Siderophore electrochemistry: Relation to intracellular iron release mechanism

(bacterial iron transport/enterobactin/hydroxamates/reduction potential)

STEPHEN R. COOPER, JAMES V. MCARDLE, AND KENNETH N. RAYMOND

Department of Chemistry, University of California, Berkeley, California 94720

ABSTRACT   Previous studies have shown that there is a major difference between the iron release mechanism of enterobactin, a catechol-based siderophore, and that of the hydroxamate-based siderophores such as ferrichrome. For ferric enterobactin there is an esterase that hydrolyzes the ligand during iron release. In contrast, iron is released by the hydroxamate-based siderophores and the ligands are reused in subsequent iron transport. It has been suggested that release of iron by hydroxamates occurs by reduction to the ferrous complex, a process that does not occur for ferric enterobactin. Cyclic voltammograms of ferrichrome A and ferrioxamine B exhibit reversible one-electron waves with pH-independent formal potentials (E_f vs. the normal hydrogen electrode) -446 and -454 mV, respectively, within the range of physiological reductants. Ferric enterobactin also shows a reversible one-electron wave (at pH > 10) with $E_f = -986$ mV vs. the normal hydrogen electrode. From the pH dependence of this potential we estimate a reduction potential of -750 mV at pH 7. In sharp contrast to the value for the ferric hydroxamates, this value is well below the range of physiological reducing agents. The results demonstrate that the observed hydrolysis of enterobactin is a necessary prerequisite to *in vivo* release of iron from the siderophore via ferric ion reduction.

Clinical studies have shown that the pathogenicity of some bacteria is closely related to the availability of iron (1), and that one defense against infection in mammals is the denial of iron to bacteria. Sequestration of serum iron by proteins such as transferrin (2, 3) reduces the ferric ion activity to levels well below those required for optimal microbial growth. Overcoming this defense mechanism by saturating serum transferrin with exogeneous iron increases dramatically the virulence of certain bacterial infections (1). For example, intraperitoneal injection of 5 mg of iron per kg of body weight decreases the number of *Escherichia coli* cells necessary to kill rats by five orders of magnitude (4). Thus the means by which microorganisms acquire iron has significant clinical ramifications.

Although the importance of iron to living systems can scarcely be overemphasized, the means by which bacteria acquire this vital element received little attention prior to the discovery by Neilands of ferrichrome, an iron-transporting chelate (siderophore), in 1952 (5). Aerobic microorganisms have a particularly severe problem acquiring iron, because in oxidizing environments iron is available only as ferric ion, for which a saturated solution is about 10^{-18} molar at physiological pH ($K_{sp} = [Fe^{3+}][OH^-]^3 \sim 10^{-39}$) (6). This problem has been circumvented in many prokaryotes by the evolution of ferrichrome and other siderophores, ligands that bind ferric ion with extreme avidity and selectivity, thereby solubilizing it for metabolic incorporation (7).

The two most common siderophore classes contain hydroxamate and catecholate functional groups. Ferrichrome, isolated from *Ustilago sphaerogena* (5), and ferrioxamine B, e.g., from *Streptomyces pilosus* (8), are typical of the hydroxamate class. Ferrichrome is a cyclic hexapeptide that includes a tripeptide sequence of δ-N-acetyl-L-δ-N-hydroxyornithine, each residue of which binds to ferric ion via the bidentate hydroxamate [$-N(OH)-CO-$] group, forming a hexacoordinate complex (7) (Fig. 1). Ferrioxamine B is a linear conjugate of alternating succinate and 1-amino-5-hydroxyaminopentane constituents (7) (Fig. 2). Among fungi, yeasts, and molds—such as *U. sphaerogena*—are found primarily hydroxamic acid-based siderophores. Among the true bacteria, however, chelates based on catechol (*o*-dihydroxybenzene) are also found, of which enterobactin [from, e.g., *E. coli* (9) or *Salmonella typhimurium* (10)] is the salient example. Enterobactin consists of a cyclic triester of 2,3-dihydroxybenzoylserine and binds iron via three bidentate catechol groups, once again yielding a hexacoordinate ferric complex (7) (Fig. 3).

Both classes of ligands are secreted into the medium only by organisms growing under low-iron stress; evidently under normal conditions only very small amounts of these siderophores are synthesized and the uncharged ligands are retained in the cell wall to scavenge adventitious iron. Under low iron concentrations, siderophore synthesis is derepressed and the chelators are secreted into the medium (7).

Despite the similarity in function and regulation, there is a basic difference in the mechanism of intracellular iron release from the complexes of the hydroxamates and enterobactin. The hydroxamate chelates are reused by the cell—after intracellular iron release the iron-free ligand is once again secreted into the medium. Intracellular release of iron from the hydroxamate chelates probably occurs by reduction to the ferrous state, converting the tightly bound ferric complex to the loosely bound ferrous complex,* from which the iron can be easily extracted and the intact ligand can be recycled (11). Thus one siderophore molecule can shuttle more than one iron ion into the organism. By contrast, the cyclic triester linkages of ferric enterobactin are cleaved by an esterase specific for the ferric complex (12, 13). Esterase-deficient mutants are colored pink due to intracellular ferri-enterobactin (in contrast to the white cells of the parent strain) but grow poorly under iron stress, establishing that esterase activity is vital to enterobactin-mediated transport. Furthermore, the bacteria are unable to use the cleavage product, 2,3-dihydroxybenzoylserine, as a substrate for enterobactin synthesis, which occurs instead via condensation of 2,3-dihydroxybenzoic acid and serine. (12). Thus each molecule of enterobactin yields one iron atom to the cell and then is destroyed. The enormous waste of metabolic energy

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Abbreviations: SCE, saturated calomel electrode; NHE, normal hydrogen electrode.
* K. Abu-Dari, S. R. Cooper, and K. N. Raymond, unpublished data.
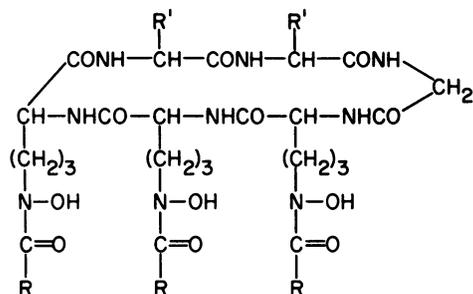
FIG. 1.   Structure of desferriferrichrome. For ferrichrome, R′ = H, R = CH₃. For ferrichrome A, R′ = CH₂OH,

$$R = $$
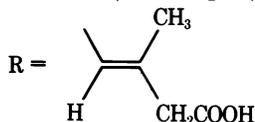
entailed in synthesis and subsequent degradation of enterobactin after one iron transport cycle suggests that such destruction must be necessary. Previous workers (12) have suggested that enterobactin cleavage is necessitated by the inordinately low reduction potential of the ferric complex, so much lower than that physiologically available that the reductive iron release mechanism of the hydroxamates is unavailable to the bacteria. This hypothesis was supported by the work of of O'Brien *et al.* (12), who were unable to observe ferric enterobactin reduction electrochemically to −1.8 V vs. the saturated calomel electrode (SCE). On the other hand, the cleavage products of enterobactin, in particular the ferric tris (2,3-dihydroxybenzoylserine) complex, are readily reduced electrochemically at potentials well within the physiological range. In order to consider this hypothesis more thoroughly we have examined the electrochemical behavior of ferric enterobactin and the representative hydroxamates ferrichrome A and ferrioxamine B.

METHODS AND MATERIALS

Ferrichrome A and ferrioxamine B were kindly supplied by J. B. Neilands and Ciba Pharmaceutical Company, respectively, and were used as received. Enterobactin was isolated from culture of *Aerobacter aerogenes* 62-1 or *E. coli* as previously described (10). All solutions were deoxygenated by bubbling with purified water-saturated nitrogen.

Electrochemistry was performed on a triple-distilled mercury pool or hanging drop electrode, with the saturated calomel electrode as reference and a platinum wire auxiliary electrode. Triangular waves were generated by the Princeton Applied Research (PAR) 175 universal programmer in conjunction with the PAR 173 potentiostat, and current–voltage curves were recorded on a Houston Omnigraphics 2000 X–Y recorder. All measurements were performed at ambient temperature (22 ± 2°) and are uncorrected for liquid junction potentials.
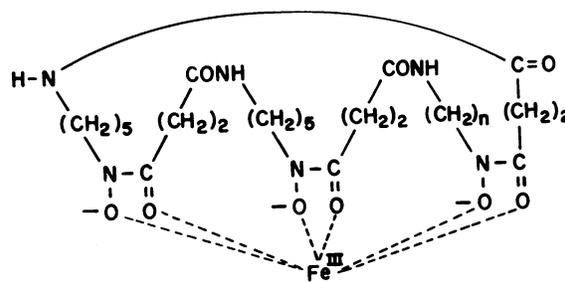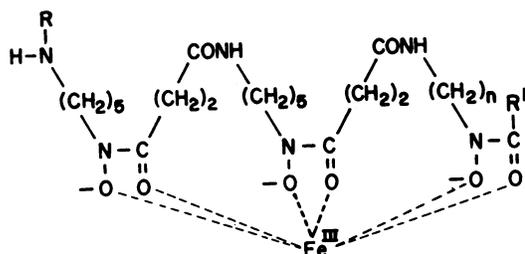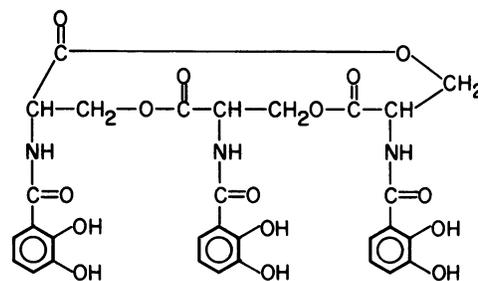
FIG. 3.   Structure of enterobactin.

RESULTS

Cyclic voltammograms of ferrichrome A and ferrioxamine B are shown in Fig. 4 top and middle. These complexes exhibit reversible one-electron oxidation–reduction waves with $E_{1/2}$ values of −690 mV and −698 mV, respectively, vs the SCE or −446 and −454 mV vs. the normal hydrogen electrode (NHE). A similar value for ferrioxamine B from polarographic data has been reported by Keller-Schierlein and coworkers (8). A separation between extrema in the cyclic voltammogram of $59/n$ mV is expected for a reversible n-electron process at 25°. The observed separation of 60 mV, its scan rate independence, and the ratio of peak cathodic to anodic currents of nearly unity are all consistent with electrochemical reversibility for both ferrichrome A and ferrioxamine B. The half-wave potentials for reversible couples can be equated to the thermodynamic midpoint potential within an excellent approximation. The midpoint potentials of the two hydroxamates are essentially independent of pH over the region 8–12, as expected for a redox process involving no protons.

Ferric enterobactin, like ferrichrome A and ferrioxamine B, yields a reversible one-electron wave, as evidenced by the scan rate-independent 60-mV separation between extrema and the peak current ratio. In sharp contrast to the hydroxamate complexes, however, is the much lower midpoint potential for this process, −1230 mV vs. SCE, or −986 mV vs. NHE (Fig. 4 bottom).

DISCUSSION

The cyclic voltammetry data on these three biological chelates demonstrate that all are reversibly reduced electrochemically. We are unable to account for previous reports (12) that ferric enterobactin is unreducible down to −1.8 V vs. SCE. In our experiments, reversible loss of the ligand-to-metal charge transfer band characteristic of the ferric complex upon exhaustive reduction proves that reduction of ferric enterobactin is occurring.

The salient feature of the cyclic voltammetry results is the striking difference in reduction potential between the hydroxamates and ferric enterobactin chelates. Ferrichrome A

FIG. 2.   Structure of linear (*Left*) and cyclic (*Right*) ferrioxamines. For ferrioxamine B, a linear ferrioxamine, R = R′ = H.
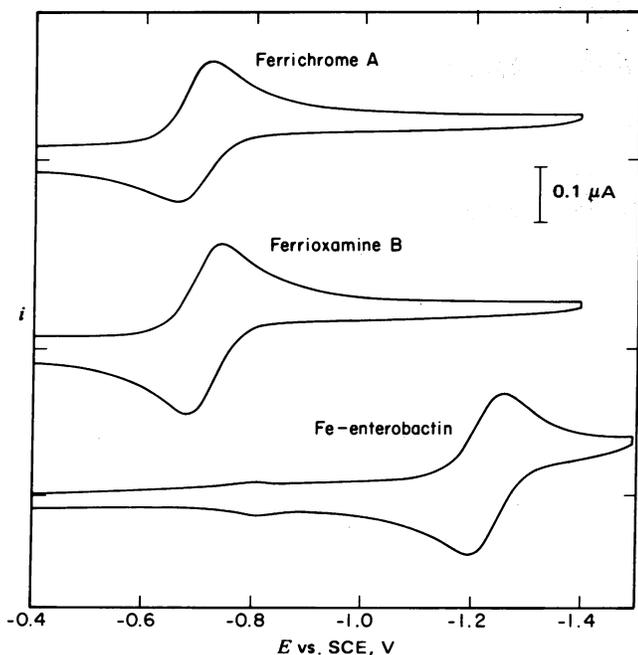
FIG. 4. Cyclic voltammograms of: (top) ferrichrome A (pH 8); (middle) ferrioxamine B (pH 8); (bottom) ferric enterobactin (pH 10.5). All are in 1 M KCl, 0.05 M sodium borate/0.05 M sodium phosphate buffer. All cyclic voltammograms were at hanging mercury drop electrode with 100 mV/sec scan rate.

and ferrioxamine B have reduction potentials within the physiological range, but that of ferric enterobactin is well below that of any known physiological reductant. Below pH 10.5 the cyclic voltammogram of ferric enterobactin exhibits peak-to-peak separations exceeding 60 mV, typical of an irreversible process. Similar behavior is found for ferric tris (catecholate), for which raising the total catechol concentration extends the pH region over which reversible waves are observed.

Although the ferric enterobactin cyclic voltammograms are not strictly reversible below pH 10, the Fe(III)-enterobactin reduction potential can be approximated as the average of the peak potentials for quasireversible waves ($\Delta E = 75$ mV). The observed increase in reduction potential with decrease in pH (Table 1) indicates that protonation of the ferrous enterobactin is occurring and is consistent with protonation and detachment of one catechol group. For such a case, the reduction potential is expected to vary as

$$E_{1/2} = E' - 59 \log(1 + K_1[H^+] + K_1 K_2[H^+]^2)$$

Table 1. pH dependence of Fe(III)-enterobactin reduction potentials

pH	E vs. SCE, mV	ΔE, mV
10.92	−1230	60
10.79	−1230	60
10.69	−1230	60
10.56	−1225	60
10.46	−1222	60
10.36	−1220	62
10.26	−1215	65
10.13	−1213	65
10.04	−1210	62
9.95	−1205	65
9.87	−1200	65
9.73	−1190	70
9.60	−1178	90

at 25°, in which $E_{1/2}$ is the observed half-wave potential, E' is the limiting high pH potential, and K_i is the acid association constant of the *i*th proton on the ferrous-enterobactin catechol. Only the beginning of the pH-dependent region is accessible before the increasing irreversibility of the Fe(III)/(II)-enterobactin couple precludes accurate determination of redox potentials. Extrapolating to pH 7, the physiologically relevant reduction potential is estimated to be −750 mV vs NHE, still well below the range of physiological reductants. O'Brien *et al.* (12) have reported, and we have confirmed, a cyclic voltammetric reduction wave at −350 mV vs NHE (pH 7) for the ferric tris(2,3-dihydroxybenzoylserine) complex, the product of complete ferric enterobactin hydrolysis. Because no corresponding oxidation wave is observable, this potential cannot be identified with the midpoint potential but does establish a lower bound for it.

These observations are consistent with intracellular iron release occurring via reduction to Fe(II), although they do not prove that this mechanism is operative *in vivo*. The hydroxamic acid chelates are thermodynamically readily reducible by physiologically available reductants such as pyridine nucleotides, yielding the weakly bound ferrous complex (log $K \simeq 9$)* from which the iron can be easily removed and the ligand recycled. In contrast, the inordinately low reduction potential of ferric enterobactin would necessitate hydrolysis of the chelate ester linkages, with concomitant increase in ferric reduction potential, as a prerequisite to physiological reduction and removal of the ferric ion.

Despite the considerable expenditure of metabolic energy to synthesize enterobactin for just one use, a large competitive advantage is conferred on the bacteria by enterobactin-mediated iron transport. The immense stability constant of ferric enterobactin (ent) [Fe(III) + ent^{6-} $\rightleftharpoons$ Fe(ent)$^{3-}$; log $K = 52$]† allows bacteria synthesizing it to acquire iron present at extremely low concentrations—probably much lower than those that will sustain microorganisms utilizing chelates with less affinity for ferric ion. Thus bacteria employing enterobactin for iron acquisition tend to deny iron to microorganisms using other ligands. In order to free the iron for their own use, the bacteria must destroy the enterobactin, which serves to make iron uptake irreversible. The relatively labile ester linkages of enterobactin may be designed specifically to facilitate this destruction, in comparison with the more robust amide linkages, for example. In this light it is interesting that the only known polycatechol chelate without labile linkages [a spermidine conjugate with 2,3-dihydroxybenzoic acid (14)] has only *two* catechols. If our hypothesis is correct, the ferric complex of this ligand should have a redox potential near those of the hydroxamic acid chelates.

CONCLUSIONS

The cyclic voltammetry results reported here show that representative microbial hydroxamate iron transport chelates (siderophores) are reduced at potentials near those of physiological reductants such as NAD(P)H. Thus iron removal from these complexes can be effected simply by iron reduction to the weakly bound ferrous state, allowing reuse of the ligand. The extremely low redox potential of ferric enterobactin precludes this mechanism and supports the suggestion that hydrolytic depolymerization of the chelate is a prerequisite for physiological reduction and removal of the iron.

† A. Avdeef, J. V. McArdle, S. R. Cooper, S. R. Sofen, and K. N. Raymond, unpublished data.

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