labeling of the cells and all subsequent steps were performed at 4°C. At this temperature, the mobility of outer membrane proteins should be minimal. Moreover, this mechanism would involve a “flipping” of the free lipoprotein in the membrane, an unlikely event thermodynamically.

Previous workers have estimated that there are approximately 10^6 copies per outer membrane of each of the major proteins I, II*, and III with 25, 14, and 10 tyrosines, respectively (mole/mole), and that there are 7.5 × 10^4 copies of the lipoprotein (1, 5, 10). It appears, therefore, that the single tyrosine residues on the lipoprotein are greatly outnumbered by the tyrosines in the other outer membrane proteins (approximately six to one), and the ease with which the lipoprotein tyrosines are iodinated indicates that they are unusually accessible or reactive. Although several interpretations of this finding are possible, there is insufficient knowledge of the nature of the active iodinating species, the surface exposure of tyrosine residues in the other outer membrane proteins, and the nature of hydrophobic and hydrophilic diffusion through the outer membrane to make such speculation fruitful at this time.

The lipoprotein molecule contains no tryptophan and only one tyrosine (2). It has thus been difficult to follow the molecule during preparative or analytical experiments using ultraviolet absorbance or the Folin reaction, and workers have used less convenient methods such as the ninhydrin reaction (3) and labeling with [^14C]palmitate or [^35S]methionine (16, 18). In situ iodination of the lipoprotein may provide a simple alternative method for tracing the molecule during separation procedures.

ACKNOWLEDGMENTS

We thank Rita McNelis and Leon Parkes for technical assistance.

This work was supported by Public Health Service grant AI 10615 from the National Institute of Allergy and Infectious Diseases. R.S.M. was a recipient of postdoctoral fellowship Public Health Service grant F22 AI07711-02 from the National Institute of Allergy and Infectious Diseases.

LITERATURE CITED