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Localization of the Major Dehydrogenases in Two Methylotrophs by Radiochemical Labeling

ANDRZEJ A. KASPRZAK AND DANIEL J. STEENKAMP*

Department of Biochemistry and Biophysics, University of California, San Francisco, California 94143, and Molecular Biology Division, Veterans Administration Medical Center, San Francisco, California 94121

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The localization of prominent proteins in intact cells of two methylotrophic bacteria, *Hyphomicrobium* sp. strain X and bacterium W_3A_1, was investigated by radiochemical labeling with [^{14}C]isethionyl acetimidate. In bacterium W_3A_1, trimethylamine dehydrogenase was not labeled by the reagent and is, therefore, an intracellular protein, whereas the periplasmic location of the methylamine and methanol dehydrogenases was evidenced by being readily labeled in intact cells. Similarly, an intracellular location of the trimethylamine and dimethylamine dehydrogenases in *Hyphomicrobium* sp. strain X was indicated, whereas methanol dehydrogenase was periplasmic.

There are several techniques available for the localization of proteins in gram-negative bacteria. Fractionation of gram-negative organisms into sedimentable spheroplast and a supernatant containing periplasmic proteins by the procedure of Witholt et al. (32) (or modifications thereof) has been used to demonstrate the periplasmic location of several oxidoreductases and electron carrier proteins (1, 2, 5, 14, 33). Unfortunately, however, many gram-negative organisms are insensitive to lysozyme-Tris-EDTA treatment, whereas others undergo uncontrolled lysis. The limited permeability of the cytoplasmic membrane to hydrophilic, polar species has also allowed for the localization of some enzymes. Thus, a comparison of the activity of oxidoreductases with viologen dyes and ferricyanide in intact and broken cells, respectively, served to localize fumarate reductase and formate dehydrogenase on the cytoplasmic side of the inner membrane of *Escherichia coli*, whereas nitrite reductase reacted on the periplasmic side (15). The lactate dehydrogenase of *Desulfovibrio desulfuricans* was localized on the cytoplasmic side of the inner membrane by virtue of the large increase in enzyme activity, with phenazine methosulfate and dichlorophenolindophenol as the electron acceptors, observed upon cell breakage (28). For enzymes which catalyze reactions associated with overall production or uptake of protons, further information on the location of the proton-consuming or -producing site may be obtained by relying on the impermeability of the inner membrane to protons (16, 28), but the results are sometimes ambiguous. Protons generated in the oxidation of succinate, for instance, are released on the outside of the mitochondrial inner membrane (3), although succinate dehydrogenase is located on the matrix side.

In studies of the dehydrogenases of methylotrophic bacteria, we found that cell breakage does not result in an increase in the activities of the methanol, dimethylamine, and trimethylamine dehydrogenases of *Hyphomicrobium* sp. strain X, as measured in the phenazine dehydrogenase-dichlorophenolindophenol-linked assays. Similar results were obtained for the trimethylamine, methanol, and methylamine dehydrogenases of bacterium W_3A_1. These studies agreed in part with results reported by Alefounder and Ferguson (2) and Jones et al. (14), who localized methanol dehydrogenase in the periplasmic space of *Paracoccus denitrificans* and *Methylophilus methylotrophus*, respectively, by preparing spheroplasts of these organisms. We tried a similar approach to obtain evidence for a periplasmic location of the methylamine, dimethylamine, and trimethylamine dehydrogenases in *Hyphomicrobium* sp. strain X and bacterium W_3A_1, but found that these organisms were not converted to spheroplasts by the procedure of Witholt et al. (32) or Jones et al. (14). Moreover, we found that *M. methylotrophus*, which also grows on trimethylamine (18), was prone to undergo spontaneous lysis.

We then resorted to radiochemical labeling of intact cells to localize some of the dehydrogenases involved in the oxidation of methyl groups in methylotrophic bacteria. Preliminary results with diazotized [^{125}I]iodosulfanilic acid indicated that this reagent reacts to a very limited extent with native proteins, as might have been expected from earlier studies (11). The labeling

of intact *Hyphomicrobium* sp. strain X cells, moreover, was very inefficient, possibly due to poor penetration of the outer membrane. Instead, we found that the imidoester isethionyl acetimidate was a very suitable reagent for labeling extracytoplasmic proteins in intact cells. This reagent reacts with amino groups in proteins to form amidines and does not penetrate membranes (6, 23, 31). Nonetheless, there appears to have been only one report on the use of isethionyl acetimidate as a nonpenetrant label of bacterial cells (23), and this compound has not yet been used for the labeling of gram-negative organisms. In this paper, we present evidence that isethionyl acetimidate penetrates the outer membrane, but not the inner membrane, of two gram-negative methylotrophs. Evidence for the cytoplasmic location of the dimethylamine and trimethylamine dehydrogenases and for a periplasmic location of methylamine dehydrogenase is also presented.

MATERIALS AND METHODS

Growth of bacteria. *Hyphomicrobium* sp. strain X was grown under anaerobic conditions at room temperature in a mineral medium (9) supplemented with 0.5% KNO_3 and 0.3% trimethylamine. Bacterium W_3A_1 and *M. methylotrophus* were grown aerobically at 30 and 37°C, respectively, in the medium of Owens and Keddie (21) with 0.3% trimethylamine, 0.5% methanol, or 0.2% methylamine as the carbon and energy source. A culture of *M. methylotrophus* was a gift from D. Byrom. The growth medium containing bacterium W_3A_1 cells was blended for 1 to 2 min at the highest speed of a Waring blender before harvesting, to decapsulate the cells. Cells were harvested in the exponential growth phase.

Isolation of enzymes. The trimethylamine and dimethylamine dehydrogenases of *Hyphomicrobium* sp. strain X and the trimethylamine dehydrogenase of bacterium W_3A_1 and its flavoprotein electron acceptor were isolated as previously described (19, 26, 27). Methanol and methylamine dehydrogenases from bacterium W_3A_1 eluted in the front in 50 mM potassium phosphate (pH 7.2) from the DEAE-cellulose column used in the preparation of trimethylamine dehydrogenase and its electron acceptor flavoprotein (27). The fractions containing methanol and methylamine dehydrogenase activities were dialyzed against 10 mM potassium phosphate (pH 7.2) and applied to a carboxymethyl cellulose-Sephadex C-50 column (3.2 by 30 cm) which had been equilibrated against the same buffer. Some colored material containing *c*-type cytochromes was not retained and was washed through the column in the starting buffer. The column was then eluted with a linear 1-liter gradient (10 to 100 mM potassium phosphate, pH 7.2). Methylamine and methanol dehydrogenases were eluted in that order and identified by their characteristic visible spectra (4, 10). The specific activities were comparable to previously described values (4, 10).

We had initially planned to use hydroxypyruvate reductase, a pyridine nucleotide-dependent enzyme, as a cytoplasmic marker. Although this eventually became superfluous, we found that methanol dehydrogenase from *Hyphomicrobium* sp. strain X copurified with hydroxypyruvate reductase in purification steps which differed only in detail from steps 2, 3, and 4 of the purification scheme of Utting and Kohn (30). Methanol dehydrogenase and hydroxypyruvate reductase were poorly resolved by gel chromatography on Bio-Gel A 0.5 (used instead of step 5 in reference 30). The two enzymes were subsequently dialyzed against 1 mM potassium phosphate (pH 7.0) containing 25% glycerol and 3 mM 2-mercaptoethanol and then applied to a carboxymethyl cellulose-Sephadex C-50 column (3.2 by 30 cm) which had been equilibrated with the same buffer. Methanol dehydrogenase was eluted with 500 ml of the starting buffer and hydroxypyruvate reductase subsequently, using a 1.5-liter linear gradient of 1 to 100 mM potassium phosphate (pH 7.0) containing 25% glycerol and 3 mM 2-mercaptoethanol.

Radiochemical labeling of cells and crude extracts. Freshly grown cells were harvested at 4°C, washed three times by centrifugation with 0.1 M sodium phosphate (pH 8.0), and resuspended with a Potter-Elvejem homogenizer in one-tenth of the original volume. A small portion was kept as "whole cells." The remainder of the cells were disrupted in a French press at 20,000 lb/in^2 (two passages for bacterium W_3A_1 and four for *Hyphomicrobium* sp. strain X, with intermittent cooling to 0°C). A stock solution of $[^{14}C]$-isethionyl acetimidate was prepared in 0.1 M sodium phosphate buffer (pH 8.0) immediately before use. Both whole and broken cells were incubated with 10 mM $[^{14}C]$isethionyl acetimidate (Amersham Corp.) at room temperature for 30 min. The concentration of the reagent in each reaction mixture was estimated by liquid scintillation counting of samples taken from the reaction mixtures and by using the specific radioactivity specified by the manufacturer (58.1 Ci/mol). After being labeled, whole cells were washed four times in a bench top centrifuge with 0.1 M sodium phosphate buffer, pH 8.0. The cells were subsequently extracted for 2 min at 100°C with the digestion buffer described by Laemmli and Favre (17) for the preparation of samples for sodium dodecyl sulfate (SDS)-polyacrylamide disc gel electrophoresis (PAGE). The solutions were subsequently clarified by centrifugation in a bench top centrifuge, and the clear supernatants were used for electrophoretic analyses. Unbound reagent was removed from the broken-cell extract by centrifugation through a 1-ml Sephadex G-25 column (22) which had been equilibrated with the digestion buffer. Radioactively labeled samples from broken and whole cells containing approximately equal amounts of radioactivity, as estimated by liquid scintillation counting, were analyzed by gel electrophoresis. Electrophoresis was performed in a vertical slab gel electrophoresis apparatus by the procedure of Laemmli and Favre (17). The gels were subsequently stained for 35 min in a solution containing 0.05% Coomassie blue G-250, 10% acetic acid, and 25% glycerol (Coomassie blue G-250 staining solution), destained in a solution containing 10% acetic acid and 1% glycerol (destaining solution), and prepared for fluorography in Autofluor (National Diagnostics) according to the instructions of the manufacturer. This entailed soaking the gel for 1 h in 0.1 M $NaHCO_3$, soaking with gentle agitation in Autofluor, and soaking for 15 min in 0.1 N acetic acid,

again with agitation. The gel was placed on filter paper, dried under vacuum, and used to expose a Kodak XAR-2 X-ray film at −70°C.

Electrophoretic analyses of bacteria grown on different substrates. Bacterium W_3A_1 and *M. methylotrophus* were suspended in 10 mM potassium phosphate (pH 7.0) containing 1 mM $MgCl_2$ (1 g of wet cells per 5 ml of buffer). A few crystals of DNase I (Sigma Chemical Co.) were added, and the suspensions were passed twice through a French pressure cell at 20,000 lb/in^2. French press lysates were obtained from either organism after growth on methanol, methylamine, or trimethylamine as the carbon source and were centrifuged at 105,000 × *g* for 1 h. The pellet was regarded as the membrane fraction and was suspended in 10 mM potassium phosphate (pH 7.2; 1 ml/1 g of cells initially taken) to obtain a protein concentration of about 30 mg/ml, estimated by a biuret method (12). Protein samples were mixed in a 1:1 ratio with a solution containing 0.125 M Tris-chloride (pH 6.8), 4% SDS, 20% glycerol, and 0.002% bromophenol blue to obtain the final sample buffer concentrations, as described by Laemmli and Favre (17). The solutions were immersed in a boiling-water bath for 30 s and immediately cooled on ice. SDS-PAGE was performed as described by Laemmli and Favre (17), except that the stacking gel was photopolymerized by using 2.5 μg of riboflavin per ml (final concentration) in the stacking gel. Samples were electrophoresed in a cold room at a current of about 1 mA/mm^2 to minimize heat generation. The gels were stained for heme as described by Thomas et al. (29), photographed, and subsequently transferred to Coomassie blue G-250 solution. After staining overnight, the gels were diffusion destained in destaining solution and stored in the same solution. The heme stain disappeared gradually and after about a week was no longer visible.

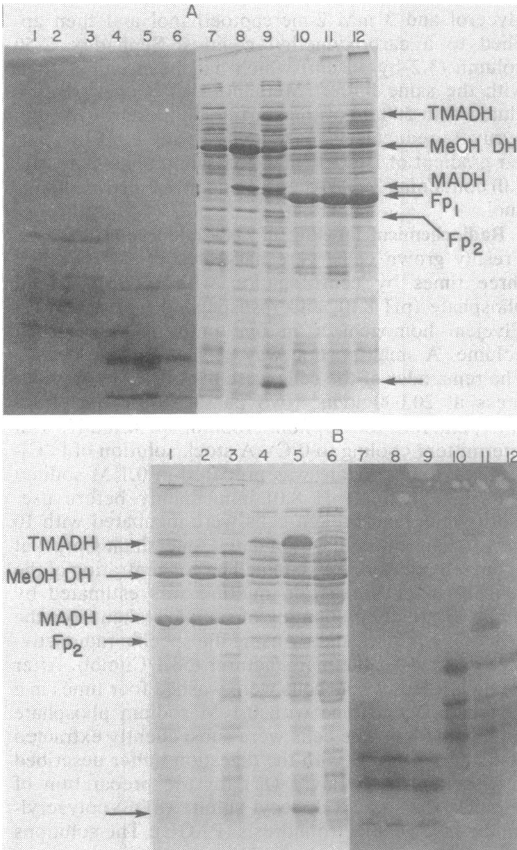

FIG. 1. Comparison of the protein and heme staining pattern after SDS-PAGE of membrane and soluble fractions of bacterium W_3A_1 and *M. methylotrophus*. The resolving gels contained 12% acrylamide and 0.032% *N,N*′-methylenebisacrylamide. (A) Results obtained with bacterium W_3A_1. Lanes 1 through 6 represent heme-staining patterns, and lanes 7 through 12 show the matching protein staining patterns for samples loaded on the same slab gel. The sample size for the protein stain was about one-half that used to obtain the heme staining pattern. Samples loaded were: lanes 1, 2, and 3, membrane fractions from cells grown on trimethylamine, methylamine, and methanol, respectively; lanes 4, 5, and 6, soluble fractions from cells grown on trimethylamine, methylamine, and methanol, respectively; lanes 7, 8, and 9, soluble fractions obtained from cells grown on methanol, methylamine, and trimethylamine, respectively; lanes 10, 11, and 12, membrane fractions from cells grown on methanol, methylamine, and trimethylamine, respectively. (B) Results obtained for *M. methylotrophus*. Lanes 1 through 6 represent the protein staining pattern, and lanes 7 through 12 show the heme staining pattern for samples loaded on the same slab gel. The sample size used in obtaining the protein staining pattern was about one-half of that used to obtain the heme stain. Samples loaded were: lanes 1, 2, and 3, membrane fractions from cells grown on methylamine, trimethylamine, and methanol, respectively; lanes 4, 5, and 6, soluble fractions from cells grown on methylamine, trimethylamine, and methanol, respectively; lanes 7, 8, and 9, soluble fractions from cells grown on methanol, trimethylamine, and methylamine, respectively; lanes 10, 11, and 12, membrane fractions from cells grown on methanol, trimethylamine, and methylamine, respectively. TMADH, trimethylamine dehydrogenase; MADH, methylamine dehydrogenase; MeOH DH, methanol dehydrogenase; Fp_1 and Fp_2, the 38-kdal and 41-kdal subunits of the electron acceptor flavoprotein, respectively. The lowermost arrow indicates the position of migration of the 13-kdal polypeptide.

RESULTS AND DISCUSSION

Electrophoretic identification of dehydrogenases and *c*-type cytochromes in bacterium W_3A_1. Major changes in the polypeptide composition of the obligate methylotrophic bacterium W_3A_1, occurring as a function of the growth substrate, were detected by PAGE in the presence of SDS (Fig. 1). The induction of the large 43-kilodalton (kdal) subunit of methylamine dehydrogenase was seen clearly in the supernatant fractions of cells grown in the presence of trimethylamine or

methylamine (Fig. 1A). In cells grown on trimethylamine, several additional polypeptides, not present in methanol- or methylamine-grown cells, were observed. These correspond to trimethylamine dehydrogenase (83 kdal), the two subunits of the electron transfer flavoprotein of trimethylamine dehydrogenase (~38 and 41 kdal), and a prominent, small peptide of about 13 kdal. In membrane fractions, the larger subunit of the electron transfer flavoprotein (~41 kdal) comigrated with major membrane components. Trimethylamine dehydrogenase and methylamine dehydrogenase were recovered mainly in the supernatant fraction, whereas the electron transfer flavoprotein and the smaller ~13-kdal peptide partition were recovered between the membrane and soluble fraction (Fig. 1A). Since the ~13-kdal peptide is a major component of trimethylamine-grown cells, it was of interest to establish whether it could perhaps be a *c*-type cytochrome, since bacterium W_3A_1, like *M. methylotrophus*, produces multiple *c*-type cytochromes. Comparison of the SDS-gel electrophoretic pattern of *c*-type cytochromes (Fig. 1), as revealed by a heme stain, with the results of the Coomassie blue G-250 staining pattern indicated that this is not the case. These experiments did, however, indicate that the *c*-type cytochromes of bacterium W_3A_1, relative to the total protein content of the cells, are not major components. Consequently, no attempt was made in the present study to localize the *c*-type cytochromes in these bacteria. In *M. methylotrophus*, an organism closely related physiologically to bacterium W_3A_1, a corresponding low-molecular-weight peptide induced by growth on trimethylamine (Fig. 1B) is also not a *c*-type cytochrome. An observation of interest, which was not apparent from previous studies (7, 8), was that the major low-molecular-weight soluble cytochromes in bacterium W_3A_1 and *M. methylotrophus* are not significantly represented in the membrane fraction. The three major *c*-type cytochromes present in the soluble fraction of *M. methylotrophus* probably correspond to cytochrome c_H, c_{LM}, and c_L (7).

Localization of oxidoreductases in bacterium W_3A_1. When whole cells of bacterium W_3A_1 were treated with [^{14}C]isethionyl acetimidate, mainly two components were labeled (Fig. 2). These correspond in molecular weight to methanol dehydrogenase and to the large subunit of methylamine dehydrogenase. Labeling of the small subunit of methylamine dehydrogenase was not observed, possibly due to its low lysine content. Labeling of a component corresponding to the subunit molecular weight of methanol dehydrogenase is in agreement with earlier reports (2, 14) on the periplasmic location of this enzyme. In broken cells, trimethylamine dehydrogenase was heavily labeled, as was the ~13-kdal peptide (Fig. 3B). Amidination of the smaller subunit of the electron acceptor flavoprotein in broken cells but not in whole cells was also observed, although the extent of labeling was relatively low (Fig. 2A). From these results it is apparent that trimethylamine dehydrogenase and its electron acceptor flavoprotein are cytoplasmic proteins, whereas the methanol and methylamine dehydrogenases are periplasmic components of bacterium W_3A_1.

Localization of oxidoreductases in *Hyphomicrobium* sp. strain X. In vitro studies have shown that the trimethylamine and dimethylamine dehydrogenases of *Hyphomicrobium* sp. strain X both reduce the electron acceptor flavoprotein

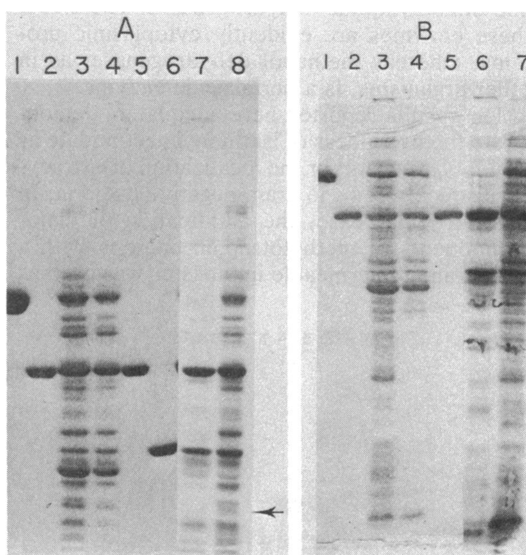

FIG. 2. Differential labeling of whole and broken cells of bacterium W_3A_1 with [^{14}C]isethionyl acetimidate. Whole and broken cells were labeled with the reagent at a concentration of 10 mM for 30 min, and SDS-PAGE in 8% (A) and 12% acrylamide (B) was performed as described in the text. Lanes 1 through 6 in (A) and 1 through 5 in (B) show Coomassie blue G-250 staining; lanes 7 and 8 in (A) and lanes 6 and 7 in (B) show the distribution of radioactivity in lanes 3 and 4 in (A) and in lanes 3 and 4 in (B) as measured by fluorography. (A) The samples loaded were: lane 1, trimethylamine dehydrogenase; lanes 2 and 5, methanol dehydrogenase; lane 3, whole cells; lane 4, broken cells; lane 6, methylamine dehydrogenase; lane 7, fluorogram of lane 3 (whole cells); lane 8, fluorogram of lane 4 (broken cells). The arrow indicates the position of migration of the smaller subunit of the electron transfer flavoprotein from bacterium W_3A_1. (B) The samples loaded were: lane 1, trimethylamine dehydrogenase; lanes 2 and 5, methanol dehydrogenase; lane 3, whole cells; lane 4, broken cells; lane 6, fluorogram of lane 3 (whole cells); lane 7, fluorogram of lane 4 (broken cells).

of bacterium W_3A_1. Nonetheless, we were unable to detect this flavoprotein in extracts of *Hyphomicrobium* sp. strain X; electron transfer to the respiratory chain is, therefore, likely to be mediated by different electron carrier proteins. For this reason, an extrapolation of our findings for the trimethylamine dehydrogenase of bacterium W_3A_1 to the trimethylamine and dimethylamine dehydrogenases of *Hyphomicrobium* sp. strain X was not warranted.

Figure 3 shows the amidination pattern of the major polypeptides of whole and broken *Hyphomicrobium* sp. strain X cells. The major polypeptide labeled in whole cells comigrates with methanol dehydrogenase during SDS-PAGE. After cell breakage, several additional proteins are labeled, the most conspicuous being polypeptides which comigrate with trimethylamine and dimethylamine dehydrogenases. Therefore, these enzymes are evidently cytoplasmic proteins, whereas methanol dehydrogenase, as in other organisms, is a periplasmic enzyme.

The results reported here adequately demonstrate the usefulness of isethionyl acetimidate as a suitable reagent for the localization of extracytoplasmic proteins in gram-negative bacteria. In this particular case, the amidination of major components of methylotrophic bacteria with a membrane-impermeable imidoester was used as

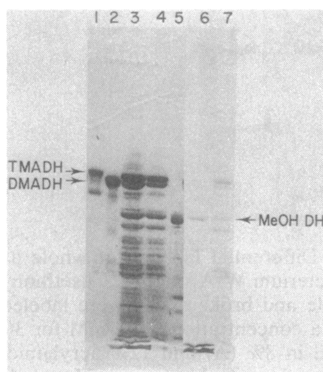

FIG. 3. Differential labeling of whole and broken cells of *Hyphomicrobium* sp. strain X with [^{14}C]isethionyl acetimidate. Whole and broken cells were labeled with the reagent at a concentration of 10 mM for 30 min and subjected to SDS-PAGE in 8% acrylamide, as described in the text. Partially purified preparations of trimethylamine dehydrogenase (TMADH), dimethylamine dehydrogenase (DMADH), and methanol dehydrogenase (MeOH DH) were used as markers. Lanes 1 through 5 show Coomassie blue G-250 staining; lanes 6 and 7 indicate the distribution of radioactivity in lanes 3 and 4. The samples loaded were: lane 1, trimethylamine dehydrogenase; lane 2, dimethylamine dehydrogenase; lane 3, whole cells; lane 4, broken cells; lane 5, methanol dehydrogenase; lane 6, fluorogram of lane 3 (whole cells); lane 7, fluorogram of lane 4 (broken cells).

a probe of their disposition relative to the cytoplasmic membrane. In conjunction with immunoprecipitation, as recently done by Hederstedt and Rutberg (13) with a diazonium reagent, the method should also give unambiguous results for minor constituents of cells. Moreover, amidination of extracytoplasmic components with isethionyl acetimidate does not seem to have some of the drawbacks encountered with other membrane-impermeable reagents. The method does not require selective disruption of the outer membrane, the conditions for which have to be empirically established for different organisms. Interference from fragments of outer membrane and wall was thought to contribute to the low extent of labeling when acetyl [^{35}S]methionyl methylphosphate sulfone was used to label nascent peptides of periplasmic proteins in spheroplasts of *E. coli* (25). In agreement with several previous reports (6, 20, 23, 31), isethionyl acetimidate seems to be membrane impermeable even at relatively high concentrations, as evidenced by the fact that most of the proteins which were heavily labeled in broken cells of the methylotrophs were not labeled in intact cells. By contrast, the penetration of cells by [^{125}I]diazobenzene sulfonic acid necessitated the use of this reagent at very low concentrations, which presumably resulted in the poor labeling of cotranslationally secreted peptide chains (24). The saturation of periplasmic components with isethionyl acetimidate should allow for the differential labeling of components, which are not extracytoplasmic, with membrane-permeable imidoesters such as ethylacetimidate, as suggested by Whiteley and Berg (31). The extent to which this objective can be achieved with intact gram-negative organisms is presently being investigated.

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