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MODIFICATION OF A FERRIC ENTEROBACTIN RECEPTOR PROTEIN FROM THE OUTER MEMBRANE OF *Escherichia Coli*.

William C. Hollifield, Jr., Ellen H. Fiss and J. B. Neilands

Department of Biochemistry, University of California, Berkeley, California 94720

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SUMMARY: Modification of a ferric enterobactin receptor protein of *Escherichia coli* was observed upon incubation of either whole membranes or Triton X-100 solubilized outer membrane at 37°C. The modification was characterized by a change in mobility of the receptor band on SDS polyacrylamide gel electrophoresis and by a decreased binding capacity for ferric enterobactin. The rate of modification was affected by temperature and a trypsin inhibitor, benzamidine. Ferric enterobactin inhibited the reaction in whole membrane. The modification affected the limited chymotrypsin digestion pattern of the receptor. The activity may represent a specific modification of the receptor, one possibly mediated by a membrane-associated enzyme.

INTRODUCTION: *Escherichia coli*, when grown in low iron ($<10^{-6}$M) media greatly increases the level of several proteins in the outer membrane (1). These include the *cbr* gene product which serves as the common receptor for ferric enterobactin and colicin B (2), and the *cir* gene product, the colicin Ia receptor (3). We shall use the designation of Braun (4), wherein the *cbr* and *cir* gene products are referred to as 81K and 74K respectively. Solubilization of the outer membrane of low iron cells by Triton X-100 and NaEDTA[1] yields a preparation capable of binding [^{55}Fe]ferric enterobactin and colicin B, an activity dependent on the presence of 81K (5). Preliminary attempts in this laboratory at purification of the ferric enterobactin receptor produced fractions with binding activity ([^{55}Fe]ferric enterobactin and/or colicin B) which upon examination after some time by SDS-PAGE showed disappearance of the 81K band and appearance of a new band migrating more slowly than the 74K band. The experimental work reported here demonstrates the conversion of the 81K protein to this new protein, to be referred to as 81K*.

[1]Abbreviations: NaEDTA, sodium ethylene diamine tetraacetic acid; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; DTT, dithiothreitol; PMSF, phenylmethyl-sulfonylfluoride; DFP, diisopropyl fluorophosphate; TPCK, L-1-tosylamido-2-phenylethylchloromethyl ketone; DPCC, diphenylcarbamyl chloride; BSA, bovine serum albumin.

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MATERIALS AND METHODS: RW193 and RWB18 are previously described *E. coli* K-12 strains (5), neither of which synthesize enterobactin. RWB18 is a mutant of RW193 resistant to colicin B and lacking 81K. BN3040 is a spontaneous mutant of RW193 resistant to colicin Ia and lacking 74K.

[^{55}Fe]ferric enterobactin was prepared as described in (5).

Cultures were grown at 37°C in Tris media (6) lacking $FeSO_4$ with a 2% inoculum from a nutrient broth culture. Cells were collected in late log to stationary phase at approximately 14 hours after inoculation, washed twice in 0.1 M Tris-HCl pH 8 for 15 minutes followed by centrifugation of the supernatant at 250,000 x g for 30 minutes. The pelleted membranes were extracted as per Schnaitman (7) using a 4:1 w/w ratio of Triton X-100 to protein. The final Triton X-100, NaEDTA extraction used a 2:1 ratio. The extraction solutions were 2% Triton X-100, 0.1 M Tris-HCl, ± 0.005 M Na_2EDTA, pH 8.

SDS-PAGE slabs were run using the Lugtenberg system (8). Gels were stained with Coomassie Blue (9). Transmission scans were performed at 600 nm on gels dried between dialysis tubing, using a Transdyne RFT Scanning Densitometer. Peak areas were determined by excising and weighing the peaks.

Limited proteolytic digesting and staining of 81K and 81K* were performed exactly as described by Cleveland *et al.* (10). The time of incubation for digestion in the gel was 30 minutes.

Protein was determined by the Wang and Smith (11) modification of the Lowry method. [^{55}Fe]ferric enterobactin binding to the receptor was measured as described in (5).

Incubation of whole membrane was performed at 37°C in 0.1 M Tris-HCl pH 8. The outer membrane was incubated at 37°C in the extraction buffer.

RESULTS: Figure 1 shows the relationship of bands 81K, 81K* and 74K as seen in solubilized outer membranes of RW193.

Upon incubation of an outer membrane preparation from BN3040 at 37°C a decrease in the level of 81K and an increase in the level of 81K* protein was found in SDS-PAGE profiles of the reaction mixtures (Figure 2). BN3040 was chosen to avoid interference between 74K and 81K* on scanning; the same effect was seen in RW193 preparations, albeit less clearly. Concomitantly a second protein band (Z) was seen to decrease. The rate of conversion of 81K varied substantially between preparations. The slight 81K* band seen in the zero minute well in Figure 2 was probably the product of the extraction procedures since little or none was seen in whole membrane profiles.

Similar incubation, followed by measurement of the ability of a RW193 preparation to bind [^{55}Fe]ferric enterobactin, produced the results shown in Figure 3A. A decrease in binding activity with time was seen at 37°C, the

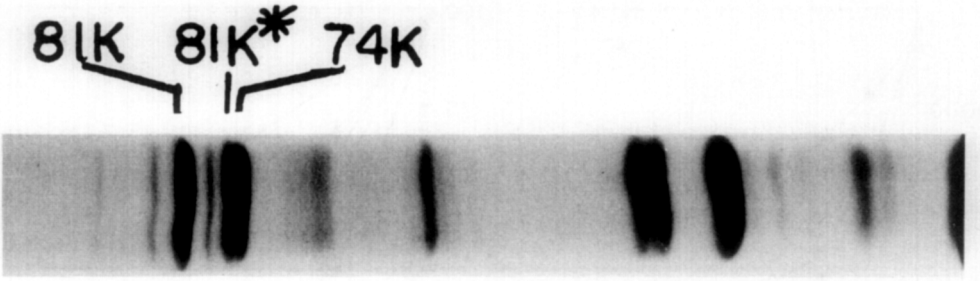

FIGURE 1: SDS-PAGE profile of RW193 solubilized outer membrane, 9% acrylamide slab gel. Decreasing molecular weight left to right.

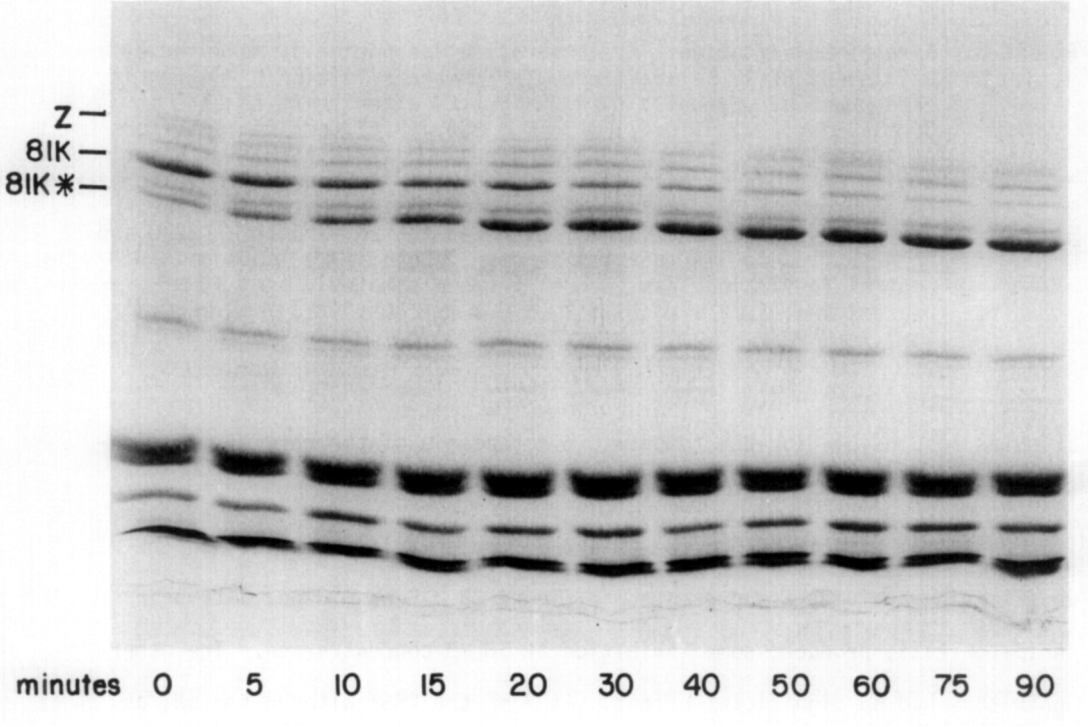

FIGURE 2: Time course of incubation of BN3040 solubilized outer membrane at 37°C, 7.5% acrylamide slab gel.

rate being unaffected by a 500% increase in protein concentration in the form of BSA. At 50°C the rate of loss of binding activity was significantly slower, but returned to normal upon readjusting the temperature to 37°C. These and other experiments ruled out either a change in pH or an irreversible denatur-

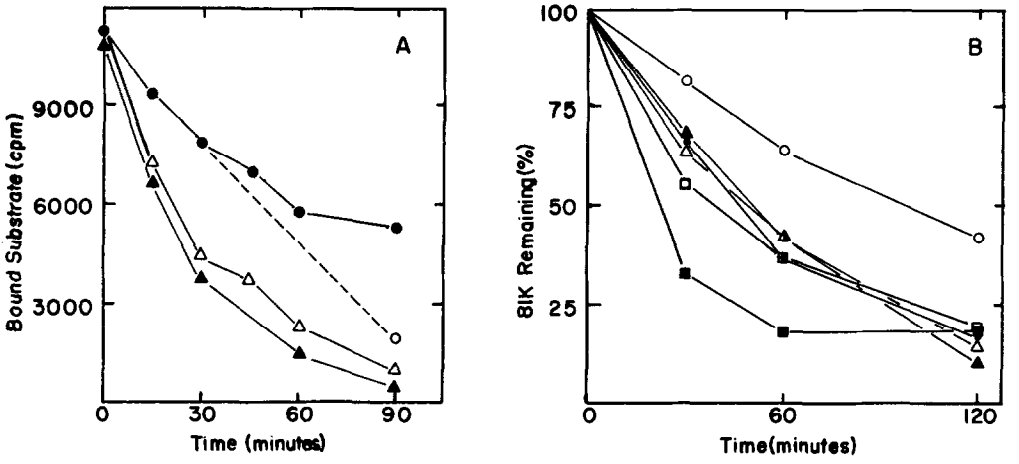

FIGURE 3: Rate of modification: A. Temperature dependence is measured by ability of RW193 solubilized outer membrane to bind [^{55}Fe]ferric enterobactin. Symbols: (●) 50°C; (○) after shift to 37°C at 30 minutes; (△) 37°C; (▲) 37°C + BSA. B. Effect of inhibitors of proteolysis as measured by conversion of 81K to 81K*. Distribution of bands normalized to 100% 81K at time zero. Inhibitor, in isopropanol, was mixed 1:9 with solubilized outer membrane from BN3040; the mixture was incubated for 20 minutes at 22°C and then shifted to 37°C at time zero. Symbols: (●) isopropanol control (deviation less than 5% between samples); (○) 10 mM benzamidine; (▲) 1 mM DTT; (△) 0.7 mM TPCK; (□) 2 mM DPCC; (■) 7 mM PMSF.

ation as responsible for the temperature dependence of the rate of modification.

Data from experiments on the effect of proteolysis inhibitors on the modification activity are presented in Figure 3B. Benzamidine, a trypsin inhibitor (12), produced some inhibition at 10 mM concentration. The other inhibitors had no significant effect. At 7 mM the PMSF altered the pH, as described by MacGregor (13), apparently as a result of its breakdown. This pH change probably accounted for the enhancement of 81K modification shown in Figure 3B. Qualitatively it was found that 1 mM DFP or PMSF had no observable effect on the rate of modification, although the chymotryptic digestion of BSA was blocked under similar conditions.

Whole membranes from BN3040 when incubated at 37°C and analyzed by SDS-PAGE also displayed the conversion of 81K to 81K* (Figure 4) which ruled out

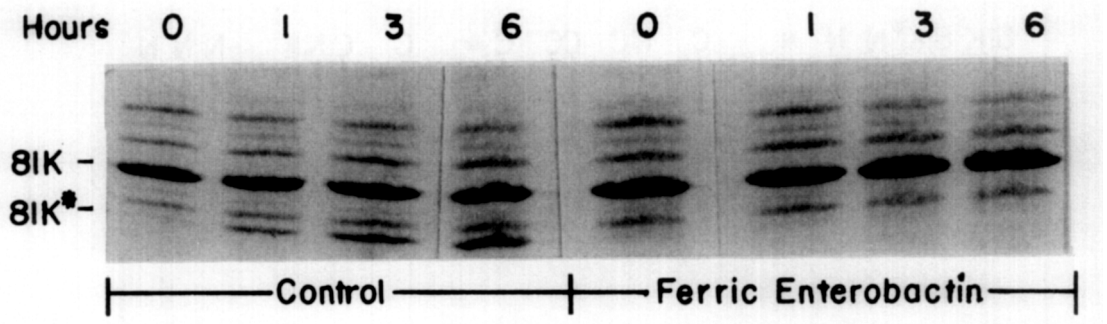

FIGURE 4: Time course of incubation of whole membrane from BN3040 at 37°C, 40 μM ferric enterobactin. Gel was 7.5% acrylamide slab. Region shown is between approximately 70K and 100K.

the modification activity as an artifact of solubilization. The time scale was much longer and conversion never exceeded 50% even after 24 hours. Figure 4 also presents the SDS-PAGE profiles of whole membranes from BN3040 incubated at 37°C in the presence of 40 μM ferric enterobactin. This totally inhibited the conversion of 81K to 81K* over a six-hour period. At 4 μM the effect was less complete. Ferrichrome, a siderophore which does not bind the ferric enterobactin receptor (5), failed to inhibit the conversion even at a concentration of 40 μM. In solubilized outer membrane preparations 40 μM ferric enterobactin did block the rate of modification of 81K, but not that of band Z.

An example of limited chymotrypsin digestion of the 81K and 81K* bands from SDS-PAGE slab gels is shown in Figure 5. Many common fragments can be seen (C), although with some variation in intensity. Several bands were associated with only one protein (N for 81K, * for 81K*). These data demonstrate a common relationship between the structures of 81K and 81K*, but also indicates their non-identity.

DISCUSSION: Evidence for a modification of the ferric enterobactin receptor has been presented. This effect was seen in both whole membrane and solubilized outer membrane preparations, and was demonstrated by several techniques. Variation in activity between otherwise equivalent preparations (criteria: SDS-PAGE, siderophore binding, colicin B binding), together with a parallel

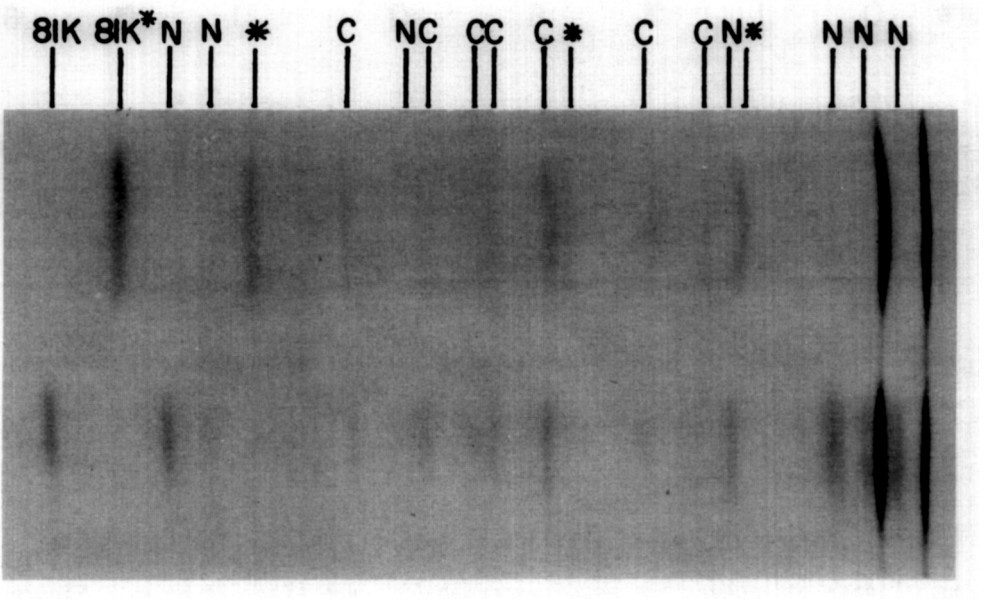

FIGURE 5: SDS-PAGE profile of limited chymotryptic digestion of 81K and 81K*, 15% acrylamide slab gel, with 0.2 µg chymotrypsin per well. Symbols: (C), fragments common to both 81K and 81K*. Decreasing molecular weight left to right.

variation in the rate of disappearance of band Z in Figure 2, suggested the causative agent to be a receptor-independent mediator. The limited modification seen in whole membrane may be the result of enzyme denaturation, enzyme immobilization, or limited exposure of the sensitive site. The inhibition of the activity by ferric enterobactin probably results from the siderophore either protecting or masking the labile site.

The limited proteolytic digestion profiles pointed to the modification as covalent in nature. The inhibition of the activity by benzamidine was suggestive of the presence of a protease, but other explanations are possible. The failure of DPCC to inhibit the rate of degradation contrasts with the strong inhibition observed with the protease described by MacGregor (13), although the differences in reaction conditions may also affect the spectrum of inhibition by various agents. The failure of DFP to inhibit at 1 mM would argue against Protease I as an explanation since 50% inhibition of this

enzyme was reported for a 0.1 mM concentration of the agent (14). The stability of DFP under the reaction conditions employed here was uncertain, but the question could not be further explored.

The modification herein reported will clearly interfere with any SDS-PAGE-dependent studies on either 81K or 74K proteins if the proper precautions are not taken. The specificity with regard to the ferric enterobactin receptor should provide a useful tool for dissecting the structure of this protein. It may represent a new addition to the expanding collection of exocytoplasmic enzymes with proteolytic-like activity. The possible association of this enzyme with the outer membrane is indicated by the retention of activity through several detergent extractions, though determination of its levels in other fractions has been frustrated by the lack of a suitable assay. The possible involvement of this activity in the mechanism of ferric enterobactin transport has been considered, but experiments in this regard have been inconclusive. A detailed investigation of both the receptor and its modification is proceeding in this laboratory.

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