Escherichia coli Iron Enterobactin Uptake Monitored by Mössbauer Spectroscopy

BERTHOLD F. MATZANKE,¹ DAVID J. ECKER,¹ T.-S. YANG,² BOI HANH HUYNH,² GERTRAUD MÜLLER,¹
AND KENNETH N. RAYMOND^{1*}

Department of Chemistry, University of California, Berkeley, California 94720,¹ and Department of Physics, Emory University, Atlanta, Georgia 30322²

Received 19 November 1985/Accepted 9 May 1986

Iron uptake by *Escherichia coli* under aerobic conditions of iron deficiency is mediated by a highly stable ferric enterobactin [Fe(ent)³⁻] siderophore complex. Mössbauer spectroscopy has been used to monitor the fate of the iron as ⁵⁷Fe(ent) was taken up by the cells. Osmotic shock experiments were used to distinguish between the iron present in the periplasmic space and that in the cytoplasm of the cell. Iron delivery by a synthetic analog of enterobactin, 1,3,5-*N,N',N''*-tris-(2,3-dihydroxybenzoyl)tri-*aminomethylbenzene* (MECAM), was also studied. Although Fe-MECAM was transported at the same rate as was Fe(ent) across the outer membrane and was apparently accumulated in the periplasmic space, the subsequent behaviors of Fe(ent) and Fe-MECAM were very different. After more than 30 min, a major fraction of the iron originally absorbed as ferric enterobactin appeared as Fe(II), apparently in the cytoplasm of the cell. However, little iron was delivered to the cytoplasm by the MECAM complex. The differences in specificity of these two stages of iron uptake by *E. coli* are discussed.

As described in our preceding paper (6), studies with synthetic analogs of enterobactin have established important correlations between the coordination chemistry of the ferric complex and recognition at the outer membrane of *Escherichia coli*. However, the use of radioactive labels or inhibition studies with synthetic analogs cannot monitor the oxidation state and coordination environment of the iron after the initial transport step. That is one purpose of the present study.

A second purpose is to probe the mechanism of iron release by ferric enterobactin. Enterobactin possesses a cyclic backbone consisting of three ester-linked serine residues. As described in the introduction of the previous paper (6), whether iron release takes place primarily by reduction of Fe³⁺ to Fe²⁺ or rather by hydrolysis of enterobactin and subsequent dissociation of the ferric complex remains unresolved (3, 23). We have recently measured the reduction potential of the ferric enterobactin Fe(ent)³⁻ in the pH range 6 to 11 (19). The potential at pH 6 is -0.56 V. Thus, reduction in an acidic environment of the cell remains a possible mechanism for iron removal from *E. coli*.

Mössbauer data (25) have shown that (in vitro) the iron in ferric enterobactin remains as Fe(III) even after precipitation of the neutral [Fe(H₃ent)]⁰ triprotonated complex. Mössbauer spectroscopy also has been employed to identify and study the diverse environments of iron in whole cells of procaryotes and eucaryotes (1, 2, 7, 18, 21, 28, 30). Here we apply this technique to obtain information concerning the mechanism of ferric enterobactin metabolism in the gram-negative bacterium *E. coli*.

Intact *E. coli* cells contain two major cellular compartments: the cytoplasm and the periplasm (the volume contained between the outer and the inner membranes). The volume of the periplasm has been recently measured (31) and found to occupy 20 to 40% of the total cell volume, depending on the composition of the growth medium. The absorbed metal complex may be distributed in either or both of these

compartments. In the cytoplasm, the pH is carefully regulated at 7.6, even when the external growth medium is as low as pH 6.4 or as high as pH 8.8 (33). The periplasm, however, is an acidic compartment of the cell (31). Less is known about pH regulation in the periplasmic compartment. The ⁵⁷Fe Mössbauer spectrum is sensitive to pH-dependent coordination changes in these metal complexes. The Mössbauer spectrum of [⁵⁷Fe]enterobactin or [⁵⁷Fe]MECAM [1,3,5-*N,N',N''*-tris-(2,3-dihydroxybenzoyl)tri-*aminomethylbenzene*] assimilated into intact cells is the sum of the signals from the metal complex in the periplasm and cytoplasm. To simplify interpretation of the Mössbauer spectra, the amount of metal complex in each compartment was estimated by the osmotic shock method. Treatment of intact cells by the mild osmotic shock method of Heppel (13) results in the release of the periplasmic contents of the cell. Cells remain viable, but are unable to perform the functions that depend upon the lost periplasmic proteins. Reynolds et al. (26) have used this method coupled with a radioactive transport assay to show that vitamin B₁₂ is accumulated in the periplasmic compartment of the cell after active transport.

MATERIALS AND METHODS

Preparation of compounds. Enterobactin and its synthetic analogs were obtained as described earlier (6). Iron complexes were prepared by adding standardized ⁵⁷Fe-nitrilotriacetic acid solution (MOPS [morpholinepropanesulfonic acid] buffer; pH 7.4; metal-to-nitrilotriacetic acid ratio, 1:2) to a methanolic solution of enterobactin or MECAM yielding an Fe-to-siderophore ratio of 1:1. Methanol was then removed in vacuo. The volume was adjusted with MOPS buffer to a final complex concentration of 0.5 mM. Concentrations were determined spectrophotometrically on an HP 8450A spectrophotometer (Hewlett-Packard Co., Palo Alto, Calif.) by using the following molar extinction coefficients: ferric enterobactin, ε₄₉₅ = 5,600 (pH 7.3); ferric MECAM, ε₅₁₆ = 3,900 (pH 7.0); MECAM, ε₃₂₁ = 10,000; enterobactin,

* Corresponding author.

$\epsilon_{319} = 11,200$. Purity of the ferric complexes was tested with D-O silica gel thin-layer chromatography (chloroform/methanol ratio = 6:5, $R_f = 0.8$) or by high-voltage paper electrophoresis (6). All manipulations in methanolic solution were performed under argon. Complexes were freshly prepared and kept under argon until used.

Growth of organisms. For all uptake studies, *E. coli* K-12 strain RW193 (8) (*pro leu trp entA purE*) was used. This strain is a mutant deficient in enterobactin synthesis. At Fe concentrations of $1 \mu\text{M}$ and higher, synthesis of enterobactin and of those membrane proteins which enable the transport of the ferric complex across the cell membrane are repressed. Thus, all glassware was washed in a solution of KOH plus isopropanol, HCl, and EDTA (1 mM) and was finally triply rinsed in deionized, distilled water to eliminate adventitiously bound iron. The iron-deficient, modified minimal medium (25) contained the following concentrations of minerals in double-distilled water: 40 mM K_2HPO_4 , 10 mM KH_2PO_4 , 10 mM NaCl, and 20 mM NH_4Cl . These mineral salts and glucose (1% final concentration) were passed separately through a Chelex 100 column to remove Fe^{3+} . Trace metals were prepared separately: 1 mM MgSO_4 , 0.1 mM CaCl_2 , 2 mg of $\text{MnSO}_4 \cdot \text{H}_2\text{O}$, 0.5 mg of $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, and 0.1 mg of CuSO_4 (final concentration) per liter. The following organic supplements were required per liter: 1.5 mM L-proline, 0.8 mM L-leucine, 0.2 mM L-tryptophan, and 0.1 mM thymine. The medium also contained 4 mM citrate. No growth was obtained if it was not added. Organic supplements and glucose were autoclaved separately.

Growth was initiated with a 0.5-ml inoculum per 100 ml of medium taken from a cell suspension grown in YM broth (complete medium) (Difco Laboratories, Detroit, Mich.) at 30°C to an optical density at 660 nm of 1.0 (HP 8450A spectrophotometer), equivalent to 0.55 mg of cell dry weight per ml. Iron-deficient cultures (2 liters) were grown in baffled 5-liter Erlenmeyer flasks in a shaking water bath (New Brunswick Scientific Co., Inc., Edison, N.J.; model G-76) at 37°C with the platform rotating at approximately 110 rpm. Cells were harvested when optical density at 660 nm was 0.9, were washed twice with 0.9% LiCl solution, and were resuspended in fresh minimal medium which was the same as described above except no citrate was added. The cells were kept on ice. At 30 min before each experiment, the cell suspensions were placed again in a water bath at 37°C and shaken.

For the study of long-term growth, an ^{57}Fe -citrate mixture (Fe-to-citrate ratio, 1:20) was added to *E. coli* cells in minimal medium, yielding a final Fe concentration of $4 \mu\text{M}$. The cells were then grown for 36 h.

Sample preparation. Depending on the experiment, [^{57}Fe]enterobactin or [^{57}Fe]MECAM was added to the culture to give a final metal complex concentration of $2 \mu\text{M}$. After an incubation time of 5 or 20 min, the cell suspension was cooled to 0°C within 45 s. Cells were washed twice with 0.9% LiCl by centrifugation. A portion of the cell material was loaded into a Delrin Mössbauer sample holder (volume, 0.3 to 0.4 ml), shock frozen to 78 K, and kept at this temperature until measurement. Samples of the residual cell material were resuspended for additional growth in baffle flasks containing minimal medium. After 30, 45, 60, 90, or 120 min of growth in a shaker bath, the cells were harvested again, and Mössbauer samples were prepared as described above.

Mössbauer measurements. The Mössbauer samples were either frozen cells or frozen aqueous solutions. Bovine serum albumin (Sigma Chemical Co., St. Louis, Mo.) was

used as a diamagnetic diluent (0.1 mg/ml) for the frozen solution to reduce the spin-spin interaction between the ferric ions. The Mössbauer spectrometer was of the constant acceleration type, operated in connection with a 512-channel analyzer in the time-scaling mode and in horizontal transmission geometry. A 50-mCi ^{57}Co source in Rh matrix was kept at room temperature. A typical run lasted about 60 h. The spectrometer was calibrated against an iron foil, and zero velocity was taken as the centroid of the room temperature Mössbauer spectrum of the iron foil.

Osmotic shock treatment. The effect of osmotic shock on the cellular retention of transported [^{59}Fe]enterobactin was examined by a modified procedure of Reynolds et al. (26). Transport experiments and liquid scintillation counting were performed with $2 \mu\text{M}$ [^{59}Fe]enterobactin as previously described (6). Before shock, cellular uptake of ^{59}Fe was monitored by removing 0.1-ml samples from the reaction mixture. After 30 min, the osmotic shock procedure was initiated by the addition of sucrose and EDTA to give final concentrations of 10% and 2 mM, respectively. After 5 min of equilibration with the sucrose and EDTA, 1 ml of the reaction mixture was carefully removed and added to another flask in the shaker bath containing 99 ml of distilled water at 37°C . Samples (10 ml) were removed after an additional 20 min to determine the amount of ^{59}Fe still associated with the cells.

A control to determine the effect of dilution without osmotic shock was prepared by removing 1 ml from the reaction mixture before the addition of sucrose and EDTA and adding it to 99 ml of the same medium at 37°C . Samples (10 ml) were removed and filtered as in the osmotic shock flask.

RESULTS

Mössbauer data. To help identify the spectral components observed for the *E. coli* cells, we measured the Mössbauer spectra of Fe(ent) in various environments, and of the Fe(III) complex of 2,3-dihydroxybenzoylserine [$\text{Fe}(\text{DHBS})_3$], the presumed metabolic breakdown product. We also repeated an experiment of Bauminger et al. (2) to obtain the spectral properties of the *E. coli* iron storage protein for comparison.

The 4.2 K spectrum of $\text{Fe}(\text{ent})^{3-}$ in frozen aqueous bovine serum albumin solution at pH 7 is shown in Fig. 1. The albumin (0.1 g/ml) was added to simulate the cell environment, which has a high concentration of proteins. The spectrum in Fig. 1A is dominated by a well-defined magnetic six-line pattern originating from the ground Kramers doublet. The outer lines are at about -8 and $+9$ mm/s. At 4.2 K, the absorptions arising from the middle Kramers doublet were also observed at about -3.8 , -0.2 , $+0.5$, and $+4$ mm/s. The spectra of $\text{Fe}(\text{ent})^{3-}$ have been analyzed by previous investigators (25, 29), who found that in aqueous solution the absorptions associated with the middle doublet were broad and featureless but in methanol the absorptions were distinct and well defined. The ill-defined absorptions observed in aqueous solution were interpreted as indicating a range of the iron environments within the sample, so that each molecule has a different g tensor and hyperfine interaction, which affect the ground doublet little but the middle doublet greatly (25). The spectrum shown in Fig. 1A resembles that of $\text{Fe}(\text{ent})$ in methanol. Thus, both methanol and serum albumin (0.1 g/ml) reduce considerably the inhomogeneity of the sample.

The spectrum of $[\text{Fe}(\text{H}_n\text{ent})]^{-3+n}$ ($n = 2$ or 3) in frozen aqueous bovine serum albumin solution at pH 3 is shown in

FIG. 1. Mössbauer spectra of iron-catechol complexes in aqueous bovine serum albumin solution and frozen *E. coli* cells. The data were recorded at 4.2°K with a magnetic field of 50 mT applied parallel to the γ beam. (A) Enterobactin, pH 7; (B) enterobactin, pH 3; (C) DHBS, pH 6.8; (D) *E. coli* cells grown in minimal medium with 4 μ M ^{57}Fe -citrate for 36 h.

Fig. 1B. A central quadrupole doublet ($\Delta E_Q = 0.8$ mm/s and $\delta = 0.5$ mm/s), indicative of fast-relaxing high-spin ferric species, and a broad and ill-defined magnetic subspectral component were observed. The buildup of fast-relaxing high-spin Fe(III) with decreasing pH has been reported earlier (25), where it was interpreted as reflecting the changes of iron environment. At pH 7, the iron is coordinated by six catecholate anions. With decreasing pH, the catecholate oxygens are sequentially protonated. The resultant mode of coordination has been assigned as a shift of the Fe(III) toward the orthophenolic oxygen and α -carbonyl oxygen; a mode of coordination similar to the bonding exhibited by salicylic acid and termed salicylate mode (24).

The spectrum of Fe(DHBS)₃ in frozen aqueous bovine serum albumin (0.1 g/ml) solution at pH 6.8 is shown in Fig. 1C. The absorptions are broad and exhibit an unresolved

magnetic structure. The overall magnetic splitting (approximately 15.4 mm/s) is considerably smaller than that of Fe(ent) (approximately 17 mm/s). Also, the center part of the spectrum is much broader than that of the fast-relaxing species shown in Fig. 1B. These spectral features indicate an inhomogeneous iron environment and may also reflect an oligomeric complex structure. Further analysis of the spectral features of Fe(DHBS)₃ would require detailed pH- and temperature-dependent Mössbauer measurements.

In accordance with the findings of Bauminger et al. (2), *E. coli* cells grown in the presence of ^{57}Fe yield a Mössbauer spectrum dominated by a quadrupole doublet characteristic of high-spin Fe(III) (Fig. 1D). The Fe(III) species has been attributed to an iron storage protein (2, 32). Since the spectrum for the $[\text{Fe}(\text{H}_n\text{ent})]^{-3+n}$ salicylate type compound is very similar to that of the iron storage protein, it is impossible to distinguish between the storage protein eventually formed and the low-pH form of ferric enterobactin. The broad absorption at approximately 2.6 mm/s observed in Fig. 1D represents an envelope of the high-energy peaks of doublets originating from various high-spin Fe(II) species. The corresponding low-energy peaks of these doublets are masked by the intense absorption of the iron storage protein.

Mössbauer spectra of frozen *E. coli* cells at 4.2 K are shown in Fig. 2. All cells were grown under conditions of severe iron deficiency (1 μ M), incubated for 20 min with $^{57}\text{Fe}(\text{ent})^{3-}$, then washed and resuspended in minimal medium for an additional growth of 30, 60, or 120 min (see above). At least three components are discernable from the spectra: a paramagnetic spectral component with resonance absorption extending from -8 to $+9$ mm/s, characteristic of slow-relaxing high-spin Fe(III) complexes; a quadrupole doublet (noted in the figure) with Mössbauer parameters ($\Delta E_Q = 3.1$ mm/s and $\delta = 1.4$ mm/s) typical of high-spin Fe(II) ions; and a central quadrupole doublet with $\Delta E_Q = 0.6$ to 0.8 mm/s and $\delta = 0.5$ to 0.6 mm/s (also noted in the figure) which may originate from fast-relaxing high-spin Fe(III) species. These three components are observed in all the spectra of frozen *E. coli* cells. The relative absorption intensities of these three components vary with the time of additional growth period. The estimated percent absorptions of these three components are listed in Table 1. Due to the complexity of the spectra, the listed percentages should be viewed as estimates and are useful only for gaining a general knowledge concerning the sequence of events during Fe(ent) metabolism.

The paramagnetic subspectral component exhibits magnetic splitting and line positions identical to those of the spectrum shown in Fig. 1A, and therefore represents ferric enterobactin (for comparison, spectrum 1A is plotted again on top of Fig. 2); however, the spectrum for the frozen cells is slightly broader, and resembles that of Fe(ent) in frozen aqueous solution without serum albumin (25). Most interest-

TABLE 1. Time dependence of Fe(ent) metabolism in *E. coli*.

Additional growth period ^a (min)	Fe(ent) (%)	Fe(II) (%)	Fe(III) fast relaxing (%)
0	66	20	14
30	62	23	15
90	65	25	10
120	50	40	10

^a See text. All incubation times with Fe(ent) were 20 min.

FIG. 2. Mössbauer spectra of frozen *E. coli* cells recorded at 4.2°K in a parallel field of 50 mT. Cells grown in iron-deficient minimum medium were incubated for 20 min in the same medium but with 2 μ M $^{57}\text{Fe}(\text{ent})$ (A). The cells were then washed and grown for an additional period of 30 min (B), 90 min (C), and 120 min (D) in minimal medium. For comparison, the spectrum of $^{57}\text{Fe}(\text{ent})$ at pH 7 is plotted on top of panel A. The bracketed area I corresponds to a quadrupole doublet characteristic of high-spin Fe(II). The area marked II is characteristic of fast-relaxing high-spin Fe(III).

ing, a significant amount (approximately 50%) of nonmetabolized iron enterobactin remains detectable after a long incubation period (120 min). Also, we found no spectral component which resembles the spectrum of $\text{Fe}(\text{DHBS})_3$ (25). Note that the percent absorption of the Fe(II) species (Table 1) increases with additional growth period, while the amount of ferric enterobactin decreases concomitantly.

Three series of parallel but independent Fe(ent) uptake experiments in *E. coli* were performed. Similar qualitative results were obtained. In all three series of experiments, cellular ferric enterobactin was observed in large quantity but decreased with additional growth. The minimum detected for ferric enterobactin in our *E. coli* cells was 30%. The amount of the fast-relaxing Fe(III) species varied, and no general trend was found; however, it appeared as a minor

species and was always less than 15% of the total iron content. The amount of high-spin Fe(II) species is found to increase with the growth period [except in one sample which had been incubated for an additional 120 min and showed first an increase and then a decrease in the amount of the Fe(II) species].

To further probe the question of whether ester bond cleavage of enterobactin is essential for iron removal from the complex, the metabolism of MECAM-bound iron was studied in vivo. Spectra representing results obtained for ^{57}Fe MECAM uptake in *E. coli* K-12 mutants are shown in Fig. 3. The spectra are dominated by two main components: a paramagnetic spectrum with broad absorptions extending from -8 to +9 mm/s indicating a slow-relaxing high-spin Fe(III) species; and a quadrupole doublet ($\Delta E_Q = 0.95$ mm/s and $\delta = 0.5$ mm/s) representing a fast-relaxing high-spin Fe(III) species. Only a trace of Fe(II) could be detected. The paramagnetic component is very similar to the spectrum of Fe-MECAM in frozen aqueous solution reported by Pecoraro et al. (25). In comparison with Fe(ent), the protonation of Fe-MECAM occurs at considerably higher pH (9 to 11). As a result, the spectrum of Fe-MECAM shows a larger contribution from fast-relaxing Fe(III) species. For example, at pH 6 the contribution of fast-relaxing Fe(III) to the total absorption area is 42 ± 5 and $15 \pm 5\%$ for Fe-MECAM and Fe(ent), respectively (25).

Osmotic shock data. After 30 min of incubation with ^{59}Fe enterobactin at 2 μ M, *E. coli* accumulated approximately 600 pmol of ^{59}Fe per mg (dry weight) (Fig. 4). Immediately after shock treatment (13), approximately 200 pmol/mg (33%) was released. Over the next 15 min, another 100 pmol/mg was released for a total of about 50% of the accumulated substrate. The control flask, used to monitor the effect of dilution without osmotic shock, showed no loss of ^{59}Fe enterobactin from the cells that was due to the dilution effect alone.

These results suggest that a substantial portion of the accumulated metal complex is in the periplasm. The osmotic shock results suggest that there is somewhat more ^{59}Fe in the cytoplasm than in the periplasmic space, but the concentration of ^{59}Fe in both compartments is roughly equal after about 30 min of incubation.

DISCUSSION

Short-term Fe(ent) uptake. Recent studies with synthetic structural analogs of enterobactin have revealed important correlations between coordination chemistry and recognition at the *fepA* outer-membrane receptor. Growth promotion tests with siderophore auxotroph mutants of *E. coli* demonstrated a clear discrimination between naturally occurring Δ cis Fe-enterobactin and the Δ cis enantiomer (22). In addition, the effect of various achiral enterobactin analogs has been probed (12), and the results suggested the primary importance of the catecholate-iron portion of the molecule for recognition at the outer membrane. However, the ferric complexes of MECAM, TRIMCAM, and 3,4-LICAMS all showed positive growth promotion tests, even in mutants lacking the ferric enterobactin outer membrane receptor (*fepA*⁻) (12). This has also been found in transport assays of the gram-positive organism *Bacillus subtilis* (20). Of the synthetic analogs studied, only ferric MECAM inhibited uptake of ^{55}Fe enterobactin (6). The other compounds did not compete with ^{55}Fe enterobactin for the outer membrane receptor. The apparent K_m value for ferric MECAM, 0.69 μ M, compared with 0.2 μ M for ferric enterobactin (6), at

FIG. 3. Mössbauer spectra of *E. coli* cells recorded under the same conditions described in the legend to Fig. 1. Cells grown in iron-depleted minimal medium were incubated for 5 min (A) and 20 min (B) in the same medium but with 2 μ M Fe-MECAM. Sample B was grown for an additional period of 90 min in the iron-depleted minimal medium.

first suggests a lower affinity for the receptor. However, when corrected for the concentration of ferric MECAM that is structurally identical with ferric enterobactin (6, 11), the calculated parameters are: $K_m = 0.21 \mu$ M, $V_{max} = 66$ pmol/mg per min, identical to the values for ferric enterobactin within experimental error.

In the early stages of iron uptake (20 min or less), the Mössbauer data show that more than 60% of the iron incorporated within the cell is still present as Fe(ent) and is exchangeable with the siderophore complex in the surrounding medium. This agrees generally with the direct observation of the periplasmic contents by the osmotic shock technique, which occurs over a longer time interval. Thus, the transport of Fe(ent) by the outer membrane protein is not driven by a concentration gradient of the Fe(ent), resulting from metabolism of the complex inside the cell. The observations are also consistent with the fact that the Fe-MECAM complex acts as a competitive inhibitor for Fe(ent) (6). The receptor protein is highly stereospecific in recognizing the iron catechol portion of Fe(ent) (Fig. 5). The effective binding constant (estimated from the K_m value of 0.2 μ M [6]) of the *fepA* outer membrane protein for ferric enterobactin is very high on the external side of the membrane. The specific chemical driving force for active transport is unknown.

Long-term Fe(ent) uptake. After 20 min of incubation with Fe(ent) and 30 min of additional growth, as much as 20% of the accumulated iron from Fe(ent) has been metabolized into several Fe(II)-containing species. During this second stage of iron uptake and metabolism, the Fe-MECAM is remarkably different from Fe(ent). Only 0 to 10% of the iron absorbed as Fe-MECAM appears as Fe(II). This is consis-

tent with an esterase activity, since the prior behavior of the MECAM and ent complexes are so similar and since the principal difference between the two ligands is the backbone of MECAM, which is not susceptible to hydrolysis. It is also consistent with an inner-membrane recognition process which recognizes only the unprotonated iron catechol complex. Although no significant amounts of Fe(ent) are protonated at pH 6, 92% of Fe-MECAM is protonated at this pH. The relative acidity of the periplasm could cause the observed discrimination. In combination, the osmotic shock and the Mössbauer data indicate that the incorporated Fe found in the cytoplasm is Fe(II) resulting from the metabolism of the Fe(ent). If this process were dependent only on a pH-dependent reduction of the complex, MECAM would be expected to have delivered Fe to the cytoplasm at rates comparable to those for ent.

Recent studies of the periplasmic compartment by Stock et al. (31) have shown that there is a Donnan equilibrium between the periplasmic compartment and the extracellular fluid, caused by high levels of anions in the periplasm that cannot penetrate the outer membrane, that results in an electrical potential across the outer membrane of about 30 mV (negative inside). A consequence is that the pH in the periplasm is significantly lower than in the external medium (31). There is other evidence that the periplasm is an acidic

FIG. 4. [^{59}Fe]enterobactin uptake followed by osmotic shock. Uptake of 2 μ M [^{59}Fe]enterobactin was monitored in *E. coli* RW-193 (1 mg/ml). At 30 min, the osmotic shock procedure was initiated by the addition of sucrose and EDTA (see the text) followed by removal of a 1-ml sample and 1/100 dilution in distilled water. The control was prepared by removal of a 1-ml sample before the addition of sucrose and EDTA, followed by a 1/100 dilution into the same medium. The results are expressed in picomoles of uptake of ^{59}Fe per milligram (dry weight) of cells. Symbols: ●, before osmotic shock; ■, osmotic shock; ▲, control.

FIG. 5. Schematic view of iron transport mediated by enterobactin in *E. coli*. Shown are the inner and outer membranes and the intervening periplasmic space. The outer protein receptor for ferric enterobactin recognizes the analog ferric MECAM equally well. Both are transported into the periplasmic space as the intact complexes. Subsequent delivery of iron, as Fe^{2+} , to the cytoplasm by ferric enterobactin is approximately 10 times faster than that by MECAM.

compartment of the cell (5), although no direct measurement of the periplasmic pH has appeared.

A low-pH compartment in the cell allows another possible explanation in the controversial matter of the pathway of cellular iron removal from ferric enterobactin. It was initially proposed, and widely accepted, that in ferric enterobactin metabolism the next step after transport is hydrolysis of the ester bonds in the backbone by an esterase (8, 27). The need for an esterase was supported by the electrochemical data (4, 10). The redox potential for reduction of unhydrolyzed Fe(III) enterobactin at pH 7 has recently been directly measured (19) and is out of the range of physiological reducing agents such as NADH and reduced flavin adenine dinucleotide. However, microorganisms (including a gram-positive organism) are known which are able to utilize iron supplied by the structural analogs of enterobactin that have similar low redox potentials but no ester bonds in the backbone. In a recent paper, Hider et al. (15) disagreed with our characterization of the ferric enterobactin pH-dependent equilibria and our proposal (24) that the pH dependence of the ferric enterobactin reduction potential might provide a mechanism for in vivo iron release. Hider et al. (17) initially characterized the product of protonating ferric enterobactin, $[\text{Fe(ent)}]^{-3}$, as the tetraprotonated ferrous species $[\text{Fe}^{\text{II}}(\text{H}_4\text{ent})]^0$. This disagreed with our earlier quantitative thermodynamic characterization of this system (4, 9, 10). Hider et al. (16) subsequently altered their formulation to the triprotonated species $[\text{Fe}^{\text{II}}(\text{H}_3\text{ent})]^0$, where ent represents the siderophore ligand in which one catechol group has been oxidized by the Fe to a semiquinone. However, it has been established that the triprotonated species isolated from aque-

ous solution contains only Fe(III) and is best characterized as $[\text{Fe}^{\text{III}}(\text{H}_3\text{ent})]^0$ (24, 25). It has been shown (25) that the data of Hider and Silver (16) are not consistent with their mechanism, but rather imply discrete reactions which begin with a single protonation and ultimately result in the neutral complex $[\text{Fe}^{\text{III}}(\text{H}_3\text{ent})]^0$. There is now agreement with this assignment of the stoichiometry and the species of the aqueous protonation of ferric enterobactin (14). Whether protonation in a low-pH region of the cell (and the subsequent increase in the ferric enterobactin redox potential) is a mechanism of in vivo iron release has not yet been demonstrated.

The preceding paper (6) and related studies have largely probed the outer membrane recognition and transport of ferric enterobactin. The present study has monitored metabolism of the iron in absorbed Fe(ent) or the Fe-MECAM analog. Although the Mössbauer data provide information about the coordination environment and oxidation state of the iron, the location within the cell of the iron species must be surmised. The results of this and the previous paper establish the following.

(i) Iron accumulation by *E. coli*, mediated by enterobactin, involves the rapid transport of Fe(ent) into the periplasmic space via the outer membrane *fepA* protein. The Fe(ent) complex is not altered in this early stage of transport and the synthetic analog Fe-MECAM , which is transported into the periplasm at the same rate, is also unaltered.

(ii) In the subsequent stages of metabolism (after 30 min or more), a major fraction of the iron originally absorbed as ferric enterobactin appears as Fe(II) in the cytoplasm of the cell. At this stage, the MECAM analog behaves very differ-

ently; little iron is delivered to the cytoplasm by the MECAM complex.

ACKNOWLEDGMENTS

This research is supported by Public Health Service grant AI-11744 from the National Institutes of Health (Berkeley) and National Science Foundation grant PCM-8305995 (Emory). We thank the American Cancer Society for a fellowship (to D.J.E.), and the National Institutes of Health for a Research Career Development Award (to B.H.H.).

LITERATURE CITED

- Bauminger, E. R., S. G. Cohen, F. Labenski de Kanter, A. Levy, S. Ofer, M. Kessel, and S. Rottem. 1980. Iron storage in *Mycoplasma capricolum*. J. Bacteriol. 141:378-381.
- Bauminger, E. R., S. G. Cohen, A. Levy, S. Ofer, and J. Yarvin. 1980. Mössbauer spectroscopy of *Escherichia coli* and its iron-storage protein. Biochim. Biophys. Acta 623:237-242.
- Bryce, G. F., and N. Brot. 1972. Studies on the enzymatic synthesis of the cyclic trimer of 2,3-dihydroxy-*N*-benzoyl-L-serine in *Escherichia coli*. Biochemistry 11:1708-1715.
- Cooper, S. R., S. V. McArdle, and K. N. Raymond. 1978. Siderophore electrochemistry: relation to intracellular iron release mechanism. Proc. Natl. Acad. Sci. USA 75:3551-3554.
- Cramer, W. A., J. R. Dankert, and Y. Uratoni. 1983. The membrane channel-forming antibiotic protein, colicin E1. Biochim. Biophys. Acta 737:173-193.
- Ecker, D. J., B. F. Matzanke, and K. N. Raymond. Recognition and transport of ferric enterobactin in *Escherichia coli*. J. Bacteriol. 167:666-673.
- Gilberman, E. 1974. Recoil-free spectra from ^{57}Co -enterochelin in *E. coli* cells. J. Physique, Colloque C6 Supplement au No 12, Tome 35, 371-374.
- Greenwood, K. T., and R. K. J. Luke. 1978. Enzymatic hydrolysis of enterochelin and its iron complex in *Escherichia coli*, K-12: properties of enterochelin esterase. Biochim. Biophys. Acta 525:209-218.
- Harris, W. R., C. J. Carrano, S. R. Cooper, S. R. Soften, A. Avdeef, J. V. McArdle, and K. N. Raymond. 1979. Coordination chemistry of microbial iron transport compounds. 19. Stability constants and electrochemical behavior of ferric enterobactin and model complexes. J. Am. Chem. Soc. 101:6097-6104.
- Harris, W. R., C. J. Carrano, and K. N. Raymond. 1979. Spectrophotometric determination of the proton-dependent stability constant of ferric enterobactin. J. Am. Chem. Soc. 101:2213-2214.
- Harris, W. R., and K. N. Raymond. 1979. Ferric ion sequestering agents. 3. The spectrophotometric and potentiometric evaluation of two new enterobactin analogues: 1,5,9-*N,N',N''*-tris(2,3-dihydroxybenzoyl)-cyclotriazatridecane and 1,3,5-*N,N',N''*-tris(2,3-dihydroxybenzoyl)-triaminomethylbenzene. J. Am. Chem. Soc. 101:6534-6541.
- Heidinger, S., V. Braun, V. L. Pecoraro, and K. N. Raymond. 1983. Iron supply to *Escherichia coli* by synthetic analogs of enterochelin. J. Bacteriol. 153:109-115.
- Heppel, L. A. 1981. The concept of periplasmic enzymes, p. 223-247. In L. I. Rothfield (ed.), Structure and function of biological membranes. Academic Press, Inc., New York.
- Hider, R. C. 1984. Siderophore mediated absorption of iron. Struct. Bonding 58:25-87.
- Hider, R. C., D. Bickar, I. E. G. Morrison, and J. Silver. 1984. Siderophore iron-release mechanisms. J. Am. Chem. Soc. 106:6983-6987.
- Hider, R. C., A. R. Mohd-Nor, J. Silver, I. E. G. Morrison, and L. V. C. Rees. 1981. Model compounds for microbial iron-transport compounds. Part 1. Solution chemistry and Mössbauer of iron(II) and iron(III) complexes from phenolic and catecholic systems. J. Chem. Soc. Dalton Trans. 609-622.
- Hider, R. C., J. Silver, J. B. Neillands, I. E. G. Morrison, and L. V. C. Rees. 1979. Identification of iron(II) enterobactin and its possible role in *Escherichia coli* iron transport. FEBS Lett. 102:325-328.
- Kaufman, K. S., G. C. Papaefthymiou, R. B. Frankel, and A. Rosenthal. 1980. Nature of iron deposits on the cardiac walls in β -thalassemia by Mössbauer spectroscopy. Biochim. Biophys. Acta 629:522.
- Lee, C.-W., D. J. Ecker, and K. N. Raymond. 1985. The pH-dependent reduction of ferric enterobactin probed by electrochemical methods and its implications for microbial iron transport. J. Am. Chem. Soc. 107:6920-6923.
- Lodge, J. S., C. G. Gaines, J. E. L. Arcenaux, and B. R. Byers. 1980. Non-hydrolytic release of iron from ferrienterobactin analogs by extracts of *Bacillus subtilis*. Biochem. Biophys. Res. Commun. 97:1291-1295.
- Matzanke, B., and G. Winkelmann. 1981. Siderophore iron transport followed by Mössbauer spectroscopy. FEBS Lett. 130:50-53.
- Neillands, J. B., T. J. Erickson, and W. H. Rastetter. 1981. Stereospecificity of the ferric-enterobactin receptor of *Escherichia coli* K-12. J. Biol. Chem. 256:3831-3832.
- O'Brien, G., G. B. Cox, and F. Gibson. 1971. Biologically active compounds containing 2,3-dihydroxybenzoic acid and serine formed by *Escherichia coli*. Biochim. Biophys. Acta 237:537-549.
- Pecoraro, V. L., W. R. Harris, G. B. Wong, C. J. Carrano, and K. N. Raymond. 1983. Coordination chemistry of microbial iron transport compounds. 23. Fourier transform infrared spectroscopy of ferric catechoylamide analogues of enterobactin. J. Am. Chem. Soc. 105:4623-4633.
- Pecoraro, V. L., G. B. Wong, T. A. Kent, and K. N. Raymond. 1983. Coordination chemistry of microbial iron transport compounds. 22. pH-Dependent Mössbauer spectroscopy of ferric enterobactin and synthetic analogues. J. Am. Chem. Soc. 105:4617-4623.
- Reynolds, P. R., G. P. Mottur, and C. Bradbeer. 1980. Transport of vitamin B₁₂ in *Escherichia coli*. J. Biol. Chem. 255:4313-4319.
- Rosenberg, H., and I. Y. Young. 1974. Iron transport in the enteric bacteria, p. 67-82. In J. B. Neillands (ed.), Microbial iron metabolism. Academic Press, Inc., New York.
- Semin, B. K., A. A. Novakova, A. Y. Aleksandov, I. I. Ivanov, A. B. Rubin, and R. N. Kuzmin. 1982. Mössbauer spectroscopy of iron metabolism and iron intracellular distribution in liver of rats. Biochim. Biophys. Acta 715:52.
- Spartalian, K., W. T. Oosterhuis, and J. B. Neillands. 1975. Electronic state of iron in enterobactin using Mössbauer spectroscopy. J. Chem. Phys. 62:3538.
- Spartalian, K., N. Smarra, and W. T. Oosterhuis. 1973. Superparamagnetism in the fungus *Phycomyces* and Mössbauer effect studies in the fungus *Phycomyces*. AIP Conf. Proc. 18:1326.
- Stock, J. B., B. Rauch, and S. Roseman. 1977. Periplasmic space in *Salmonella typhimurium* and *Escherichia coli*. J. Biol. Chem. 252:7850-7861.
- Yariv, J., A. J. Kalb, R. Sperling, E. R. Bauminger, S. G. Cohen, and S. Ofer. 1981. The composition and the structure of bacterioferritin of *Escherichia coli*. Biochem. J. 197:171-175.
- Zilberstein, D., V. Agmon, S. Schuldiner, and E. Padan. 1982. The sodium/proton antiporter is part of the pH homeostasis mechanism in *Escherichia coli*. J. Biol. Chem. 257:3687-3691.