As pointed out above, the recombination process 7 is of sufficient exergonicity to leave molecular oxygen in the $^1\Delta_g$ electronic state ($E \simeq 1$ eV) but insufficient to form $Ru(bpy)_3^{2+}$ in its excited state ($E \simeq 2.1$ eV).[2] Liu et al.[22] showed that exergonic electron-transfer reactions between $Ru(bpy)_3^{3+}$ and several Co(I) complexes resulted in preferential population of electronically excited states of the Ru(II) and/or Co(II) products with minimal ground-state production. The relative slowness of the reaction to form ground-state products was attributed to the very high exergonicity of this process, placing it in the inverted region ($-\Delta G^0 > \lambda$, where λ is the reorganization parameter).[22] In our case, there is a relatively large reorganization energy related to the difference in solvation of O_2 and $O_2^{\cdot-}$ and the different bond lengths in the two species.[23] The value of the overall barrier seems likely to be about the same as the value of ΔG^0 for the reaction leading to O_2 ($^3\Sigma_g^-$), i.e. $-\Delta G^0 \simeq \lambda$. According to this assumption, the O_2 ($^3\Sigma_g^-$) forming process would be barrierless (i.e., very fast) whereas the O_2 ($^1\Delta_g$) forming one, having a smaller driving force, is expected to proceed more slowly because it lies in the normal free energy region. Thus, the lack of singlet oxygen formation is not a surprising result.

Acknowledgment. This research was supported in part by Consiglio Nazionale delle Ricerche of Italy, in part by National Institutes of Health (Grant GM 24235), and in part by Ministero of Pubblica Istruzione of Italy (Quota 40%). The Center for Fast Kinetics Research is jointly supported by the Biomedical Research Technology Program of the Division of Research Resources of the NIH (Grant RR 00886) and by the University of Texas at Austin. The technical assistance of G. Gubellini is appreciated. We thank Prof. M. Maestri for making available to us the continuous-photolysis apparatus and for discussion of the results obtained. Q.G.M. thanks the staff of CFKR for making his visit to the Center (Spring 1987) a most rewarding one.

Registry No. MV^{2+}, 1910-42-5; $ADPA^{2-}$, 113273-49-7; $Ru(bpy)_3^{2+}$, 15158-62-0; $Ru(bpy)_3^{3+}$, 18955-01-6; O_2, 7782-44-7; $O_2^{\cdot-}$, 11062-77-4.

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Substituted Complexes of Enterobactin and Synthetic Analogues as Probes of the Ferric–Enterobactin Receptor in *Escherichia coli*†

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Abstract: The structural requirements for recognition of ferric–enterobactin by the ferric–enterobactin receptor in *Escherichia coli* have been examined with model complexes as probes in mediation, and inhibition, of transport. Kinetically inert, air-stable rhodium(III) tris ligand complexes of catechol and 2,3-dihydroxy-*N*,*N*-dimethylbenzamide (DMB) and a rhodium(III) complex of a close structural analogue of enterobactin, 1,3,5-tris[[(2,3-dihydroxybenzoyl)amino]methyl]benzene (MECAM), were used as probes to study the importance of the ligand amide functional group in complex recognition. The Rh(III)–MECAM complex is a competitive inhibitor of ferric–enterobactin transport. The ferric complex of the same ligand is a transport substrate in *E. coli*. The simple tris(catecholate) complex of rhodium(III), $[Rh(cat)_3]^{3-}$, is *not* an inhibitor of ferric–enterobactin uptake, but the tris complex of the catechoylamide (DMB) *is* potent as an inhibitor. These results indicate that although it is the tris(catecholate) portion of the ferric–enterobactin complex that is recognized by the receptor protein, the amide regions of enterobactin, which link the catechol groups to the cyclic backbone, are also required components in recognition. Consistent with these observations, the ferric complex of the enterobactin analogue TRIMCAM (1,3,5-tris[(2,3-dihydroxybenzyl)carbamoyl]benzene, a structural isomer of MECAM in which the positions of the methyene, carbonyl, and NH groups are reversed) is not recognized by the ferric–enterobactin receptor. Replacement of the amide protons of MECAM with methyl groups does not change the iron transport properties of the ligand. The ferric complex of the N-methylated derivative Me_3MECAM (1,3,5-tris[[methyl(2,3-dihydroxybenzoyl)amino]methyl]benzene) is a substrate for iron(III) transport in vivo. A third derivative of MECAM, MECAM-Me (1,3,5-tris[[(2,3-dihydroxy-4-methylbenzoyl)amino]methyl]benzene), was synthesized to probe the sensitivity of the ferric–enterobactin receptor to changes in the ligand structure near the catechol groups. No iron uptake was observed when *E. coli* cells were given $[Fe(MECAM\text{-}Me)]^{3-}$ or the ferric complex of a sulfonated derivative of MECAM, MECAMS (1,3,5-tris[[(2,3-dihydroxy-5-sulfobenzoyl)amino]methyl]benzene). These experiments demonstrate that it is the tris(catechol)iron(III) portion of the ferric–enterobactin complex that is recognized by the protein receptor, not the enterobactin triserine ring. Furthermore, the carbonyl group is essential for recognition; however, the proton on the amide nitrogen is not. In addition, we have observed that any substitution on the catechol rings opposite the region of attachment to the enterobactin triserine ring (or similar model structure) blocks recognition. Labile trivalent metal ion complexes of enterobactin, as analogues of Fe(III), were prepared to study the effect of the physical properties of the metal ions on the recognition process. These complexes of enterobactin ranged from relatively effective inhibitors of ferric–enterobactin uptake to ineffective inhibitors in the series (with the Fe(III) complex as the reference) Fe > Sc > In ≫ Al > Ga.

Enterobactin is a low molecular weight iron chelating agent (siderophore) produced and excreted by *Escherichia coli* and other enteric bacteria to bind and assimilate extracellular iron.[2,3] After iron complexation, the extracellular ferric–enterobactin complex interacts with a specific receptor in the outer cell membrane, and the complex is taken into the cell by active transport. It has been shown that the ferric complexes of some synthetic analogues of

† Part 39. Coordination Chemistry of Microbial Iron Transport. See ref 6 for the previous paper.

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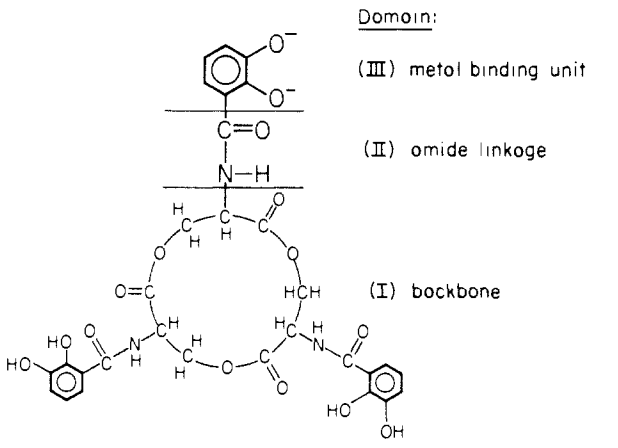


Figure 1. Structural diagram of enterobactin with one arm of the ligand emphasized. Domains of possible significance for receptor recognition are delineated: domain I, ligand backbone; domain II, amide linkage; domain III, catechol group (metal-binding unit).

enterobactin can act as iron sources for *E. coli*,[4] and we have recently reported kinetic and inhibition studies that probed structural requirements of the ferric–enterobactin outer-membrane receptor using some of these analogues.[1,5] These results suggested that the metal-binding portion of the molecule is recognized by the receptor, while the ligand platform (the triserine lactone ring) is not specifically recognized.

In this paper we further examine the requirements for recognition by the ferric–enterobactin receptor, focusing on three aspects of the recognition process. We seek to determine whether the amide groups (labeled domain II in Figure 1), which link the metal-binding catechol groups (domain III) to the central ligand backbone (domain I), are necessary for recognition by the receptor protein. In addition, we will examine the sensitivity of the receptor to small structural changes in the ligand near the catecholate rings. Finally, we will describe the effect of varying the central metal ion on the ability of metal–enterobactin complexes to inhibit competitively the uptake of ferric–enterobactin.

Two strategies have been used to probe the importance of the amide carbonyl and NH groups (domain II) in receptor recognition. First, kinetically inert rhodium model complexes of catechol, DMB, and MECAM (see Figure 2 for the chemical structures and complete names) have been prepared as models and tested as inhibitors of ^{59}Fe–enterobactin or ^{55}Fe–enterobactin uptake. Our second approach was to prepare synthetic analogues, which differ structurally in the amide region from enterobactin and MECAM. Ferric complexes of TRIMCAM, an isomer of MECAM in which the amide and methylene groups are reversed in orientation, and Me_3MECAM, an N-methylated derivative of MECAM, were used as probes in iron transport studies.

We have further examined the importance of ligand structure in domain III on complex recognition by the ferric–enterobactin receptor using ferric complexes of catechol-ring-substituted derivatives of MECAM as probes. We have also investigated the inhibitory effect of complexes of enterobactin with various other trivalent metals on labeled ferric–enterobactin uptake. Surprisingly, large differences in biological activity are found between metal ions with similar physical properties. The enterobactin complexes of Al(III) and Ga(III) show almost no inhibition of uptake in spite of the fact that gallium(III) has a charge to ionic radius ratio very close to that of iron and forms many complexes that are structurally nearly identical with those of iron.[6] On the other hand, complexes of Sc(III) and In(III) with enterobactin are quite potent inhibitors of ferric–enterobactin uptake.

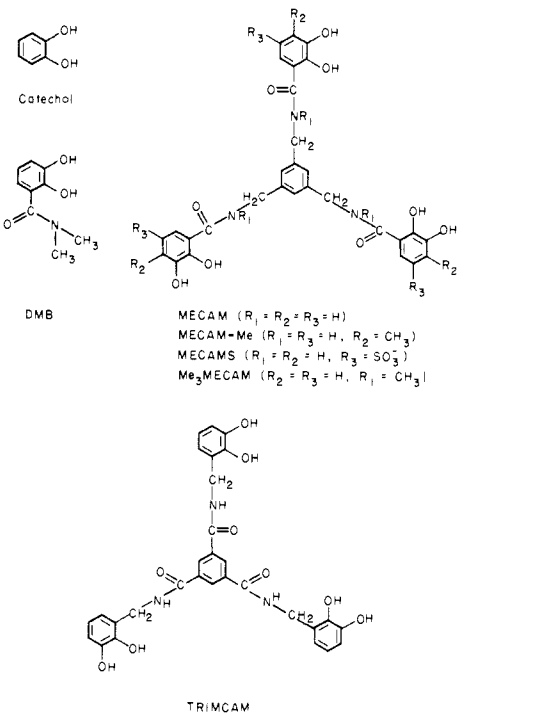
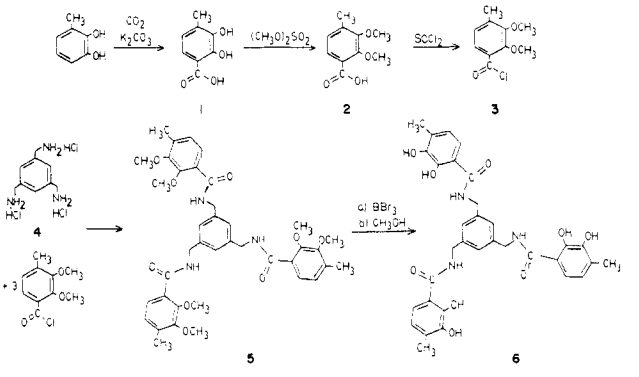


Figure 2. Synthetic ligands used in this study: catechol = 1,2-dihydroxybenzene, DMB = *N*,*N*-dimethyl-2,3-dihydroxybenzamide, MECAM = 1,3,5-tris[[(2,3-dihydroxybenzoyl)amino]methyl]benzene, TRIMCAM = 1,3,5-tris[(2,3-dihydroxybenzyl)carbamoyl]benzene, MECAM-Me = 1,3,5-tris[[(2,3-dihydroxy-4-methylbenzoyl)amino]-methyl]benzene, MECAMS = 1,3,5-tris[[(2,3-dihydroxy-5-sulfobenzoyl)amino]methyl]benzene, Me_3MECAM = 1,3,5-tris[[methyl-(2,3-dihydroxybenzoyl)amino]methyl]benzene.

Scheme I



Experimental Procedures

Physical Techniques. Ultraviolet and visible spectra were measured on a Hewlett-Packard 8450-A spectrophotometer. Infrared spectra were measured on a Perkin-Elmer FT-IR. Electrophoresis was performed by a Savant TLE-20 thin-layer tank with a Bio-Rad 3000/300 power supply. High-pressure liquid chromatography (HPLC) separations were carried out with a Beckman system (Model 421 controller, two Model 112 pumps, and a Model 153 detector). Fast atom bombardment (FAB) mass spectra were measured by the Analytical Services Laboratory, University of California, Berkeley. NMR spectra were recorded on the UCB-200 200-MHz FT-NMR at the Univeristy of California, Berkeley, NMR Facility.

Materials. The ligands MECAM, MECAMS, TRIMCAM, DMB, and Me_3MECAM (see Figure 2 for IUPAC names and structures) were synthesized as previously reported.[7-10] The synthesis of MECAM-Me is described below. Enterobactin (ent) was isolated from cultures of *E.*

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coli AN311 as previously described.[11] The following compounds were obtained from commercial sources (suppliers indicated): catechol and scandium trichloride (Aldrich); rhodium trichloride trihydrate and indium chloride tetrahydrate (Alfa); ferric chloride hexahydrate (Mallinckrodt); $^{59}FeCl_3$ and $^{55}FeCl_3$ (New England Nuclear); gallium and aluminum, as the pure metals (J. T. Baker). Buffers were obtained from Calbiochem: CHES [2-(cyclohexylamino)ethanesulfonic acid, pK_a 9.5]; HEPES [*N*-(2-hydroxyethyl)piperazine-*N*'-2-ethanesulfonic acid, pK_a 7.55]. Metal solutions were prepared by dissolving the metal in acid and diluting to the desired concentration with distilled, deionized water; they were standardized by titrations with EDTA according to the methods of Welcher.[12]

Synthesis of 1,3,5-Tris[[(2,3-dimethoxy-4-methylbenzoyl)amino]-methyl]benzene (MECAM-Me). The reaction scheme for the synthesis of MECAM-Me is shown in Scheme I. 2,3-Dihydroxy-4-methylbenzoic acid (**1**) was prepared from 3-methylcatechol (CTC-Organics) by a published procedure[13] and recrystallized from ethanol (140 mL) and water (240 mL). 2,3-Dimethoxy-4-methylbenzoic acid (**2**) was previously prepared by the reaction of 2,3-dihydroxybenzoic acid and dimethyl sulfate in aqueous NaOH.[14] Here, **2** was synthesized from a protocol analogous to that used for the preparation of 2,3-dimethoxyterephthalate:[15] 2,3-Dihydroxy-4-methylbenzoic acid (**1**) (14.48 g, 8.62×10^{-2} mol), K_2CO_3 (35.68 g, 0.259 mol), and dimethyl sulfate (24.5 mL, 0.259 mol) were boiled at reflux in 200 mL of acetone for 24 h under argon; acetone was removed by rotoevaporation, and the crude product was boiled at reflux for 10 h in 200 mL of aqueous NaOH (0.1 N). The resultant solution was acidified with concentrated HCl (14 mL) to pH 1, and the product was extracted into ethyl acetate and dried over Na_2SO_4. Ethyl acetate was removed byt rotoevaporation. The product was recrystallized from hot ethanol (50 mL) to which 70 mL of distilled water was added; yield 90%. IR (C=O): 1668 cm^{-1}. ^1H NMR (CD_3OH): δ 7.44 (d, 1, Ar H), 6.98 (d, 1, Ar H), 4.93 (br s, H_2O), 3.88 (s, 3, OCH_3), 3.81 (s, 3, OCH_3), 3.30 (m, CH_3OH), 2.27 (s, 3, CH_3). ^{13}C NMR (CD_3OD): δ 169.2 (C=O), 154.5, 153.4, 138.9, 127.0, 126.8, 124.9 (ar C's), 62.0, 60.7 (OCH_3), 16.3 (CH_3). ^{13}C NMR ($CDCl_3$): δ 166.5 (C=O), 152.4, 150.9, 139.5, 127.0, 126.6, 120.3 (ar C's), 62.0, 60.7 (OCH_3), 16.2 (CH_3). Acid chloride **3** was prepared as follows: **2** (6.75 g, 3.4×10^{-2} mol) was refluxed in distilled benzene (50 mL), thionyl chloride (20 mL, 0.274 mol), and 2 drops of DMF for 10 h under argon. Benzene and excess $SOCl_2$ were removed in vacuo by trap-to-trap distillation. The product was purified by vacuum distillation [90 °C (1 mm)] to yield a yellow viscous oil; yield 66%. IR (C=O): 1778 cm^{-1}. ^{13}C NMR ($CDCl_3$): δ 164.0 (C=O), 153.4, 152.1 (Ar COH), 140.6, 128.1, 126.2, 125.6 (Ar C), 61.4, 60.2 (OCH_3), 16.5 (CH_3). 1,3,5-Tris[[(2,3-dimethoxy-4-methylbenzoyl)amino]methyl]benzene (**5**) was synthesized by a Schotten–Baumann procedure: **3** (1.01 g, 4.70×10^{-3} mol) in 110 mL of distilled CH_2Cl_2 and 110 mL of aqueous 0.5 M NaOH were each added dropwise with stirring over the course of 2 h to a solution of 1,3,5-tris(aminomethyl)benzene trichloride (**4**) (0.431 g, 1.57×10^{-3} mol, synthesized by a published procedure),[8] in H_2O (150 mL) and CH_2Cl_2 (40 mL) and cooled to 0 °C. The solution was stirred for an additional 5 h at 0 °C. The CH_2Cl_2 layer was collected, washed with water, dried over Na_2SO_4, and then rotoevaporated to yield product (88%). The crude product was purified by flash chromatography on Kieselgel 60 [230–400 mesh silica, 20 cm × 20 mm column, eluent (30/12/3 petroleum ether/ethyl acetate/methanol, product R_f 0.31)]; yield 58%. ^1H NMR ($CDCl_3$): δ 8.35 (br t, 3, NH), 7.77 (d, 3, Ar H, J = 8 Hz), 7.27 (s, 3, central ring Ar H), 6.99 (d, 3, Ar H, J = 8 Hz), 4.66 (d, 6, CH_2), 3.8 (s, 9, OCH_3), 3.79 (s, 9, OCH_3), 2.28 (s, 9, CH_3). ^{13}C NMR ($CDCl_3$): 165.1 (C=O), 151.6, 151.2 (Ar $COCH_3$), 139.8, 136.9, 126.2, 126.0, 125.7, 124.2 (Ar C's), 61.2, 60.2 (OCH_3), 43.3 (CH_2), 16.1 (CH_3). Deprotection of **5** (0.36 g, 5.14×10^{-4} mol) in 20 mL of dried distilled CH_2Cl_2 (at 0 °C) was accomplished by the slow addition of boron tribromide (3 mL, 7.95 g, 3.17×10^{-2} mol) in 15 mL of dry distilled CH_2Cl_2. The reaction mixture was stirred for 48 h. Distilled H_2O (19 mL) was added, and the reaction mixture was stirred for several hours to hydrolyze all boron compounds. Solvents were removed by rotoevaporation. A total of 12 times the solid product was dissolved in 40 mL of methanol, heated to boiling, and then rotoevaporated to remove methanol and any volatile boron compounds. The product (**6**) was recrystallized from methanol; yield 93%. ^1H NMR (DMSO-d_6): δ 13.00 (s, 3, OH), 9.35 (t, 3, NH), 8.67 (br s, 3, OH), 7.23 (d, 3, Ar H), 7.19 (s, 3, Ar H central ring), 6.59 (d, 3, Ar H), 4.45 (d, 6, CH_2), 3.46 (br, H_2O), 2.15 (s, 9, CH_3). ^{13}C NMR (DMSO-d_6): δ 170.1 (C=O), 149.4, 143.7, 139.4, 128.8, 124.8, 119.9, 116.4, 112.1 (Ar C's), 42.2 (CH_2), 16.2 (CH_3). MS: ($^+$FAB): parent ion peak m/e 616 ($M + H^+$). Anal. Calcd for $C_{33}H_{33}O_9N_3$ (found): C, 64.42 (64.08); H, 5.40 (5.29); N, 6.83 (5.73).

Bacteria and Transport Studies. The *E. coli* K12 strain RW193 (ATCC33475), a mutant that is deficient in the synthesis of enterobactin (leu^-, trp^-, thi^-, $purE^-$, $entA^-$) was used for all transport studies. The experimental details for the preparation of cells and substrates for transport experiments have been reported in detail elsewhere.[5] Briefly, iron-deficient *E. coli* RW193 was suspended in a growth medium designed for transport studies at about 1 mg/mL cell concentration. Radiolabeled substrates were added at 1 μM concentration. Aliquots were removed at various intervals and filtered through 0.45 μM pore size filters. The cell-containing filters were rinsed and transferred to 20-mL glass scintillation vials. The cells and filters were homogenized and counted in a Searle Mark III liquid scintillation counter as previously described.[5] Inhibitors were prepared in advance and their concentrations determined spectroscopically from absorbances in the UV, assuming a molar extinction at 340 nm of 5000 per catechol ring. The effect of transport inhibitors was measured two ways. First, the potential inhibitor was incubated with the cells 5 min before the start of the transport experiment. Second, the transport experiment was started, and at least four data points were collected before the inhibitor was added. The active and passive transport of the ferric iron complexes of MECAMS, MECAM-Me, and Me_3MECAM were studied in the presence and in the absence of added glucose as an energy source. The protocol was previously published.[3]

Preparation of K_3[Rh(cat)$_3$]. The preparation of $K_3[Rh(cat)_3]$ was accomplished as previously described, with modifications.[16] All procedures were carried out under oxygen-free conditions, using standard Schlenk glassware with degassed solvents, under inert atmosphere. The rhodium(III) perchlorate [*Caution*! Hazardous and potentially explosive material!] was prepared from rhodium trichloride trihydrate by a published procedure.[17] Freshly sublimed catechol (0.38 mmol) and K_2CO_3 (1.5 mmol) were mixed dry and dissolved in 30 mL of water. To this solution was added $Rh(ClO_4)_3 \cdot 6H_2O$ (0.114 mmol) already dissolved in 2–3 mL of water. The solution was adjusted to pH 10.0 with 0.1 N KOH, and the reaction was heated to reflux for 1 h with stirring. An initial precipitate that formed on addition of the $Rh(ClO_4)_3 \cdot 6H_2O$ largely dissolved when refluxed. The reaction was concentrated to approximately 3 mL by rotary evaporation, filtered, and applied to a Biogel P2 column that was preequilibrated with degassed water. The $K_3[Rh(cat)_3]$ eluted as a sharp golden band that was collected, dried, and stored under nitrogen. The UV–vis spectrum of the product was identical with that previously reported.[16] On high-voltage paper electrophoresis on Whatman 3-mm paper, with 50 mM potassium carbonate buffer at pH 10, the compound moved in a sharp band as a trivalent anion with a mobility slightly greater than that of ferric–enterobactin.

Preparation of K_3[Rh(DMB)$_3$]. The ligand DMB (1.74 mmol) was dissolved in 20 mL of water along with a slight excess of KOH (3.5 mmol). Rhodium perchlorate hexahydrate (348 μmol) already dissolved in 20 mL of water was added to the basic ligand solution. This canary yellow, colloidal mixture was boiled at reflux for 18 h, during which time it turned deep brown and all material dissolved. The volume was reduced by 50% under vacuum and the solution allowed to cool slowly overnight. Well-formed cubic crystals of $KClO_4$ (200 mg) were removed from the reaction mixture. This process was repeated and another, much smaller, crop of salt crystals removed. The solvent was removed under vacuum, and the brown solid remaining was washed three times with THF, dried, and taken up in minimum H_2O. The product was applied to a Biogel P2 column preequilibrated with degassed water, pH 10. Two colored fractions were obtained and dried. The first, which ran in the void volume, appeared to be a $K_2[Rh_2(DMB)_4]$ dimer as shown by its FAB mass spectrum (parent ion at m/e 1001, and a possible fragmentation product at m/e 501), its slower movement electrophoretically, and a peak in the IR at 540 cm^{-1} (an M–O stretching band characteristic of an M–O–M dimer).[18] The second band was the desired $(DMB)_3$ complex of rhodium(III). This was characterized by electrophoresis (it ran with Fe–ent), by IR (identical with Fe–MECAM), by FAB mass spectrum (parent ion at m/e 644 for the fully protonated form), and by UV spectroscopy (λ_{max} 328 nm, similar to Fe–MECAM).

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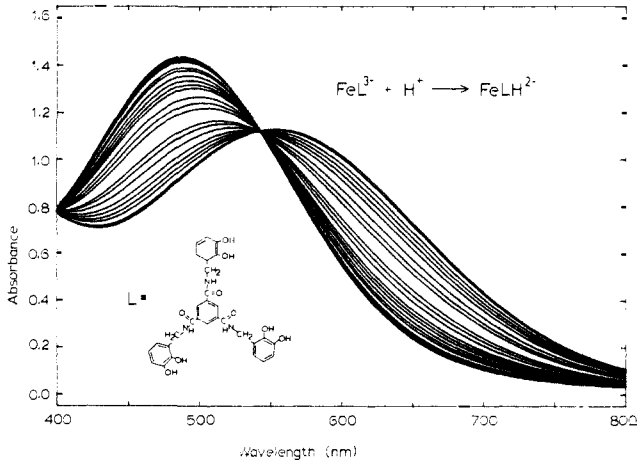

Figure 3. Visible spectra recorded in the spectrophotometric titration of Fe–TRIMCAM; 22 spectra in the isosbestic region of the titration between pH 9.55 and 7.39.

Preparation of K_3[Rh(MECAM)]. MECAM (0.6 mmol) and K_2CO_3 (6 mmol) were dissolved in 90 mL of water. Rhodium perchlorate hexahydrate (0.6 mmol) was dissolved in 10 mL of water. The buffered ligand solution was added slowly to the metal with stirring. The reaction was boiled at reflux for 12 h during which time it changed from light yellow to golden brown. Electrophoresis (pH 10 carbonate) showed several bands, one of which moved as a trivalent anion with the same mobility as a K_3[Fe(MECAM)] standard. The UV spectrum of this fraction showed a shift of the ligand-based absorbance from 331 to 328 nm, and a shoulder grew in at 386 nm. The reaction products were concentrated by rotary evaporation, filtered, and chromatographed on Biogel P2 as described above for the rhodium tris(catecholate) complex. The greenish gold band was then dried and dissolved in minimum $MeOH/H_2O$ (2:1). This was chromatographed on a 7-mm reversed-phase HPLC column at a flow rate of 3.5 mL/min, with monitoring at 254 nm. The solvent system was ramped in 10 min from 100% water to 90% MeOH, both containing either 0.01 M triethylammonium formate, pH 10, or 0.1 M valine ethyl ester, pH 7.5, as ion-pairing agents. The peak eluting at the same retention time as Fe–MECAM was collected and dried by rotary evaporation. The Rh–MECAM peak had a UV–vis spectrum identical with that reported for Rh–ent (no longer showing a shoulder at 380 nm) and migrated on high-voltage electrophoresis (vide infra) at a rate identical with that of Fe–ent.[16]

All three compounds, K_3[Rh(cat_3)], K_3[Rh(DMB)$_3$], and K_3[Rh(MECAM)], were stable for hours in aqueous solution in the presence of air, as determined by a lack of spectral or electrophoretic changes. For longer term storage the compounds were stored dry under inert atmosphere or frozen at liquid nitrogen temperature.

Preparation of Fe–TRIMCAM. The Fe–TRIMCAM complex was prepared in an oxygen-free environment by Schlenk techniques. The TRIMCAM (15.380 mg, 2.684×10^{-5} mol) was weighed on a microanalytical electrobalance and placed in a 25-mL Schlenk flask. Degassed 0.1016 M KOH (3.167 mL) and standardized $FeCl_3$ (0.050 29 (8) M, 0.1000 M in HCl, 537 μL, 2.684×10^{-5} mol), respectively, were added to the stirred mixture under a flow of argon. The resultant Fe–TRIMCAM solution was kept under argon and used for spectrophotometric titration work shortly after the preparation was complete.

Spectrophotometric Titration of Fe–TRIMCAM. An aliquot (200 μL) of the Fe–TRIMCAM solution was transferred by a 2.000-mL Gilmont buret to 50.00 mL of 0.1 N KCl (prepared from low iron KCl and boiled degassed, deionized distilled water) in a spectrophotometric titration cell. The pH of the solution was raised to pH 10.35 with 15 μL of concentrated KOH. The solution temperature was maintained at 25.0 ± 0.5 °C with a circulating water bath. The solution was titrated with 0.1000 N HCl (prepared from a Baker Dilut-It ampule and stored under argon) with an automatic spectrophotometric titrator. Descriptions of the cell and automatic titrator are provided in ref 19 and 20. Spectra were recorded on a Hewlett-Packard 8540A UV–vis multichannel digital spectrophotometer approximately every 0.1 pH unit and were stored along with pH and volume data on disk. Data (85 points) were taken over the course of 22 h from pH 10.35 to 4.99. The isosbestic region of the titration from pH 9.55 to 7.39 is shown in Figure 3. A factor analysis and nonlinear least-squares refinement program, REFSPEC,[19-20] was used to determine the first protonation constant of Fe–TRIMCAM from 22 spectra taken in the isosbestic region. The first protonation constant for the single-proton protonation of Fe–TRIMCAM to HFe–TRIMCAM was determined to be log K_{MLH} = 7.76 (R = 0.0106) from 11 cycles of least-squares refinement. Molar extinction coefficients for the Fe–TRIMCAM (λ_{max} 488 nm, ϵ 3900 cm L mol^{-1}) and HFe–TRIMCAM (λ_{max} 582 nm, ϵ 3300 cm L mol^{-1}) were also determined from REFSPEC. A second data set gave a similar refinement (log K_{HML} = 7.78).

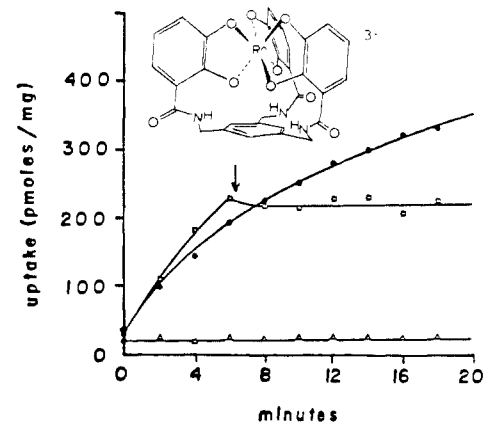

Figure 4. Inhibition of 2 μM ^{59}Fe–ent uptake by [Rh(MECAM)]$^{3-}$. Control (closed circles) indicates uptake observed with no addition of inhibitor. K_3[Rh(MECAM)] was added at 35-fold excess either at t = 6 min (open squares) or as a control, 5 min before the start of the experiment (open triangles). See text for details on inhibition experiments.

Preparation of ^{59}Fe–Enterobactin, ^{59}Fe–MECAM, and ^{59}Fe–TRIMCAM. Radiolabeled solutions of ^{59}Fe–ent, ^{59}Fe–MECAM, and ^{59}Fe–TRIMCAM were prepared as reported previously.[5]

Preparation of ^{55}Fe–Enterobactin. A ^{55}Fe–enterobactin solution was prepared from the addition of 100 μL of 10 mM NTA solution, 0.42 μmol of enterobactin (137 μL of an enterobactin solution in methanol, the concentration of which was determined spectrophotometrically at λ 316 nm, ϵ 9500 L mol^{-1} cm^{-1}), 8.0 μL of a 48.88 mM solution of $FeCl_3$, and 6.8 μL of the standard $^{55}FeCl_3$ solution (total moles Fe 0.40 μmol). This mixture was brought to 2 mL volumetrically with 1 M (pH 7.4) HEPES buffer.

Preparation of ^{55}Fe–MECAM-Me. A ^{55}Fe–MECAM-Me solution was prepared from 100 μL of 10 mM NTA, 0.42 μmol of MECAM-Me (148 μL of an MECAM-Me solution in methanol), 8.0 μL of a 48.88 mM solution of $FeCl_3$, and 6.8 μL of the standard $^{55}FeCl_3$ solution (total moles Fe 0.40 μmol). The mixture was brought to 2 mL volumetrically with 1 M (pH 7.4) HEPES buffer. Preliminary thermodynamic analysis of complex formation shows that the trianionic hexacoordinate complex, [Fe(MECAM-Me)]$^{3-}$ is the principal form (70%) at pH 7.4.

Preparation of ^{55}Fe–MECAMS. A ^{55}Fe–MECAMS solution was prepared from 50 μL of 10 mM NTA, 0.21 μmol of MECAMS (514 μL of a 8.18×10^{-4} M MECAMS solution in 10% methanol and 90% 1 M HEPES), 4.0 μL of a 48.88 mM solution of $FeCl_3$, and 3.4 μL of the standard $^{55}FeCl_3$ solution (total moles Fe 0.20 μmol). The mixture was brought to 1 mL volumetrically with 1 M (pH 7.4) HEPES buffer.

Preparation of ^{55}Fe–Me$_3$MECAM. A ^{55}Fe–Me$_3$MECAM solution was prepared from 50 μL of 10 mM NTA, 0.21 μmol of Me$_3$MECAM, 4.0 μL of a 48.88 mM solution of $FeCl_3$, and 3.4 μL of the standard $^{55}FeCl_3$ solution (total moles Fe 0.20 μmol). The solution was brought to 1 mL volumetrically with 1 M (pH 7.4) HEPES buffer.

Preparation of Metal Complexes of Enterobactin. A stock solution of enterobactin in MeOH was prepared and standardized spectrophotometrically (ϵ(316 nm) 9500 L mol^{-1} cm^{-1}). This solution, if stored at −8 °C, could be used for about 2 weeks without appreciable degradation. The desired metal, as the trichloride in HCl, was combined with a stoichiometric amount of enterobactin in MeOH. The pH and concentration were adjusted with 1 M HEPES buffer, pH 7.4.

Results

Inhibition by Rhodium Complexes. Since there is no commercially available radioisotope of rhodium, the interactions of kinetically stable rhodium(III) complexes with the ferric–enterobactin receptor were examined by transport inhibition experiments. The inhibition of transport of ^{59}Fe–enterobactin or ^{55}Fe–enterobactin was measured in the presence of the rhodium

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(20) Scarrow, R. C. Ph.D. Thesis, University of California, Berkeley, 1985.

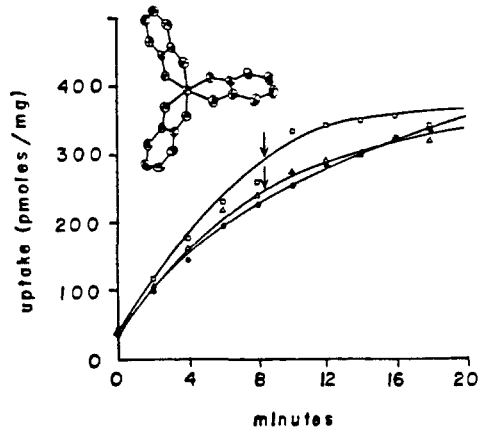

Figure 5. Inhibition of 2 μM ^{59}Fe–ent uptake by $[Rh(cat)_3]^{3-}$. Control (closed circles) represents uptake of 1 μM label with no inhibitor added. Inhibition experiments: $K_3[Rh(cat)_3]$ added at t = 6 min in 10-fold excess (open squares) or 100-fold excess (open triangles).

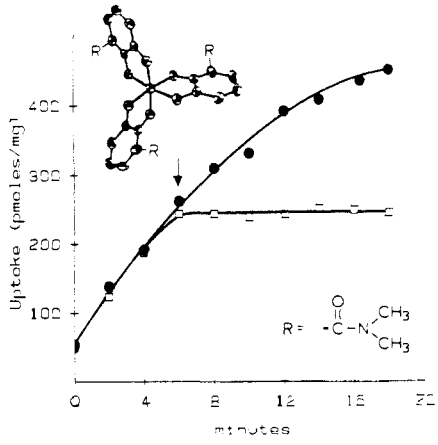

Figure 6. Inhibition of 2 μM ^{55}Fe–ent uptake by $[Rh(DMB)_3]^{3-}$. Control (closed circles) represents uptake with no inhibitor added. $K_3[Rh(DMB)_3]$ is added at t = 6 min in 50-fold excess (open squares).

complexes. The results obtained in the inhibition experiments by the $[Rh(MECAM)]^{3-}$ complex are shown in Figure 4. When added at 35 μM concentration either 5 min before the start of the experiment or immediately after removing the 8-min aliquot, the rhodium complex completely inhibits $[^{59}Fe(ent)]^{3-}$ uptake. At 10 μM concentration a significant reduction in the rate of transport of $[^{59}Fe(ent)]^{3-}$ is observed. The $[Rh(MECAM)]^{3-}$ complex does not appear to displace transported $[^{59}Fe(ent)]^{3-}$ from inside the cell. This would be indicated by a much larger decrease in counts associated with the cells, as is observed when an excess of unlabeled $[Fe(ent)]^{3-}$ or $[Fe(MECAM)]^{3-}$ is added in a transport experiment after a period of cellular uptake.[5] These observations suggest that although the $[Rh(MECAM)]^{3-}$ complex is competing for the binding site on the outer membrane receptor with ^{59}Fe–enterobactin, it is not being transported into the cell.

The $[Rh(cat)_3]^{3-}$ does not inhibit the uptake of ^{59}Fe–enterobactin (Figure 5). No effect is seen on the rate of ^{59}Fe–enterobactin (1 μM) uptake by $[Rh(cat)_3]^{3-}$ at any concentration up to 350 μM. Both inhibition experiments were performed: (1) adding the $[Rh(cat)_3]^{3-}$ in the middle of the transport assay and (2) adding the compound to the cells 5 min before the start of the transport assay. No inhibition of ^{59}Fe–enterobactin transport is observed in either case.

In contrast with the results obtained with the tris(catecholate) complex, $[Rh(DMB)_3]^{3-}$ *is* a potent inhibitor of ^{55}Fe–ent uptake (Figure 6). When added at 50-fold excess, the rhodium–DMB complex completely stops uptake of the labeled ferric complex. Lower relative concentrations of inhibitor affect uptake of label less powerfully (data not shown).

Fe–TRIMCAM Uptake. The first pK_A of the Fe–TRIMCAM complex is 7.8. (See the discussion of the spectrophotometric

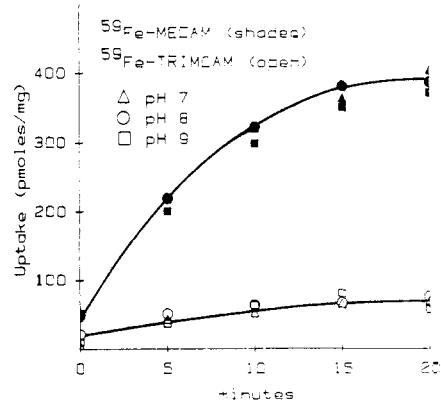

Figure 7. Uptake of ^{59}Fe–MECAM and 2 μM ^{59}Fe–TRIMCAM by *E. coli* RW193 as a function of the pH of the uptake medium. See text and ref 5 for details of uptake experiments: ^{59}Fe–MECAM (closed symbols) and ^{59}Fe–TRIMCAM (open symbols) at pH 7 (triangles), 8 (circles), and 9 (squares).

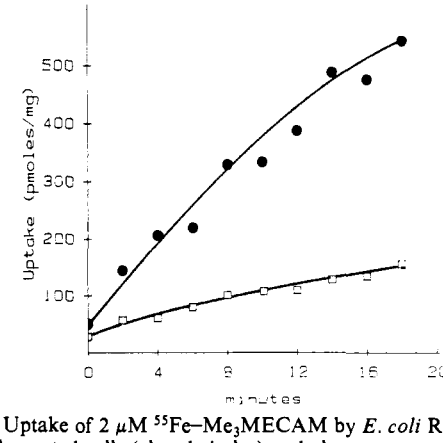

Figure 8. Uptake of 2 μM ^{55}Fe–Me_3MECAM by *E. coli* RW193: glucose-supplemented cells (closed circles) and glucose-starved cells (open squares).

titration of Fe–TRIMCAM at the end of this section.) At the pK_a (i.e., at pH 7.8), 50% of Fe–TRIMCAM is a tris(catecholate) complex, and 50% is in the monoprotonated form. Transport experiments were performed with ^{59}Fe–TRIMCAM at pH 7–9, where 15, 61, and 94%, respectively, of the complex is in the ferric–tris(catecholate) form: $[Fe(TRIMCAM)]^{3-}$. This complex is not significantly transported into the cell under any of these conditions, while controls showed no change in the rate of cellular uptake of ^{59}Fe–ent or ^{59}Fe–MECAM at pH 7–9 (Figure 7). The small amount of cell-associated ^{59}Fe–TRIMCAM, approximately 60–80 pmol/mg by 20 min, may represent a low rate of uptake or nonspecific cellular adsorption of oxidized ^{59}Fe–TRIMCAM. Glucose-starved cells were found to bind approximately the same amount of ^{59}Fe–TRIMCAM (not shown), suggesting that it is more likely nonspecific adsorption than receptor-mediated uptake.

The lack of effect of pH as high as 9.0 on the rate of transport of ^{59}Fe–ent and ^{59}Fe–MECAM was an unexpected result. Although the coordination chemistry of these complexes is unaffected at neutral pH and higher,[21] it might seem likely that there would be an effect on energy-dependent cellular processes, which require maintenance of pH gradients, including active transport. Zilberstein et al., however, have recently reported studies showing that *E. coli* recovers energetically within minutes after the pH is adjusted as high as pH 8.8.[22] The results of Zilberstein, along with the current transport studies, indicate that *E. coli* is able to perform active transport up to a pH at least as high as 9.0.

Fe–Me_3MECAM Uptake. In accordance with growth studies on *E. coli* strains AB2847 and AN92, $[^{55}Fe(Me_3MECAM)]^{3-}$ (2

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(22) Zilberstein, D.; Agmon, V.; Schuldiner, S.; Padan, E. *J. Biol. Chem.* **1982**, *257*(7), 3687.

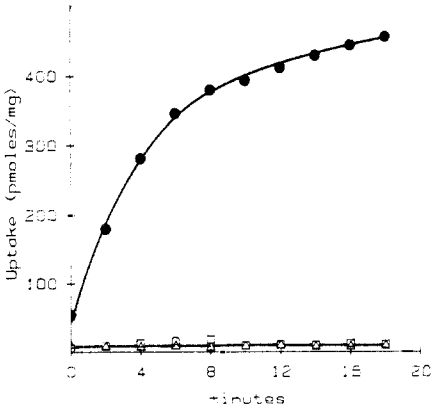

Figure 9. Uptake of ^{55}Fe–MECAMS by RW193 *E. coli*: control, 2 μM $[^{55}Fe(ent)]^{3-}$ as substrate (closed circles); 2 μM $[^{55}Fe(MECAMS)]^{6-}$ (5.6 mM in glucose) as substrate (open triangles); 2 μM $[^{55}Fe(MECAMS)]^{6-}$ (no added glucose) as substrate (open squares).

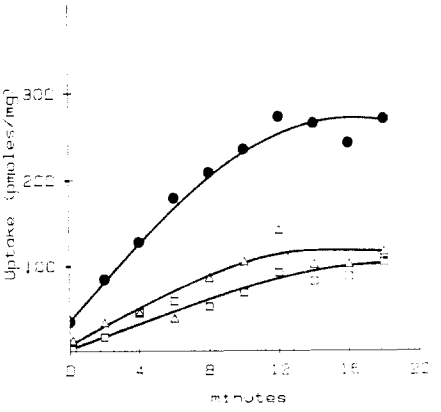

Figure 10. Uptake of ^{55}Fe-MECAM-Me by RW193 *E. coli*: control, 2 μM $[^{55}Fe(ent)]^{3-}$ as substrate (closed circles); 2 μM $[^{55}Fe(MECAM-Me)]^{3-}$ (5.6 mM in glucose) as substrate (open triangles); 2 μM $[^{55}Fe(MECAM-Me)]^{3-}$ (no added glucose) as substrate (open squares).

μM) was taken up by *E. coli* RW193 cells. Glucose-starved cells took up label to a much smaller extent. The uptake of radiolabeled ^{55}Fe in the absence and presence of glucose is shown in Figure 8.

Fe–MECAMS Uptake. The inability of $[Fe(MECAMS)]^{6-}$ to support growth of *E. coli* was previously established.[4] Correspondingly, we observed no uptake of radiolabeled ^{55}Fe when *E. coli* RW193 cells were given $[^{55}Fe(MECAMS)]^{6-}$ (2 μM) as an iron source. A comparison of the ^{55}Fe uptake by *E. coli* when given $[^{55}Fe(ent)]^{3-}$ and $[^{55}Fe(MECAMS)]^{6-}$ in both the presence and absence of added glucose is shown in Figure 9.

Fe–MECAM-Me Uptake. A comparison of the uptake of $[^{55}Fe(ent)]^{3-}$ (2 μM) and $[^{55}Fe(MECAM-Me)]^{3-}$ (2 μM) by *E. coli* is shown in Figure 10. Within experimental error, no difference is observed between the active and passive uptake of radiolabel when the cells were given $[^{55}Fe(MECAM-Me)]^{3-}$ as an iron source. The relatively large amount of uptake of ^{55}Fe by glucose-starved cells (approximately 100 pm/mg of cells) suggests that the $[Fe(MECAM-Me)]^{3-}$ complex binds nonspecifically to the cells. In addition, a very high background count of radiolabel was observed in a control experiment for $[^{55}Fe(MECAM-Me)]^{3-}$ in the absence of cells (approximately 12 times higher than the background count for $[^{55}Fe(ent)]^{3-}$), indicating that the complex also binds to some extent to the filter paper.

M^{3+}–Enterobactin Inhibition. Competitive inhibition of labeled ferric-enterobactin uptake by ent complexes of a number of M(III) ions is shown in Figure 11. Two complexes, $[Al(ent)]^{3-}$ and $[Ga(ent)]^{3-}$, had no measurable effect on ferric–ent uptake (1 μM) when added in 10-fold excess and only slight effect at over 30-fold excess. The complexes of scandium and indium were much more effective, with the latter (the most effective) showing complete inhibition at only 20-fold excess.

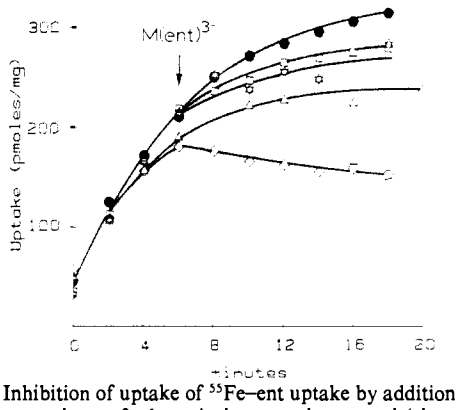

Figure 11. Inhibition of uptake of ^{55}Fe–ent uptake by addition of excess enterobactin complexes of other trivalent metals: control (closed circles), uptake of 1 μM ^{55}Fe–ent with no inhibitor added. Inhibitors added at t = 6 min: (open squares) Ga–ent, 35-fold excess; (stars) Al–ent, 30-fold excess; (open triangles) Sc–ent, 30-fold excess; (open diamonds) In–ent, 20-fold excess.

Spectrophotometric pH Titration of Fe–TRIMCAM. The visible spectrum of Fe–TRIMCAM at pH >9.6 (Figure 3) is essentially identical with that reported for the ferric complex formed with three catecholate ligands $[Fe(cat)_3]^{3-}$.[21] As the pH is lowered, the λ_{max} shifts from 488 to 582 nm. The spectral shift occurs with retention of three sharp isosbestic points at 543, 402, and 362 nm over the entire pH region until pH 7.4 is reached. The isosbestic spectral changes indicate an equilibrium between only two metal complexes with different visible spectra. The data refined on a model of a single protonation of $[Fe(TRIMCAM)]^{3-}$ to $[HFe(TRIMCAM)]^{2-}$ with a log K_{HML} of 7.8. The single proton stoichiometry for the protonation of $[Fe(TRIMCAM)]^{3-}$ was an unexpected result. We have previously reported the synthesis and thermodynamic evaluation of an analogue of TRIMCAM where each of the three catecholate groups is sulfonated in the 5-position, 1,3,5-tris[(2,3-dihydroxy-5-sulfobenzoyl)carbamido]benzene (TRIMCAMS).[6] Protonation of the ferric tris(catecholate) form of TRIMCAMS $[Fe(TRIMCAMS)]^{6-}$ was earlier reported to occur by a two-proton step with log K_{HML} = 13.66. The coordination structure of the monoprotonated $[HFe(TRIMCAM)]^{2-}$ was not further investigated.

Discussion

We have previously reported a detailed kinetic and inhibition study of the outer membrane receptor for ferric–enterobactin with respect to the specificity of the receptor and the mechanism of transport across the outer membrane.[5] We found that Fe–enterobactin and the structural analogue Fe–MECAM are actively transported in *E. coli* at identical rates under saturating substrate concentrations but that a higher concentration of $[Fe(MECAM)]^{3-}$ is required to achieve maximum transport velocity. We concluded that the primary reason a higher concentration of Fe–MECAM is required to achieve maximum velocity is that only half of the complex forms the appropriate coordination isomer. We further concluded that the part of the molecule recognized by the receptor is the metal-binding end, but the ligand platform to which these functionalities are attached is not important in receptor recognition.

The exact meaning of "metal-binding end" and "ligand platform" is probed in the current study. The question arises as to which category the adjacent amide portion of the molecule falls. In principle, the simplest way to do this would be to examine the transport of tris(catecholato)ferrate(III), $[Fe(cat)_3]^{3-}$, which is identical with $[Fe(ent)]^{3-}$ around the metal center but does not have the adjacent amide function. Unfortunately, that is not possible because the tris complex exists only in high-pH solutions in the presence of a very large excess of catechol. Under the conditions used in transport experiments with live cells (micromolar concentrations and neutral pH), the bis(catecholate)–ferric complex predominates.

We have circumvented this problem by using two strategies. Since the ligand field stabilization of rhodium(III) compounds

makes them among the most ligand-exchange-inert complexes,[23] the first method of probing the importance of the amide functions of enterobactin was to test [rhodium(III)tris(catecholate ligand)]$^{3-}$ complexes as inhibitors of ^{59}Fe–ent or ^{55}Fe–ent uptake. Although these complexes are prepared under conditions of high concentration, pH, and temperature, they can be used at low concentrations under physiological conditions where they are thermodynamically unstable because they dissociate very slowly. Thus, transport inhibition studies can be completed before significant breakdown of the complex is observed.

The results clearly show that [Rh(MECAM)]$^{3-}$ is an inhibitor of [^{59}Fe(ent)]$^{3-}$ uptake. At 10 μM concentration there is significant inhibition of uptake of 1 μM [^{59}Fe(ent)]$^{3-}$. At 35 μM [Rh(MECAM)]$^{3-}$, transport of [^{59}Fe(ent)]$^{3-}$ is completely inhibited. That [Rh(MECAM)]$^{3-}$ is an effective inhibitor of ^{59}Fe–enterobactin uptake is an important positive control for the [Rh(cat)$_3$]$^{3-}$ experiment. It is conceivable that changing the coordinated metal ion in these complexes could change interactions between the metal–ligand complex and the receptor. This has been shown to be true, in fact, for complexes between enterobactin and various trivalent metals, vide infra. It was necessary, therefore, to first prove that the change from iron to rhodium does not itself interfere with recognition by the receptor. The [Rh(MECAM)]$^{3-}$ results show that it is possible for a complex containing rhodium(III) to be recognized by the *E. coli* ferric–enterobactin receptor.

In contrast to the rhodium–MECAM results, [Rh(cat)$_3$]$^{3-}$ appears not to be recognized by the ferric–enterobactin receptor. At up to 350-fold excess over the [^{59}Fe(ent)]$^{3-}$ concentration (at this concentration the cell culture actually takes on the golden color of the rhodium complex), no inhibition of uptake is observed. When the [Rh(DMB)$_3$]$^{3-}$ complex is used, the result is strikingly different. This complex, which has amide functionalities on the ligands (and so resembles ferric–ent more closely than does the tris(catechol) complex), is very effective at inhibiting ^{55}Fe–ent uptake. A higher relative concentration of inhibitor is required to achieve complete inhibition than in the Rh–MECAM case, but this may be accounted for by their structural differences. In addition to stereoisomers, [Rh(DMB)$_3$]$^{3-}$ theoretically can exist as two different geometrical isomers: the cis (or facial) isomer, which has all three amide groups on one side of the molecule, and the trans (meridianal) isomer, in which one of the amides is on the opposite side. Of the four possible combinations—cis and trans configurations each with Λ and Δ isomers—only one matches the known solution structure of ferric–ent, which is Δ-cis.[24] If the requirement by the receptor for this isomer is absolute, then the observed inhibition is due entirely to the Δ-cis isomer of [Rh(DMB)$_3$]$^{3-}$.

The second strategy for probing the importance of the amide groups was to substitute the structural analogues [Fe(TRIMCAM)]$^{3-}$ and [Fe(Me$_3$MECAM)]$^{3-}$ for [Fe(ent)]$^{3-}$ in uptake studies. The ferric complex of TRIMCAM more closely resembles that of [Fe(cat)]$^{3-}$ than both the ferric complexes of MECAM and enterobactin, which have carbonyl groups conjugate to the catechol binding subunits. The TRIMCAM ligand forms a stable tris(catecholate) complex with ferric ion, which is *not* transported by *E. coli*. The Me$_3$MECAM ligand, on the other hand, does mediate iron transport in vivo. Uptake of radiolabeled ^{55}Fe is observed when cells are given [^{55}Fe(Me$_3$MECAM)]$^{3-}$ as an iron source.

The results from these two different strategies are in agreement: the amide functionalities (domain II of Figure 1) adjacent to the catechol rings appear to be necessary for recognition by the outer membrane receptor for ferric–enterobactin in *E. coli*. Figure 12 illustrates our explanation. The ligand backbones of enterobactin and MECAM (Figure 12, bottom view) are very different, yet the ferric complexes of both these ligands are taken up, indicating

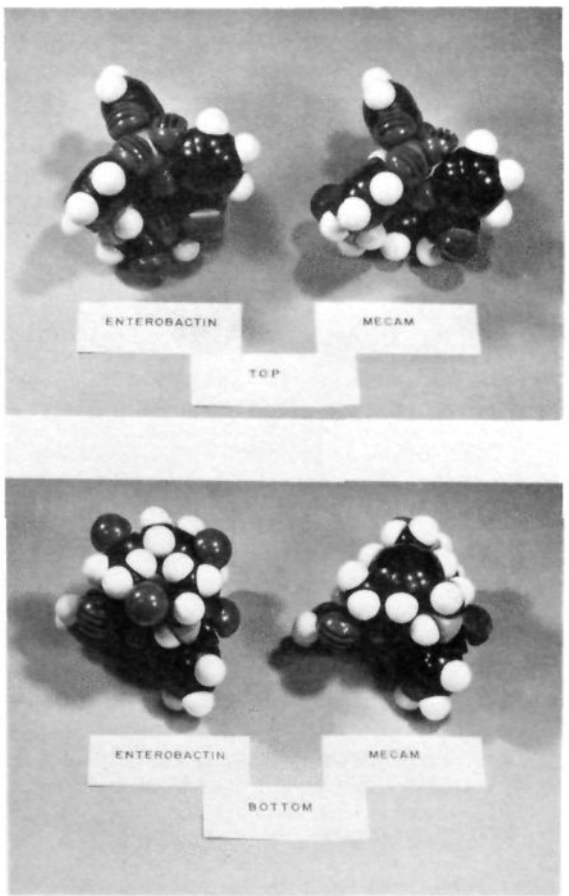

Figure 12. Top and bottom views of CPK models of ferric–enterobactin.

that the receptor does not bind significantly to this region (domain I). The regions of the metal complexes that must be recognized by the receptor are shown in the top view of Figure 12. The salient features of both complexes from this viewpoint are (1) the metal–catechol core (domain III) and (2) the adjacent carbonyls of domain II. The latter may be recognized by a receptor protein through hydrogen bonding with the carbonyl oxygen. The amide nitrogens are not as obvious in these views; they are less accessible from this orientation than are the carbonyls. These experiments indicate that the molecular structure at the nitrogen is not significant for recognition, since both secondary amides ([Fe(ent)]$^{3-}$ and [Rh(MECAM)]$^{3-}$), which can engage in hydrogen bonding, and tertiary amides ([Rh(DMB)$_3$]$^{3-}$ and [Fe(Me$_3$MECAM]$^{3-}$), which cannot hydrogen bond, act as transport substrates. In conclusion, the carbonyl groups (domain II) adjacent to the catechol-binding subunits of enterobactin and synthetic analogues are required for recognition by the ferric enterobactin receptor.

Small structural changes in the metal-binding unit (domain III) of the ligand were shown to reverse the activity of the synthetic enterobactin analogues as iron-transport agents. No uptake of radiolabel was observed when *E. coli* cells were given [^{55}Fe(MECAM-Me)]$^{3-}$ or [^{55}Fe(MECAMS)]$^{6-}$ as an iron source. Note that the [Fe(MECAM-Me)]$^{3-}$ complex has the same charge as, and is only slightly larger than, the transported [Fe(MECAM)]$^{3-}$ complex. This result reinforces the importance of ligand structure in domain III to the recognition of ferric complexes by the ferric–enterobactin receptor. In addition, this result suggests a strategy to prepare synthetic analogues of enterobactin, which like enterobactin, are effective iron chelators, however, unlike enterobactin, do not support growth of *E. coli* or related organisms.

The inhibition of ^{55}Fe–ent uptake by complexes of different metal ions does not appear to be consistent with a simple single-step binding and transport model. Results using complexes of six different metals, all in the same oxidation state (+3), are addressed in this paper: Fe, Rh, Sc, Al, Ga, and In. Previous results have

(23) Basolo, F.; Pearson, R. G. *Mechanism of Inorganic Reactions*, 2nd ed.; Wiley: New York, 1961.
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shown that different isotopes of ferric–ent competitively inhibit uptake of each other, as would be expected. The results with rhodium have been obtained with MECAM, rather than enterobactin, since enterobactin is hydrolytically unstable under the reaction conditions used to make K_3[Rh(MECAM)] and K_3-[Rh(cat)]$_3$, and at pH 7 the reaction between Rh(III) and enterobactin is so slow that the competing ligand hydrolysis reaction yields only minuscule amounts of Rh–enterobactin.[16] The Rh–MECAM complex should, however, be a good model given the similarities between Fe–ent and Fe–MECAM in recognition by the outer membrane receptor. To make a fair comparison, it is probably necessary to divide the concentration of Rh–MECAM by 2 in order to compensate for the fact that Rh–MECAM is a racemic mixture of Λ and Δ coordination isomers while complexes of enterobactin assume the Δ (right-handed) isomer required for recognition by the receptor.[24] The rhodium complex, then, is a highly effective inhibitor. Two other metal complexes—those of scandium and indium—are also potent inhibitors, while the remaining two—those of aluminum and gallium—are ineffective.

Plaha and Rogers et al. have reported the bacteriostatic effects of complexes of enterobactin and various metal ions.[25-27] On the basis of our experience with the syntheses of metal complexes of enterobactin and synthetic analogues, we question whether the desired enterobactin complexes were actually formed with some of these metal ions, in particular with Co(II), Rh(III), Ru(III), Ti(IV), and VO^{2+}. Some of these ions (Co^{2+} and VO^{2+}) are labile and do not form stable catechol complexes under these conditions. Others (Rh(III) and Ru(III)) form inert complexes, which are difficult to prepare. For example, $[Rh(ent)]^{3-}$, reportedly formed upon addition of an acidic 0.01 M $RhCl_3$ solution to enterobactin in 1-butanone and then upon increase to pH 7 with solid $NaHCO_3$, showed no antibacteriostatic effects.[27] In contrast to these results, we find the $[Rh(MECAM)]^{3-}$ complex inhibits the uptake of $[Fe(ent)]^{3-}$. We found, however, that the Rh(III) complex of MECAM is formed only under vigorous conditions, and isolation of a 1:1 complex requires purification by size-exclusion chromatography. Similarly, Rogers et al. observed no antibacteriostatic effects for a Ti(IV) complex of enterobactin formed analogously to their Rh(III) complex.[27] We have observed only small quantities of a 1:1 monomer to be formed from the reaction of $TiCl_4$ and MECAM and, as expected, the dimeric Ti(IV) complex of MECAM does not compete with the uptake of ferric–enterobactin (data not shown).

Unlike rhodium(III), a number of these metal ions do form enterobactin complexes essentially on mixing. For these labile metals, our inhibition results are in agreement with the growth inhibition studies performed by Plaha and Rogers.[26] The enterobactin complexes of Al^{3+} and Ga^{3+} are ineffective as inhibitors, whereas those of Sc^{3+} and In^{3+} are quite effective.

We cannot explain the observed differences in biological discrimination between these metal ions. These differences contrast with similar studies performed on other systems, for example, metal substitution into ferrichrome.[28] We have previously shown that the ferric–enterobactin complex is taken into the cell intact,[5] so this discrimination appears to occur against intact complexes at the level of the outer membrane receptor. These metal ions are similar in ionic radii (all within 0.2 Å), and they form virtually identical complexes with a number of ligands.[6] It seems unlikely that the different biological activity of these iron analogues is based on redox properties, since all of them (except for iron itself) are highly stable in the 3+ oxidation state. Also, ligand-exchange kinetics do not appear to be the determining factor, as suggested in a recent review[29] since the potent inhibitors incorporate metal ions ranging from the extremely labile (In^{3+}, with kinetics of water exchange on the order of 10^5 s^{-1}) to the extremely inert (Rh^{3+}, aquo exchange kinetics $<10^{-7}$ s^{-1}).[23] We are currently undertaking a study of the physical inorganic chemistry and solution thermodynamics of each of these model complexes of enterobactin to further understand these biological observations.

In summary, through the use of kinetically inert Rh(III) complexes and synthetic analogues of enterobactin, the various domains of enterobactin (Figure 1) have been examined as determinants in the recognition and transport of ferric enterobactin by the fepA outer membrane protein receptor of *E. coli*. These results confirm that domain I, the triserine backbone of the Fe(ent)$^{3-}$, is not recognized. Domain III, the tris(catechol)iron(III) center, is necessary, but not sufficient, for recognition. Any change made to domain III (e.g., by functionalizing the catechol rings) blocks recognition of the complex. In addition to the three catechol groups immediately surrounding the iron (domain III), the carbonyl group of enterobactin (domain II) is required for recognition by the ferric–enterobactin receptor. The proton on the amide nitrogen, however, is not necessary for recognition. While the structure of the receptor protein is not yet known, the composite of these results gives a sketch of what the Fe(ent)$^{3-}$ binding site must look like: a relatively rigid pocket for receiving the ferric catecholate portion of the complex and proton donor groups out and around this pocket positioned to hydrogen bond to the carbonyl oxygens of the ferric–enterobactin amide groups.

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Registry No. 1, 3929-89-3; **2**, 77869-39-7; **3**, 77869-41-1; **4**, 638-09-5; **5**, 113323-56-1; **6**, 113323-57-2; K_3[Rh(cat)$_3$], 67577-17-7; cat, 120-80-9; K_3[Rh(DMB)$_3$], 113351-85-2; K[Rh(DMB)$_2$], 113351-86-3; K_3[Rh-(MECAM)], 113351-87-4; MECAM, 69146-59-4; Fe-TRIMCAM, 113351-88-5; TRIMCAM, 71353-09-8; ^{55}Fe-MECAM-Me, 113351-90-9; ^{55}Fe-MECAMS, 113351-91-0; ^{55}Fe-Me$_3$MECAM, 113351-92-1; ^{55}Fe-ent, 113351-89-6; $^{-}$Ga-ent, 113351-93-2; Al-ent, 113351-94-3; Sc-ent, 113429-11-1; In-ent, 113351-95-4; Fe-Me$_3$MECAM, 113378-55-5; Fe-MECAMS, 113378-57-7; Fe-MECAM-Me, 113378-56-6; $^{-}$Fe-ent, 61481-53-6; K_2CO_3, 584-08-7; Rh(ClO$_4$)$_3$, 15979-23-4; KOH, 1310-58-3; $FeCl_3$, 7705-08-0.

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