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Aerobactin-Mediated Utilization of Transferrin Iron†

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ABSTRACT: Aerobactin and enterobactin, hydroxamate- and catechol-type siderophores, respectively, were found capable of removing iron(III) from transferrin in buffered solution. Although under these conditions aerobactin displaced the iron much more slowly than did enterobactin, the rate for the former could be accelerated by addition of pyrophosphate as mediator. Transfer of iron(III) from transferrin to aerobactin appeared to proceed via a ternary complex. Cells of *Escherichia coli* BN 3040 Nal^R *iuc* containing transport systems for both enterobactin and aerobactin, the genetic determinants for the latter specified on a ColV-type plasmid, took up iron from [^{55}Fe]transferrin in minimal medium. In this case aerobactin was effective at a much lower concentration, although enterobactin still displayed superior ability to transfer the iron. In serum, however, the rate measured with aerobactin exceeded that found with enterobactin. The results indicate that aerobactin, in spite of its relatively unimpressive affinity for iron(III) as a siderophore, is nonetheless equipped with structural features or properties that enhance its ability to remove the metal ion from transferrin, especially when receptor-bearing cells of *E. coli* are present to act as a thermodynamic sink for the iron. These attributes of the aerobactin system of iron assimilation may account for its status as a virulence determinant in hospital isolates of *E. coli*.

Aerobactin, the prototypical member of the hydroxamic acid–citrate family of siderophores, is a conjugate of 6-(*N*-acetyl-*N*-hydroxyamino)-2-aminohexanoic acid and citric acid. It is produced by *Aerobacter aerogenes*—in addition to the catechol-type siderophore enterobactin—under conditions of low iron stress. The high-spin octahedral iron(III)–aerobactin complex is formed with the two bidentate hydroxamate groups, the central carboxylate, and probably the citrate hydroxyl group. The stability constant for ferric aerobactin is many orders of magnitude below that of ferric enterobactin and approximates that of ferric transferrin (Harris et al., 1979). The question arises as to why the microorganism should produce the thermodynamically inferior chelator aerobactin when it can form the more powerful chelator enterobactin. Thus far there is no convincing answer to this question, though certain observations would tend to favor aerobactin as the more efficient chelator in a biological milieu.

Enterobactin is chemically unstable and is destroyed enzymatically following its iron transport function, whereas there is no evidence to show that aerobactin cannot be recycled. It

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has been suggested that the iron(III) can be more easily reduced and removed from hydroxamate siderophores than from enterobactin (Cooper et al., 1978). Enterobactin has low aqueous solubility, and its aromatic character causes it to adhere to proteins and to act as a haptene for synthesis of antibodies. Antibodies may also be raised to the surface receptor for ferric enterobactin (Neilands, 1981). Recently, it has been shown that antibodies to enterobactin occur in normal human serum, which presumably act together with transferrin to hinder iron assimilation by enterobactin-producing pathogens (Moore et al., 1980; Moore & Earhart, 1981).

A correlation between virulence and iron assimilation has been established for a large number of animal pathogens, including enteric bacteria (Weinberg, 1978). Recently, it has been shown that certain clinical isolates and invasive strains of *Escherichia coli* bearing the ColV plasmid synthesize both catechol- (enterobactin) and hydroxamate-type siderophores, genetic determinants for the latter residing on the plasmid (Williams, 1979). Virulence is not associated with synthesis of enterobactin or colicin V but with the noncatechol siderophore (Williams & Warner, 1980), now identified as aerobactin (Warner et al., 1981). Plasmid-specified synthesis may be a general feature of aerobactin formation in *E. coli* strains (Bindereif et al., 1981a,b); the origin of the siderophore in certain other enteric bacteria is still unresolved.

Bacterial proliferation in vivo is dependent upon mobilization of iron from sources that are normally unavailable to the microorganism. The pool of low molecular weight iron compounds, the iron-storage protein ferritin, and the iron-transport protein transferrin are three potential sources of iron. The concentration of free iron in human plasma is of the order of 10^{-12} μM or less, and it has been assumed that the production of siderophores of either the hydroxamate- or catechol-types might facilitate microbial growth by removal of iron from transferrin. It seems possible that the invasive strains of *E. coli* producing aerobactin can in some way remove iron from the metal complexes, e.g., transferrin. The present paper described the kinetics of iron transfer from transferrin to aerobactin in vitro as well as the rate of aerobactin-mediated bacterial utilization of the transferrin-bound iron in serum. Our results show that aerobactin can remove iron from transferrin in buffer and can transfer the iron of [^{55}Fe]-transferrin to *E. coli* incubated in minimal medium or in human serum.

Materials and Methods

Materials. $^{55}FeCl_3$ in 0.5 M HCl was purchased from ICN Corp. AG1-X4 anion-exchange resin and Bio-Gel P-2 were obtained from Bio-Rad Laboratories. Aerobactin (Bindereif, 1980), deferriferrichrome A (Emery & Neilands, 1960), and enterobactin (Wayne et al., 1976) were isolated and purified by published methods. Deferrioxamine B mesylate (Desferal) was purchased from Ciba Pharmaceuticals. Human apotransferrin (Sigma) was saturated with iron as described by Bates & Schlabach (1975) with a freshly prepared solution of 5 mM ferric nitrilotriacetate at pH 7.4. Labeled ferric aerobactin was prepared by mixing a solution of 20 mM aerobactin with $^{55}FeCl_3$ and $FeSO_4$ in 20 mM Tris-HCl[1] buffer, pH 7.0. [^{55}Fe]Ferric aerobactin was purified by chromatography on a Bio-Gel P-2 column. Human blood was collected from healthy adults. Serum was separated from the clot by centrifugation and was sterilized by filtration through a bacteria-retaining filter. The haemolysis void serum was heated at 56 °C for 30 min to inactivate the complement and then stored at −20 °C. Serum used for the measurements of the transferrin iron uptake was enriched with the addition of [^{55}Fe]transferrin.

Bacterial Strains and Growth Media. Two bacterial strains derived from *E. coli* K12 were used: BN 3040 Nal^R carrying the ColV-K 30 *iuc* plasmid and the plasmid-less parent strain BN 3040 Nal^R (F$^-$, *proC*, *leuB*, *trpE*, *thi*, *entA*, *cir*) (Hollifield et al., 1978). The BN 3040 Nal^R *iuc* strain was obtained by conjugation with *E. coli* LG 1522 (Williams & Warner, 1980); the latter strain was obtained through the courtesy of Peter H. Williams. The ColV strain can utilize both enterobactin and aerobactin while the parent can utilize only enterobactin. Both strains are blocked in synthesis of the two siderophores. L broth (LB; 10 g of tryptone, 5 g of yeast extract, 5 g of NaCl, distilled water to 1.0 liter) and phosphate-buffered M9 salts medium (Warner et al., 1981) supplemented with 30 mM succinate, thiamine (5 μg/mL), and the required amino acids were employed.

Kinetics of Iron Removal from Transferrin. Kinetic studies were performed with a Beckman Model 25 spectrophotometer at 20 °C in 10 mM Hepes/KOH buffer, pH 7.4. Solutions were clarified by centrifugation in an Eppendorf microcentrifuge. Time zero was taken at the moment of addition of the transferrin solution to the remainder of the reaction mixture. By use of a large excess of the competing ligand, pseudo-first-order conditions and linear plots of ln ($[TfFe]_0 - [TfFe]_t/[TfFe]_0$) vs. time were obtained. The transferrin concentration $[TfFe]_t$ was found by means of Cramer's rule, with evaluation of the determinants by the method of pivotal condensation (Bauman, 1962). The data were corrected for ligand absorption, and values of the apparent first-order rate constant (k_{obsd}) were obtained by linear regression analysis. The k_{obsd} vs. ligand concentration data were analyzed by a nonlinear least-squares computer program (HP-97, Standard Pac).

Chromatography. Separation of the reaction products was carried out on AG1-X4 anion-exchange resin equilibrated with 0.05 M KCl/0.05 M Hepes at pH 7.5. One-hundred percent saturated [^{55}Fe]transferrin (0.2 mg of protein/mL) and aerobactin or other microbial siderophores were incubated at 20 °C in a solution buffered with 10 mM Hepes/KOH, pH 7.4. At appropriate time intervals, 200-μL aliquots were taken as counting standards, and 1-mL aliquots were passed through a 1.5 × 6.0 cm column. Elution was with 20 mL of 0.05 M KCl/0.05 M Hepes and then with 20 mL of 1 M KCl/0.05 M Hepes at pH 7.5. Transferrin was eluted with 0.05 M KCl, and the iron–aerobactin or ferrichrome A complexes were eluted with 1 M KCl. Fractions of 1 mL were collected. ^{55}Fe radioactivity was measured with a scintillation counter, type Searle Delta 300. The range of ^{55}Fe recovery was about 90% of the total counts applied to the column.

Uptake Experiments. *E. coli* BN 3040 Nal^R *iuc* and BN 3040 Nal^R were cultured for 4–6 h at 37 °C in L broth and then inoculated (1%) into appropriately supplemented M9 medium. After two serial transfers in this medium, the cells were collected by centrifugation, washed, and resuspended in M9 medium to give an absorbance of 1.0 at 650 nm, corresponding to approximately 10^9 cells/mL. The cell suspensions were shaken in a water bath at 37 °C for 30 min, at which time [^{55}Fe]ferric aerobactin or [^{55}Fe]transferrin was added.

[1] Abbreviations: Tf, apotransferrin; PP_i, pyrophosphate; k_{obsd}, the apparent first-order rate constant; K_{eq}, the equilibrium constant; 2,3-DHBA, 2,3-dihydroxybenzoic acid; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; 3,4-LICAMS, 1,5,10-tris(5-sulfo-2,3-dihydroxybenzoyl)-1,5,10-triazadecane; Tris, tris(hydroxymethyl)aminomethane.

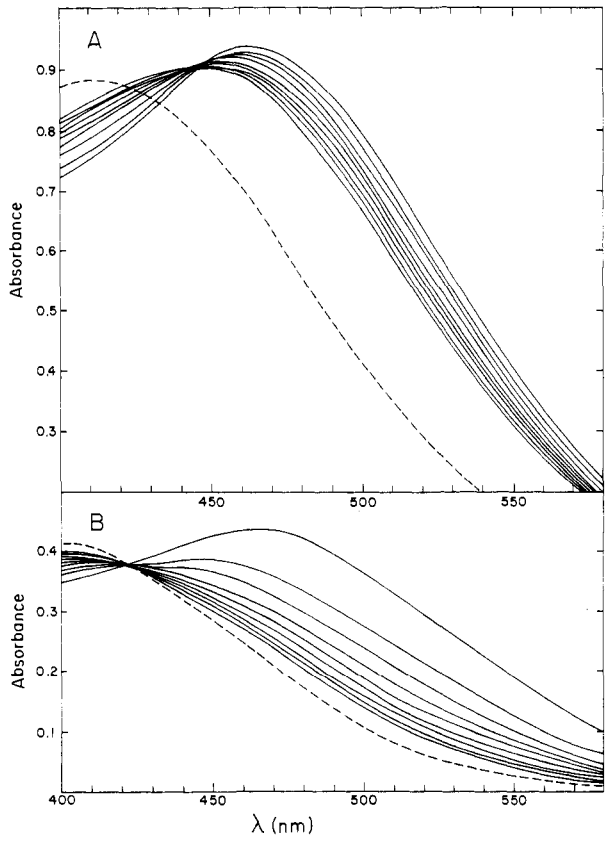

FIGURE 1: Spectral changes associated with exchange of transferrin-bound iron with aerobactin in 10 mM Hepes/KOH buffer, at pH 7.4. (A) Reaction mixture contained 0.2 mM 100%-saturated transferrin and 10.8 mM aerobactin. (B) Reaction mixture contained 0.1 mM 100%-saturated transferrin, 10.8 mM aerobactin, and 10 mM PP_i. Time points were taken at 15-min intervals for 2 h (solid lines) and after 24 h (dashed lines).

Further additions are indicated in the figure legends. In [^{55}Fe]ferric aerobactin uptake experiments, samples of 0.1 mL were withdrawn at timed intervals, and the cells were collected on 0.45-μm Millipore filters. [^{55}Fe]Ferric aerobactin uptake was also determined by using the cells resuspended in human serum. In siderophore-mediated uptake of ^{55}Fe from [^{55}Fe]transferrin, samples of 0.2 mL were withdrawn at timed intervals, and the cells were spun out, washed, resuspended, and collected on 0.45-μm Millipore filters that had been soaked overnight in 30 μM apotransferrin. The filters were dried and counted in 10 mL of scintillation fluid. In experiments in which serum was used, the cells were resuspended in [^{55}Fe]-transferrin–serum and shaken in a water bath at 37 °C for 30 min, at which time aerobactin or enterobactin was added. Samples of 0.1 mL were withdrawn at timed intervals, and the radioactive iron uptake was measured as described.

Results

Kinetics of Iron Removal from Transferrin. The addition of aerobactin to a solution of iron transferrin resulted in the spectral changes seen in Figure 1A. The upper curve corresponds essentially to the spectrum of unreacted transferrin. Subsequent spectra illustrate the decrease in intensity of the transferrin peak at 465 nm, with a concomitant increase in absorbance at 400 nm indicating that the iron was now at least partially coordinated to the aerobactin moiety. These spectral changes are consistent with the ferric aerobactin spectrum at neutral pH [λ_{max} 398 nm, ϵ 2170 L/(mol·cm)] (Harris et al., 1979). The time elapsed between the first and last spectral traces was 24 h, at which point approximately 54% of the iron was removed. An isosbestic point was not observed, indicating the presence of more than two absorbing species in solution as equilibrium was approached. The transfer of iron from transferrin to deferriferrichrome A and desferal was also measured (data not shown); both show a signficantly slower rate of iron removal.

Plots of ln ($[TfFe]_0 - [TfFe]_t/[TfFe]_0$) vs. time gave straight lines over 120 min for concentrations of aerobactin from 2.0 to 10.0 mM. This represents a 20–120-fold excess of ligand over transferrin. There was no evidence for loss of iron from ferric aerobactin when the siderophore was allowed to react with apotransferrin in a molar ratio of 10:1.

Mechanism of Iron Removal from Transferrin by Aerobactin. Previous results indicated that the rate of iron removal from transferrin shows a hyperbolic dependence on concentration for some chelators and a linear dependence for others. The group giving hyperbolic kinetics have ligands that are hydroxamates, phosphates, or catechols, all of which have high intrinsic affinities for Fe(III). The hyperbolic kinetic data seemed to support a ternary complex formation, although the anticipated spectral evidence was lacking (Bates, 1981).

In the reaction between transferrin and aerobactin we propose that the exchange reaction proceeds through initial formation of a ternary complex in which iron is bound to both transferrin and aerobactin, followed by iron removal and dissociation of the complex. This scheme is outlined in eq 1

$$\mathrm{TfFe} + \mathrm{L} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \mathrm{TfFeL} \xrightarrow{k_2} \mathrm{LFe} + \mathrm{Tf} \qquad (1)$$

where TfFe is iron transferrin, L is aerobactin and Tf is apotransferrin. If the concentration of aerobactin is much greater than that of transferrin, it will remain essentially constant, so that the equilibrium constant K_{eq} will be given by

$$K_{eq} = [\mathrm{TfFe}]/([\mathrm{L}]_0[\mathrm{TfFe}]) \qquad (2)$$

where $[L]_0$ represents the initial concentration of aerobactin. A hyperbolic relationship has been obtained between k_{obsd} and the concentration of competing ligand, with k_{obsd} approximately 0.9×10^{-3} min^{-1} at an aerobactin concentration of 4.0 mM with both 100% and 40% saturation of transferrin.

The k_{obsd} for appearance of ferric aerobactin is thus obtained for solutions in which [TfFe] is kept constant but $[L]_0$ is varied, always keeping $[L]_0$ much greater than [TfFe]. A plot of ln ($[TfFe]_0 - [TfFe]_t/[TfFe]_0$) vs. time should give a straight line with a slope equal to k_{obsd}. A plot of $1/k_{obsd}$ vs. $1/[L]_0$ should give a straight line with a slope equal to $2.303/(k_2K_{eq})$ and an intercept of $2.303/k_2$ (Kidani et al., 1976; Kidani & Hirose, 1977):

$$k_{obsd} = \frac{k_2K_{eq}[\mathrm{L}]_0}{2.303 + 2.303K_{eq}[\mathrm{L}]_0} \qquad (3)$$

$$1/k_{obsd} = 2.303/(k_2K_{eq}[\mathrm{L}]_0) + 2.303/k_2 \qquad (4)$$

The apparent K_{eq} was 1.2×10^2 M^{-1}, and the rate constant k_2 was 6.6×10^{-3} min^{-1}. The values found for 3,4-LICAMS by this method were $K_{eq} = 4.1 \times 10^2$ M^{-1} and $k_2 = 6.6 \times 10^{-2}$ min^{-1} (Carrano & Raymond, 1979). The effect of the concentration of transferrin on the rate of reaction was tested at 10.8 mM aerobactin. The k_{obsd} was independent of transferrin concentration over approximately an 8-fold range from 0.025 to 0.2 mM, indicating that the rate was first order with respect to transferrin (Table I). Under the pseudo-first-order conditions observed here ([L] $\gg$ [Tf]) the initial rate was found directly proportional to [TfFe] and insensitive to the concen-

Table I: Reaction of Aerobactin with Various Concentrations of Iron Transferrin[a]

iron transferrin concn (mM)	obsd rate constant (k_{obsd}) ($\times 10^{-3}$ min^{-1})
0.025	1.75
0.05	2.1
0.1	2.1
0.2	1.8

[a] The concentration of aerobactin was 10.8 mM in all cases.

Table II: Reaction of Iron Transferrin with Various Concentrations of Aerobactin[a]

aerobactin concn	obsd rate constant (k_{obsd}) ($\times 10^{-3}$ min^{-1})
2.7	16.2
5.4	16.0
8.1	16.7
10.8	13.5

[a] The concentrations of transferrin and pyrophosphate were in all cases 0.09 and 10.8 mM, respectively.

tration of aerobactin. In fact, the initial rate found was 0.6×10^{-6} M Fe removed per min over the range of aerobactin concentration of 4.5–11.5 mM at 0.09 mM transferrin. The absence of an isosbestic point suggests the existence of a reaction intermediate.

Kinetics of Reaction of Transferrin with Aerobactin in the Presence of PP_i. Addition to PP_i to the reaction mixture facilitates iron transfer from transferrin to aerobactin and other siderophores. The spectral changes observed when 10 mM PP_i was added to a mixture of transferrin and aerobactin are shown in Figure 1B. The top trace corresponds essentially to the spectrum of unreacted transferrin and the bottom curve represents the product, ferric aerobactin. The time elapsed between the first and last spectral traces is 24 h, at which time practically 100% of the iron has been removed. The isosbestic point at 422 nm indicates the presence of two absorbing species in solution as equilibrium is approached. These are transferrin and the final product, ferric aerobactin.

Plots of $\ln([TfFe]_0 - [TfFe]_t/[TfFe]_0)$ vs. time give straight lines, and their slopes afford the first-order rate constants (k_{obsd}). The linear relation of k_{obsd} to PP_i concentration at 0.1 mM transferrin and 2.7 mM aerobactin was observed. The relation between the first-order rate constant and the concentration of aerobactin is given in Table II. It can be seen that in the presence of 10 mM PP_i the rate constant is independent of the concentration of aerobactin. The value of k_{obsd} was 1 order of magnitude greater than that obtained at 10.8 mM aerobactin in the absence of PP_i.

The most likely sequence leading to the transfer of iron from transferrin to aerobactin in the presence of PP_i is

$$TfFe + L \xrightarrow{PP_i} TfFe^* + L \xrightarrow{k_d} Tf + LFe \qquad (5)$$

where the abbreviations are as for eq 1 except that TfFe* represents a conformational state in which the iron of transferrin is partially exposed and k_d is the dissociation rate constant of TfFe* and is given by (Kidani & Hirose, 1977)

$$k_{obsd} = k_d/2.303 \qquad (6)$$

The relation between the dissociation rate constant and the concentration of PP_i is presented in Table III. The ternary complex [TfFeL] is not considered to be present in any appreciable amount, as is the case in the absence of PP_i.

Table III: Reaction of Iron Transferrin with Aerobactin in the Presence of Various Concentrations of Pyrophosphate (PP_i)[a]

PP_i concn	dissoc constant (k_d) ($\times 10^{-2}$ min^{-1})
1.0	0.61
2.5	1.25
5.0	2.26
7.5	3.29
10.0	4.15

[a] The concentrations of iron transferrin and aerobactin were in all cases 0.1 and 2.7 mM, respectively.

Reaction between [^{55}Fe]Transferrin and Siderophores. The results obtained by using anion-exchange chromatography confirm the data obtained by the spectral analysis. At an 80-fold excess of aerobactin, 27% and 38% of the iron was removed from transferrin at 24 and 48 h, respectively. Under comparable conditions the values for deferriferrichrome A were 6% and 8% for 24 and 48 h, respectively. Mobilization of iron by 2,3-DHBA alone results in very slow exchange with virtually no iron removal after 48 h. PP_i has the ability to remove iron directly from transferrin (Konopka et al., 1980). The addition of 10 mM 2,3-DHBA to the incubation mixtures resulted in a more effective iron transfer from transferrin to either aerobactin or deferriferrichrome A; after 24 h 41% and 27% of ^{55}Fe was bound to aerobactin and ferrichrome A, respectively. The addition of 10 mM PP_i to the incubation mixtures led to significant iron removal from [^{55}Fe]transferrin, and the released iron was transferred to siderophores. Thus after 24 h virtually no ^{55}Fe appeared in the 0.05 M KCl eluate in the presence of a 20–100-fold excess of both siderophores.

Uptake Experiments. Direct measurements of the uptake of radioactive iron into bacterial cells demonstrated that the plasmid-less parent strain BN 3040 Nal^R is defective in aerobactin utilization. Under the same growth and uptake conditions, transport of ^{55}Fe into cells of the BN 3040 Nal^R *iuc* strain was found (Figure 2A), and after a 2-h incubation practically 100% of the radioactive iron was accumulated by the cells. In experiments in which the cells of the BN 3040 Nal^R *iuc* strain were resuspended in human serum, the extent of ^{55}Fe uptake from [^{55}Fe]ferriaerobactin was nearly identical with that in M9 medium, and 86% of the radioactive iron was accumulated by the cells (data not shown).

The uptake experiments using [^{55}Fe]transferrin (Figure 2B) confirmed the previous observations based on in vitro studies of exchange of iron between iron transferrin and aerobactin. The BN 3040 Nal^R strain does not contain the plasmid-specified transport system of ferric aerobactin. This strain, upon incubation with [^{55}Fe]transferrin in the presence or absence of aerobactin, was unable to accumulate radioactive iron. The BN 3040 Nal^R *iuc* strain assimilated iron from [^{55}Fe]transferrin only in the presence of added aerobactin or enterobactin. Enterobactin was approximately twice as efficient as aerobactin in removal of iron from [^{55}Fe]transferrin when the bacteria were incubated in M9 medium. However, in contrast to the modest rate observed in vitro aerobactin is relatively effective in removing iron from transferrin when the ultimate repository of the metal ion is the cells of *E. coli*. Thus an effect was observed at a concentration of 5 and 10 μM at 10 μM [^{55}Fe]transferrin, a ratio at which aerobactin was ineffective in vitro in mobilizing iron from transferrin. At a 1:1 aerobactin:transferrin ratio 0.46 μM of transferrin-bound iron was accumulated by the cells after 2 h. Saturation kinetics

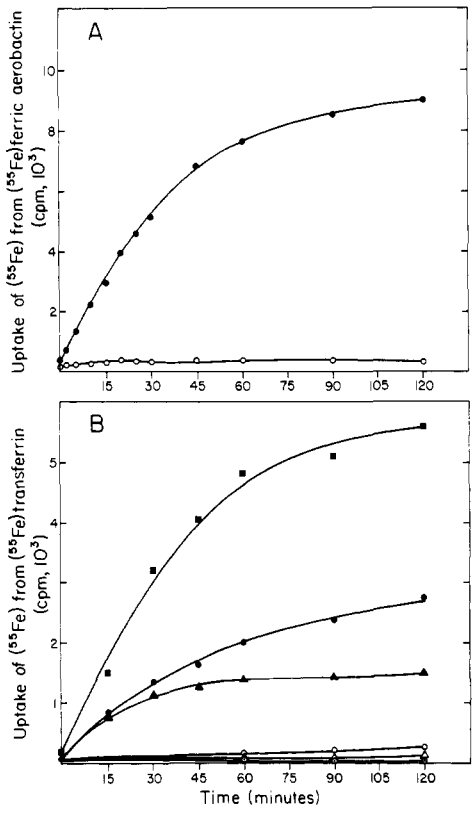

FIGURE 2: Uptake experiments. (A) Uptake of ^{55}Fe from [^{55}Fe]ferric aerobactin by the BN 3040 Nal^R *iuc* strain (●) and the BN 3040 Nal^R strain (○). Cells were shaken at 37 °C with 0.63 μM [^{55}Fe]ferric aerobactin. (B) Uptake of ^{55}Fe from [^{55}Fe]transferrin: the BN 3040 Nal^R *iuc* strain without siderophores (△), plus 5 μM aerobactin (▲), plus 10 μM aerobactin (●), and plus 10 μM enterobactin (■); the BN 3040 Nal^R strain without siderophores (○) and with 10 μM aerobactin (⊙). Specific activity 30.0 cpm/pmol of Fe.

were observed for aerobactin concentrations above 10 μM at 10 μM [^{55}Fe]transferrin (data not shown).

For investigation of the influence of serum on the uptake of transferrin-bound iron by the BN 3040 Nal^R *iuc* strain, experiments were performed with a serum suspension of the cells in the presence of aerobactin and enterobactin. These experiments showed that the rate and the extent of iron uptake in human serum were different from those in medium M9 (Figure 3). Thus siderophore mediated uptake of ^{55}Fe from [^{55}Fe]transferrin was much slower in serum than in medium M9, and aerobactin was much more efficient than enterobactin in transport of iron to cells. In the presence of 0.3 mM aerobactin (at a 10:1 aerobactin:transferrin ratio), 0.37 μM transferrin-bound iron was accumulated in the cells after 2 h.

Discussion

Possible mechanisms of removal of iron from diferric transferrin by synthetic and natural tricatecholate ligands have been discussed by Carrano & Raymond (1979). One compound, 3,4-LICAMS, was examined in detail. The reaction exhibited saturation kinetics with regard to the chelator, but the spectral changes accompanying iron transfer indicated a gradual monotonic shift from product to reactant with no suggestion of a kinetic intermediate. It was suggested that, in contrast to the catecholate compounds, the hydroxamate-based chelating agents are kinetically incapable of iron removal from transferrin.

The usual strains of *E. coli* do not make or transport aerobactin, but certain invasive *E. coli* strains bearing the ColV

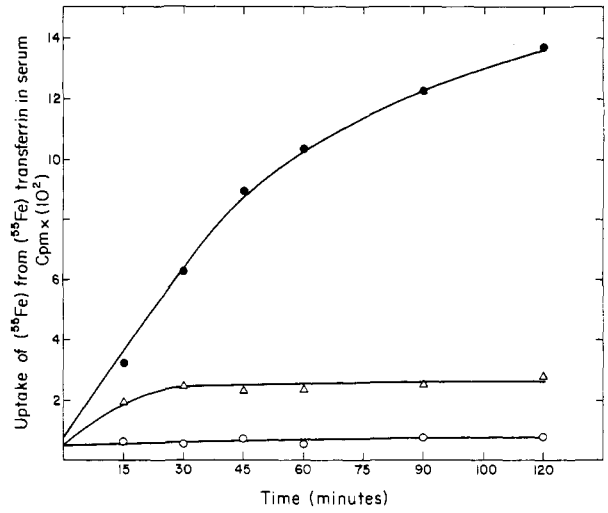

FIGURE 3: Uptake of ^{55}Fe from [^{55}Fe]transferrin by the BN 3040 Nal^R *iuc* strain in human serum: without siderophores (○), plus 0.3 mM aerobactin (●), and plus 0.3 mM enterobactin (△). Cells were shaken at 37 °C in human serum enriched with [^{55}Fe]transferrin. Specific activity 36.5 cpm/pmol of Fe.

plasmid have been shown to produce this siderophore and to grow in the presence of transferrin (Williams, 1979). These observations prompted our examination of the efficiency of aerobactin to remove iron from transferrin.

Several lines of evidence indicated immediately that aerobactin could indeed remove iron from transferrin. The spectra (Figure 1A) show progressive changes in the λ_{max} from 465 nm of transferrin to the characteristic orange-colored complex of ferric aerobactin with the λ_{max} at approximately 400 nm as the reaction proceeds, indicating that the iron is now coordinated to the hydroxamate siderophore and has been released from transferrin. The removal of iron from transferrin by aerobactin took place through the formation of a ternary complex involving iron transferrin and aerobactin. It is of interest that not only the stability constants but also the chemical structure of the competing ligand plays an important role in the rate of iron mobilization from transferrin. Both deferriferrichrome A and deferriferrioxamine B are hydroxamate chelators with p[M] values many orders of magnitude higher than those for aerobactin and apotransferrin (Harris et al., 1979). However, despite their superior affinity for ferric iron, they show a significantly slower rate of iron removal from transferrin than does aerobactin. As found previously (Pollack et al., 1977), this kinetic barrier of deferriferrioxamine B can be overcome by the addition of certain anions to the reaction mixture. In the present study we have confirmed this observation for the kinetically inert hydroxamate siderophores by using 2,3-DHBA and PP_i. The former has no ability to remove iron directly from transferrin, and the mechanism of its labilizing effect is unknown. Thus steric factors as well as intrinsic binding affinity are probably involved in determining the rate of intermediate complex formation and release of the protein-bound iron.

PP_i is by far the best chelating agent for removal of iron from transferrin in the presence or absence of an iron acceptor (Kojima & Bates, 1979). The chelation reaction rate profile follows a hyperbolic dependence on PP_i concentration. No spectral evidence for a reaction intermediate was detected. The explanation for the hyperbolic kinetics observed with transferrin and PP_i is that iron transferrin undergoes a conformational change resulting in exposure and labilization of iron. The hyperbolic dependence of rate on chelator concentration reflects a competition between chelator attack on the exposed

iron and rewrapping of apotransferrin about the iron (Bates, 1981). We assume that in the presence of PP_i a path to the ternary complex of transferrin–iron–aerobactin does not occur or the intermediate must be present in a low steady-state concentration throughout the course of the reaction. A third absorbing species, if present in significant concentration, would destroy the observed isosbestic point. Presumably, in the presence of PP_i the iron transferrin converts from the "closed" stable state to the "open", apotransferrin-like conformation (TfFe*) (Bates, 1981). The labilized Fe(III) is released and then forms a stable complex with aerobactin. In this case there is no competition between transferrin and PP_i for the exposed Fe(III), and the hyperbolic dependence of rate on PP_i concentration is not observed.

With regard to the mechanism of siderophore-mediated iron assimilation, it is difficult to construct a meaningful comparison of our results with those already in the literature. Different investigators have employed a variety of bacterial species, siderophores, and media. By restricting ourselves to *E. coli*, it appears that studies comparable to those recorded here with aerobactin have not been previously reported. Rogers (1973) demonstrated the capacity of enterobactin to remove ^{59}Fe from transferrin and to supply the labeled element to cells of *E. coli* 0111. In contrast, Mellencamp et al. (1981) concluded that siderophores such as enterobactin and rhodotorulic acid stimulate enteric bacteria (*E. coli*, *Salmonella typhimurium*) to use stored iron and do not function to shuttle the metal ion from transferrin to the cells. However, in their experiments the amount of ^{59}Fe acquired by the bacteria, some 2–4% of the total supplied, may have sufficed to account for the normal growth that they observed. Tidmarsh & Rosenberg (1981) showed that an enterobactin producing strain of *Salmonella paratyphi* B could obtain iron from transferrin when the protein was sequestered within a dialysis bag. They concluded that contact between the ferric enterobactin (colicin B) receptor and transferrin was not required for siderophore-mediated removal of the iron.

The data of Williams & Warner (1980) and Stuart et al. (1980) showing that virulence is dependent on synthesis of a hydroxamic acid type siderophore, subsequently identified as aerobactin, rather than the common catechol-type siderophore, enterobactin, suggested strongly that only the former can capture iron in vivo. Our experiments confirm this suggestion and indicate that aerobactin, despite its relatively humble affinity for Fe(III) among the siderophore series, must be endowed with special structural features favoring its adoption as a plasmid-specified iron carrier in clinical isolates of *E. coli*. This aspect of the problem will be the subject of a future paper.

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