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ABSENCE OF FERRIC ENTEROBACTIN RECEPTOR MODIFICATION ACTIVITY IN MUTANTS OF *Escherichia coli* K-12 LACKING PROTEIN *a*

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SUMMARY: The modification activity for the ferric enterobactin receptor in the Triton X-100 solubilized outer membrane of *Escherichia coli* K-12 was adsorbed to a column of *p*-aminobenzamidine-//-sepharose and eluted with free benzamidine. Recombination of the dialyzed eluate with the filtrate from the column reinstituted conversion of the receptor from 81K to 81K*, the latter exhibiting an apparent molecular weight of 74,000 daltons in sodium dodecyl sulfate polyacrylamide gel analysis. The eluate from the *p*-aminobenzamidine column was shown to contain a component, coincident on gels with both protein and modification activity, which by mutational and other analyses appears to be identical with protein *a* of the outer membrane.

INTRODUCTION: We have described the presence of a modification activity in either whole membranes or Triton X-100 solubilized outer membrane which results in conversion of the SDS-PAGE[1] molecular weight of the receptor for ferric enterobactin, the high affinity iron transport compound (siderophore) of *Escherichia coli* K-12, from 81K to 74K (1). Benzamidine was identified as an effective inhibitor of this transformation. We now report that this activity can be adsorbed reversibly to a *p*-aminobenzamidine-//-sepharose column and we present evidence suggesting its identity with component *a*, a well known major outer membrane protein of previously undescribed biochemical activity.

MATERIALS AND METHODS: The characteristics of the strains of *E. coli* K-12 used in this work are recorded in Table 1. The cells were grown to late log to stationary phase at 37° in low iron Tris medium and the pelleted membranes extracted

[1] Abbreviations: SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; Tris, trihydroxymethylaminomethane; *entA*⁻, mutant in enterobactin (enterochelin) biosynthesis at gene A; *colIa*ʳ, mutant lacking colicin Ia receptor; *cbr*⁻, mutant lacking colicin B receptor; *a*⁻, mutant lacking protein *a*; *cbr*, colicin B receptor = ferric enterobactin receptor.

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TABLE 1

Characteristics and Sources of Strains of Escherichia coli K-12

Strain*	Relevant characteristics	Source (Ref.)
BN3040	entA⁻, collIa^r	(1)
UT2300	entA⁻, cbr⁻	(2)
UT4400	entA⁻, cbr⁻, a⁻	(2,3)
UT5600	entA⁻, cbr⁻, a⁻	(2,3)

*All strains derived from RW193 (4).

as described previously (1,5). SDS-PAGE slabs were run in the Lugtenberg system and stained with Coomassie Blue (6,7). Analyses on 7% polyacrylamide gel under non-denaturing conditions were performed by substitution of Triton X-100 for SDS (6). Protein was assayed by the Wang and Smith (8) modification of the Lowry procedure.

Benzamidine was obtained from the Aldrich Chemical Company and aminocaproyl-p-aminobenzamidine-//-sepharose^R 4B was purchased from Pierce Chemical Company. The affinity adsorbent was suspended in buffer consisting of 2% Triton X-100, 100 mM NaCl and 100 mM Tris pH 8.0 and was contained in a column of 6 ml bed volume.

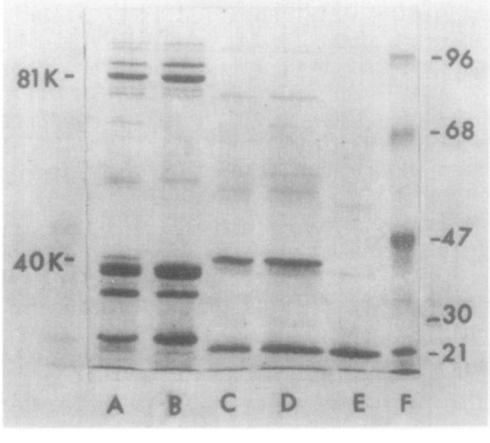

FIGURE 1: SDS-PAGE analysis of outer membrane preparation of E. coli BN3040 following adsorption to a p-aminobenzamidine-//-sepharose^R 4B column and elution with free benzamidine. Lane A, solubilized outer membrane; lane B, filtrate from p-aminobenzamidine-//-sepharose^R 4B column; lanes C and D, free benzamidine eluate, 5 and 10 µg of protein, respectively; lane E, 5 µg of eluate heated at 37° for 30 min.; lane F, molecular weight standards.

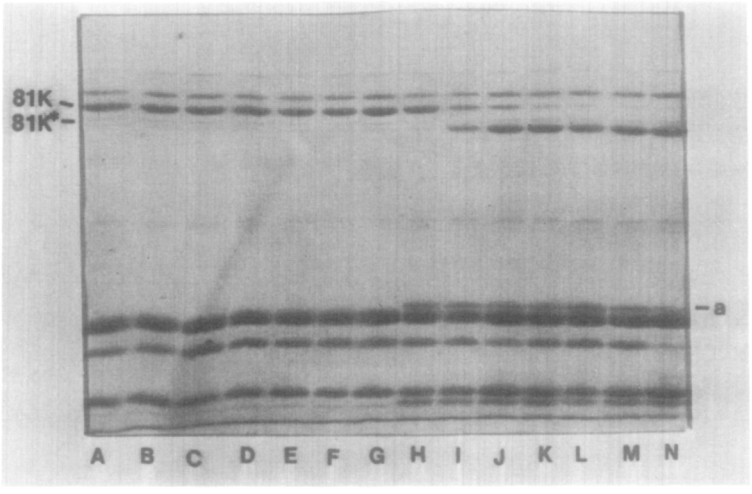

FIGURE 2: A time course profile by SDS-PAGE of resolved and reconstituted outer membrane preparation of E. coli BN3040. Lanes A-G, filtrate from p-aminobenzamidine-//-sepharose^{R} 4B column incubated at 37^{o}C and analyzed at 30 min. intervals from zero time (A) up to 3 hrs (G); lanes H-N, combined filtrate and dialyzed eluate from p-aminobenzamidine column incubated at 37^{o}C and analyzed at 30 min. intervals from zero time (H) up to 3 hrs (N).

RESULTS: Figure 1 shows outer membrane profiles from BN3040, a strain which lacks the colicin Ia receptor and is defective in enterobactin biosynthesis. Growth of this siderophore-minus organism in Tris medium assures strong induction of cbr at 81K; the colicin Ia receptor, if it were present, would for reasons unknown also be induced at low iron and would occur in the 74K region of the gels, where it would obscure the transition of 81K to 81K*. Figure 1 illustrates that the simple expedient of filtration through the p-aminobenzamidine column eliminates a prominent band with apparent molecular weight of about 40K (lanes A and B). The figure also shows that elution of the column with free benzamidine followed by exhaustive dialysis to remove the inhibitor afforded a gel with a staining pattern reminiscent of that reported in the literature (9) for protein a (lanes C and D). Since the migration rate of protein a in SDS-PAGE is dependent upon the solubilization conditions (9), we incubated the eluted fraction at 37^{o}C for 30 min. instead of at 100^{o}C for 5 min. As shown in Figure 1 (lane E), solubilization at 37^{o} resulted in a more rapid migration of a larger proportion of the major band in the eluate from the p-aminobenzamidine column. Thus the band at 40K in the benzamidine eluate has chemical and physical properties ascribed to protein a.

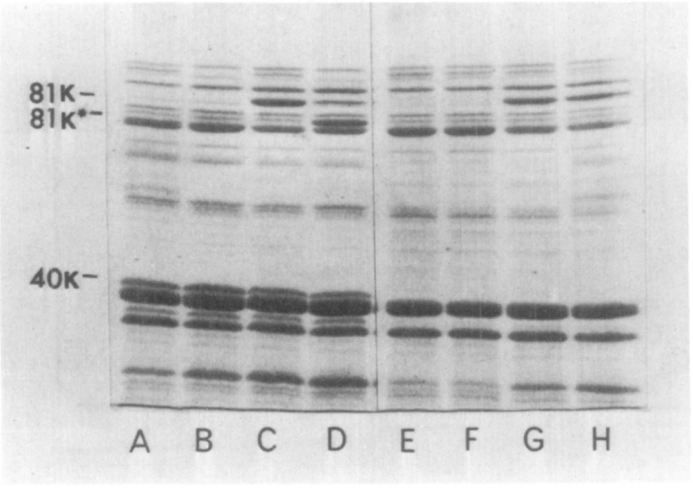

FIGURE 3: SDS-PAGE analysis of outer membrane preparation of E. coli UT2300 (A-D) and UT4400 (E-H). Lane A, zero time; lane B, after incubation 5 hrs at 37°C; lane C, as in lane A after addition of filtrate from p-aminobenzamidine column of preparation derived from BN3040 and containing 81K, zero time; lane D, as in lane C after incubation at 37°C for 5 hrs; lanes E-H, identical sequence of analyses applied to UT4400.

We next examined the stability of the ferric enterobactin receptor following its passage through the p-aminobenzamidine column. From the data presented in Figure 2 it is apparent that this filtration step removes the factor(s) responsible for the modification activity (lanes A-G). Furthermore, as also shown in Figure 2, reconstitution of the eluted and dialyzed fraction with the filtrate restores the activity (lanes H-N). This demonstrates that the gradual conversion of 81K to 81K* is not an intrinsic property of the ferric enterobactin receptor. It also provides a stable preparation of the receptor against which various fractions and extracts of mutants can be examined for the presence of processing activity.

In an effort to correlate precisely protein a with the processing activity we have investigated a series of mutants carrying lesions in the region of the iron operon at minute 13 of the E. coli K-12 chromosome. Protein a has recently been mapped in this area (3). The data are shown in Figure 3. In UT2300, which is replete with respect to protein a, the addition of receptor results in its processing (lanes C and D). This was not observed in UT4400 (lanes G and H) or in UT5600, a larger deletion (data not shown), which the gel profiles reveal to lack the bands at both 81K and 40K.

The use of membrane preparation solubilized with Triton X-100 in the absence of SDS enabled a further correlation of the major protein band in the eluate from the p-aminobenzamidine column with the modification activity. In data not shown here in detail, aliquots of dialyzed benzamidine eluate from the p-aminobenzamidine-//-sepharose column were applied to several 0.5 x 10 cm tubes containing 7% polyacrylamide gel. Separation at 100 v. for 6 hrs sufficed to move the only band stainable with Coomassie Blue to the center of the gel. A simultaneously prepared, unstained gel was divided into 5 mm sections, individual extracts of which were incubated with the cbr-containing filtrate from the p-aminobenzamidine column. Activity was detected only in the single gel segment exactly opposite the band in the companion gel which stained with Coomassie Blue.

DISCUSSION: The exact nature of the processing or modification activity in the outer membrane of E. coli K-12 resulting in the conversion of the ferric enterobactin receptor to 81K*, a component behaving on gels as though it had a molecular weight some 7000 daltons less than 81K, has not yet been established (1). Its inhibition with benzamidine and affinity for a p-aminobenzamidine-//-sepharose column suggests that it is a protease.

It has been established that a proteolytic step is required in the maturation of exported proteins in both prokaryotic and eukaryotic cells (10). However, protein a is unlikely to be an exclusive signal peptidase since in its absence most of the common proteins of the outer membrane of E. coli K-12 appear to occur in normal abundance. It may bear a more intimate relationship to the ferric enterobactin receptor, particularly since mutants lacking only protein a (and not also cbr) have so far not been reported. Thus protein a could be the signal peptidase for cbr and retain some residual action upon the finished receptor. It must be added, however, that we have no indication that the processing activity we describe occurs in vivo. The activity is significant nonetheless since it appears to inactivate the protein as a receptor for ferric enterobactin but not for colicin B (1).

The relationship of ferric enterobactin processing to the various proteolytic activities recently identified in the outer membrane of E. coli is similarly obscure.

These include proteases for the solubilization of nitrate reductase (11), the maturation of alkaline phosphatase (12), the cleavage and activation of colicins (13) and the degradation of casein (14).

Protein a is obviously present in substantial quantities when E. coli K-12 is cultured at low iron in minimal media. Its location at 12.5 minutes on the chromosome places it in or very close to the iron operon centered at minute 13.

Although the ferric enterobactin receptor is the component which most obviously suffers processing it is likely that additional proteins of the outer membrane are affected. Thus the band at about 96K ("Z") appears to be similarly acted upon (1).

Current research is aimed at elucidation of the mechanism of action and biological significance of the modification activity herein assigned to protein a.

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