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Metal sequestering agents in bioinorganic chemistry: enterobactin mediated iron transport in *E. coli* and biomimetic applications

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Abstract - Iron transport into the cells of microbes is mediated by low-molecular-weight sequestering agents (siderophores) which are powerful and highly specific sequestering agents for Fe(III). The current status of iron uptake by E. coli as mediated by the tricatechol siderophore enterobactin will be reviewed. Recently the synthesis of kinetically inert Rh(III) complexes of enterobactin analogs have allowed the recognition process of the ferric enterobactin protein receptor to be characterized. The "salicylate" mode of bonding which occurs when ferric enterobactin is protonated has now been structurally characterized in a model system. Biomimetic approaches to metal-ion-specific sequestering agents based on enterobactin have been applied to two problems. The first is the synthesis of specific sequestering agents for actinide(IV) ions, including plutonium. The second is the synthesis of ferric ion sequestering agents of possible therapeutic utility. The structural characterization of one of these has provided an unprecedented example of a trigonal-prismatic Fe(III) complex.

INTRODUCTION

The mobilization and uptake of iron by microorganisms is mediated by low molecular weight chelating agents known as siderophores. Enterobactin (Fig. 1a), also referred to as enterochelin, a siderophore produced by E. coli, is the most powerful iron complexing agent known ($K_f = 10^{52}$) (1). With this ligand as a model, two goals of our research are the development of ion specific chelating agents for iron(III) and the chemically similar and highly toxic plutonium(IV) ion. Based on the tremendous affinity and specificity of enterobactin for iron(III), and the similarity of plutonium(IV) to iron(III), we have developed an approach to the design of structurally similar chelating agents for these metal ions. In the following paper, we present our study of the chemistry and biochemistry of ferric enterobactin and our most recent work in the design and synthesis of specific ion chelating agents for iron(III) and plutonium(IV).

Enteric bacteria such as Escherichia coli and Salmonella typhimurium synthesize and release enterobactin in response to iron deprivation (2,3). Enterobactin has three catechoylamide binding subunits which are appended to a tri-L-serine ligand backbone. Ferric iron is bound through the six catechol oxygens forming the trianionic complex shown in Fig. 1b. Seven gene products (entA through entG) are involved in the biosynthesis of enterobactin, while five gene products (fepA, fepB, fesB, tonB, and exbB) are involved in transport and utilization of ferric enterobactin (4,5). Production of these gene products is regulated by the availability of exogenous iron, and also apparently by the growth rate of the cell (6).

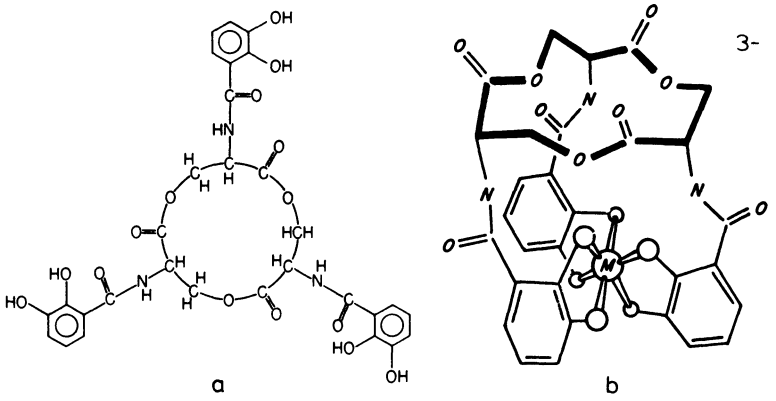

Fig. 1. Structures of a) enterobactin and b) ferric enterobactin.

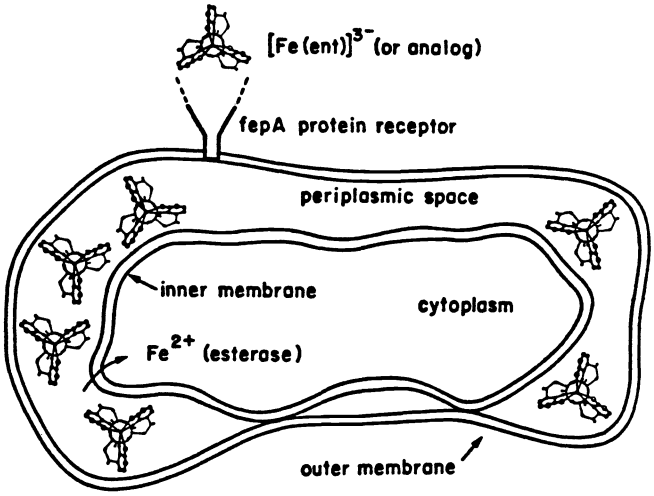

Fig. 2. Schematic representation of iron uptake in E. coli.

In gram-negative bacteria nutrients must traverse both the outer and inner membranes and the intervening periplasmic space to be utilized intracellularly (See Fig. 2 for a schematic representation of the iron transport process in E. coli). The outer membrane receptor protein for ferric enterobactin is the product of the fepA gene. Initial characterization of the structural requirements for recognition of ferric enterobactin by the fepA receptor protein was examined using the ferric complex of MECAM (1,3,5-N,N',N"-tris(2,3-dihydroxybenzoyl)triaminomethylbenzene, Fig. 3), a close structural analog of enterobactin (7,8,9). Like enterobactin, MECAM has three catechoyl binding subunits; however, the catechoylamide groups are appended to 1,3,5-triaminomethylbenzene rather than to the tri-L-serine ligand backbone of enterobactin. Ferric-MECAM was shown to be a substrate for transport in vivo, indicating that ferric enterobactin and ferric MECAM are recognized at the structurally similar metal chelate regions of the molecules (10). In addition, recognition of ferric enterobactin by the fepA receptor has been shown to be stereoselective. Enterobactin forms exclusively the Δ-cis isomer of iron(III), whereas enantioenterobactin, synthesized and characterized by Rastetter and coworkers, forms exclusively the Λ-cis isomer. Ferric enantioenterobactin is not recognized by the fepA receptor protein (11).

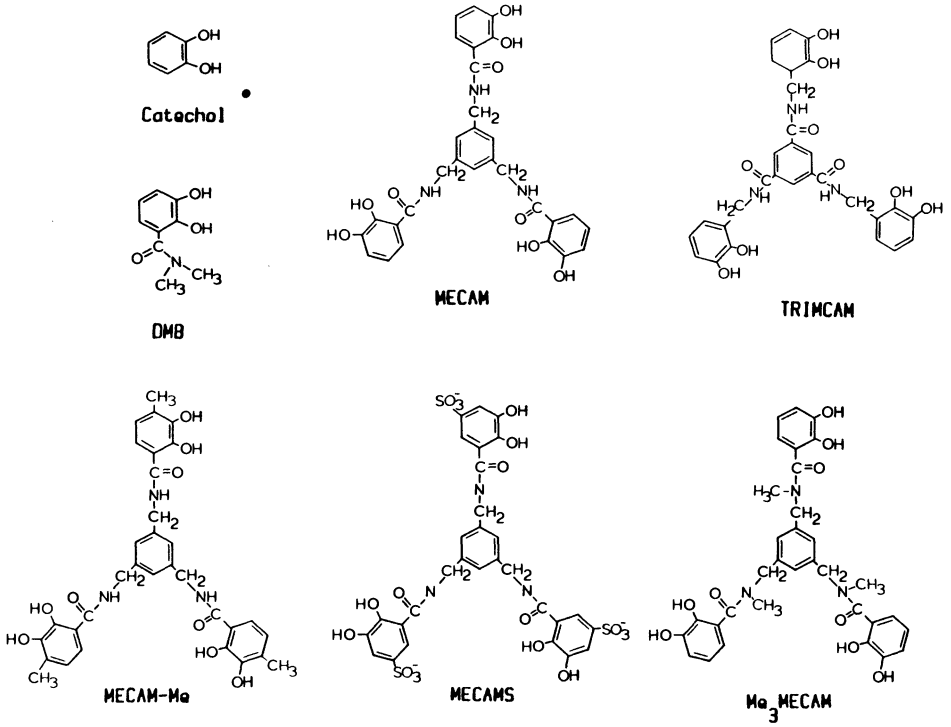

Fig. 3. Synthetic ligands used in this study.

Once recognized by the receptor protein, ferric complexes of enterobactin and synthetic analogs are actively transported through the outer membrane. Transport experiments carried out with the complex of ^{3}H-MECAM and ^{59}Fe confirmed that both the ligand and metal are transported through the outer membrane at identical rates (10). Although it is known that iron from the complex is eventually found as iron(II) (presumably in the cytoplasm) the mechanism of transport through the inner membrane and mechanism of iron release are not well understood. It is known however that four additional gene products are required for enterobactin-mediated iron uptake and utilization (4). The tonB and exbB gene products are required for a variety of high affinity transport processes, including transport of iron enterobactin. The fepB gene product is the cytoplasmic membrane permease. Mutants lacking this gene product are unable to utilize iron from enterobactin or synthetic analogs (12). Spheroplasts (cells in which the outer membrane has been physically removed) that have the fepB protein do show enterobactin-mediated iron uptake (4), however a detailed examination of the structural requirements for recognition using synthetic analogs has not been carried out. The fesB gene product, which is located in the cytoplasm, is an esterase which hydrolyses both enterobactin and ferric enterobactin (4). Tritium labeling studies indicate that once internalized, enterobactin is hydrolyzed and not reused for the acquisition of ferric iron (13). Mutants lacking the fesB gene product transport ferric enterobactin, however grow poorly on culture media where iron enterobactin is the sole iron source (4). Hydrolysis of the ferric enterobactin complex raises the electrochemical potential for the reduction of Fe(III) to Fe(II) which may be essential for release of iron in the cytoplasm (14,15,16).

FURTHER CHARACTERIZATION OF THE FERRIC ENTEROBACTIN RECEPTOR

We have extended our study of the structural requirements for recognition of ferric enterobactin by the fepA receptor protein to determine if only the metal tris-catechol (iron(III) plus Domain III, Fig. 4) portion of the molecule is required for recognition. Kinetically inert, air stable, rhodium(III) tris ligand complexes of catechol (See Fig. 3 for structures of ligands used in this study) and 2,3-dihydroxy-N,N-dimethylbenzamide (DMB), and a rhodium(III) complex of MECAM were used as probes to study the importance of the ligand amide function group in recognition of the complex (17). The ability of these rhodium complexes to interact with the ferric enterobactin receptor was judged by their capacity to competitively inhibit ^{55}Fe-enterobactin uptake. The tris-catecholate complex of rhodium-(III), $[Rh(cat)_3]^{3-}$ (which lacks amide functional groups), was observed to have no inhibitory effect on ferric enterobactin uptake by E. coli (Fig. 5a). In contrast the $[Rh(DMB)_3]^{3-}$ and $[Rh(MECAM)]^{3-}$ complexes, which both are catechoylamides, are competitive inhibitors of ferric-enterobactin transport. The inhibition of ferric enterobactin uptake by $[Rh(DMB)_3]^{3-}$is shown in Fig. 5b. Consistent with these observations, the ferric complex of the enterobactin analog TRIMCAM [1,3,5-tris(2,3-dihydroxybenzoyl)carbamidebenzene, a structural isomer of MECAM in which the positions of the methylene, carbonyl, and NH groups are reversed)] is not recognized by the receptor protein. Furthermore, 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[N,N',-N"-methyl-N,N',N"-(2,3-dihydroxybenzoyl)]triaminomethylbenzene], which has tertiary rather than secondary amide nitrogens is transported in vivo (uptake of $[^{55}Fe(Me_3MECAM)]^{3-}$ by E. coli is shown in Fig. 6). These experiments indicate that, in addition to the three catechol groups immediately surrounding the iron, the carbonyl group of enterobactin is important for recognition by the ferric-enterobactin receptor. The proton on the amide nitrogen however, is not necessary for recognition of the synthetic analogs of ferric-enterobactin (17).

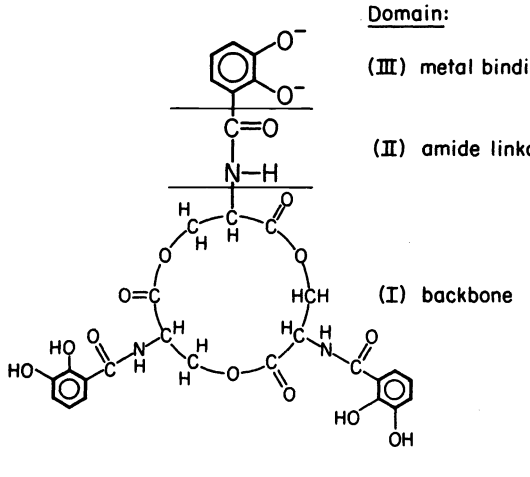

Fig. 4. A structural diagram of enterobactin with one arm of the ligand emphasized. Domains of possible significance for receptor recognition are delineated: Domain I - ligand backbone, Domain II - amide linkage, Domain III - catechol group (metal binding unit).

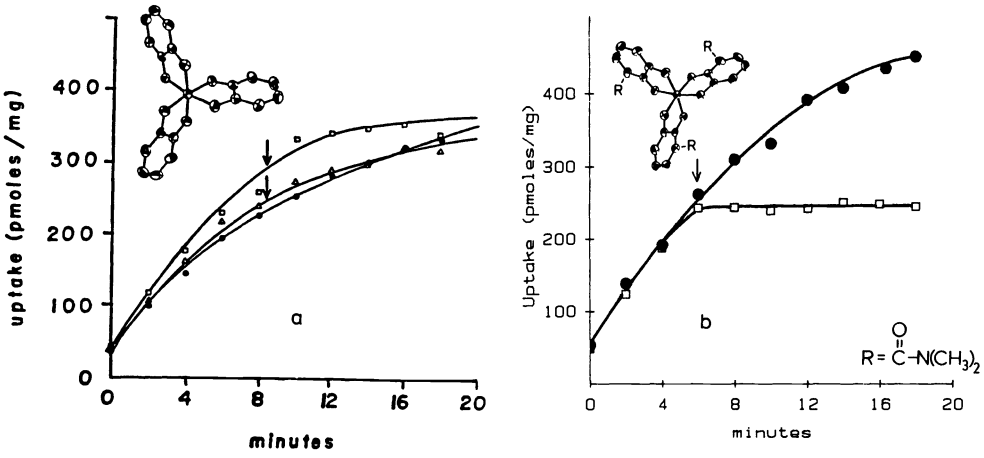

Fig. 5. a) Inhibition of 2μM ^{59}Fe-ent uptake by $[Rh(cat)_3]^{3-}$. Control (closed circles), represents uptake of 1 μM label with no inhibitor added. Inhibition experiments: $K_3[Rh(cat)_3]$ added at t = 6 min in 10-fold excess (open squares), or 100-fold excess (open triangles). b) Inhibition of 2μM ^{55}Fe-ent uptake by $[Rh(DMB)_3]^{3-}$. Control (closed circles), uptake with no inhibitor added. $K_3[Rh(DMB)_3]$ added at t = 6 min in 50-fold excess (open squares).

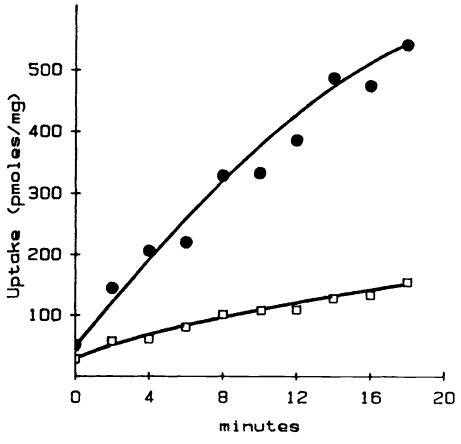

Fig. 6. Uptake of 2μM ^{55}Fe-Me_3MECAM by E. coli RW193: glucose supplemented cells (closed circles) and glucose starved cells (open squares).

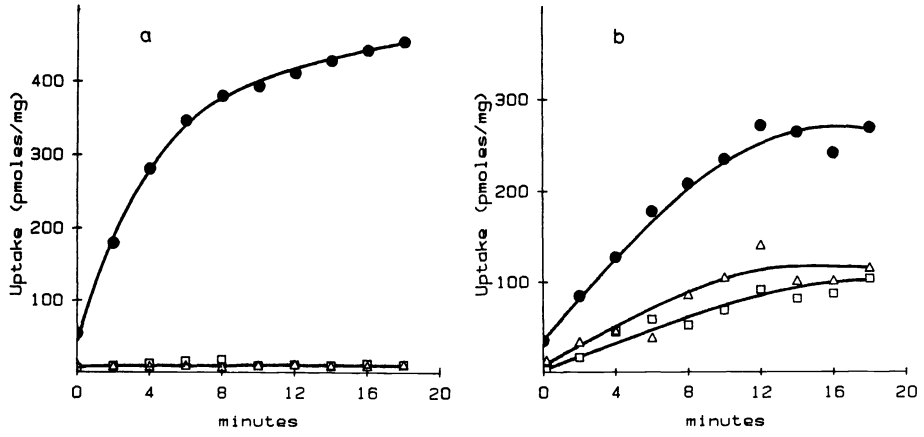

Fig. 7. a) Uptake of ^{55}Fe-MECAMS by RW193 E. coli. Control; 2μM ^{55}Fe-$(ent)^{3-}$as substrate (closed circles), 2μM ^{55}Fe$(MECAMS)^{6-}$ (5.6mM in glucose) as substrate (open triangles), and 2μM ^{55}Fe$(MECAMS)^{6-}$ (no added glucose) as substrate (open squares). b) Uptake of ^{55}Fe-MECAM-Me by RW193 E. coli. Control; 2μM ^{55}Fe$(ent)^{3-}$ as substrate (closed circles), 2μM ^{55}Fe$(MECAM\text{-}Me)^{3-}$ (5.6mM in glucose as substrate (open triangles), and 2μM ^{55}Fe$(MECAM\text{-}Me)^{3-}$ (no added glucose) as substrate (open squares).

Catechol ring substituted derivatives of MECAM do not mediate iron transport in vivo. An alkyl derivative, MECAM-Me (1,3,5-N,N',N"-tris(2,3-dihydroxy-4-methylbenzoyl)triaminomethylbenzene), was synthesized to probe the sensitivity of the receptor protein to changes in the ligand structure near the catechol groups (Domain III). No iron uptake was observed when E. coli cells were given $[Fe(MECAM-Me)]^{3-}$ or the ferric complex of a sulfonated derivative of MECAM, MECAMS [1,3,5-N,N',N"-tris(2,3-dihydroxy-5-sulfobenzoyl)triaminomethylbenzene] (see Fig. 7) (17). These experiments demonstrate the sensitivity of the ferric enterobactin receptor to structural changes on the binding subunits of enterobactin and its analogs, and suggest a strategy for the preparation of synthetic analogs of enterobactin which, like enterobactin, are effective iron chelators, but which do not support growth of E. coli or other related organisms.

MECHANISMS OF IRON RELEASE FROM FERRIC ENTEROBACTIN

The mechanism by which iron is released from ferric enterobactin remains uncharacterized, but is under investigation. It has been proposed that once inside the cell the ester linkages of the ferric complex are hydrolyzed, and the hydrolyzed complex is subsequently reduced by a reductase. This mechanism was suggested on the basis of the role of the fesB gene product in utilization of ferric enterobactin and on the basis of the reduction potentials of the Fe(III)/Fe(II) redox couple for the intact and hydrolyzed complexes. Tris(2,3-dihydroxybenzoylserine)-iron(III), the hydrolyzed form of the complex, has a reduction potential approximately 400mV higher (-0.35V vs NHE) (14,15) than that of ferric enterobactin (-1.03V vs SCE; -0.79V vs NHE at physiological pH (7.4)) (16). Only when hydrolyzed is the Fe(III)/Fe(II) redox couple within the range of physiological reductants. In apparent contradiction to a mechanism for iron release requiring ligand hydrolysis, synthetic analogs of enterobactin lacking the ester linkages have been observed to support growth of E. coli (8,12). More recently, we have shown by Mössbauer spectroscopy on intact cells that the subsequent fates of ferric enterobactin and of ferric MECAM (which lacks ester linkages) diverge after the initial stages of transport. Only a small percentage of the Fe(III) taken up as ferric MECAM is later found as Fe(II), while significant quantities of iron from ferric enterobactin are reduced (18).

Protonation of ferric enterobactin also facilitates reduction of iron(III). We have observed a pH dependence of the formal electrode potential of the Fe^{3+}/Fe^{2+} redox couple in ferric enterobactin. Four pH regions involving proton transfers concurrent with reduction of Fe(III) have been identified. The cyclic voltammogram of iron(III)enterobactin at pH 7.0 and a plot of pH vs E (in V vs SCE) is shown in Figure 8. Above pH 10.4 no protonation of the complex occurs as it is reduced. Between pH 10.4 and near pH 7 a single protonation accompanies reduction of Fe(III). Near pH 7 protonation in either two-proton or three-proton steps accompanies the one-electron reduction process. The overlapping two- and three-proton reactions have protonation constants sufficiently close as to make determination of their independent protonation constants relatively uncertain. Although at physiological pH (7.4) the measured potential is out of range of physiological reductants, the formal potential rises to -0.57V at pH 6, and may be estimated to be +0.17V at pH 4 (16). We propose that the iron release process might occur by protonation in a low pH environment in vivo.

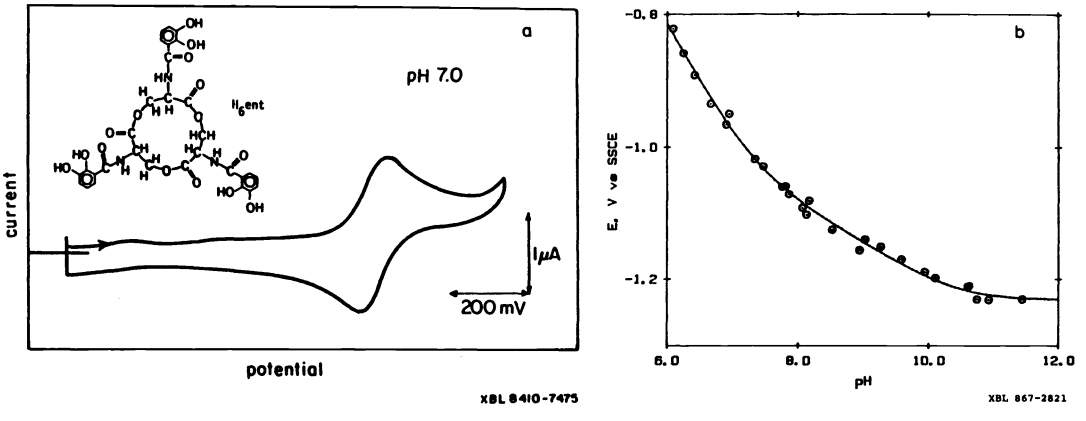

Fig. 8. a) Cyclic voltammogram of 0.2 mM Fe^{3+}(ent) in 2M NH_4Cl at pH 7.0 (20 mM phosphate). b) pH dependence of formal potentials of iron(3+, 2+) enterobactin complexes. The line is the theoretical curve calculated from the determined protonation constants from least squares refinement (16).

Previously we suggested a salicylate mode of bonding for the protonated complexes of enterobactin and of the structurally similar catechoylamide ligands, based on a comparison by IR spectroscopy of the protonated and unprotonated complexes (19). We propose that when protonated, coordination of iron is shifted from the two catechol (phenolate) oxygens to the ortho-phenolate oxygen and the carbonyl oxygen of the amide group. Recently we have obtained a single crystal X-ray structure of the first salicylate mode of bonding for a catechoylamide ligand analog. In the $[Li(MDMB)_2Ga(OH)\cdot THF]_2^{z+}$ complex (Fig. 9), two Ga(III) ions each bind to the carbonyl and ortho-phenolate oxygens of the 2-hydroxy-3-methoxy-N,N-dimethylbenzoylamide ligands (20). Further investigation of the low pH forms of ferric catechoylamides is in progress.

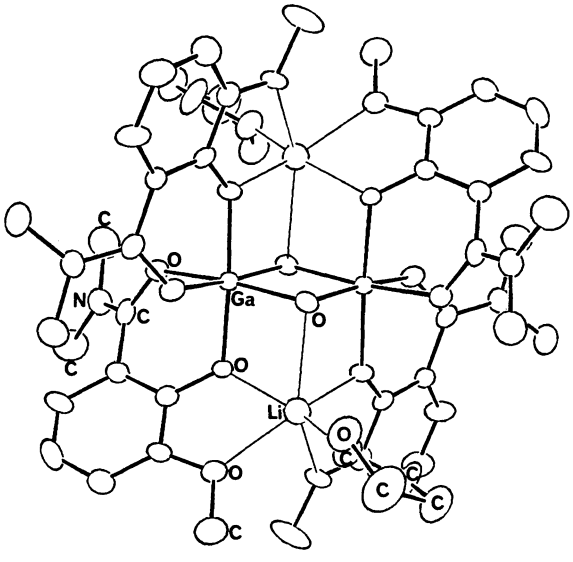

Fig. 9. Molecular Structure of the $[Li(MDMB)_2Ga(OH)\cdot THF]_2^{z+}$ Dication.

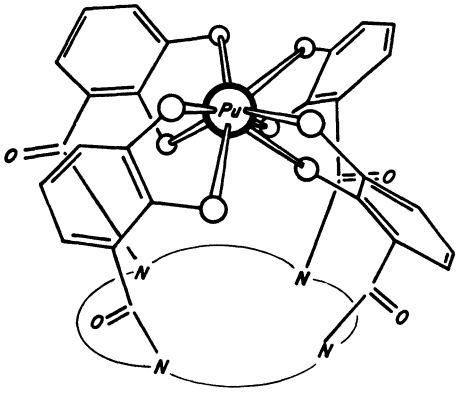

Fig. 10. Schematic Representation of a Plutonium Sequestering Agent.

DESIGN OF PLUTONIUM(IV) SEQUESTERING AGENTS

Much of the biological hazard posed by plutonium(IV) stems from its similarity to iron and thus its affinity for the mammalian iron transport and storage proteins. We have therefore extended our concept of the design of ion specific sequestering agents as analogs of siderophores to the development of Pu(IV) chelating agents (Reviewed in 21). The first class of compounds studied were catechoylamide ligand analogs of enterobactin. Since the actinide (IV) ions are typically eight coordinate, rather than six coordinate, the Pu(IV) chelating agents were designed to incorporate four, rather than three, catechol binding subunits (Fig. 10). The most effective of these chelating agents (3,4,3-LICAMC, Fig. 11) was reported to remove 75% of trace amounts of plutonium injected into mice (21). Solution thermodynamics studies have since shown that these ligands are not octadentate for Pu(IV) and Ce(IV) at neutral pH due to the weak acidity of the catechol groups (22). Therefore, new ligands similar to, but more acidic than, catechoylamides are being explored. The hydroxamates and hydroxypyridones are structurally similar to catechols, however they differ in two important respects: they are monoprotic acids, and they have significantly lower pK_a's (and are therefore much more effective ligands at lower pH). Examples of ligands which proved to be effective plutonium(IV) removal agents in mice are DFO-HOPO, a mixed hydroxamate/hydroxypyridone ligand, and DFOCAM, a ligand with hydroxamate and catecholate binding subunits (Fig. 11). These two ligands removed respectively 86% and 73% of trace amounts of Pu(IV) from contaminated mice. A new chelating agent, 3,4,3-LIHOPO (Fig. 11) has recently been synthesized and found to be the most effective Pu(IV) sequestering agent when administered as the ferric complex (23). Currently we are investigating the development of alternative Pu(IV) removal agents which utilize catechol and hydroxypyridone binding subunits to form eight-coordinate complexes with plutonium at physiological pH.

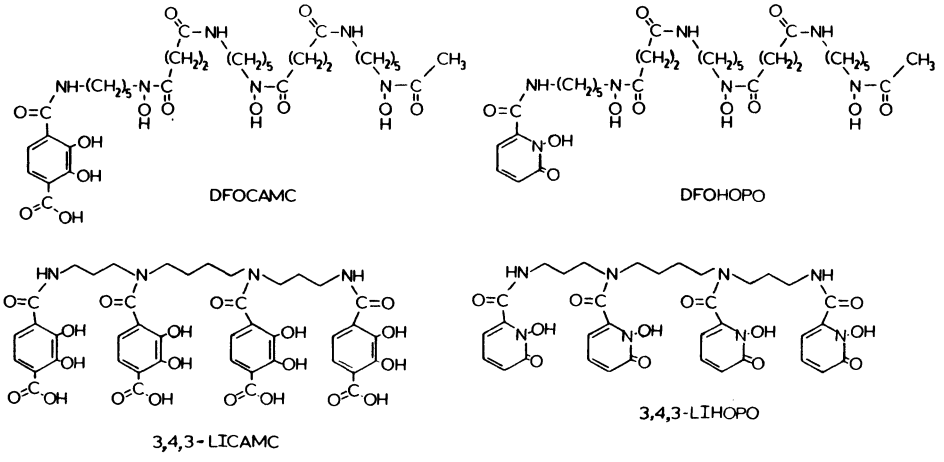

Fig. 11. Structures of Plutonium(IV) Sequestering Agents.

DESIGN OF IRON SEQUESTERING AGENTS

Using enterobactin as a model iron sequestering agent, we have sought to develop analogs which are nontoxic, soluble in aqueous solution at physiological pH, and have a high affinity and specificity for ferric iron. However, unlike enterobactin, we hope to prepare iron chelators which do not promote growth in E. coli and which lack the hydrolytically unstable ester linkages in the central backbone. Several of our sulfonated and carboxylated derivatives of the catechoylamide ligands have been tested as iron removal agents in mice (24). The DFO-HOPO (Fig. 11) and TRENCAM (1,3,5-N,N',N''-tris(2,3-dihydroxybenzoylamide)tri(aminoethyl)amine) ligands are among the most potent and least toxic iron removal agents studied in iron overloaded mice. More recently, we have looked to the development of macrocyclic and macrobicyclic analogs of the siderophore enterobactin (25,26,27). In principle, macrocyclic and macrobicyclic ligands might be expected to form stronger and more inert complexes with Fe(III). Examples of our macrocyclic and macrobicyclic ligands are shown in Figures 12 and 13. Recently, an X-ray crystal structure determination has been carried out on the ferric complex of bicapped TRENCAM (Fig. 14), a macrobicyclic ligand which consists of two tri(aminoethyl)amine backbones bridged by three 2,3-dihydroxyterephthaloyl binding subunits (26). The ligand imposes an unusual trigonal prismatic geometry about the iron(III) metal center. This is the first reported X-ray structure of a ferric complex of any of the hexadentate catechoylamide or terephthaloyl ligands and the first trigonal prismatic Fe(III) complex. Determination of the formation constants for the iron complexes of these macrobicyclic ligands is in progress.

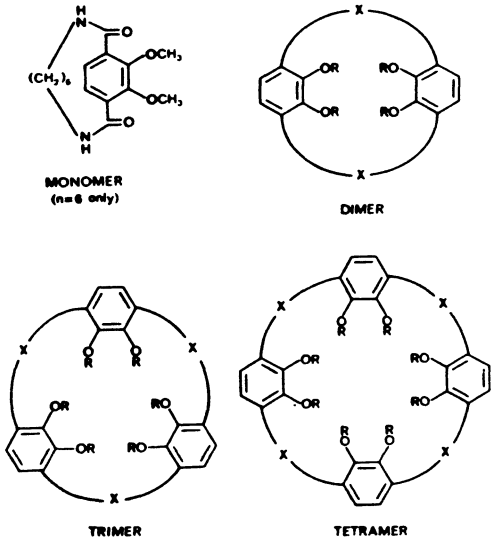

Fig. 12. Structures of Macrocyclic Terephthaloyl Ligands (x= $CONH(CH_2)_nNHCO$, R= CH_3, H, n=2, 4, 6).

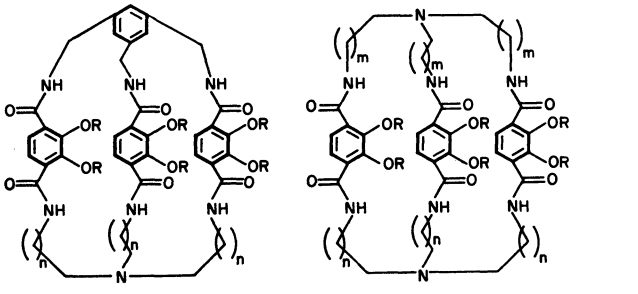

Fig. 13. Structures of Macrobicyclic Terephthaloyl Ligands.

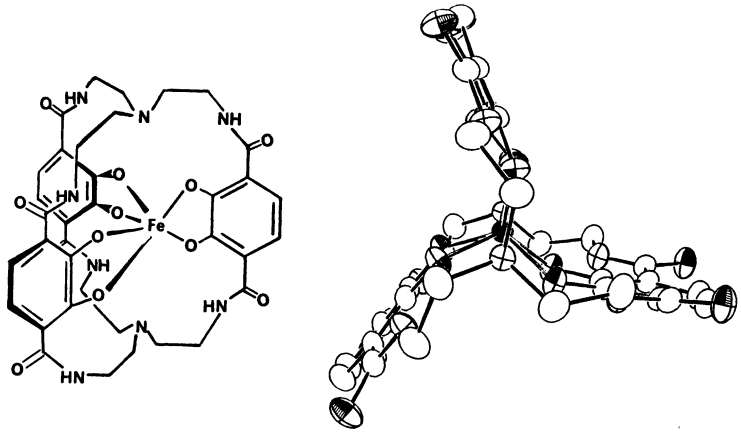

Fig. 14. Schematic Representation and ORTEP Drawing of the Ferric-Bicapped TRENCAM Anion.

Acknowledgments

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