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Influence of Complex Structure on the Biodegradation of Iron-Citrate Complexes

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The biodegradation of iron-citrate complexes depends on the structure of the complex formed between the metal and citric acid. Ferric iron formed a bidentate complex with citric acid, $[Fe(III)(OH)_2\ cit]^{2-}$ involving two carboxylic acid groups, and was degraded at the rate of 86 $\mu M\ h^{-1}$. In contrast, ferrous iron formed a tridentate complex with citric acid, $[Fe(II)\ cit]^-$, involving two carboxylic acid groups and the hydroxyl group, and was resistant to biodegradation. However, oxidation and hydrolysis of the ferrous iron resulted in the formation of a tridentate ferric-citrate complex, $[Fe(III)OH\ cit]^-$, which was further hydrolyzed to a bidentate complex, $[Fe(III)(OH)_2\ cit]^{2-}$, that was readily degraded. The rate of degradation of the ferrous-citrate complex depended on the rate of its conversion to the more hydrolyzed form of the ferric-citrate complex. Bacteria accelerated the conversion much more than did chemical oxidation and hydrolysis.

Citric acid, a naturally occurring compound, is found in soils and plant exudates and is produced by microorganisms, especially fungi (3, 9, 13). It is also widely used in foods and beverages as an acidulation agent and preservative. In addition to its ubiquity in nature, it is found in domestic and radioactive wastes (15). Citric acid is a multidentate chelating agent which forms stable complexes with metal ions. Mixed and axenic cultures of bacteria are known to metabolize several metal-citrate complexes at different rates and to different extents. For example, sewage microorganisms degraded Ca-citrate more rapidly than Fe-, Al-, or Mg-citrate, whereas *Pseudomonas* sp. degraded Ca- and Fe-citrate, but not Al- or Mg-citrate; *P. pseudoalcaligenes* mineralized Ca-, Fe-, and Al-citrate but had little effect on Mg-citrate; *P. fluorescens* degraded Ca-, Fe(III)-, and Ni-citrate complexes but not Cd-, Cu-, Pb-, and U-citrate complexes; and *Klebsiella* sp. degraded Mg-citrate but not Cd-, Cu-, and Zn-citrate complexes (4, 8, 11). Partial degradation of iron-citrate in soil was reported (2).

The type of complex formed between the metal and citric acid plays an important role in determining its biodegradability (8). The presence of a free hydroxyl group of the citric acid is the key determinant in effecting biodegradation of the metal complex. Metals which form mononuclear bidentate complexes are readily biodegraded, whereas those which form mononuclear tridentate, binuclear, and polynuclear complexes involving the hydroxyl group of the citrate are not biodegraded. Similarly, the presence of a hydroxyl group in aliphatic acids retards their degradation in soils (6). In this communication, we report that the tridentate iron-citrate complex was readily biodegraded only when it was converted to the bidentate form.

MATERIALS AND METHODS

Culture conditions. A *P. fluorescens* biovar II (ATCC 55241) capable of degrading citric acid as a sole carbon source was isolated from a leachate sample collected from the low-level radioactive waste disposal site at West Valley, N.Y. The choice of culture medium is critical in elucidating the biodegradation of metal-citrate complexes because the components of the culture medium must not alter the complex. Therefore, we used a modified mineral salts medium (11) that was developed in accordance with equilibrium calculations by using the MINEQL computer program (21), so that the cation complex to be studied was the predominant species. The medium consisted of the following chemicals (per liter): NH_4Cl, 35.8 mg; $CaCl_2 \cdot 2H_2O$, 2.75 mg; $MgCl_2 \cdot 6H_2O$, 6.25 mg; PIPES [piperazine-*N*,*N*′-bis(2-ethanesulfonic acid)] buffer (disodium salt; Sigma Chemical Co., St. Louis, Mo.), 1.47 mg; glycerolphosphate, 1.74 mg; $FeSO_4 \cdot 7H_2O$, 1.49 mg; $MnSO_4 \cdot H_2O$, 1.155 mg; $CuCl_2 \cdot 2H_2O$, 0.101 mg; $NaMoO_4 \cdot 2H_2O$, 0.0945 mg; $ZnSO_4 \cdot H_2O$, 0.103 mg; $CoCl_2 \cdot 6H_2O$, 0.151 mg; citric acid (anhydrous; Sigma Cell Culture Reagent), 100 mg. The ionic strength of the medium was adjusted to 0.1 M by adding 7.4 g of KCl, and the pH was adjusted to pH 6.1 with KOH. The culture sample in triplicate was incubated at 26 ± 1°C in the dark on a rotary shaker at 150 rpm. The phosphate buffer and P_i present in the original medium were replaced with PIPES buffer and glycerolphosphate, respectively, to prevent the precipitation of iron.

Iron-citrate complex. A stock solution containing 13.0 mM citric acid was prepared with deionized water, and 2 ml of this stock solution was diluted to 50 ml. The ionic strength of the stock solution was adjusted to 0.1 M with KCl and was standardized by titration with 0.100 N NaOH solution (Acculute). Stock solutions were prepared containing 13.0 mM ferrous iron ($FeSO_4 \cdot 7H_2O$; Mallinckrodt) or ferric iron [$Fe(NO_3)_3 \cdot 9H_2O$; Mallinckrodt]. The stock solution of ferrous iron was prepared in a glove box filled with N_2 to prevent oxidation. Deionized water was prereduced before use by boiling while purging with filtered ultra-high-purity N_2. The solution was maintained under anoxic conditions until further use. The ferric iron solution turned deep yellow with a concurrent drop in pH, indicating extensive hydrolysis of iron. Equimolar iron-citrate complexes were prepared by adding 2 ml each of 13.0 mM citric acid and iron(II) or iron(III) solution and diluted to a final concentration of 0.52 mM. The ionic strength was adjusted to 0.1 M by the addition of KCl, and the pH was adjusted to 6.1. All complexes were prepared under low light and kept in the dark to prevent photodecomposition. The concentrations of

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iron(II), iron(III), and total iron were determined by the *o*-phenanthroline method (1).

Potentiometric titration. The number of hydrogens released from the citric acid during complex formation and hydrolysis reactions with iron was determined by potentiometric titration. The change in pH of a solution containing 0.52 mM Fe-citrate complex due to the incremental addition of 0.01 N NaOH was measured at 26 ± 1°C by using a Futura II pH electrode (Beckman Instruments, Inc., Fullerton, Calif.). Iron(II)-citrate complex prepared under anaerobic conditions was titrated in a glove box filled with N_2.

Spectrophotometric measurements. Iron-citrate complexes (0.2 mM) were prepared with Fe(II) and Fe(III) perchlorate. The pH was adjusted to 6.1, and the ionic strength was maintained at 0.1 M by adding sodium perchlorate. The UV-visible light absorption spectra for ferrous and ferric iron-citrate complexes were determined at 25°C by using a Beckman DU-7 scanning spectrophotometer. Absorption spectra for the Fe(II)-citrate complex prepared under anaerobic conditions also were determined.

Degradation of iron-citrate complex. Degradation of equimolar 1:1 ferrous and ferric iron-citrate complexes by *P. fluorescens* was determined under aerobic conditions. The treatments consisted of (i) 0.52 mM uncomplexed citrate inoculated with the bacteria, (ii) 0.52 mM Fe-citrate complex inoculated with bacteria, and (iii) uninoculated 0.52 mM Fe-citrate complex (control). Iron-citrate complexes were filter sterilized by passing them through a 0.22-μm-pore-size Millex filter and then added aseptically to acid-washed 500-ml Erlenmeyer flasks containing 180 ml of mineral salts medium. The final pH of the medium was adjusted to 6.1 by adding filter-sterilized base or acid. A 1-ml sample of an early-logarithmic growth phase of bacterial culture grown in modified mineral salts medium was added to the medium, and the samples in triplicate were incubated in the dark on a reciprocating shaker at 26 ± 1°C. Samples were taken periodically and analyzed for pH, iron, and citrate. The numbers of bacteria were determined by direct microscopic counts with DAPI (4′,6-diamidino-2-phenylindole) stain (14).

Citrate analysis. Citrate degradation was monitored by high-pressure liquid chromatography (Spectra Physics) with a Bio-Rad HPX-87H column using a UV-visible light detector at 210 nm. The samples were filtered through a 0.22-μm-pore-size Millex filter before analysis.

RESULTS

Potentiometric titration. Figure 1 shows the number of hydrogen ions titrated from uncomplexed and complexed citric acid. The uncomplexed citric acid has three ionizable hydrogens from the carboxylic acid groups with $pK_1 = 3.08$, $pK_2 = 4.39$, and $pK_3 = 5.49$ in 0.1 M KCl, and a fourth ionizable hydrogen involving the hydroxyl group with $pK_4 > 11$ (12). The titration curve for citric acid alone exhibited an inflection point at 3 mM OH^-/mM metal, indicating that the three acid hydrogens of the citric acid were completely neutralized (Fig. 1). The fourth hydroxyl proton was not titrated. The ferric iron-citrate complex solution was yellow, indicating the hydrolysis of iron. Three carboxylic acid hydrogens, as well as the hydrogen ion released from the first hydrolysis step of the ferric iron, were titrated up to pH 3.5 when the [Fe(III)OH cit]$^-$ complex was formed. Between pH 4 and 7, 5 mM OH^- was consumed with the further hydrolysis of iron to the [Fe(III)$(OH)_2$ cit]$^{2-}$ complex.

The ferrous iron-citrate complex showed neutralization of

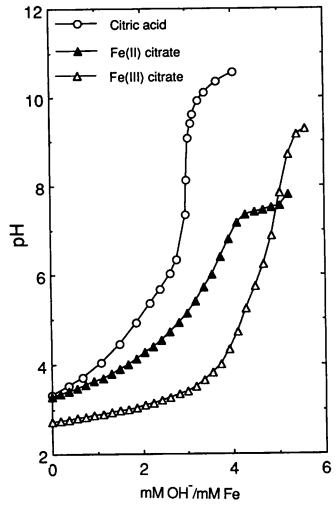

FIG. 1. Potentiometric titrations of citric acid, ferrous iron-citrate, and ferric iron-citrate complexes.

three acid hydrogens from the citrate at pH 5.0 with the formation of the [Fe(II) cit]$^-$ complex. Between 3 and 4 mM OH^-/mM metal, the solution turned yellow very slowly due to oxidation and hydrolysis of the ferrous iron, leading to the formation of the [Fe(III)OH cit]$^-$ complex. At 5 mM OH^-, the titration curve intercepted the Fe(III)-citrate complex, reflecting the formation of the [Fe(III)$(OH)_2$ cit]$^{2-}$ complex. The titration of the ferrous iron took over 1 week because of the slowness of the hydrolysis reaction.

The titration curve for the Fe(II)-citrate complex prepared and titrated under anaerobic conditions was similar to that of the aerobic sample up to 3 mM OH^-/mM metal at pH 5 (data not shown). Above this pH, a light green solution was formed due to the hydrolysis of ferrous iron and formation of [Fe(II)OH cit]$^{2-}$ corresponding to titration of a fourth proton. Oxidation of ferrous iron to ferric iron was not observed.

Spectrophotometric analysis. Figure 2 shows the absorption spectra of the iron-citrate complexes. The absorption spectrum for the [Fe(II) cit]$^-$ complex (curve A) was broad and featureless, and no ferric iron was detected in the sample under anoxic conditions. Upon exposure to air, an increase in absorption was noted after 2 h, with oxidation of all

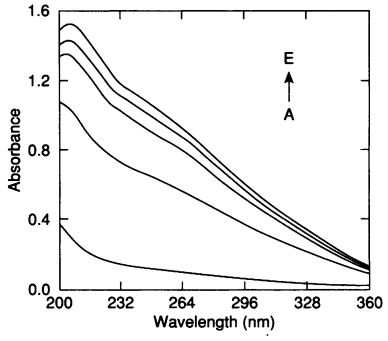

FIG. 2. Changes in the absorption spectra of ferrous iron-citrate complex undergoing oxidation and hydrolysis reactions upon exposure to air (curves A to D) and absorption spectra of ferric iron-citrate complex (curve E).

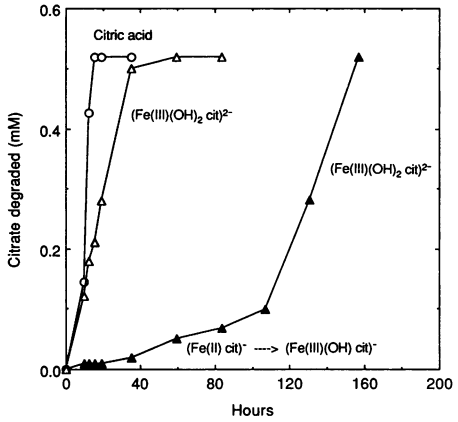

FIG. 3. Degradation of citric acid, ferric iron-citrate complex, and ferrous iron-citrate complex by *P. fluorescens*.

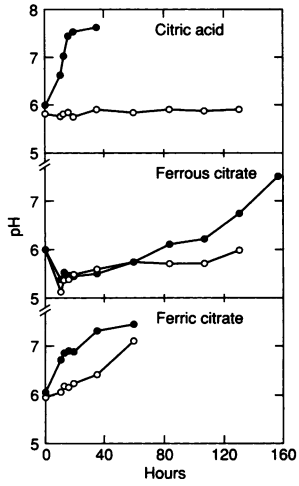

FIG. 4. Changes in pH of the culture medium containing citric acid, ferrous iron-citrate, and ferric iron-citrate complexes. Symbols: ○, uninoculated medium, control; ●, inoculated medium.

ferrous iron to the ferric form along with hydrolysis of ferric iron (curve B). The predominant form of the complex was [Fe(III) cit]. The absorption spectrum of the complex gradually increased after 24 h (curve C) and 96 h (curve D), with an increase in intensity of yellow color. The pH of the sample changed from 6.1 to 4.9 due to the oxidation of ferrous iron, which was followed by slow hydrolysis of the ferric iron to the $[Fe(III)OH\ cit]^-$ complex. The absorption maximum of this complex gradually shifted to increasing wavelengths. The ferric iron-citrate $[Fe(III)(OH)_2\ cit]^{2-}$ complex (curve E) exhibited a broad spectrum with maximum absorption at 205 nm and a small shoulder with a maximum at 260 nm. Adjustment of the pH of the complex (curve D) to 6.1 gave a spectrum identical to $[Fe(III)(OH)_2\ cit]^{2-}$ (curve E). There were no isosbestic points in the spectrum, suggesting that the conversion of the ferrous-citrate complex to ferric-citrate is continuous and no other species in equilibrium is involved.

Degradation of citrate complexes. The biodegradation of iron-citrate complexes is shown in Fig. 3. Uncomplexed citric acid was completely metabolized by *P. fluorescens* in less than 15 h at a rate of 305 ± 20 μM h^{-1}. Degradation of iron(II)-citrate was very slow initially, but after 100 h, it was metabolized rapidly. About 85% of the ferrous iron was oxidized at the start of the experiment, and conversion to ferric iron was complete within 2 h. The rate of biodegradation of this complex was 5.0 ± 0.1 μM h^{-1}. The biodegradation of iron-citrate complex increased to 42 ± 1 μM h^{-1} with an increase in pH of the medium; this corresponded to a shift in the complex equilibrium from $[Fe(III)OH\ cit]^-$ to $[Fe(III)(OH)_2\ cit]^{2-}$. Citrate was degraded in ~157 h. Iron(III)-citrate was degraded at a rate of 86 ± 8 μM h^{-1}, and degradation was complete in about 40 h (Fig. 3).

Changes in the pH of the culture medium are presented in Fig. 4. The pH of the inoculated medium containing citric acid alone increased from 6.0 to 7.6. The uninoculated medium showed no change. In medium containing ferrous-citrate, the pH decreased initially from 6.1 to 5.3 in both inoculated and uninoculated samples, which was due to the oxidation and hydrolysis of ferrous iron. However, in the inoculated sample, the pH increased gradually to 6.2 at 100 h and then rapidly to 7.5 at 157 h, concurrent with the degradation of the complex. The pH of the uninoculated sample showed no change. The bacteria completely metabolized citrate in ~15 h when the initial pH of the medium was 5.3. The pH of the medium containing ferric-citrate increased from 6.1 to 7.1 in the uninoculated sample due to the release of hydroxyl ion from equilibrium reactions (19). The pH of the inoculated sample increased to 7.5, and the rate of increase was much faster than in the uninoculated sample. The standard error of the mean (±1) was less than 0.1 pH unit for all samples analyzed.

There was an increase in the numbers of bacteria in the culture medium commensurate with citrate degradation (Fig. 5). There were slight differences in the growth of bacteria in medium containing citric acid and ferric iron-citrate. However, in the presence of ferrous-citrate, a lag period was followed by an increase in growth of the bacteria that followed closely the disappearance of citrate from the medium.

Degradation of the iron-citrate complexes resulted in the release of iron which then precipitated from solution (Fig. 6). A decrease in the concentration of iron from solution followed a pattern similar to the increase in growth and utilization of citrate by the bacteria.

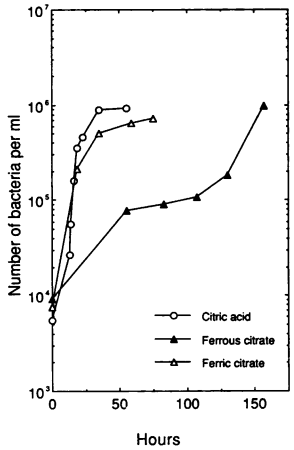

FIG. 5. Growth of *P. fluorescens* in media containing citric acid, ferric iron-citrate complex, and ferrous iron-citrate complex.

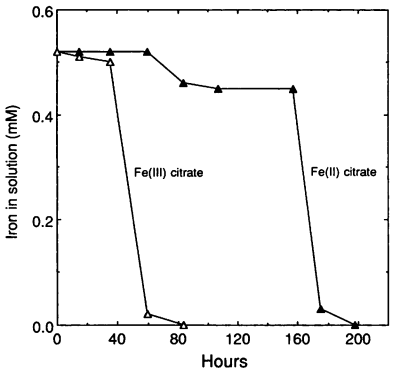

FIG. 6. Concentration of iron in culture medium containing ferrous and ferric iron-citrate complexes, after biodegradation by *P. fluorescens*.

DISCUSSION

Citric acid forms a mononuclear bidentate complex, [Fe (III)$(OH)_2$ cit]$^{2-}$, with ferric iron involving two carboxylic acid groups and a tridentate complex, [Fe(II) cit]$^-$, with ferrous iron involving two carboxylic acid groups and the hydroxyl group. Although the characterization of these complexes is not straightforward, potentiometric and spectrophotometric studies with ferric iron-citrate complexes have shown that a tridentate mononuclear complex is formed below pH 3 (17), and magnetic susceptibility studies have shown the formation of a bidentate dimer complex at neutral pH (5). Nuclear magnetic resonance studies of ferrous-citrate confirmed the formation of a tridentate complex involving the hydroxyl group (16).

In the presence of oxygen, the ferrous-citrate complex undergoes oxidation and hydrolysis, leading to the formation of ferric iron-citrate complex. The intermediates involved in the conversion of ferrous-citrate to ferric-citrate are presented in Fig. 7. The degradation of ferrous iron-citrate by the bacteria depended on the rate of oxidation and hydrolysis of the ferrous-citrate complex. In the presence of oxygen, the tridentate ferrous iron-citrate [Fe(II) cit]$^-$ complex (log K = 4.4) was oxidized to the neutral ferric iron-citrate [Fe(III) cit] complex (log K = 11.4) with no change in structure of the complex (10). The first hydrolysis of the ferric iron to [Fe(III)OH cit]$^-$ (log K = 9.4) is the rate-limiting step, as shown by the absorption spectrum. The absorption maximum at 205 nm has been attributed to the presence of the $FeOH^{2+}$ species (18). The second hydrolysis step results in the formation of [Fe(III)$(OH)_2$ cit]$^{2-}$, and the log K is dramatically reduced to 1.9 to 2.6 (10). This significant loss of stability of the tridentate complex suggests the formation of a weaker bidentate complex.

The influence of metal-ligand conformation on the biodegradation of complexes has not been intensively studied. Warren (20) showed that Ca, Fe(III), and Mn, which formed weak tridentate complexes with nitrilotriacetic acid, were readily biodegraded, whereas Cd, Cu, Ni, and Zn, which formed tetradentate complexes, were degraded slowly. Firestone and Tiedje (7) found a similar pattern but attributed the slow degradation of the tetradentate complexes to the effects of toxicity on the bacteria. Recently, we (8) reported that Ca, Fe(III), and Ni, which formed mononuclear bidentate complexes with citric acid, were readily biodegraded, whereas, Cd, Cu, Fe(II), and Pb, which formed mononuclear tridentate complexes, and U, which formed a binuclear complex involving the hydroxyl group of the citrate, were not biodegraded. The lack of degradation was not due to toxicity. Citric acid in bacteria is transported across the cytoplasmic membrane into the cell, and this transport is affected by the presence of divalent cations (22). However, the mechanisms by which the bacteria discriminate between conformations of metal-ligand complexes is not known. Various explanations include the transport of different chemical species at different rates by single or multiple transport systems, and the presence of a transport protein which carries the metal-free ligand into the cell after dissociation of the metal from the ligand. We believe that the metabolism of the tridentate ferrous-citrate complex is more likely to be limited by the lack of transport into the cell (unpublished data).

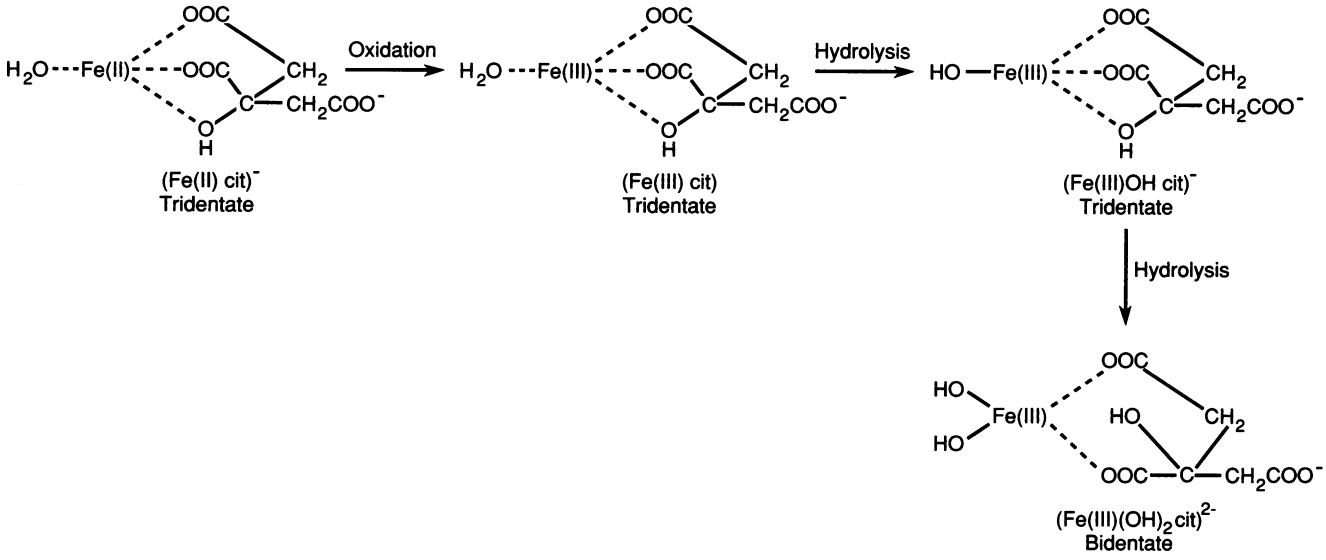

FIG. 7. Oxidation and hydrolysis of tridentate ferrous iron-citrate complex to bidentate ferric iron-citrate complex.

In this study, we found that the bidentate ferric iron-citrate complex was rapidly degraded, while degradation of the tridentate ferrous iron-citrate complex depended on its oxidation and hydrolysis to form the ferric iron-citrate $[Fe(III)(OH)_2\ cit]^{2-}$ complex. These results show that the structural characteristics of the metal-citrate complex play a major role in determining its biodegradability.

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