Coordination Chemistry of Microbial Iron Transport Compounds. 10.[1] Characterization of the Complexes of Rhodotorulic Acid, a Dihydroxamate Siderophore

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Abstract: The Fe^{3+}, Al^{3+}, and Cr^{3+} complexes of the fungal iron chelator, rhodotorulic acid (H_2RA), have been isolated and characterized. The iron complex exists as a dimer of formulation, Fe_2RA_3, at pH values from 4 to 11. Below pH 4 the dimer dissociates into a monomeric cationic species, $[FeRA]^+$, in which both of the hydroxamate groups of the RA are coordinated to a single ferric ion. The ferric ions in the dimer are each octahedrally coordinated by the hydroxamate groups of three RA ligands; the absolute configuration about each ferric ion has been determined to be Δ-cis.

Introduction

For aerobic organisms the availability of iron, an essential element for growth, is limited by the pronounced insolubility of ferric hydroxide ($K_{sp} \simeq 10^{-39}$)[2] and the formation of high molecular weight hydroxy-bridged ferric polymers well below physiological pH. This engendered the evolution of specific chelating agents for ferric ion, called siderophores.[3-5] These low molecular weight multichelate compounds are iron transport agents which form octahedral high-spin complexes of Fe(III), usually using hydroxamate or catecholate functional groups.[3,5]

The pathogenicity and virulence of certain microbial infections has been found to be associated with iron transport in these organisms, and several siderophores have been found to be potent, broad-spectrum antibiotics.[6,7] In all cases, the biological activity of the siderophores centers on their unique ability to chelate iron and, following chelation, to take up the iron via a specific cellular transport system.

Among the hydroxamate siderophores are the ferrichromes and ferrioxamines.[3] The ferrichromes are produced by fungi and are composed of a cyclic hexapeptide of which three amino acid residues (N^{δ}-acyl-N^{δ}-hydroxyl-*l*-ornithines) terminate with hydroxamic acid groups. The ferrioxamines are produced by bacteria of the *Nocardia* and *Streptomyces* families and are made up of repeating units of *l*-amino-ω-hydroxyaminoalkane and succinic or acetic acid.

A third type of hydroxamate siderophore, and one of the more unusual natural iron chelators, is the dihydroxamate, rhodotorulic acid (H_2RA) (Figure 1). This cyclic dipeptide or diketopiperazine of δ-*N*-acetyl-L-(*S*)-δ-*N*-hydroxyornithine has been isolated from low iron cultures of *Rhodotorula pilimanae* and related yeasts.[8] This siderophore is unique in several respects. First, it contains only two hydroxamate groups per molecule, making it unable to satisfy the six coordination of iron as a simple one to one complex. Second, the biosynthesis of the compound by the yeast proceeds at an amazingly high level under conditions of low iron stress—ultimately 40–50% of the nitrogen in the media is incorporated into H_2RA, and yields of 3–4 g/L are often obtained.[8] The presence of two hydroxamate groups in H_2RA, its siderophore activity in the Arthrobacter assay, and the repression of its biosynthesis by iron all suggest an iron transport role for this substance, although direct proof of this contention has been lacking until recently.[9] Very little of the coordination chemistry of H_2RA is known. It has been suggested that the iron complex is polymeric, based on an apparent molecular weight, via gel filtration, of several thousand.[8]

Rhodotorulic acid is both one of the more promising potential antidotes for acute iron poisoning and a potential therapeutic agent in the treatment of Cooley's anemia or β-thalassemia major, for which H_2RA is presently undergoing limited clinical tests.[10] However, neither the composition of the ferric-RA complex(es) nor a measure of the affinity of RA for ferric ion is known.

This research was begun in order (1) to characterize the complexes of ferric ion formed by H_2RA, including both their stoichiometry and geometry, and (2) to determine the affinity of RA for ferric ion, from titrimetric and electrochemical data. Both types of information are necessary to characterize the role of H_2RA in iron mobilization in microbes or in man. This paper reports the characterization of the ferric RA complex formed at physiological pH, a dimer, $Fe_2(RA)_3$, and a second complex, $[Fe(RA)]^+$, which forms in acidic media. The spectroscopic and other physical properties of these complexes are presented and are used to assign the structure and absolute configuration of the ferric RA complexes. These results are contrasted with those for ferrichrome A,[11] ferrichrysin,[12] and mycobactin[13]—all of which have been characterized as Λ-cis by single-crystal x-ray diffraction and CD spectra. The formation constants and electrochemical behavior of the RA complexes[14] and the role of H_2RA in uptake of iron in yeasts[9] will appear in subsequent papers.

Experimental Section

Ultraviolet–visible spectra were obtained with a Cary Model 118 spectrophotometer, and circular dichroism (CD) spectra were measured with a Jasco J-20 automatic recording spectropolarimeter. NMR spectra were recorded in D_2O or trifluoroacetic acid on a Varian T-60 spectrometer using tetramethylsilane or sodium 2,2-dimethyl-2-silapentane-5-sulfonate as a standard. Infrared spectra were obtained as KBr pellets on an Perkin-Elmer spectrometer, Model 283. Chemical analyses were performed by the Microanalytical Laboratory, Department of Chemistry, University of California, Berkeley, Calif.

Materials. Reagent grade chemicals were used throughout. $CrCl_3 \cdot 3THF$ was prepared by literature methods.[15] Rhodotorulic acid was isolated from low iron cultures of *R. pilamanae* as described by Neilands.[8] The H_2RA so obtained was crystallized at least three times from hot water. Yields from 4 L of media averaged 4 g. Gel filtration was performed on Bio-Gel P-2 (200–400 mesh, Bio-Rad Laboratories). Ion exchange chromatography was performed on the cation exchange resins AG 50W-X8 (200–400 mesh) or Bio-Rex 70 (200–400 mesh, sodium form) and anion exchange resin AG-1 X-4 (200–400 mesh); all are products of Bio-Rad Laboratories.

Thin Layer and Paper Chromatography. Baker silica gel G/HR was used for TLC and column chromatography of the chromium complexes. The solvent system was 70% methanol/water. Paper chromatography on Whatman no. 1 was performed on all complexes using several solvent systems. The one found best for general use was 1-butanol/1-propanol/water (1:1:1). Spots were detected visually, stained with iodine vapor or $FeCl_3$ in methanol.

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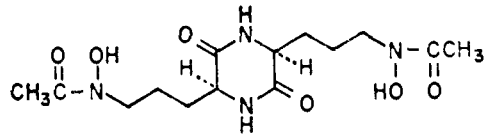

Figure 1. The structure of rhodotorulic acid.

Paper Electrophoresis. Paper electrophoresis was performed in the laboratory of Dr. J. B. Neilands, Department of Biochemistry, University of California, Berkeley, Calif. Complexes were spotted on Whatman no. 1 paper and run at 16 mA, 2000 V dc, for 45 min using phosphate (pH 6.9) or formate (pH 1.96) buffers. Ferrichrome A was used as a standard and glucose as a marker for the endosmotic flow.

Magnetic Susceptibility. The magnetic susceptibility was determined in solution by the Evans method[16] over a pH range of 1–10.

Molecular Weight Determinations. Molecular weight determinations were done via gel filtration on Bio-Gel P-2 using 0.1 M Na_2CO_3 (pH 11) or 0.1 M HCl (pH 1.1) as eluents. A flow rate of 1 mL/min was used and elution volumes were calibrated by using blue dextran (Pharmacia) and *l*-[Co(EDTA)]$^-$.

Ferric Rhodotorulate (Fe_2RA_3). One millimole (0.344 g) of rhodotorulic acid was dissolved in 20 mL of hot H_2O. To this was added an excess of freshly precipitated and carefully washed $Fe(OH)_3$. The mixture was stirred and kept at ~80 °C for 2 h, during which time most of the ferric hydroxide dissolved and the solution turned a dark orange-red. The solution was filtered through paper and then membrane filtered (Unipore, 1 μm, Bio-Rad Laboratories) to remove colloidal $Fe(OH)_3$ and polymers. The pH of the resulting clear orange-red solution was 6.8. Following concentration by rotary evaporation, the material was precipitated from solution with acetone. After the precipitate was allowed to stand at 0 °C overnight, the fluffy, orange solid was removed by filtration and dried over P_2O_5 under vacuum for 24 h: yield 0.298 g (79%); mp 227 °C. (The complex decomposes almost explosively.) The resulting orange solid gave a ratio of ligand to metal of 1.48 ± 0.03 (average of six analyses); the expected ratio for Fe_2RA_3 is 1.50.

Anal. Calcd for $C_{42}H_{66}N_{12}O_{18}Fe_2 \cdot H_2O$: C, 43.61; H, 5.92; N, 14.53; Fe, 9.65. Found: C, 43.21; H, 5.93; N, 14.20; Fe, 9.99.

The iron complex showed only a single spot on paper chromatography in several solvents: R_f (1-butanol/1-propanol/H_2O, 1:1:1) 0.12.

Aluminum Rhodotorulate (Al_2RA_3). Aluminum hydroxide was freshly prepared from $Al(NO_3)_3 \cdot 9H_2O$ using dilute NaOH. The resulting gel was washed twice with H_2O and filtered. An excess of the above was mixed with 1/3 mmol (0.114 g) of RA in warm H_2O. The mixture was heated with stirring to 90 °C for 15 min. The excess $Al(OH)_3$ was removed and the solution was concentrated by rotary evaporation to a volume of 2 mL. The solution was then chromatographed on a Bio-Rex 70 column using water as an eluent. Those fractions which gave a positive test with $Fe(ClO_4)_3$ were combined and evaporated to dryness to yield 0.080 g (67%) of the aluminum complex. The clear solid is very hygroscopic and the analysis given is for the wet material.

Anal. Calcd for $C_{42}H_{66}N_{12}O_{18}Al_2 \cdot 9H_2O$: C, 40.61; H, 6.73; N, 13.53; Al, 4.34. Found: C, 40.87; H, 6.22; N, 13.49; Al, 4.17.

The aluminum complex appears to be pure by the observation of only a single spot on paper chromatography. The R_f value of the aluminum complex is similar to that of the iron.

Chromic Rhodotorulate. One-half gram of RA and 0.54 g of $CrCl_3 \cdot 3THF$ were dissolved in 50 mL of methanol. The solution immediately turned a bright green and after 1 h of stirring under reflux, 0.5 g of $NaHCO_3$ was added. The color changed to blue-green and the solution was kept at reflux for another 2 h. The mixture was allowed to stand overnight after which it was filtered and the complex was isolated from the filtrate and chromatographed on sodium cation exchange resin and then on Bio-Gel P-2 as described above. TLC on silica gel indicated a very impure mixture; so it was again chromatographed on silica gel G-HR using 70% MeOH/H_2O. Two bands were observed to separate, although much of the material remained fixed to the column. Two bluish-green fractions were collected and their visible and CD spectra recorded. The first fraction appeared to be pure and give a single spot on TLC with an R_f of 0.57. The second fraction had one main spot with R_f 0.34 and a trace with R_f 0.57. Both gave a single spot on paper chromatography (1-butanol/1-propanol/H_2O) with the same R_f as the iron complex. Although the solutions appeared to be pure, repeated attempts to isolate a solid for analysis were unsuccessful.

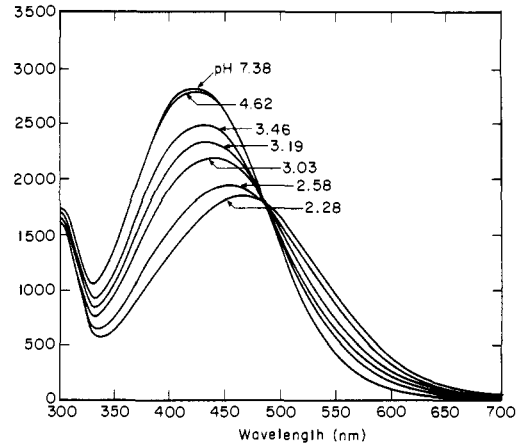

Figure 2. The visible absorption spectra of ferric rhodotorulate as a function of pH.

Results and Discussion

Ferric Rhodotorulate. Examination of the visible spectra of ferric rhodotorulate at pH values from 1 to 11 revealed changes previously described qualitatively by Neilands.[8] As the pH is raised from 1 to 11 the λ_{max} is observed to shift from 480 to 425 nm. This charge transfer band is typical of ferric hydroxamates but the pH dependence of the λ_{max} is not like that of the other naturally occurring siderophores. A sharp color change is observed from red to orange at about pH 3. No further changes are seen until approximately pH 10.5 when the color lightens to yellow. Above pH 12 the iron is observed to precipitate as the hydroxide. Analysis of Jobs plots[17] at pH 2.0, using the method of continuous variations, clearly revealed that the species with λ_{max} 480 nm (ϵ 1750) was a simple 1:1 complex between Fe^{3+} and RA. The same analysis at pH 6.8 did not give unequivocal results. The maximum in the plot did not correspond to an integral value of ligand:metal ratio nor was the plot symmetrical, suggesting a polynuclear complex at this pH. The simplest complex that fit the data was one with L/M ratio of 3:2. There was no evidence for a series of complexes other than the monomer and dimer. Examination of the visible absorption spectra at various pH values revealed a good isosbestic point at 488 nm indicating that only two absorbing species exist in the range pH 1 to 11 (Figure 2). At physiological pH ±3 the dimer is the predominant species; the monomer only becomes important at pH values below 3.

Further support for a dimeric formulation comes from estimations of molecular weight. At pH 1.1 an apparent mol wt of 407 was obtained; the calculated value for the monomer Fe(RA) is 398. At pH 11 the observed mol wt was 900; that calculated for the dimer Fe_2RA_3 is 1138. In addition, analytical data confirm the Fe_2RA_3 formulation, as do equilibrium titration data which will be reported in a subsequent paper.[14]

The dimeric complex, based on the formula Fe_2RA_3, should be neutral while the monomer could be either a mono- or dication (depending on the coordination of one or two hydroxamate groups). Electrophoresis and ion exchange chromatography confirm that the orange dimeric compound is indeed neutral. When the endosmotic flow is taken into account there is no net movement of the complex at pH 6.8 upon electrophoresis, nor is it retarded by either anion or cation exchange resins. At pH 1.1, however, the complex turns red and moves

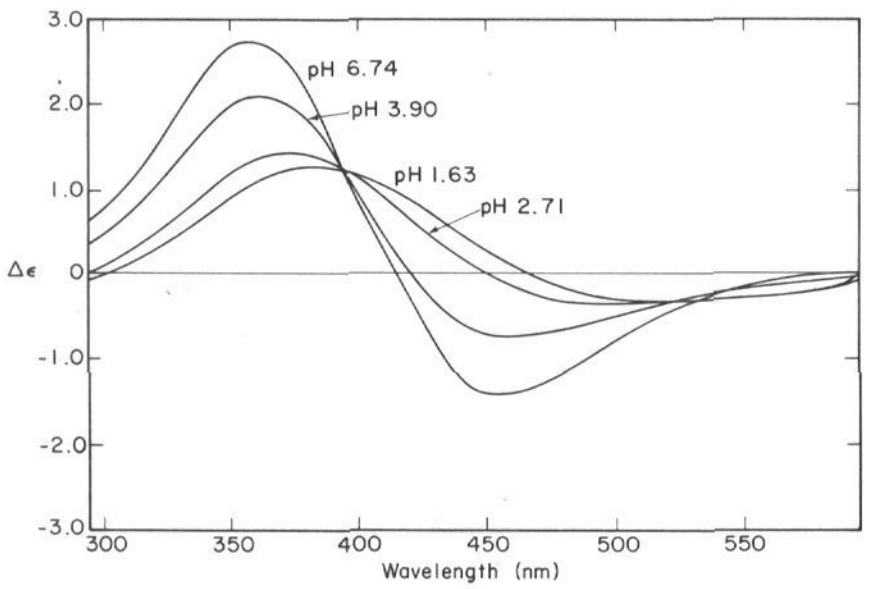

Figure 3. The circular dichromism spectra of ferric rhodotorulate as a function of pH.

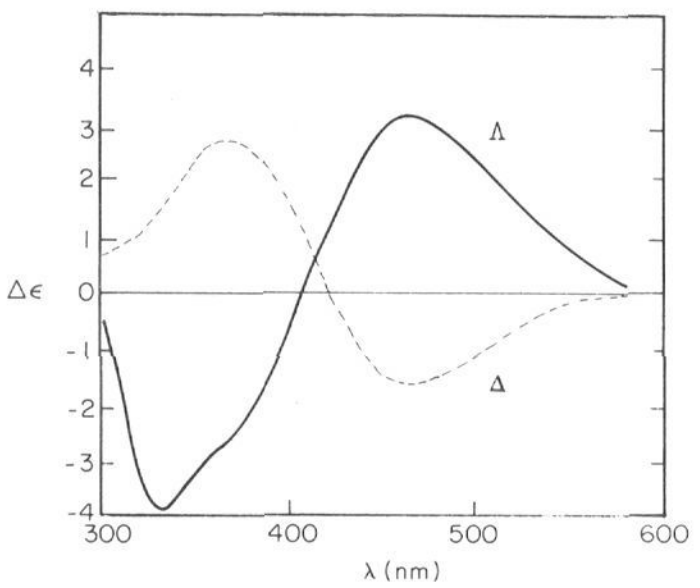

Figure 4. The circular dichromism spectra of ferrichrome (——) and ferric rhodotorulate (- - - -), at neutral pH.

as a monocation on paper electrophoresis and has an elution profile similar to that of monocations on AG 50 X8 resin.

Solution magnetic data indicate a value of $\mu = 6.0 \pm 0.1\ \mu_B$ at all pH values. The iron is thus high-spin iron(III) in both the monomer and dimer.

The circular dichroism spectra were also obtained as a function of pH (Figure 3). At neutral pH two CD peaks are observed: 464 (−1.41) and 372 nm (+2.73) ($\Delta\epsilon$). As the pH is lowered, the peak at 372 nm is reduced in intensity and shifts to longer wavelength so that at pH 1.9 λ_{max} is385 nm. The peak at 464 nm also shifts to longer wavelength. Ferrichrome A, which is a trihydroxamate iron(III) complex, crystallizes in the Λ-cis form, and has a positive CD band at 465 nm (in the region of the strong absorption at 440 nm). Likewise the Λ-cis isomer of tris(benzohydroxamato)iron(III) has been resolved and has a positive CD band at 455 nm (absorption band is at 435 nm).[18] Ferric RA has a *negative* CD band at 462 nm (absorption maxima at 425 nm) as does Δ-cis tris(benzohydroxamato)iron(III). The CD spectra of Fe_2RA_3 and ferrichrome A are compared in Figure 4. They are clearly enantiomorphic. Consequently we assign a Δ absolute configuration to both ferric ions in the neutral pH complex, Fe_2RA_3. This is the first example of the Δ isomer predominating in a hydroxamate siderophore. The close similarity of the CD spectra of the ferrichromes, the simple tris(benzohydroxamato)iron(III) complexes, and ferric rhodotorulate also establishes that each iron in Fe_2RA_3 is octahedrally coordinated by three hydroxamate groups. The structure of the proposed dimer is shown in Figure 5.

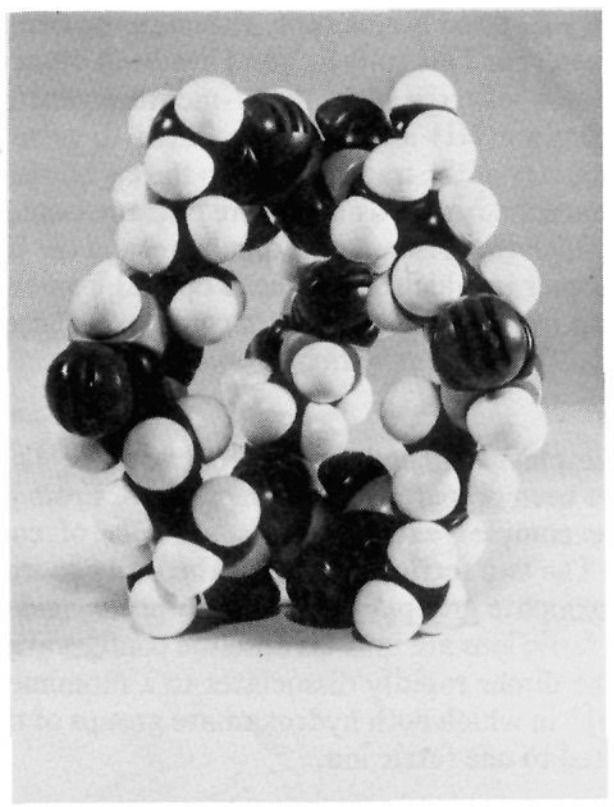

Figure 5. CPK model of the proposed structure of ferric rhodotorulate. The model has pseudo-threefold symmetry with each iron atom octahedrally coordinated by three hydroxamate groups.

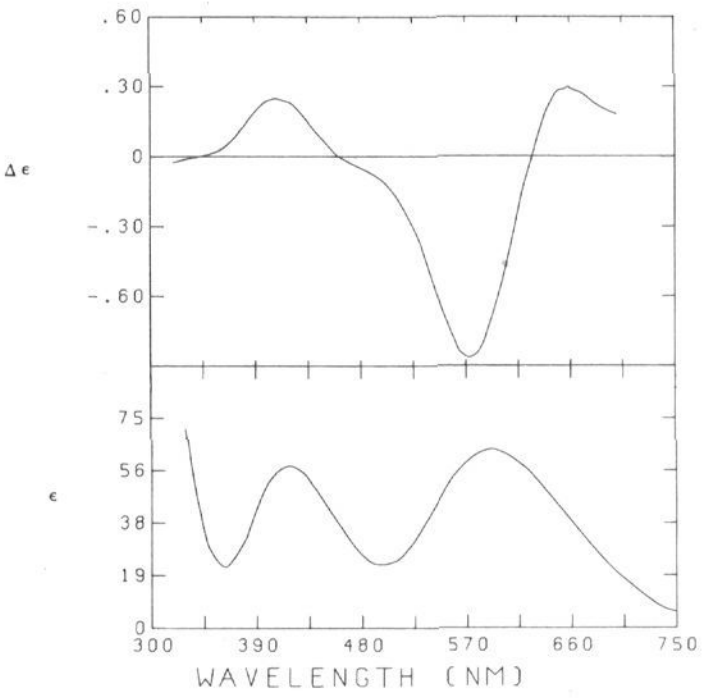

Figure 6. Circular dichromism and electronic adsorption spectra of one of the Δ-cis isomers of chromic rhodotorulate.

Chromic Rhodotorulate. Assuming that the Cr complex is isostructural with the iron chelate, three enantiomeric pairs of geometrical isomers of RA are possible. These are the Δ-cis,cis, Δ-cis,trans, and Δ-trans,trans, and their mirror images, the Λ optical isomers. Silica gel chromatography of the chromic rhodotorulate results in two blue-green bands. The complexes were found to be neutral via paper electrophoresis and ion exchange chromatography. The more abundant isomer, which elutes first, has a ratio of extinction coefficient maxima, λ_{max} 592/417 nm, of approximately one, while in the slower moving isomer this ratio is greater than one. The CD spectra of the isomer with the larger R_f has bands at λ_{max} 658 (+0.31), 573 (−0.92), 408 nm (+0.26) ($\Delta\epsilon$) (Figure 6). Based on analogy with the simple chromic hydroxamates[19-21] the band at 658 nm is assigned as the $^4A_2 \rightarrow {}^4A_1$ transition, the band at 573 nm as the $^4A_2 \rightarrow {}^4E_a$, and that at 408 nm as the $^4A_2 \rightarrow {}^4E_b$ transition. The bands in the second isomer are located at λ_{max} 662 (+0.37), 570 (−1.26), 450–410 nm (+0.39) ($\Delta\epsilon$), and the

high-energy E_b band is now split, although the two bands are not well resolved. This split E_b band has been observed in the trans isomer of tris(*N*-methyl-*l*-menthoxyacetohydroxamato)chromium(III) and was speculated to arise from the lower symmetry of the trans relative to the cis isomer. Thus we assign the second isomer as a mixture of trans isomers, both Λ and Δ in which the Δ isomers predominate, and the first isomer as a cis isomer in which again the Δ optical isomer predominates. Both iron and the chromium complexes therefore exist predominantly as the Δ *optical isomers.*

Summary

The complexes of rhodotorulic acid with Fe^{3+}, Al^{3+}, and Cr^{3+} have been prepared and characterized. From pH 4 to 10 the ferric complex exists only as a dimer of composition Fe_2RA_3. The two ferric ions are octahedrally coordinated by the hydroxamate groups of three rhodotorulic acid molecules and both ferric ions are in Δ-cis absolute configurations. Below pH 3.5 the dimer rapidly dissociates to a monomeric cation $[Fe(RA)]^+$ in which both hydroxamate groups of the RA are coordinated to one ferric ion.

Acknowledgment. We thank Professor J. B. Neilands for helpful discussions and the use of his electrophoresis equipment, and Mr. Kamal Abu-Dari for a gift of $CrCl_3 \cdot 3THF$. This research is supported by the NIH.

References and Notes

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The Relationship of Thermodynamic Data for Base Adduct Formation with Cobalt Protoporphyrin IX Dimethyl Ester to the Corresponding Enthalpies of Forming Dioxygen Adducts with Implications to Oxygen Binding Cooperativity

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Abstract: Enthalpies for the binding of a wide variety of axial bases to cobalt(II) protoporphyrin IX dimethyl ester, CoPPIXDME, have been determined by spectrophotometric titration methods. The enthalpies for the subsequent binding of dioxygen to the resulting base CoPPIXDME adducts have also been determined. For similar donor types, enthalpies of dioxygen binding are found to increase with the base-binding enthalpies. Implications of the above result to the complex problem of cooperative effects in hemoglobin are presented and discussed in terms of a "modified restraint theory". The *E* and *C* model has been successfully used to correlate enthalpies of base adduct formation to CoPPIXDME. We have shown theoretically and experimentally that the *E* and *C* equation can be extended to include the enthalpies of dioxygen binding enabling us to predict the O_2–cobalt bond strength for some 50 base adducts. The EPR spectra of several adducts have been investigated and interpreted in terms of the electron transfer model we proposed earlier.

Introduction

Understanding the processes whereby the reactivity of dioxygen is enhanced or inhibited by metal ions has important implications in fuel cell design, improving commercial catalytic oxidations, and understanding oxidations as well as oxygen transport in biological systems. The factors that influence the nature and strength of interaction between the metal and the dioxygen as well as the nature of the bound dioxygen are essential features for understanding the above problems. One of the key features regarding the nature of the bound dioxygen is the amount of metal electron density transferred into it upon coordination. This is an important property that is expected to influence the susceptibility of attack on dioxygen by nucleophiles or electrophiles. Variation of the metal and coordinated ligands is expected to have a pronounced effect on the electron density transfer into the bound dioxygen. In view of both the relative stability of the dioxygen adducts of cobalt(II) toward irreversible decomposition and the EPR probe provided by the existence of one unpaired electron in the molecule, most of the research in the area of dioxygen binding has involved this metal center.

We have recently proposed a spin-pairing model to account for the binding of dioxygen to a series of cobalt(II) complexes.[1]