Properties and Proteolysis of Ferric Enterobactin Outer Membrane Receptor in *Escherichia coli* K12†

Ellen H. Fiss, Pat Stanley-Samuelson, and J. B. Neilands*

ABSTRACT: A protein with a relative subunit molecular weight of 81 000 (81K) has been isolated in virtually pure form from the outer membrane of low iron grown cells of *Escherichia coli* K12. The 81K protein, which is part of the receptor complex for translocation of the siderophore ferric enterobactin, displays activity in vitro for binding both ferric enterobactin and colicin B. The dissociation constant for the 81K–ferric enterobactin compound at 4 °C in 2% Triton–0.1 M Tris, pH 7, was determined to be 10 nM. The N-terminal amino acid was identified as phenylalanine, and the amino acid composition was shown to be similar to that published for the ferric aerobactin–cloacin receptor of *Enterobacter cloacae*. A plasmid-bearing strain of *E. coli* was employed to confirm that degradation of 81K to a slightly smaller, inactive form (81K*) is performed by a second outer membrane component, protein a. The endoproteolytic action of protein a was verified by the finding of alanine as the N-terminal residue of 81K*. A survey of enteric species suggests that the 81K–protein a interaction is confined to the K12 strain of *E. coli*.

The strict requirement for iron in microorganisms has necessitated the expression of complex iron sequestration and transport systems under low iron conditions. The ability to sequester iron, which is either insoluble or bound to proteins such as transferrin, has been suggested as one of the factors determining virulence of an organism. Enteric bacteria, such as *Escherichia coli* K12, when grown in iron-poor media, produce the siderophore enterobactin. The large formation constant for iron(III) enables enterobactin to solubilize iron from ferric oxyhydroxide polymers and to compete effectively against iron-binding proteins (Neilands, 1981).

Due to the size, in excess of 700 daltons, and the hydrophilic nature of ferric enterobactin, *E. coli* requires an outer membrane component to transport the iron ligand into the cell. The first step in the transport of ferric enterobactin involves the binding of the ligand to an outer membrane receptor, which also serves as the binding site for colicins B and D (Wayne et al., 1976). This receptor has been referred to as the product of the *cbr* gene, later renamed the *fepA* gene. It has also been specified by its relative subunit molecular weight (M_r)[1] of 81K, as estimated by $NaDodSO_4$–polyacrylamide gel electrophoresis. This paper will conform to the latter designation.

In a previous communication, an assay was described (Hollifield & Neilands, 1978) that measures the binding of [^{55}Fe]ferric enterobactin to receptor solubilized in Triton. So that better understanding of the the process of ferric enterobactin transport could be gained, the receptor, still retaining binding activity, was purified to ~90% electrophoretic homogeneity. The present communication describes the isolation and partial characterization of the 81K protein, which is at least an essential component of the receptor.

While elaborating the purification procedure, a novel proteolytic activity was identified in the outer membranes that preferentially acts upon the receptor in vitro (Hollifield et al., 1978). The receptor 81K is inactivated by cleavage to a smaller form, called 81K*, migrating on $NaDodSO_4$–polyacrylamide gel electrophoresis with a M_r of 74K. This activity has been ascribed to protein a (Fiss et al., 1979), an outer membrane component also known as protein 3b (Bassford et al., 1977). In this paper we describe further the action of protein a on 81K.

Materials and Methods

Materials. $^{55}FeCl_3$ in 0.5 M HCl was purchased from ICN Corp. DEAE-cellulose was purchased from Whatman Corp. in the form of DE-52. Enterobactin was isolated from *E. coli* strain AN102 as previously described (Wayne et al., 1976). [^{55}Fe]Ferric enterobactin, prepared according to the procedure for formation of ferric catecholates, and colicin B were obtained by the methods given by Hollifield & Neilands (1978). Bis(2,3-dihydroxybenzoyl)lysine was synthesized in this laboratory by T. Peterson and A. Chimiak via a modification of the method of Corbin & Bulen (1969). Bio-Gels A5M and A.5M were obtained from Bio-Rad Laboratories.

Bacterial Strains. Bacterial strains derived from *E. coli* K12 include BN3040 and UT4400 and have been previously described (Fiss et al., 1979). Plasmid DNA pGGC110 is a pBR322 derivative of pMC44 (Gayda & Markovitz, 1978), while *E. coli* B, *E. coli* B/r and *Salmonella typhimurium* LT2 are from laboratory stocks.

Assay Procedures. The assay measuring the ability of the receptor to bind [^{55}Fe]Ferric enterobactin has been described (Hollifield & Neilands, 1978). The colicin B protection assay was a modification of the triple layer plate assay developed by Davies & Reeves (1975). The first layer contained just enough colicin B to kill an entire plate of sensitive cells. Two microliters of sample were spotted onto this layer. The plate was then overlayed with top agar seeded with 0.1 μL of an overnight culture of sensitive cells.

Outer Membrane Isolation. E. coli K12 BN3040 cells were passaged and grown in minimal salts Tris media lacking $FeSO_4$ (Simon & Tessman, 1963). The cells were grown to late log phase at 37 °C, harvested by centrifugation, and washed in 10 mM Tris, pH 8, and the cells were broken by sonication with a Branson sonifier (a total of 4 min in an ice–salt bath). The cell debris and unbroken cells were removed by low-speed centrifugation, and the membranes contained in the supernatant were pelleted by ultracentrifugation at 38 000 rpm for

† From the Department of Biochemistry, University of California, Berkeley, California 94720. *Received March 9, 1982.* This work was supported by Grants AI04156, AM17146, and ES07075 from the National Institutes of Health.

[1] Abbreviations: M_r, relative subunit molecular weight; $NaDodSO_4$, sodium dodecyl sulfate; DEAE, diethylaminoethyl; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetate; TPCK, L-1-(tosylamido)-2-phenylethyl chloromethyl ketone; Mo-diDHB-Lys, molybdenum(VI) bis(2,3-dihydroxybenzyl)lysine.

0006-2960/82/0421-4517$01.25/0

30 min. The pellet was washed in Tris buffer containing 10 mM $MgCl_2$. The solubilization of the outer and cytoplasmic membranes was based upon the procedures described by Schnaitman (1971) with 2% Triton–10 mM Tris, pH 8, minus and plus 5 mM EDTA.

Protein Determination. Protein concentration was measured by the method of Lowry et al. (1951) or, for solutions containing Triton X-100, by a procedure developed by Dudley & Grieve (1975).

$NaDodSO_4$–Polyacrylamide Gel Electrophoresis. The protein composition of samples was determined with analytical 10% $NaDodSO_4$–polyacrylamide gel electrophoresis as previously described (Fiss et al., 1979).

Affinity Resin. The resin was prepared by coupling molybdenum(VI) bis(2,3-dihydroxybenzyl)lysine to Affigel 102 with 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide hydrochloride at pH 5.0 at room temperature overnight. The ligand was combined with the resin in the presence of the coupling agent with a 2:1:10 molar basis, respectively. Coupling was monitored by absorbance, the molybdenum chelate imparting an orange-yellow color to the resin.

Additional Procedures. The binding constant of 81K for [^{55}Fe]ferric enterobactin was determined by the method of Hummel & Dryer (1962). The K_d was calculated from the slope of the *Scatchard* plot (Fairclough & Fruton, 1966). The amino acid composition was determined by using the procedure of Brown & Howard (1980) on a Beckman Spinco Model 120B amino acid analyzer equipped with an autolab AA system integrator. Carbohydrate content was measured according to the protocol of Fairbanks et al. (1971).

Processing Activity. The method for assaying processing activity has been peviously described (Fiss et al., 1979). Partial purification of the protease activity by use of an aminocaproyl-*p*-aminobenzamidine–Sepharose 4B column has also been described (Fiss et al., 1979).

Bacterial Transformation. Plasmid DNA pGGC110 was a generous gift of R. Gayda. Strains BN3040 (*fepA*$^+$, protein a$^+$) and UT4400 (*fepA*$^-$, protein a$^-$) were transformed with the plasmid by following the procedure of Berg et al. (1976)

Tryptic Fingerprint Mapping of 81K and 81K.* Purified 81K and 81K* were isolated from preparative $NaDodSO_4$–polyacrylamide gel electrophoresis slabs. The bands of interest were identified by phosphorescence (Isenberg et al., 1975), and the protein was removed from the gel by electrophoresis with an Isco concentrator, dialyzed extensively against distilled water, and lyophilized to dryness. The proteins were digested wih trypsin–TPCK and chromatographed in two dimensions (Stephens, 1978). The peptide spots were treated with 0.025% U/V fluorescamine in acetone, followed by 5% triethylamine in acetone, and viewed under UV light. The amino termini were determined by following the procedures of Gray (1972) and Weiner et al. (1972).

Results

Purification of 81K. E. coli BN3040 was selected as the source of receptor for two reasons. The first is that it is *entA*$^-$ and thus unable to synthesize the siderophore enterobactin. This means that the cells, when grown in an iron-poor environment, are unable to relieve their iron starvation and because of this overproduce the receptor. The second reason is that the colicin Ia receptor (74K) copurifies with 81K on the DE-52 column. In the colicin Ia resistant mutant *E. coli* BN3040, the colicin Ia receptor is absent. Also, this helped to make the conversion of 81K to 81K* more obvious since 81K* migrates very similarly with the colicin Ia receptor on $NaDodSO_4$–polyacrylamide gel electrophroresis.

Table I: Purification of Ferric Enterobactin Outer Membrane Receptor

	units/mL	total units ($\times 10^{-4}$)	protein (mg/mL)	units/mg
whole membranes	1730	69.2	5.1	339
soluble outer membranes	1102	24.0	2.45	450
DE-52 column 1	2090	14.6	2.46	839
DE-52 column 2	2819	6.8	2.1	1432
Mo-diDHB-Lys	250	1.8	0.1	2500

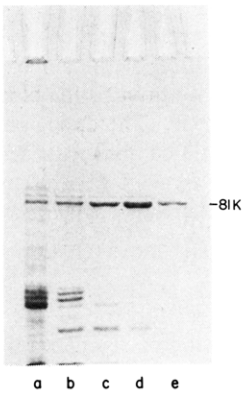

FIGURE 1: Purification of ferric enterobactin outer membrane receptor. Protein composition of the various steps in the procedure: (a) whole membranes, (b) Triton–EDTA-solubilized outer membranes, (c) pooled fractions from the first DE-52 column, (d) pooled fractions from the second DE-52 column, and (e) pooled fractions from the affinity column.

Table I and Figure 1 summarize the purification scheme utilized to isolated 81K. One unit of activity equals 1 pmol of ^{55}Fe-enterobactin bound. After the cells were disrupted and the membranes isolated, the cytoplasmic membranes were removed by solubilization with 2% Triton X-100–100 mM Tris, pH 8, via two 20-min incubations at 18 °C. It was observed that incubating the membranes twice with Tris–Triton X-100 rather than once led to a higher yield of receptor when the outer membranes were solubilized at a later step in the Tris–Triton X-100–EDTA buffer. The outer membranes were solubilized generally 3 times in order to recover most of the activity. Gel filtration chromatography (Bio-Gel A5M and A.5M) of this solubilized outer membrane fraction demonstrated that the receptor was in the form of mixed micelles migrating with a molecular weight of approximately 300K daltons. The data given in Table I are that of a typical purification. The less than 10-fold increase in specific activity is typical of the procedure. Whole membranes were assayed by suspending the membrane pellet in the Tris–Triton–EDTA buffer. The high value of total units and specific activity may be a reflection of binding to nonprotein molecules such as lipopolysaccharide. Isolated solubilized cytoplasmic membranes did not bind ferric enterobactin.

First DE-52 Chromatography. From analysis of two-dimensional polyacrylamide gel electrophoresis (O'Farrell, 1975), we determined that the p*K* of 81K was in the range of 5 and therefore would be negatively charged at pH 7.2. For this reason we chose the anion-exchange resin DE-52. The Triton–EDTA solubilized outer membrane preparations were dialyzed against 2% Triton X-100–50 mM Tris (pH 7.2)–5 mM EDTA and applied to a Whatman DE-52 column equilibrated with the same buffer. The ratio of bed volume to total protein applied was 0.3 mL/mg. The column was washed with 4–6 bed volumes of buffer, followed by a salt gradient of 0–0.1 M NaCl in 2% Triton X-100–50 mM Tris

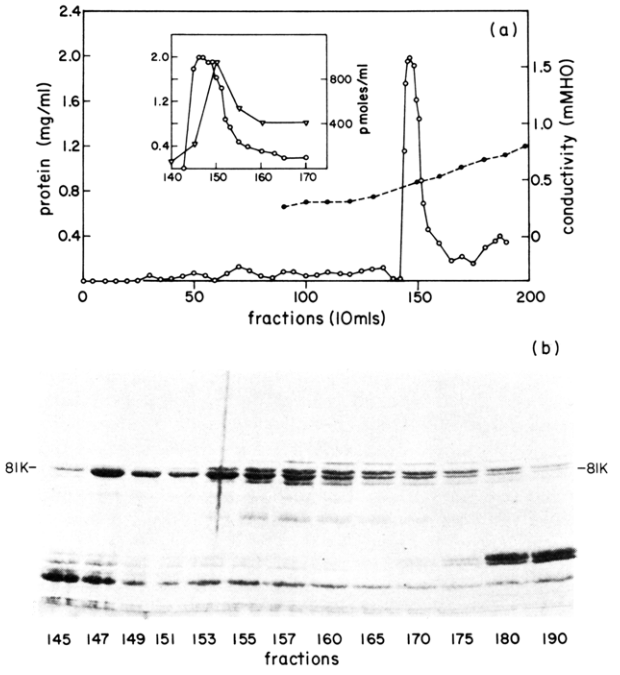

FIGURE 2: DEAE-cellulose chromatography of receptor. (a) Chromatography of Triton-solubilized outer membranes (540 mg) with a Whatman DE-52 column (2.5 × 34 cm) equilibrated with 2% Triton X-100–50 mM Tris (pH 7.2)–5 mM EDTA: (○) protein, (●) conductivity, and (▽) activity. (b) Protein composition of the DEAE column peak fractions. Aliquots from the DE-52 column were analyzed by 10% $NaDodSO_4$–polyacrylamide gel electrophoresis.

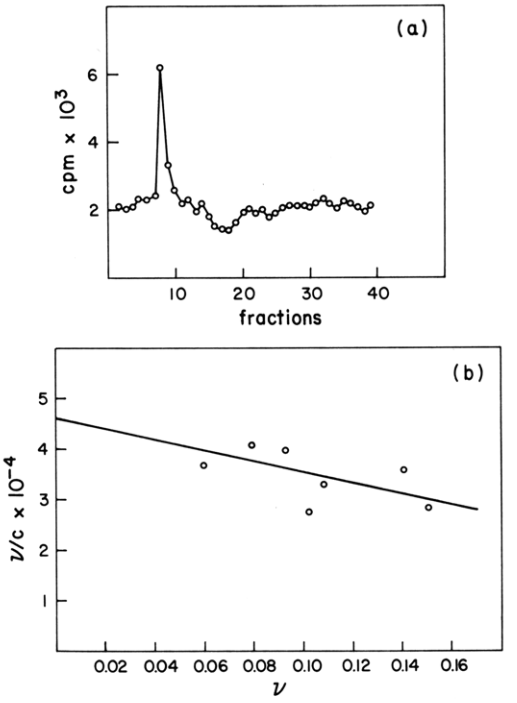

FIGURE 3: Determination of dissociation constant. (a) Representative elution diagram for the binding of [^{55}Fe]ferris enterobactin to receptor. Amount of receptor was 0.042 nmol; concentration of [^{55}Fe]ferric enterobactin was 3.26×10^{-6} nM; Bio-gel P4 column (200–400 mesh) (0.7 × 19.5 cm) in 2% Triton–0.1 M Tris, pH 7.0, at 4 °C. (b) Scatchard plot. The concentration of ligand varied from 1.62×10^{-6} to 5.3×10^{-6} mM.

(pH 7.2)–5 mM EDTA applied in 6 bed volumes. Figure 2 shows the elution profile from the first DE-52 column step. Since the porins account for 35% of the outer membrane proteins, separation from these major outer membrane proteins resulted in substantial purification of the receptor.

Second DE-52 Column Chromatography. The peak of activity pooled from the first column was dialyzed against 2% Triton X-100–50 mM Tris (pH 7.2)–5 mM EDTA and applied to a second DE-52 column equilibrated in the same buffer; the ratio of bed volume to protein was 0.3 mL/mg. The column was washed with 3 volumes of buffer, followed by elution with a salt gradient of 0–0.15 M NaCl in the same buffer of 5 bed volumes. The peak of activity was pooled. The second DE-52 column step was used as a means of concentrating the sample since this step did not significantly increase the specific activity. After these steps, the receptor was ~86% pure as measured by densitometric analysis of the $NaDodSO_4$ gel.

Affinity Chromatography. Affinity resins based on the binding properties of the receptor were constructed in order to separate it from contaminating proteins. As an initial screening procedure, in collaboration with R. C. Hider, the ligand, molybdenum(VI) bis(2,3-dihydroxybenzoyl)lysine, was compared with [^{55}Fe]ferric enterobactin for ability to bind to the receptor in the column assay (Hollifield & Neilands, 1978). Molybdenum(VI) was used instead of iron(III) as the coordinated metal ion as the latter has a tendency to form polymeric species that would be unavailable for binding to the receptor. The results of the competition studies (not shown) demonstrated that the molybdate ligand was able to compete effectively with [^{55}Fe]ferric enterobactin for the receptor.

The peak of activity from the second DE-52 column was dialyzed and a sample applied to the column equilibrated in 2% Triton X-100–10 mM Tris (pH 7.2)–2 mM Na_2MoO_4. The receptor was eluted with a salt gradient of 0–0.2 M NaCl applied in the same buffer. The yield of 81K from 150 L of culture with A_{650} of 0.8 was approximately 50 mg after the second DE-52 column and 19 mg after the affinity column. The protein is insoluble in water but dissolves readily in buffer containing at least 0.5% Triton X-100 or 0.1% $NaDodSO_4$. Examination of 81K in the electron microscope revealed extensive aggregation into long filaments following dilution of detergent.

Dissociation Constant. Figure 3 shows a typical profile from a Hummel & Dryer (1962) column and the Scatchard plot derived therefrom. The K_d was determined to be ~10 nM with a binding ratio $\eta = 0.42$.

Amino Acid Composition. Table II shows the amino acid composition of purified 81K. The published data for the cloacin receptor of *Enterobactin cloacae* are listed for comparison (Oudega et al., 1979). The p*I* of 81K was determined from two-dimensional polyacrylamide gel electrophoresis (O'Farrell, 1975) to be 5.5, which is similar to that reported for the cloacin receptor. With the staining procedure of Fairbanks et al. (1971), no carbohydrate was detectable in association with 81K.

Characterization of a Processing Activity. We previously correlated protein a with the 81K–81K* proteolytic activity by its molecular weight and by the use of deletion mutants (Fiss et al., 1979). For further testing of our belief that protein a was the processing agent, plasmid pGGC110 was transformed into BN3040 (protein a^+, $protease^+$) and UT4400 ($protease^-$, protein a^-). Plasmid pGGC110 is a pBR322 derivative containing a 1.5-Mdalton piece of DNA encoding the structural gene for protein a (Gayda & Markovitz, 1978). Outer membranes were then isolated from these transformed strains, with the untransformed strains as control, incubated at 37 °C for up to 2 h, and examined for the presence of processing activity. The results are shown in Figure 4. UT4400, which originally lacked protease activity, when transformed, became capable of converting 81K to 81K*.

Table II: Amino Acid Analyses (Mole Percent)

amino acid type	amino acid	81K	cloacin receptor[a]	*E. coli* cell supernatant[b]
basic	Lys	4.4	5.7	6.4
	His	1.4	1.4	2.0
	Arg	4.9	2.8	4.1
		10.7[c]	9.9[c]	12.5[c]
acidic	Asx	14.9	13.3	10.4
	Glx	10.0	9.3	11.2
		24.9[c]	22.6[c]	21.6[c]
neutral	Thr	8.0	6.5	5.7
	Ser	6.9	7.9	4.7
	Pro	3.9	3.2	4.2
	Gly	11.9	13.8	8.5
	Ala	7.6	8.0	10.2
	½-Cys	0	0	1.1
		38.3[c]	39.4[c]	34.4[c]
hydrophobic	Met	2.4	1.3	2.6
	Val	5.3	6.0	7.9
	Ile	4.4	3.8	5.9
	Leu	7.1	6.1	8.1
	Tyr	4.4	4.6	2.8
	Phe	2.5	3.5	3.5
		26.1[c]	25.3[c]	30.8[c]

[a] Calculated from data reported by Oudega et al. (1979). [b] Data reported by Spahr (1962). [c] Value represents sum.

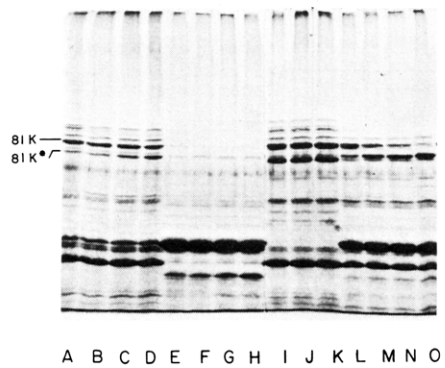

FIGURE 4: Assay of strains containing plasmid-encoded protein a. $NaDodSO_4$–polyacrylamide gel electrophoresis analysis of Triton–EDTA-solubilized outer membrane preparations of *E. coli* BN3040 (A–D), BN3040/pGGC110 (E–H) UT4400 (I–K), and UT4400/pGGC110 (L–O). Since UT4400 is *fepA* (lacking 81K), membranes from the latter two strains (20 μg) were combined with partially purified 81K receptor (10 μg). Membranes were incubated at 37 °C and analyzed at 0 min (A, E, I, and L), 30 min (B, F, and M), 60 min (C, G, J, and N), and 120 min (D, H, K, and O). Each lane contained 10 μg of protein.

*Characterization of 81K and 81K**. 81K and 81K* were further characterized in order to better understand the degradation event. Whereas 81K is still active with respect to binding to ferric enterobactin and colicin B, 81K* is devoid of activity. Limited proteolysis using *Staphylococcus aureus* V8 protease (Cleveland et al., 1977) verified that the two proteins were virtually identical (see Figure 5). Composite tracings from the two-dimensional tryptic mapping (not shown) demonstrated that only two spots were consistently different between 81K and 81K*. The amino-terminal residues of 81K and 81K* were identified as phenylalanine and alanine, respectively.

Specificity of Protein a. As a means of analyzing the significance of the protease in iron transport regulation, *E. coli* K-12 was compared with *E. coli* B, *E. coli* B/r, and *S. typhimurium* LT2. While the 81K protein for *E. coli* K12 was rapidly degraded, there was no evidence of proteolysis of the equivalent proteins in *E. coli* B and B/r, within the same time period. Likewise, no proteolysis was observed in *S. typhimurium* (data not shown).

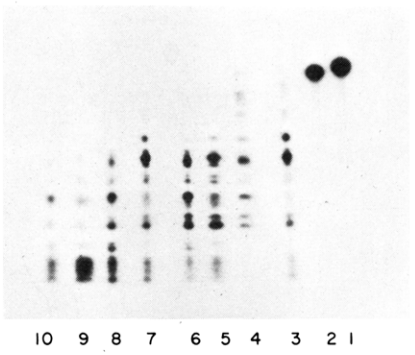

FIGURE 5: Limited proteolysis. Limited proteolytic digests of [^{3}H]leucine-labeled 81K and 81K* using *Staphylococcus aureus* V8 protease and 15% $NaDodSO_4$–polyacrylamide gel electrophoresis: (lanes 1, 3, 5, 7, and 9) digest of 81K; (lanes 2, 4, 6, 8, and 10) digests of 81K*. Lanes 1 and 2, 3 and 4, 5 and 6, 7 and 8, and 9 and 10, contained 0, 0.02, 0.05, 0.10, and 0.20 μg of *S. aureus* protease, respectively. Outer membrane was prepared as described in the text and in the legend to Figure 4.

Table III: Cross-Reactivities of Ferric Enterobactin Receptor and Protease in *Escherichia coli* Strains K12 BN3040 and B[a]

receptor source	protease source	proteolysis[b]
K12 (P)[c]	K12 (P)	+
K12 (P)		–
K12 (P)	B (P)	+
	B (P)	–
B (P)		–
B (P)	K12 RWB18 (OM)[d]	–
B (P)	B (P)	–
B (OM)	K12 RWB18 (OM)	–
K12 (P)	K12 RWB18 (OM)	+
K12 (P)	B (OM)	+

[a] Mixing experiments were performed by incubating 20 μg of receptor with 100 μL of protease at 37 °C overnight. The time points were analyzed on 10% $NaDodSO_4$–polyacrylamide gels for conversion of 81K to 81K*. [b] (+) or (–) = conversion of 81K to 81K* overnight as visualized by gel analysis. [c] P = purified. The receptor was purified as described in the text. The protease was purified by passage through a *p*-aminobenzamidine column. [d] OM = outer membrane.

So that the question of whether these other bacteria contained no active protease or, alternatively, an insensitive ferric enterobactin receptor could be answered, mixing experiments were performed. Solubilized outer membranes from *E. coli* K12, B, and B/r were isolated. Outer membranes from *E. coli* K12 strain RWB18, which is *fep*$^-$ (lacks 81 K) and protein a$^+$ (protease positive), were used as an *E. coli* K12 source of protease. The proteases from *E. coli* K12 and B were purified by using affinity chromatography. $NaDodSO_4$–polyacrylamide gel electrophoresis of the purified proteases confirmed the presence of a band with M_r of 40K in *E. coli* B. Receptors and proteases were then mixed and incubated. The results of mixing *E. coli* B and K12 are listed in Table III. Identical results were seen with *E. coli* B/r. Although *E. coli* B and B/r contain active proteases that convert the *E. coli* K12 receptor 81K to 81K*, the receptors from *E. coli* B and B/r are insensitive to the proteolysis from all three strains. This suggests that the ferric enterobactin receptor from *E. coli* K12 differs in structure from those of *E. coli* B and B/r. Results with *S. typhimurium* LT2 suggest that not only is this receptor insensitive to the K12 protease but it also does not contain a protease capable of converting the 81K receptor of K12 to 81K*. However, the possibility also exists that the protease

is present but is not Triton extractable.

Discussion

Transport of hydrophilic ligands across the outer membrane of enteric bacteria can be described as falling into two classes (Nikaido, 1979). The first class involves the binding of the ligand to the receptor via specific recognition and is represented by the transport of vitamin B_{12} and the siderophores. In the second class the ligand displays little or no binding, and transport proceeds through water-filled pores with a definite size exclusion limit. This is represented by the porins and the λ receptor (Nikaido, 1979; Luckey & Nikaido, 1980). In this paper we have demonstrated that even in a highly purified state the receptor for ferric enterobactin is capable of attaching the ligand with a high binding constant. A K_d of 10 nM is consistent with earlier studies reporting an apparent K_M of 0.1 μM (Frost & Rosenberg, 1973) and is only 1 order of magnitude larger than that estimated in situ for the B_{12} receptor (White et al., 1973).

There are many similarities between the uptake systems for vitamin B_{12} and ferric enterobactin. Both have outer membrane receptors that exhibit tight binding and both require an energized membrane state as well as the *tonB* gene function (Reynolds et al., 1980). Although an inner membrane receptor has been implicated for both systems, binding of the ligand to the inner membrane has not been observed in the B_{12} (White et al., 1973) or our own system (results not shown). The B_{12} system apparently requires the presence of a periplasmic binding protein. Although no comparable protein has been described in the ferric enterobactin system, its existence has not been ruled out. All of these similarities suggest that the transport mechanism for the two systems may be very similar.

The inducible nature of 81K allowed for a purification procedure comprised of relatively few steps. This circumvented an immediate need to clone the receptor, although attempts to encode the enterobactin genes on a plasmid are now in progress. In this study, we used the purified receptor to determine its binding constant and amino acid composition. In comparing the amino acid composition with other proteins, we were impressed with its similarity to the cloacin receptor. The latter is believed to act as the receptor for the hydroxamate siderophore, ferric aerobactin (Tiel-Menkveld et al., 1981), and, like 81K, this receptor is induced under iron restrictive conditions. Both receptors also have similar molecular weights and isoelectric points. Just as we found no carbohydrate in association with purified 81K, so did Oudega et al. (1979) report that carbohydrate was not required for the activity of the cloacin receptor. Thus the similarity between these receptors is extensive. The amino acid composition of the two proteins appears more similar to each other than to the mixture of proteins present in the cell supernatant of *E. coli* (Table II). There is, however, little to suggest by the nature of their composition that these proteins are membrane associated. This is consistent with reports from other workers [for a review, see Dunn & Maddy (1976)].

The low dissociation of the receptor–siderophore compound indicates the feasibility of future attempts to crystallize the complex. The tight binding may be the consequence of multiple electrostatic attachment of the siderophore triple anion to cationic centers in the protein, perhaps reinforced by hydrophobic bonding to the catechol aromatic rings. Evidently the protein recognizes the metal ion side of the ferric siderophore. Thus the Fe(III) complexes of both a carbocyclic (Hollifield & Neilands, 1978) and an aromatic (Venuti et al., 1980) analogue of enterobactin support the growth of mutants. Ferric enterobactin is exclusively, or predominantly, the Δ-cis coordination isomer (Raymond et al., 1980). Enantioenterobactin, prepared synthetically from D-serine, affords the Δ-cis isomer, a product which binds iron in a form unavailable to *E. coli* (Neilands et al., 1981).

The data presented in Figures 4 and 5 provide convincing evidence that protein a is the agent responsible for the processing of 81K to 81K* in *E. coli* K12. That protein a performs an endolytic cleavage close to the amino terminus is evident from the finding of phenylalanine and alanine as the N-terminal residues in 81K and 81K*, respectively. As 81K* is devoid of activity for either ferric enterobactin or colicin B, it is tempting to speculate that the active site of the protein residues near the amino end, although other explanations for loss of activity such as loss of overall conformation following scission are, of course, possible.

Protein a was cloned by a group attempting to clone the *lon* gene (Gayda & Markovitz, 1978). It was selected because it complemented one of the phenotypes of the *lon* mutation, i.e., the regulation of the capsular polysaccharide. The *lon* gene, also known as *deg* or *capR*, has been shown to be involved in the degradation of nonsense peptide fragments (Gottesman & Zipser, 1978) and has also recently been shown to be an ATP-dependent protease (Zehnbauer et al., 1981). Thus, it is not surprising that proteins a, which functionally replaced *lon* in this selection, is also a protease.

Further experiments will include testing protein a for activity toward substrates known to be cleaved by those proteases already characterized. With respect to inhibitors, protein a is not inhibited to EDTA, phenylmethanesulfonyl fluoride, or L-1-(tosylamido)-2-phenylethyl chloromethyl ketone and is sensitive to *p*-aminobenzamidine (Hollifield et al., 1978). Of those proteases reported, the inhibition profile of protein a correlates with protease I (Pacaud et al., 1976), an outer membrane esterase (Pacaud, 1982), the protease reported by MacGregor et al. (1979), and the activity reported to degrade colicin A (Brey, 1982). In terms of cellular localization and molecular weight, protein a also resembles protease IV (Regnier, 1981).

It is known that protein a and the iron-regulated outer membrane proteins share a similar pathway as regards their posttranscriptional processing. All four proteins require the *perA* gene function. It has been shown that *perA* mutants are blocked in the transport of a number of periplasmic proteins and outer membrane proteins including Ia, the three iron-related proteins 83K, 81K, and 74K, and protein a (Wanner et al., 1979; Lundrigan & Earhart, 1981). The exact nature of this block is unknown. An interesting observation we have made is that protein a while moderately expressed in cultures grown in L broth, in low-iron Tris media protein a is the major protein appearing in the membrane (see, for example, Figure 4, lane E). This regulation is being studied further.

Two general conclusions can be drawn from our survey of the cross-reactivity of the receptor and protein a (Table III). The first is that the receptor from strain K12 appears uniquely sensitive to cleavage, suggesting minor differences in the primary structures of the receptors among *E. coli* strains. The second conclusion is that the proteolytic function of protein a, despite the map location of its gene at 12.5 min immediately adjacent to the enterobactin operon, may not be requisite to the normal function of the ferric enterobactin system. The latter system is presumably functional in *E. coli* strains B and B/r, strains in which we have not detected cleavage of the endogenous 81K. However, an important feature of protein a is its utility as a surgical tool with which to probe the active site of 81K.

Acknowledgments

We are indebted to Dr. R. C. Hider for advice, Dr. R. Gayda for the generous gift of his strains, and Dr. F. H. Carpenter for advice and the determination of the amino acid composition.

References

Bassford, P. J., Diedrich, D. L., Schnaitman, C. L., & Reeves, P. (1977) *J. Bacteriol. 131*, 608–622.

Berg, P. E., Gayda, R., Avni, H., Zehnbauer, B., & Markovitz, A. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73*, 697–701.

Brey, R. N. (1982) *J. Bacteriol. 149*, 306–315.

Brown, N. E., & Howard, G. C. (1980) *Anal. Biochem. 101*, 294–298.

Cleveland, D. W., Fischer, S. G., Kirschner, M. W., & Laemmli, U. K. (1977) *J. Biol. Chem. 252*, 1102–1106.

Corbin, J. L., & Bulen, W. A. (1969) *Biochemistry 8*, 757–762.

Davies, J. L., & Reeves, P. (1975) *J. Bacteriol. 123*, 96–117.

Dudley, J. R., & Grieve, P. A. (1975) *Anal. Biochem. 64*, 136–141.

Dunn, M. J., & Maddy, A. H. (1976) in *Biochemical Analysis of Membranes* (Maddy, A. H., Ed.) pp 197–251, Chapman and Hall, London.

Fairbanks, G., Steck, T. L., & Wallach, D. F. H. (1971) *Biochemistry 10*, 2606–2617.

Fairclough, G. F., & Fruton, J. S. (1966) *Biochemistry 5*, 673–683.

Fiss, E. H., Hollifield, W. C. H., & Neilands, J. B. (1979) *Biochem. Biophys. Res. Commun. 91*, 29–34.

Frost, G. E., & Rosenberg, H. (1973) *Biochim. Biophys. Acta 330*, 90.

Gayda, R. C., & Markovitz, A. (1978) *J. Bacteriol. 136*, 369–380.

Gottesman, S., & Zipser, D. (1978) *J. Bacteriol. 133*, 844–851.

Gray, W. R. (1972) *Methods Enzymol. 25*, 121–138.

Hollifield, W. C. H., & Neilands, J. B. (1978) *Biochemistry 17*, 1922–1928.

Hollifield, W. C. H., Fiss, E. H., & Neilands, J. B. (1978) *Biochem. Biophys. Res. Commun. 83*, 738–746.

Hummel, J. P., & Dryer, W. J. (1962) *Biochim. Biophys. Acta 63*, 530–532.

Isenberg, I., Smerdon, M. J., Cardenas, J., Miller, J., Schaup, H. W., & Bruce, J. (1975) *Anal. Biochem. 69*, 531–535.

Lowry, O. H., Rosenbrough, N. J., Farr, A. L., & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265–275.

Luckey, M., & Nikaido, H. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 167–171.

Lundrigan, M., & Earhart, C. F. (1981) *J. Bacteriol. 146*, 804–807.

MacGregor, C. H., Bishop, C. W., & Blech, J. E. (1979) *J. Bacteriol. 137*, 574–583.

Neilands, J. B. (1981) *Annu. Rev. Nutr. 1*, 27–46.

Neilands, J. B., Erickson, T. J., & Rastetter, W. H. (1981) *J. Biol. Chem. 256*, 3831–3832.

Nikaido, H. (1979) *Angew. Chem., Int. Ed. Engl. 18*, 337–350.

O'Farrell, P. H. (1975) *J. Biol. Chem. 250*, 4007–4021.

Oudega, B., Aldenziel-Werner, W. J. M., Klaasen-Boor, P., Rezee, A., Glas, J., & deGraaf, F. K. (1979) *J. Bacteriol. 138*, 7–16.

Pacaud, M. (1982) *J. Bacteriol. 149*, 6–14.

Pacaud, M., Sibilli, L., & LeBlas, G. (1976) *Eur. J. Biochem. 69*, 141–151.

Raymond, K. N., Kamal, A. D., & Sofen, S. R. (1980) in *Stereochemistry of Optically Active Metal Compounds* (Douglas, B. E., & Saitoy, Y., Eds.) ACS Symp. Ser. No. 119, pp 113–167, American Chemical Society, Washington, DC.

Regnier, P. (1981) *Biochem. Biophys. Res. Commun. 99*, 1369–1376.

Reynolds, P. R., Mottur, G. P., & Bradbeer, C. (1980) *J. Biol. Chem. 255*, 4313–4319.

Schnaitman, C. A. (1971) *J. Bacteriol. 108*, 553–563.

Simon, E. H., & Tessman, I. (1963) *Proc. Natl. Acad. Sci. U.S.A. 50*, 536–532.

Spahr, P. F. (1962) *J. Mol. Biol. 4*, 395.

Stephens, R. E. (1978) *Anal. Biochem. 84*, 116–126.

Tiel-Menkveld, G. J., Oudega, B., Kepmers, O., & deGraaf, F. K. (1981) *FEMS Microbiol. Lett. 12*, 373–380.

Venuti, M. C., Rastetter, W. H., & Neilands, J. B. (1980) *J. Org. Chem. 45*, 5011–5012.

Wanner, B. L., Sarthy, A., & Beckwith, J. (1979) *J. Bacteriol. 143*, 1374–1383.

Wayne, R., Frick, K., & Neilands, J. B. (1976) *J. Bacteriol. 126*, 7–12.

Weiner, A. M., Platt, T., & Weber, K. (1972) *J. Biol. Chem. 247*, 3242–3251.

White, J. C., DiGirolamo, P. M., Fu, M. L., Preston, Y. A., & Bradbeer, C. (1973) *J. Biol. Chem. 248*, 3978–3986.

Zehnbauer, B. A., Foley, E. F., Henderson, G. W., & Markovitz, A. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 2043–2047.