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Synthesis, Solution Behavior, Thermal Stability, and Biological Activity of an Fe(III) Complex of an Artificial Siderophore with Intramolecular Hydrogen Bonding Networks

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Previously, an artificial siderophore complex, the iron(III) complex with tris[2-{(*N*-acetyl-*N*-hydroxy)glycylamino}-ethyl]amine (TAGE), was constructed in order to understand the effect of intramolecular hydrogen bonding interaction on the siderophore function, and its structural characterization in the solid state was reported (*Inorg. Chem.* **2001**, *40*, 190). In this paper, the solution behavior of the M(III)–TAGE (M = Fe, Ga) system has been investigated using ^{1}H NMR, UV–vis, and FAB mass spectroscopies in efforts to characterize the biological implication of hydrogen bonding networks between the amide hydrogens and coordinating aminohydroxy oxygens of the complex. The temperature dependence of ^{1}H NMR spectra for Ga(III) complex of TAGE indicates that hydrogen bonding networks are maintained in polar solvents such as DMSO-d_6 and D_2O. The UV–vis spectra of the Fe(III)–TAGE system under various pH conditions have shown that TAGE forms a tris(hydroxamato)iron(III) complex in an aqueous solution in the pH range 4–8. By contrast, tris[2-{(*N*-acetyl-*N*-hydroxy)propylamido}ethyl]amine (TAPE; TAGE analogue that is difficult to form intramolecular hydrogen bonding networks), which has been synthesized as a comparison of TAGE, forms both of bis- and tris(hydroxamato)iron(III) complexes in the same pH range. Both the stability constants (log β_{FeTAGE} = 28.6; β_{FeTAGE} = [Fe^{III}TAGE]/([Fe^{3+}][$TAGE^{3-}$])) and pM (–log[Fe^{3+}]) value for Fe^{III}TAGE (pM 25) are comparable to those of a natural siderophore ferrichrome (log β = 29.1 and pM 25.2). The kinetic study of the TAGE–Fe(III) system has given the following rate constants: the rate of the ligand exchange reaction between Fe^{III}TAGE and EDTA is 6.7×10^{-4} s^{-1}, and the removal rates of iron from diferric bovine plasma transferrin by TAGE are 2.8×10^{-2} and 6.0×10^{-3} min^{-1}. These values are also comparable to those of a natural siderophore desferrioxamine B under the same conditions. In a biological activity experiment, TAGE has promoted the growth of the siderophore-auxotroph Gram-positive bacterium *Microbacterium flavescens*, suggesting that TAGE mimics the activity of ferrichrome. These results indicate that the artificial siderophore with intramolecular hydrogen bonding networks, TAGE, is a good structural and functional model for a natural ferrichrome.

Introduction

Most living organisms require iron for their growth and development. For microorganisms such as bacteria and fungi, the availability of iron is uncertain because of the low solubility of ferric ions. Under aerobic conditions, the iron ion precipitates in the form of ferric oxyhydroxide with a solubility product constant of 10^{-39} M,[4] which limits the concentration of ferric ions to approximately 10^{-18} M at pH 7. This is far below the level of demand for the iron supply of living cells (approximately 10^{-7} M).[1] To overcome this obstacle, microorganisms produce and release several kinds of chelating compounds, siderophores, in order to sequester ferric ion from the environment.[2−4] Siderophores are mainly classified into hydroxamate- and phenolate (catecholate)-

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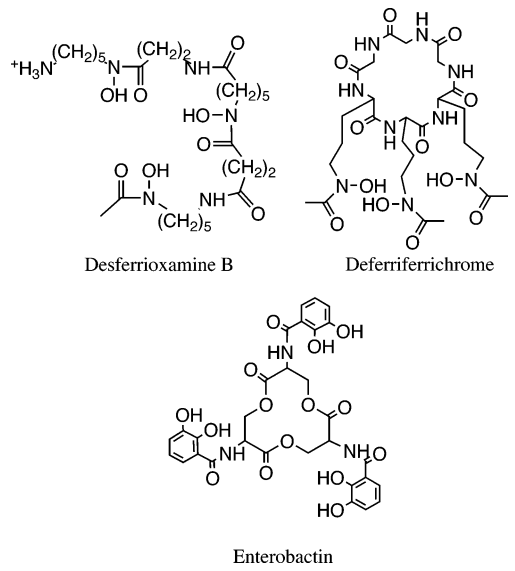
Chart 1



containing compounds which function as iron specific chelating agents.[2] Once siderophores form the complexes with the iron(III) ion, and possess high stability constants (log $\beta \approx 30$ and 49 for hydroxamte-types and catecholate-types, respectively),[3] they are efficiently taken up into the cell. The highly specific receptors on the outer membrane of bacteria recognize unique structural characteristics of siderophores, such as chirality, and transport the ferric complexes into the periplasm in an active and energy-dependent manner. Release of iron from ferric siderophores in the cell often proceeds through degradation of the ligand or reduction of ferric to ferrous ion which has a lower affinity for the siderophore ligand.[2,3] Indeed, the thermodynamic stability of the Fe(II)-siderophores (log $\beta = 10-12$) is much lower than those of Fe(III)-siderophores (log $\beta \approx 30$).[5] Therefore, it is reasonable that the reduction of the Fe(III) complex to the Fe(II) state plays an important role in biological processing of iron by bacteria.

Several approaches have been taken in the field of biomimetics of siderophores and have clarified the characteristics of Fe(III)-siderophores and the mechanisms of transportation of Fe(III) ion into the cell.[6] Deferriferrichrome, which is a siderophore containing three hydroxamate groups as iron binding sites on an 18-membered cyclic hexapeptide ring, was first isolated from the fungus *Ustilago sphaerogena* as a ferric complex (ferrichrome) by Neilands in 1952.[7] The other trihydroxamate-type siderophores were subsequently identified.[8] Examples of natural trihydroxamate-type siderophores are shown in Chart 1. In order to develop synthetic hydroxamate siderophores, Olsen et al. have synthesized and characterized retrodeferriferrichrome, a deferriferrichrome analogue in which the carbonyl groups and aminohydroxyl groups on the hydroxamates are exchanged.[9] This investigation revealed that this modification of the hydroxamate groups had little influenced on its biological activity, although retroferrichrome is more labile than ferrichrome. Thus, many synthetic retrohydroxamates were developed as the analogues for hydroxamate-type siderophores.[5b,6] Raymond and coworkers characterized many Fe(III) complexes containing catecholates and hydroxamates[10] and prepared a thermodynamically stable Fe(III) complex using a tripodal trihydroxamate ligand (TRENDROX) with a tris(2-aminoethyl)amine (TREN) tether (log $\beta = 32.9$).[10a] The thermodynamic stability of the complex is greater than that of ferrichrome (log $\beta = 29.1$)[3] and is comparable to ferrichrome A (log $\beta = 32.0$),[3] although the ligand TRENDROX and its iron(III) complex are insoluble at physiological pH.

Single crystal X-ray structure analyses performed on natural siderophore complexes[12] have revealed the existence of intramolecular hydrogen bonds between the coordinating oxygen and the amide hydrogen with the O···(H)N distances of 2.73−2.81 Å. In light of this finding, Shanzer and coworkers have reported some tripodal compounds which include an intramolecular hydrogen bond and found that the amide-containing compounds tend to induce a propeller-like conformation via formation of intramolecular hydrogen bonds in nonpolar solvents such as $CHCl_3$.[11] The iron(III) complexes of trihydroxamate ligands with a TREN tether did not exhibit significant biological activity.[11e] Thus, other types of tripodal retro-trihydroxamate ligands with 1,1,1-tris{(2-carboxyethoxy)methyl}propane or 1,1,1-tris{(carboxymethoxy)methyl}propane were employed as the tripodal tethers.[11b] The Fe(III) complex with 1,1,1-tris{(2-carboxyethoxy)methyl}propane as its tether has intramolecular hydrogen bonding networks to form a five-membered ring, and promoted growth of the siderophore-deficient mutants, but the Fe(III) complex with 1,1,1-tris{(carboxymethoxy)methyl}propane, which does not form such a hydrogen bond, did not promote the growth.[11a,b] This result suggests that

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Chart 2

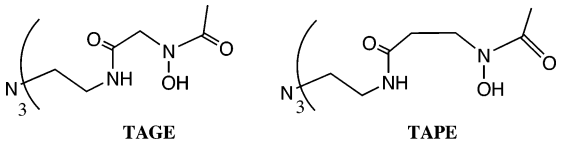
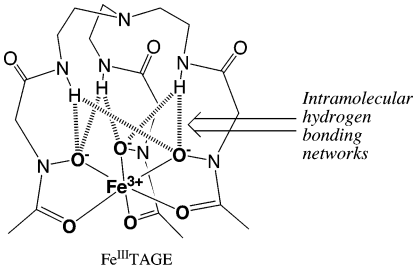


Chart 3



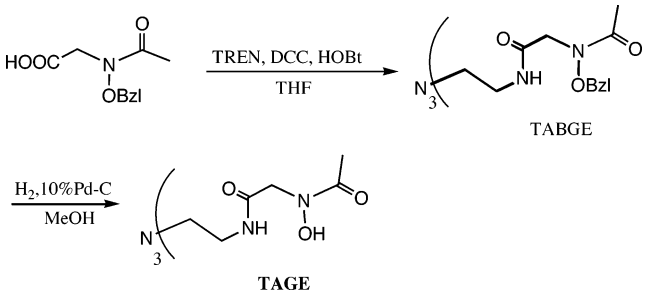
formation of intramolecular hydrogen bonds is an important factor in uptake of the Fe(III)-siderophores by microorganisms.

Several other artificial siderophores have been reported.[6] However, the replacements of the N-terminal hydroxamate with various functional groups are not recommended from the requirement of the alkyl- or arylhydroxylamines because such hydroxylamines are limited and their syntheses are diffcult. By contrast, the introduction of hydroxamate group as a C-terminal substituent is more easily accomplished than for an N-terminal one. On the basis of the above experimental findings, the ligand, tris[2-{(*N*-acetyl-N-hydroxy)glycylamino}-ethyl]amine (TAGE: Chart 2, left), was synthesized. This ligand consists of three components: a terminal acyl group, an *N*-hydroxy-α-amino acid, and a TREN group as a tripodal anchor, which can form intramolecular hydrogen bonds with a six-membered ring between the aminohydroxyl oxygen and amide hydrogen in the same strand when the Fe(III) complexes are formed. Furthermore, many kinds of siderophore analogues can be synthesized by replacing the terminal acyl groups and/or *N*-hydroxy-α-amino acid residues by other functional groups. Another ligand, tris[{2-(*N*-acetyl-N-hydroxy)propylamido}ethyl]amine (TAPE: Chart 2, right), was also synthesized as a ligand with poor intramolecular hydrogen bonds between the amide hydrogen and amino-hydroxyl oxygen due to the long distance. We have recently reported the crystal structure of Fe^{III}TAGE, which revealed that not only multiple intramolecular hydrogen bonds but also intramolecular hydrogen bonding networks were formed in the complex (Chart 3). The redox potential of the complex was increased.[13] In this Article, we report the solution structure, thermal stability, kinetics, and biological activity of the TAGE complex with an iron(III) ion and discuss the contribution of intramolecular hydrogen bonding networks in the siderophore function.

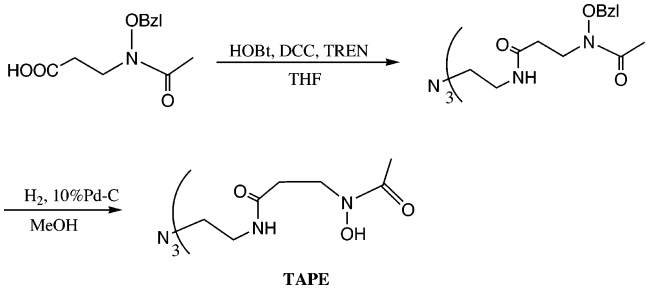
Experimental Section

General. Reagents and solvents were purchased from commercial suppliers and used without further purification. Elemental analyses were performed for C, H, and N elements on a LECO CHN-900 analyzer. FAB-mass spectra were measured on SHIMADZU KRATOS. Measurements of UV−vis spectra were performed with a JASCO U-best 35 spectrophotometer. ^{1}H NMR spectra (TMS or DSS reference) were taken with a HITACHI R-90 spectrometer equipped with a HITACHI 672 graph plotter or by a JEOL JMN-Lambda 500 Fourier transformation spectrometer equipped with AIR-VT thermocontroller. Infrared spectra were recorded using a JASCO FT/IR-410 Fourier transformation spectrometer.

Scheme 1



Scheme 2



Syntheses of Ligands. The ligands employed, TAGE and TAPE, were synthesized according to Schemes 1 and 2. Details of the syntheses are as follows.

Tris[2-{(*N*-acetyl-*N*-benzyloxy)glycylamino}ethyl]amine (TABGE). *N*-Acetyl-*N*-benzyloxyglycine was synthesized by the literature methods.[14,15] *N*-Acetyl-*N*-benzyloxyglycine (2.17 g, 9.72 mmol) and 1-hydroxybenzotriazol (HOBt; 1.31 g, 2.94 mmol) were dissolved in 180 mL of THF at −3 °C. Furthermore, 20 mL of THF solution containing tris(2-aminoethyl)amine (0.43 g, 2.94 mmol) was added to this solution. THF (20 mL) solution containing dicyclohexylcarbodiimide (DCC; 2.01 g, 9.72 mmol) was then added dropwise to this solution. The reaction mixture was stirred for 2 h at −3 °C and then 2 h at room temperature. The solvent was evaporated, and the residue was dissolved in 80 mL of ethyl acetate. An insoluble dicyclohexyl urea precipitate was filtered off. The filtrate was washed with 5% aqueous solution of $NaHCO_3$, and then the organic layer was treated with an aqueous 10% citrate solution, which was prepared with sodium bicarbonate to pH 8 and re-extracted three times with 50 mL of ethyl acetate. The organic layer was dried with anhydrous magnesium sulfate and evaporated. Pure TABGE was obtained as a pale yellow amorphous solid (1.80 g; 80.3% yield). IR (KBr pellet): ν 1659, 3291 cm^{-1}. ^{1}H NMR (90 MHz, $CDCl_3$): δ 2.0 (s, CH_3CO), 2.5 (br, NCH_2CH_2), 3.3 (br, NCH_2CH_2), 4.2 (s, $NHCOCH_2$), 4.9 (s, $PhCH_2$), 6.3 (m, Ph-H), 7.8 (s, NHCO).

Tris[2-{(*N*-acetyl-*N*-hydroxy)glycylamino}ethyl]amine (TAGE). TABGE (1.35 g, 1.77 mmol) dissolved in methanol (100 mL) was hydrogenated with a 10% Pd/C catalyst under 3 atm H_2 gas for

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several hours at room temperature. This hydrogenation was monitored by silica gel TLC. When the reaction was completed, the methanol solution was filtered through Celite to remove the Pd/C and then evaporated. TAGE was obtained as pale yellow amorphous solid (0.84 g; 96.6% yield). IR (KBr pellet): ν 1649, 3285 cm^{-1}. ^1H NMR (500 MHz, DMSO-d_6): δ 2.02 (s, COCH_3), 2.5 (overlapping the solvent peak, NCH_2CH_2), 3.13 (td, NCH_2CH_2), 4.11 (s, NHCOCH_2), 7.72 (br s, NHCO), 9.87 (br s, NOH). Found: C, 43.56; H, 6.79, N; 18.56%. Calcd for $C_{18}H_{33}N_7O_9\cdot 0.75CH_3OH\cdot 0.25H_2O$: C, 43.31; H, 7.07; N, 18.85%. FAB-mass spectrum (*m*/*z*): 492 ($[M + H]^+$) for TAGE.

Tris[2-{(*N*-acetyl-*N*-hydroxy)propylamido}ethyl]amine (TAPE). TAPE was synthesized in a manner similar to that for TAGE using 3-(*N*-benzyloxyamino)propionic acid instead of *N*-benzyloxyglycine. TAPE was obtained as a pale orange oil. ^1H NMR (90 MHz D_2O): δ 2.1 (s, CH_3), 2.5 (t, HONCH_2CH_2CO), 3.2 (t, NCH_2CH_2), 3.5 (t, NCH_2CH_2), 3.9 (t, HONCH_2CH_2CO). FAB mass spectrum (*m*/*z*): 534 ($[M + H^+]$) for TAPE.

Synthesis of Fe(III) Complex of TAGE, [Fe^{III}(TAGE)]. To a methanol solution (10 mL) containing 0.1 mmol of TAGE was added tris(acetylacetonato)iron(III) (Fe^{III}(acac)$_3$; 0.1 mmol). The deep red crystal of the tris(hydroxamato)iron(III) complex was precipitated after standing for a few hours and then isolated by filtration. The compound thus obtained was identified by elemental analysis and mass spectral measurements. Found: C,39.32; H, 5.92; N, 17.37%. Calcd for $C_{18}H_{30}N_7O_9Fe\cdot 0.25H_2O\cdot 0.5CH_3OH$: C,39.34; H, 5.80; N, 17.36%. FAB-mass spectrum (*m*/*z*): 545 ($[M + H]^+$) for **[Fe^{III}(TAGE)].**

Preparation of Ga(III) Complex of TAGE, [Ga^{III}(TAGE)]. Ga(III) complex of TAGE was prepared according to the same procedure as that of Fe^{III}TAGE using Ga^{III}(acac)$_3$ instead of Fe^{III}(acac)$_3$. Found: C, 38.66; H, 5.81; N, 16.61%. Calcd for $C_{18}H_{30}N_7O_9Ga\cdot CH_3OH$: C, 38.71; H, 5.56; N, 16.66%. FAB-mass spectrum (*m*/*z*): 558 and 560 ($[M + H]^+$) for [^{69}Ga^{III}TAGE] and [^{71}Ga^{III}TAGE], respectively.

UV−Vis Spectral Measurements. Sample solutions containing Fe^{III}−TAGE or Fe^{III}−TAPE system were prepared to a final concentration of 2.8×10^{-5} M in 50 mL volumetric flasks using the stock solutions (7.14×10^{-4} M) containing $NH_4Fe(SO_4)_2\cdot 12H_2O$ and ligand (TAGE or TAPE). The ionic strength and pH values were kept constant with 0.1 M sodium perchlorate solution ($NaClO_4$) and 0.01 M buffer solutions as follows: $HClO_4$−$NaClO_4$ (pH 1−3), CH_3COOH−CH_3COONa (pH 3−5), HEPES−NaOH (pH 6−8). The absorption spectra of the solutions were measured after standing for 1 day.

Potentiometric Titration. A titrant solution was prepared using deionized and distilled water that was further purified by passing through a Millipore Milli-Q reverse osmosis cartridge system (resistivity 18 MΩ cm). The system was flushed with CO_2-free N_2 gas prior to and during the titration. A carbonate-free 0.1 M KOH solution was standardized by titrating against potassium hydrogen phthalate. A 0.1 M HNO_3 solution was standardized by titrating against KOH. About 0.1 mmol of TAGE was dissolved into 40 mL of an aqueous solution containing 0.100 M of KNO_3. The sample solution was acidified by addition of 1 mL of 0.1 M HNO_3 to fully protonate the ligand. The acidic TAGE solution was titrated in triplicate with the standardized carbonate-free KOH solution. All the titrations were performed using a Dosimat 665 (Metrohm) piston buret and an AC-15 pH meter (Iwaki Glass) equipped with a BECKMAN 39419 reference electrode and a BECKMAN 39321 glass electrode, at a constant temperature of 25 °C using a Hakke circular thermostat model F-3. The protonation constants were estimated by least-squares calculations for the titration data using the SUPERQUAD program system.[16]

Spectrophotometric Titration of the Fe(III) Complex of TAGE with EDTA. The stability constant of the tris(hydroxamato)-iron(III) complex of TAGE was spectrophotometrically investigated by a competition reaction with *N*,*N*,*N*′,*N*′-ethylenediaminetetraacetic acid (Na_2H_2edta) at 25 °C. The eight solutions were prepared in 50 mL volumetric flasks containing Fe^{III}TAGE (final concentration: 3.88×10^{-5} M) and Na_2H_2edta (final concentration: 1.16×10^{-5} to 7.74×10^{-4} M) with the ionic strength kept constant with 0.1 M of sodium perchlorate ($NaClO_4$). The pH values of the mixed solutions were maintained constant at 6.1 with a 10 mM hexamine−$HClO_4$ buffer system. The absorption spectra of the solutions were measured after standing for a few days until equilibrium was completed for the ligand-exchange reaction.

Kinetics. (a) Ligand-Exchange Reaction between [Fe^{III}-(TAGE)] and Na_2H_2EDTA. Stock solutions containing Fe^{III}TAGE (1.23×10^{-5} M) prepared from equimolar amounts of $NH_4Fe(SO_4)_2\cdot 12H_2O$ and TAGE and 10−40 excess amounts of Na_2H_2-EDTA for Fe^{III}TAGE, which were prepared in 0.01 M acetate buffer (pH 5.3, I = 0.1 M $NaClO_4$) to a final concentration of 9.8×10^{-4} M (Fe^{III}TAGE). The kinetic experiments were monitored using the absorbance (Abs) at 420 nm at 25 °C. The pseudo-first-order rate constant (k_{obs}) was evaluated from a slope of the plots of $\ln[(\mathrm{Abs} - A_\infty)/(A_0 - A_\infty)]$ as a function of time (t), where A_0 and A_∞ are the absorbance at $t = 0$ and that of Fe^{III}EDTA$^-$, respectively. The second-order rate constants were calculated from the linear relationship between the concentration of Na_2H_2EDTA and k_{obs}.

(b) Removal of Iron(III) Ion from Bovine Plasma Transferrin Using TAGE. The iron(III) removal reactions were carried out using a commercially available bovine plasma iron saturated transferrin (Tf) (99.999%, Nacalai tesque). The stock solutions composed of Fe_2Tf (1 mL, 1.00×10^{-3} M), L = TAGE or desferrioxamine B (DFO B) (1 mL, 2.00×10^{-2} M), and 0.1 M HEPES buffer (1 mL, I = 0.1 M KNO_3) were prepared, and then the absorbance (Abs) of the solutions was followed by monitoring the band at 420 nm. The pseudo-first-order rate constants (k_1, k_2) were calculated from a slope of the plots of $\ln[(A_\infty - \mathrm{Abs})/(A_\infty - A_0)]$ for the times, where A_0 and A_∞ are the initial and final absorbances for the respective reactions.

Biological Activity. Biological activity was investigated by observing the growth of the siderophore-auxotrophic bacterium, *Microbacterium flavescens* (ATCC No. 25091), in a siderophore-free liquid medium. *M. flavescens* was purchased from American Type Culture Collection (ATCC). Siderophore-free ATCC no. 424 broth was prepared by the following method: bactopeptone (1 g), yeast extract (1 g), and K_2HPO_4 (0.2 g) were dissolved in 100 mL of water, and then the solution was adjusted at pH 7.4 using 1 M NaOH prior to the sterilization. A 25 mL flask containing 8 mL of siderophore-free ATCC no. 424 broth with desferrioxamine mesylate (20 μg/L) was inoculated with 5 μL of the stock culture solution and incubated for 20 h at 30 °C. After 20 h, the bacterium in 1 mL of the culture solution was centrifuged for 15 min at 2000*g* and washed with 1 mL of the siderophore-free ATCC no. 424 broth. The washing of the cell was repeated to completely remove the siderophore within the preincubated medium. The 25 mL flasks were filled with 7.5 mL of the siderophore-free ATCC no. 424 broth containing a final concentration of 10 μM Fe^{III}TAGE and then were inoculated with 0.5 mL of the preincubated culture and incubated at 30 °C and 100 rpm. The degree of growth of the

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siderophore-auxotrophic bacterium was examined by monitoring the optical density of the solutions that were measured using the absorption at 660 nm. Each treatment was performed in duplicate. The biological activity was examined simultaneously for the siderophore-free broth and the ferrichrome solution by the same procedure as the reference for Fe^{III}TAGE.

Results and Discussion

Intramolecular Hydrogen Bonds in Polar Solvents As Examined by ^{1}H NMR. Previously, we reported the crystal structure of Fe^{III}TAGE, which revealed the formation of intramolecular hydrogen bonding networks between the amide hydrogens and the coordinating aminohydroxyl oxygens.[13] In order to study the solution behavior of the iron(III) complex, ^{1}H NMR measurements of the TAGE complex with Ga(III) in the place of Fe(III) have been performed, because Ga(III) ion has the same ionic charge as Fe(III) ion and has an ionic radius similar to that of Fe(III) (0.62 and 0.65 Å for Ga(III) and Fe(III), respectively, in an octahedral geometry)[17] and because the diamagnetic Ga(III) complex yields a clear NMR spectrum which is easily interpreted in contrast to that of the paramagnetic Fe(III) complexes. The ^{1}H NMR spectra of the Ga(III) complex of TAGE measured in DMSO-d_6 and D_2O indicated that the complex formed a 1:1 Ga(III)−tris(hydroxamato) species, which was determined also by the elemental analysis and FAB-mass spectrum.

The ^{1}H NMR spectrum of the metal-free TAGE ligand in DMSO-d_6 exhibits a simple pattern suggesting C_3 symmetry. The signal observed at 3.13 ppm was assigned to the methylene protons attached to the amide group of the TREN cap. The other methylene protons of the TREN were not found by overlap with the signals of a solvent. The methylene peak of the glycine moiety was observed at 4.11 ppm as a singlet peak. The two remaining signals in the lower field region were assigned to the amide (7.72 ppm) and hydroxide protons (9.87 ppm).

The ^{1}H NMR spectrum of the Ga(III) complex of TAGE in DMSO-d_6 also indicated C_3 symmetry. The acetyl proton signal shifted to the lower field region compared to that of metal-free TAGE. However, we failed in assignment of the methylene protons of the TREN cap because the signal overlaps with the solvent. The methylene protons of the glycine moiety is split with an AX pattern (δ 4.19, 4.64 ppm; J = 17.6 Hz), suggesting the steric hindrance of rotation around the methylene carbon. The amide proton signal of TAGE, which appeared at 7.72 ppm, also shifted to the lower-field region (7.93 ppm) upon complexation with Ga(III) ion. The chemical shift showed limited temperature dependence (temperature coefficients for the Ga(III) complex: 0.39 ppb/K), in contrast to the temperature-dependent higher field shift observed for the metal-free TAGE (−4.6 ppb/K) (Figure S1). These results indicate that the amide protons of the TAGE complex form extremely strong intramolecular hydrogen bonding networks even in a polar solvent.

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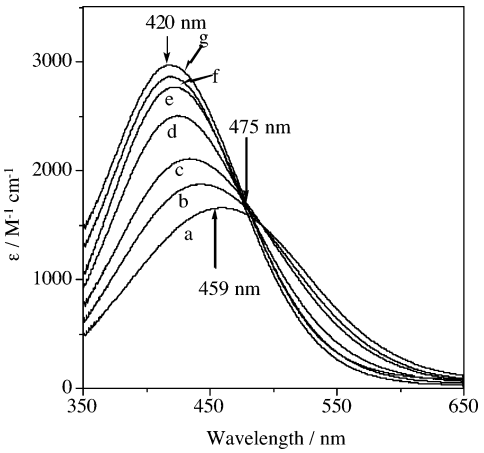

Figure 1. UV−vis spectral changes of Fe^{III}TAGE under various pH conditions: (a) pH 2.4, (b) pH 2.9, (c) pH 3.1, (d) pH 3.6, (e) pH 4.1, (f) pH 7.2, (g) pH 7.5. Conditions: $[Fe^{III}] = 2.8 \times 10^{-5}$ M, [TAGE] = 2.9×10^{-5} M, ionic strength = 0.1 M $NaClO_4$, cell length = 50 mm.

The ^{1}H NMR spectrum of TAGE also exhibited C_3 symmetry in D_2O and DMSO-d_6. The signals corresponding to the amide and aminohydroxyl protons were not detected because of the fast NH/ND exchange reaction. For the Ga(III) complex of TAGE, the four ethylene protons of the TREN anchor, which are not detected in DMSO-d_6 because of overlap with the solvent peaks, are observed at 2.43, 2.58, 2.76, and 3.66 ppm as nonequivalent signals, and the methylene proton signals of the glycyl moiety are split with an AX pattern as well as in DMSO-d_6. Interestingly, the spectrum of the Ga(III) complex measured immediately after the preparation in D_2O clearly indicated the amide proton peak at 8.20 ppm, which disappeared when the sample stood for a few minutes at room temperature. This finding indicates that the amide proton is more inert for the NH/ND exchange reaction than the ligand TAGE, suggesting that the amide protons are well shielded from attack of the solvent. Thus, the intramolecular hydrogen bonding networks in the Fe^{III}TAGE complex, as shown in the crystal structure,[13] are maintained even in polar solvents such as DMSO and D_2O.

Complexation Behavior of Fe(III)−Artificial Siderophore Compound Systems in an Aqueous Solution. The complexation behavior of ferric artificial siderophores was studied using UV−vis spectral measurements as a function of pH. The ferric hydroxamate complexes give a characteristic ligand-to-metal charge transfer (LMCT) band in the visible region, which is characteristic of the number of hydroxamate groups bound to the iron(III) ion.[18] The UV−vis spectra of Fe^{III}−TAGE and Fe^{III}−TAPE systems under several pH's in an aqueous solution are represented in Figures 1 and 2, respectively.

In the Fe^{III}−TAGE system, the UV−vis spectra under an acidic condition (pH 2.4) showed the absorption band corresponding to a bis(hydroxamato)iron(III) complex at 459 nm. Increasing the pH from 3 to 5 causes a blue shift of the band with an isosbestic point at 475 nm, and a new absorbance maximum at 420 nm (ϵ_{max} = 2860 M^{-1} cm^{-1}) at pH 4. This observation is indicative of formation of a tris(hydroxamato)iron(III) complex (Table 1). This finding indicates that the conversion from the bis(hydroxamato)-

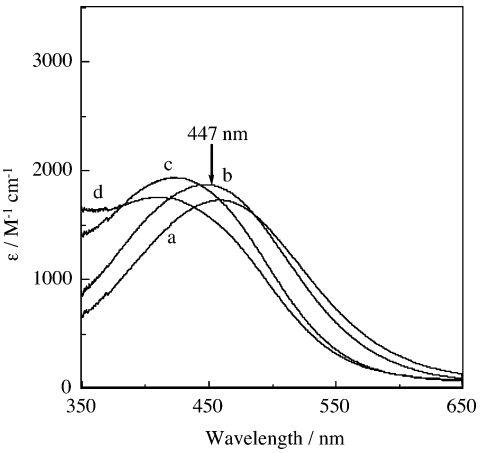

Figure 2. UV−vis spectral changes of Fe^{III}TAPE under various pH conditions: (a) pH 2.4, (b) pH 4.2, (c) pH 6.7, (d) pH 7.5. Conditions: $[Fe^{III}] = 2.8 \times 10^{-5}$ M, $[TAPE] = 3.0 \times 10^{-5}$ M, ionic strength = 0.1 M $NaClO_4$, cell length = 50 mm.

iron(III) complex to the tris(hydroxamato)iron(III) complex occurs in this pH range. The absorption band of the tris(hydroxamato)iron(III) complex is maintained from pH 4 to 8 for the Fe^{III}−TAGE system, whose behavior is similar to other synthetic trihydroxamates.[26] However, this range is narrow in comparison with those of natural trihydroxamate siderophores[5a,19] (pH 2−11) and an artificial siderophore TRENDROX (pH > 2).[10a] The difference in complexation behavior between TAGE and natural trihydroxamates could be explained as follows: the tertiary amine of the TAGE ligand is protonated under acidic conditions (pH 2) (Table 2). As shown in the crystal structure of Fe^{III}TAGE,[13] the lobe of the lone pair of electrons of the tertiary amine and the three amide protons for TAGE are directed toward the inside of the molecule, suggesting that protonation at the tertiary amine nitrogen would impede the formation of the tris(hydroxamato)iron(III) complex because of the steric and electrostatic repulsions. By contrast, under more basic conditions (>pH 8), such intramolecular hydrogen bonding networks may be difficult to maintain because of intermolecular hydrogen bonds by surrounding hydroxyl ions. It is also clear from the fact that the iron(III) complex of TAPE, which is the TAGE analogue bearing poor intramolecular hydrogen bonds, cannot form a stable tris(hydroxamato)iron(III) complex as described above. Actually, the UV−vis spectra of the iron(III) complex with TAPE exhibited the characteristic absorption band suggesting formation of a bis(hydroxamato)iron(III) complex in the range pH 2−7. Over pH 7, the formation of a tris-type complex has been barely observed.

Although TRENDROX, which has structure similar to TAGE and TAPE, can form a stable Fe(III)−tris(hydroxamato) complex even in acidic conditions, the neutral ferric-TRENDROX starts to precipitate above pH 3 because of the hydrophobic *p*-tolyl groups of its molecule.[10a] However, the neutral ferric TAGE complex without such a hydrophobic substituent is soluble even at neutral pH. The higher water solubility of the ferric TAGE complex might be advantageous for the biological availability.

Protonation and Stability Constants. Protonation constants (pK_n) were evaluated via potentiometric titration. The titration data were treated with SUPERQUAD,[16] and the resultant protonation constants of TAGE are listed in Table 2.

The three basic constants observed in the range pH 7−9 were assigned to the protonation constants of the three hydroxamate groups, which are almost identical in magnitude with those of ferrichrome[24d] and its analogues with three hydroxamate groups[10a,25,26c] (Table 2). The average value of the protonation constants calculated for the hydroxamate groups of TAGE, $\log K = 8.93$, is in excellent agreement with that of the monomeric hydroxamate compound, *N*-methylacetohydroxamic acid (*N*-methylAHA) ($\log K = 8.95$).[20] The difference in successive protonation constants is nearly equal to 0.9 log units, which is larger than the statistical value ($\log 3 = 0.48$),[21] indicating that the protonation of one hydroxamate group influences those of the others. The constant obtained in the most acidic region ($\log K_4$) has been assigned to that of the tertiary amine, which is consistent with that of a tri-acetylated TREN-type compound, TRENAC ($pK_a = 5.98$).[22] The lower basicity of the amine in comparison with a simple amine must be attributed to the five-membered ring formed by hydrogen bond between the amine nitrogen and the amide N−H hydrogen atoms. These results suggest that a weak hydrogen bond occurs between the strands and tertiary amine even in metal-free ligand.

The stability constant was spectrophotometrically estimated for Fe^{III}−TAGE system by means of the competition reaction with *N*,*N*,*N*′,*N*′-ethylenediaminetetraacetic acid disodium salts (Na_2H_2EDTA) at pH 6.1. The competition reaction is defined as shown in eq 1.

$$Fe^{III}TAGE + H_2EDTA^{2-} + H^+ \overset{K_{eq}}{\rightleftharpoons} Fe^{III}EDTA^- + H_3TAGE \quad (1)$$

Here, the species other than Fe^{III}TAGE did not exhibit such an absorption band in the pH region examined. Upon an increase in the concentration of EDTA, the absorption band at 420 nm gradually decreased with an isosbestic point at 354 nm, indicating that the reaction proceeds without other side reactions as defined in eq 1. The overall formation

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Table 1. Coordination Properties of Artificial and Natural Siderophores

	λ_{max} (nm)	ϵ_{max} (M^{-1} cm^{-1})	log β	pM[k]	ligand-exchange rate k_{obs}(s^{-1})	iron removal rate k_1, k_2 (min^{-1})[m]	$E_{1/2}$ (mV vs NHE)
Fe^{III}TAGE	420[i]	2860[i]	28.6(3)[j]	25	6.7×10^{-4} [l]	2.8×10^{-2}, 6.0×10^{-3}	−230[g,n]
ferrichrome[a]	425	2895	29.1	25.2	6.1×10^{-4} [d]		−400[h]
ferrioxamine B[a]	428	2800	30.5	26.6	0.71×10^{-5} [e]	3.8×10^{-2}, 9.0×10^{-3} [f]	−468[h]
Fe^{III}TRENDROX[b]	442	2934	32.9	27.8			
Fe^{III}−ligand **6**[c,o]	425	2880	26.4	22.5			
Fe^{III}−ligand **3**[d,p]	425	2800	28	24	2.7×10^{-2}		

[a] Reference 3. [b] Reference 10a. [c] Reference 25. [d] Reference 26c. [e] Reference 26b. [f] In this work. [g] Reference 13. [h] Reference 6. [i] Conditions: pH 7, ionic strength $I = 0.1$ M $NaClO_4$. [j] Conditions: pH 6.1, $I = 0.1$ M $NaClO_4$, 25 °C. [k] The pM value is estimated under the experimental condition that the concentrations of the Fe(III) ion (C_{Fe}) and ligand (C_L) are 1.0 and 10.0 μM, respectively, at pH 7.4. [l] Conditions: pH 5.3 (0.01 M acetate buffer), $I = 0.1$ M $NaClO_4$, 25 °C. [m] Conditions: pH 7.4 (0.01 M HEPES buffer), $I = 0.1$ M KNO_3, 25 °C. [n] Conditions: pH 7, surpporting electrolytes 0.1 M $NaClO_4$. [o] Ligand **6**: $CH_3C[CH_2O(CH_2)_3N(OH)COCH_3]_3$. [p] Ligand **3**: [Ac-Ala-Ala-β(HO)Ala-Ala-$NHCH_2CH_2$]$_3$N.

Table 2. pK_n's of Artificial and Natural Siderophores

	log K_1	log K_2	log K_3	log K_4
TAGE[a]	9.790(3)	8.948(3)	8.066(3)	5.459(3) (NR_3)
deferriferrichrome[b]	9.83	9.00	8.11	
desferrioxamine B[c]	10.79 (RNH_2)	9.70	9.03	8.39
TRENDROX[d]	10.30	9.33	8.58	6.51 (NR_3)
ligand **6**[e,h]	9.69	9.02	8.39	
ligand **3**[f,i]	9.69	9.03	8.26	
N-methylAHA[g]	8.95			

[a] Conditions: 25 °C, ionic strength $I = 0.1$ M KNO_3. [b] Reference 24d. [c] Reference 24c. [d] Reference 10a. [e] Reference 25. [f] Reference 26c. [g] Reference 20. [h] Ligand **6**: $CH_3C[CH_2O(CH_2)_3N(OH)COCH_3]_3$. [i] Ligand **3**: [Ac-Ala-Ala-β(HO)Ala-Ala-$NHCH_2CH_2$]$_3$N.

constant in the equilibrium eq 2 is given in eq 3 on the basis of material balances and the absorbance changes

$$Fe^{3+} + TAGE^{3-} \overset{\beta}{\rightleftharpoons} Fe^{III}TAGE \qquad (2)$$

$$\beta_{FeTAGE} = \left(\frac{Z}{1-Z}\right)\left(\frac{C_{EDTA} - (1-Z)C_{Fe}}{C_{TAGE} - ZC_{Fe}}\right)\left(\frac{\alpha_{TAGE}}{\alpha_{EDTA}}\right)\beta_{FeEDTA} \qquad (3)$$

$$\left(Z = \frac{A - A_E}{A_0 - A_E}\right)$$

where A = absorbance of the competing system in the equilibrium state, A_E = absorbance of $Fe^{III}EDTA^-$, A_0 = initial absorbance of Fe^{III}TAGE, α_{TAGE} and α_{EDTA} [23] are the side reaction coefficients for protonations of TAGE and EDTA, respectively, C_{EDTA}, C_{TAGE}, and C_{Fe} are the total concentrations of EDTA, TAGE, and Fe(III) ion, respectively, and β_{EDTA} is the stability constant of $Fe^{III}EDTA^-$. Since $Fe^{III}EDTA^-$ does not show any significant absorption at 420 nm, the term of A_E is negligible. The protonation and stability constants of EDTA are obtained from the literature[24a,b] (log $K_{1EDTA} = 10.19$, log $K_{2EDTA} = 6.13$, and log $\beta_{EDTA} = 25.1$). The stability constant of Fe^{III}TAGE (log β_{FeTAGE}) using eq 3 was calculated to be 28.6(3), which is comparable to those of ferrichrome (log $\beta = 29.1$)[3] and other synthetic trihydroxamates (log $\beta \approx 26-32$)[10a,25,26] (Table 2). This value, however, is lower than that of TRENDROX (log $\beta = 32.9$),[10a] which is supposed to be attributed to the difference in the hydroxamate substituent groups of both ligands. Because TRENDROX has a *p*-tolyl group that strongly stabilizes the resonance state of the hydroxamate anion,[6] the methyl groups of the hydroxamates in TAGE do not contribute to stabilization of the resonance structure of hydroxamate anion. Therefore, the high stability of Fe^{III}TAGE without such a *p*-tolyl group might be attributed to the intramolecular hydrogen bonding networks around the iron(III) ion.

The stability constant is very useful to compare the overall thermodynamic stability of tris(hydroxamato)iron(III) complexes. However, it does not provide an evaluation of the binding strength of the Fe(III) ion under specified or physiological conditions, because the competition of the Fe(III) ion with a proton is not negligible. Therefore, the pM value (=−log[$Fe^{3+}(H_2O)_6$]) has been adopted in order to estimate the binding strength of the Fe(III) ion under specified conditions.[3] The pM value for Fe^{III}TAGE is given by eq 4 and was determined under the experimental condition that the concentrations of the Fe(III) ion (C_{Fe}) and ligand (C_L) are 1.0 and 10.0 μM, respectively, at pH 7.4.

$$pM = -\log[Fe^{3+}] = -\log\left(\frac{C_{Fe}}{\frac{C_L\beta_{FeL}}{\alpha_L} + 1}\right) \qquad (4)$$

The pM value obtained is listed in Table 1. The pM of Fe^{III}TAGE (25) is equivalent to that of ferrichrome (25.2).[3] This value is slightly larger in comparison with those of other ferric trihydroxamato complexes except for TRENDROX (Table 1).[25,26] This result suggests that the ligand TAGE can strongly bind to the Fe(III) ion even in a neutral aqueous solution.

Kinetics. In order to investigate the kinetic lability of Fe^{III}TAGE, ligand exchange reactions with EDTA (20 times excess) were performed at pH 5.3 by UV−vis spectroscopy. The UV−vis spectral changes of Fe^{III}TAGE after addition of EDTA are shown in Figure 3. The characteristic absorption band at 420 nm, which is characteristic of an iron(III)−tris(hydroxamato) complex, decreases with an isosbestic point at 350 nm, indicating that the iron removal from Fe^{III}TAGE by EDTA proceeds according to eq 1.

The observed pseudo-first-order rate constant (k_{obs}) of Fe^{III}TAGE has been estimated to be 6.7×10^{-4} s^{-1} (Table 1). This value is comparable to that of the natural siderophore complex, ferrichrome, as measured under the same conditions (Table 1), which indicates that the complex is 10−1000 times more inert than the previously reported ferric artificial siderophore complexes.[26] These findings suggest that in-

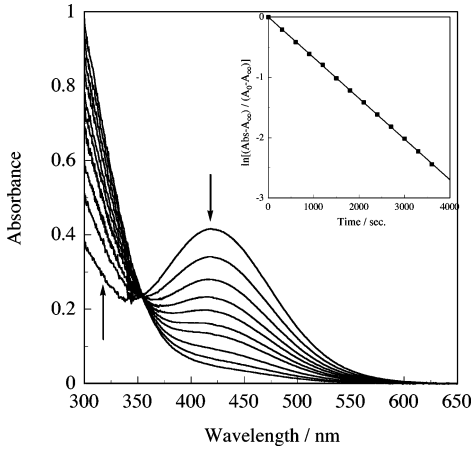

Figure 3. Time course of UV−vis spectra for the reaction of Fe^{III}TAGE with a 20-fold excess of EDTA. The inset shows the plot of ln[(Abs − A_∞)/(A_0 − A_∞)] vs time on ligand exchange reaction of Fe^{III}TAGE with EDTA, where A_0 and A_∞ are initial and final absorbances, respectively. Conditions: [Fe^{III}TAGE] = 9.8×10^{-5} M, [EDTA] = 2.0×10^{-3} M, ionic strength = 0.1 M $NaClO_4$, pH 5.3 (0.01 M acetate buffer), cell length = 20 mm, temperature = 25 °C.

tramolecular hydrogen bonds are important for formation of inert iron(III) complexes. A linear relationship was found between k_{obs} and the concentrations of EDTA (Figure S2), the second-order rate constant of which was 0.34 M^{-1} s^{-1}, indicating a simple bimolecular reaction. In contrast, the iron release reaction from ferrioxamine B to EDTA has exhibited saturation kinetics for high concentrations of the ligand.[27] These observations suggest that the more inert Fe^{III}TAGE complex results from limited access of EDTA rather than pre-equilibrium between Fe^{III}TAGE and EDTA. In fact, it is known that the iron release from transferrin is kinetically slow because of the closed conformation of the apo-protein wrapping the iron ion, although its thermodynamic stability constant (log $\beta \approx 20$)[28] is lower than that of $Fe^{III}EDTA^-$ (log β = 25.1).[24] As shown in the crystal structure of Fe^{III}TAGE,[13] the iron(III) ion is wrapped in the tripodal strands fixed by the intramolecular hydrogen bonding networks. Therefore, the lesser lability of Fe^{III}TAGE may result from the fact that the central metal ion is protected from attack of EDTA by the TAGE ligand.

Next, the iron removal reaction from the iron-containing compounds is also one of the important functions expected for artificial siderophores. In order to investigate the functionality as the iron removal agent, the iron removal reactions from bovine plasma transferrin (Fe_2Tf) as an iron containing compound by TAGE were studied by UV−vis spectroscopy.[9] As shown in Figure 4, the absorption band around 420 nm, which indicates the formation of the iron(III)−tris(hydroxamato) complex, increased with time. Initially, the isosbestic point was observed at 355 nm, and later another one was detected at 363 nm. This result indicates that the iron removal reactions from transferrin by

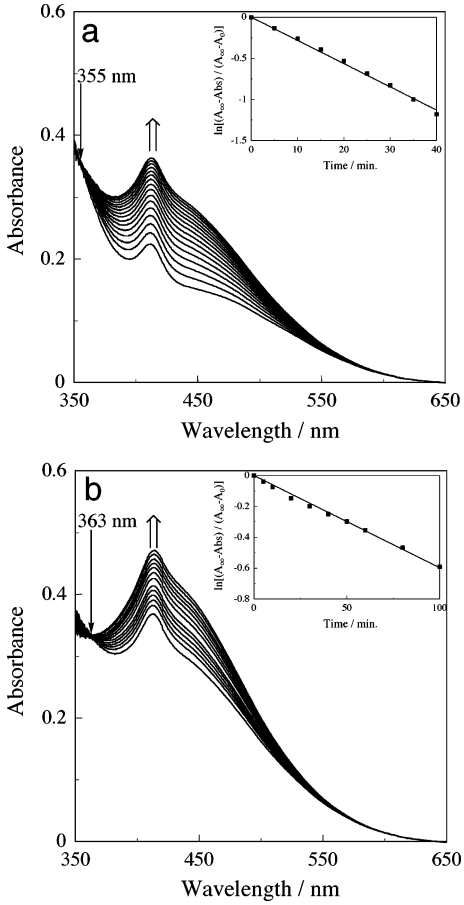

Figure 4. Time course of UV−vis spectra for the reaction of Fe^{III}_2Tf with a 20-fold excess of TAGE. The inset shows the plot of ln[(A_∞ − Abs)/(A_∞ − A_0)] vs time on the iron removal reaction from Fe^{III}_2Tf by TAGE, where A_0 and A_∞ are the initial and final absorbances in each reaction, respectively. Conditions: [Fe^{III}_2Tf] = 3.3×10^{-5} M, [TAGE] = 6.7×10^{-4} M, ionic strength = 0.1 M KNO_3, pH 7.4 (0.01 M HEPES buffer), cell length = 10 mm, temperature = 25 °C.

this artificial siderophore proceed in the successive two step reactions.

$$Fe_2Tf + L \xrightarrow{k_1} FeTf + FeL$$

$$FeTf + L \xrightarrow{k_2} Tf + FeL$$

The two observed pseudo-first-order rate constants, k_1 and k_2, at pH 7.4 and 25 °C were 2.8×10^{-2} and 6.0×10^{-3} min^{-1}, respectively. These rate constants are comparable to those for a natural siderophore desferrioxamine B (DFO B, 3.8×10^{-2} and 9.0×10^{-3}, respectively) under the same conditions (Table 1), suggesting that TAGE is able to abstract iron from the iron sources such as transferrin in the environment. Transferrin has two iron-binding sites at the C- and N-terminal lobes. Generally, the binding ability of C-terminal lobe is larger than that of the N-terminal. On the basis of the above structural information, the faster (k_1) and slower (k_2) reactions may correspond to the releasing rates of iron ions from N- and C-terminal lobes, respectively.

Biological Activity. The growth-promoting activity of Fe^{III}TAGE was studied using the siderophore-auxotroph gram-positive bacterium *Microbacterium flavescens*, which

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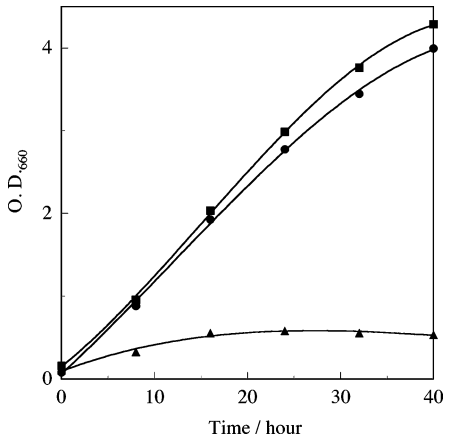

Figure 5. Growth curves of *M. flavescens* in liquid media, as followed by monitoring the increase in optical density at 660 nm (▲, no siderophore; ●, 10 μM ferrichrome; ■, 10 μM Fe^{III}TAGE). Conditions: temperature = 30 °C, shaking speed = 100 rpm.

is known not to be able to synthesize any siderophore by itself and can use only hydroxamate-type siderophores for the growth. *M. flavescens* in the siderophore-deficient medium did not grow even in the presence of excess iron. By contrast, in the presence of 10 μM Fe^{III}TAGE, the microbe grew with the growth rate similar to that in the ferrichrome-containing media (Figure 5). Interestingly, TAGE as well as Fe^{III}TAGE showed the growth promoting activity for *M. flavescens*, suggesting that TAGE functioned in the same manner as a natural siderophore. We previously reported that Fe^{III}TAGE exhibited a much higher redox potential value (−230 mV vs NHE)[13] relative to that of natural siderophores (Table 1) and that the high redox potential of Fe^{III}TAGE is attributed to the intramolecular hydrogen bonding networks.[13] However, the influence of the redox potentials of the complexes on biological activity was not observed despite the difference in the redox potentials between them; Fe^{III}TAGE and ferrichrome did not express different growth rates. This may be explained as follows: Since Fe^{III}TAGE tends easily to be reduced to iron(II), the inefficient recognition of the iron(III) complex and/or its transportation might be overcome by quick reduction of the complex, or only the iron ion might be taken up into the cell through the reduction of the iron(III) complex on the cell surface as can be seen in fungi. These results suggest that the TAGE compound is a good functional model for ferrichrome.

Conclusion

With a view to understanding the effect of intramolecular hydrogen bonding networks in the natural siderophores with hydroxamate groups, the artificial siderophore TAGE with such networks was synthesized. Also, it was shown that the intramolecular hydrogen bonding networks, as previously demonstrated in the crystal structure of Fe^{III}TAGE,[13] are formed even in aqueous and DMSO-d_6 solutions using Ga^{III}TAGE by ^{1}H NMR. TAGE complexes iron(III) ion as a tris-hydroxamato complex with a 1:1 ratio in the range pH 4−8 in solutions, as measured by UV−vis spectroscopy. By contrast, TAPE, the TAGE analogue bearing poor intramolecular hydrogen bonds, is unlikely to form a tris-hydroxamato complex at pH 7. Furthermore, it was demonstrated that the thermodynamic and kinetic properties of Fe^{III}−TAGE system are similar to those of natural siderophores and other synthetic trihydroxamates. These results indicate that TAGE has a higher affinity for an iron(III) ion than an iron(II) ion, although the redox potential of Fe^{III}TAGE (−230 mV vs NHE)[13] is nearly 200 mV higher than those of ferric natural trihydroxamates. TAGE also promoted the growth of the siderophore-auxotroph gram-positive bacterium *Microbacterium flavescens*, indicating that TAGE has the siderophore function comparable to the natural ferrichromes. These results indicate that the artificial siderophore TAGE is a good structural and functional model for ferrichrome, and that the intramolecular hydrogen bonding networks stabilize the iron(III) complex and increase the redox potential even in an aqueous solution. These observations highlight the importance of intramolecular hydrogen bonding networks for the coordination properties of the iron(III) complex.

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Supporting Information Available: Additional figures. This material is available free of charge via the Internet at http://pubs.acs.org.

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