Crystal Structure of Ferrichrome and a Comparison with the Structure of Ferrichrome A

Dick van der Helm,* J. R. Baker, D. L. Eng-Wilmot, M. B. Hossain, and R. A. Loghry

Contribution from the Department of Chemistry, The University of Oklahoma, Norman, Oklahoma 73019. *Received November 15, 1979*

Abstract: The ferric ionophore (siderophore), ferrichrome ($FeC_{27}H_{42}N_9O_{12}$), for the smut fungus, *Ustilago sphaerogena*, has been isolated from low-iron cultures, and purified by anion exchange and silica gel chromatography. The chelate was crystallized from an aqueous methanol solution equilibrated with methanol in the space group $P2_12_12_1$ ($Z = 4$) of the dimensions: $a = 16.255$ (18), $b = 29.571$ (15), and $c = 8.894$ (4) Å; $V = 4275.2$ Å^3 at -135 ± 2 °C; $\rho_{obsd} = 1.425$; $\rho_{calcd} = 1.322 - 1.420$ g/cm^3 (depending on the degree of solvation). Intensity data (4303 unique reflections) were collected on a CAD-4 automatic diffractometer at -135 ± 2 °C. The structure was solved by direct methods and Fourier syntheses. The final R value was 0.108 for the 3067 observed reflections. In the coordination sphere, six oxygen atoms of the N^{δ}-acetyl-N^{δ}-hydroxy-L-ornithine residues coordinate the central Fe^{3+} ion in a distorted octahedron; upon complexation, a minimum of 8000 cal of strain energy is imparted to the bond and conformational angles of the alkane portion of the three ornithine residues. The molecule assumes the Λ-cis absolute configuration in the crystalline state and in solution, as determined by anomalous dispersion and solution and single-crystal circular dichroism. In the cyclic hexapeptide ring, there is only one intramolecular hydrogen bond. Two of the six peptide nitrogen atoms are sterically accessible. Conformationally, ferrichrome and ferrichrome A are quite similar, although minor differences exist in the hexapeptide ring.

Introduction

The availability of iron to aerobic organisms is limited by the extreme insolubility of ferric hydroxide. This problem has been solved by these organisms through the production of siderophores (or siderochromes), chelating agents, which solubilize ferric iron and transport or facilitate the transport of the iron into bacteria and fungi.[1,2] Ferrichrome is one of the first siderophores discovered in nature and is perhaps the most studied compound of this type.

Ferrichrome was originally isolated[3a] in 1952, from the fungus *Ustilago sphaerogena*, and has subsequently been detected in cultures of *U. maydis*,[3b] *Aspergillus niger*,[3b] *A. quadricintus*,[3c] *A. duricaulis*,[3c] and *Penicillium resticolosum*.[3b] The chemical structure was proven[4] by degradation and partial synthesis, and later confirmed by a complete chemical synthesis[5a,b] and a single-crystal X-ray diffraction structure determination of ferrichrome A.[6] More recently, the structure determination of the closely related siderophore ferrichrysin[7] was reported, as was the chemical synthesis of *ent*-ferrichrome.[8]

Both ferrichrome and ferrichrome A are produced by the *U. sphaerogena*, but only the first is actively transported into the cell,[9] while the latter has the larger complexation constant for Fe(III). An extensive study has been made of the solution conformation of alumichrome, alumichrome A, and desferriferrichrome[10] using H, D, ^{13}C, and ^{15}N NMR experiments. In the interpretation of these spectra, considerable weight was given to the crystal structure and conformation of ferrichrome A.[6] The relevance of the interpretation of the NMR data with respect to ferrichrome is based on two assumptions: (a) that the conformations of ferrichrome and ferrichrome A are similar, and (b) that the conformations of the Al(III) and Fe(III) complexes are similar or the same. These assumptions were made plausible because of the similarity of both the complexation strengths of iron(III) and aluminum(III) hydroxamates,[11] and the NMR spectra of alumichrome and alumichrome A.[10b] We have attempted to verify point b by determining the structure of alumichrome A and reexamining the structure of ferrichrome A, both at low temperature.[12] The results show that the only significant difference between the two molecules is in the chelate rings. One of the reasons for the structure determination of ferrichrome was to examine the validity of point a, and at the same time obtain a better, more structural understanding of the differences in the transport properties of ferrichrome and ferrichrome A. The structure of ferrichrome is also of considerable importance in membrane physiology and molecular biology, as it has been firmly established[13a] that a number of enterobacteria, including most notably *Escherichia coli* K-12 and *Salmonella typhimurium* LT-2, have specific outer membrane receptors for ferrichrome, despite the fact that these microbes do not synthesize this siderophore. In *E. coli* K-12, this receptor, called *Ton* A, is also the receptor for bacteriophages T1, T5, and φ80, the ferrichrome-like antibiotic albomycin, and the protein, colicin M,[13a-e] and there is substantial evidence for competition between phage, protein, and siderophore for siderophore membrane receptors in other enteric bacteria. It is apparent, then, that each of these competitors possesses certain configuration features complementary with ferrichrome, which allows them to be accommodated by the membrane receptor.[13a]

Experimental Section

Production, Isolation, and Purification. A starter culture of *Ustilago sphaerogena* was obtained from the collection of Dr. Thomas Emery, Department of Chemistry and Biochemistry, Utah State University, Logan.

Axenic stock (50 mL) and production (500 mL) cultures were maintained in a modified minimal salts medium described elsewhere[14] at 24 ± 1 °C on a rotary incubator (New Brunswick Scientific). For siderophore production, the medium was deferriated prior to trace element, magnesium, and sucrose supplementation and sterilization, by passage through a bed of Chelex-100 chelating resin (Na^+ form, Bio-Rad Laboratories).

Ferrichrome and ferrichrome A were prepared in the cell-free growth medium from their respective ligands and isolated via extraction by the established procedures.[14]

Purification of ferrichrome was achieved in several stages of chromatography, using a modified procedure of Tadenum and Sato.[15] The aqueous extract was applied to an anion exchange column (Cellex D, Bio-Rad Laboratories). Ferrichrome A was exchanged, while ferrichrome was eluted with water. The exchanged species were then eluted with phosphate buffer, 0.1 M, pH 7. Fractions containing ferrichrome were pooled, lyophilized, and then applied to a bed of silica gel H (TLC grade, E. Merck). The column was eluted with a stepwise gradient of water-saturated chloroform and ethanol. Ferrichrome, which eluted as a sharp band in the 65:35 (v/v) fraction, was collected, evaporated to dryness, dissolved in a small amount of water, and reextracted as detailed above.

The purity of the preparation was evaluated on the basis of a single sharp spot on silica gel TLC plates (type 60, E. Merck), and high-voltage paper electrophoresis. Using several elution systems, including

0002-7863/80/1502-4224$01.00/0

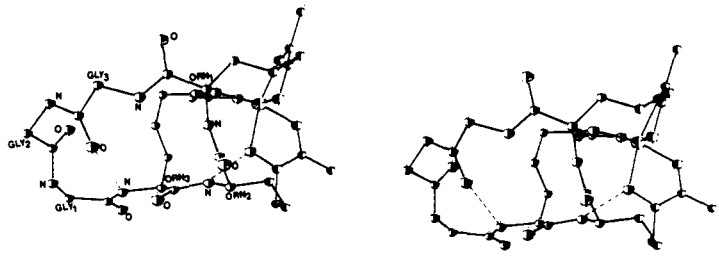


Figure 1. A stereoscopic drawing of the ferrichrome molecule. Intramolecular hydrogen bondings are shown by dashed lines.

4:1:1 1-butanol-acetic acid-water, 4:1:1 1-propanol-acetic acid-H_2O, 65:25:4 $CHCl_3$-CH_3OH-H_2O, and 4:1 CH_3OH-$CHCl_3$, pure ferrichrome exhibited R_f values of 0.13, 0.23, 0.52, and 0.38, respectively. Electrophoresis was performed on Whatman No. 3MM medium flow paper using a pyridine-acetic acid-water buffer, pH 5.2 (14:10:930) at 1000 V for 2 h. Detection systems included iodine vapors and 5% $FeCl_3$ in 0.5 N HCl.

Spectroscopy. Ultraviolet-visible spectra were measured on a Cary Model 118 recording spectrophotometer. Circular dichroism measurements were made using a Cary Model 61 recording spectropolarimeter. Single-crystal CD measurements were made using the procedures described previously.[16,17]

Crystallization and Data Collection. Single crystals of ferrichrome were obtained by two separate methods: (1) room temperature evaporation of a dimethylformamide solution and (2) an aqueous methanol solution (minimum amount of H_2O) equilibrated with pure methanol. Both systems yielded dark red, needle-shaped single crystals, the majority of which were too small for diffraction studies. A crystal with dimensions of 0.06 × 0.11 × 0.55 mm was obtained by the latter method, and used for data collection. The crystal data are: $FeC_{27}H_{42}N_9O_{12}$; mol wt 740.54; space group $P2_12_12_1$; $Z = 4$; unit cell parameters at 24 °C—a = 16.323 (4), b = 29.704 (8), c = 9.000 (2) Å, V = 4363.7 Å^3; at −135 (2) °C—a = 16.255 (18), b = 29.571 (15), c = 8.894 (4) Å, V = 4275.2 Å^3; λ for room temperature 2θ values = 1.54051 Å (Cu $K\alpha_1$); for low-temperature 2θ data, λ = 0.7093 Å (Mo $K\alpha_1$); for intensity measurements at −135 (2) °C λ = 0.7121 Å (Mo $K\bar{\alpha}$); μ(Mo $K\alpha$) = 4.25 cm^{-1}; ρ_{obsd} = 1.425, ρ_{calcd} = 1.322 to 1.420 g/cm^3 (depending on amount of solvation).

The cell parameters were determined by a least-squares fit to the 2θ values of 64 reflections at room temperature, and of 36 reflections at low temperature. Each set of reflections was chosen to be evenly distributed throughout reciprocal space. The density was measured by the flotation method using carbon tetrachloride-hexane. The variance in the calculated density reflects the effect of the number of solvent molecules included in the unit cell. The range is reported for four to six molecules of methanol.

The intensities of all 4303 unique reflections with $2\theta \leq 50°$ were measured on a Nonius CAD-4 automatic diffractometer using the θ-2θ scanning technique. The Mo $K\bar{\alpha}$ radiation used for recording these intensities was first monochromatized using a graphite crystal. The scan angle used for each reflection was calculated from the formula $\theta° = (0.65 + 0.12 \tan\theta)°$. The receiving aperture, located 173 mm from the data crystal, had a variable width, which was calculated as (3.0 + 0.86 tan θ) mm, while the height of the aperture remained constant at 6 mm. The maximum scan time per reflection was 90 s, with $^2/_3$ of the time spent scanning the peak (P) and the remaining $^1/_3$ divided equally between the high- and low-θ backgrounds (RH and LH). The intensity of a standard reflection was monitored every 1800 s of X-ray exposure and was later used to normalize the data. The orientation of the crystal was checked after every 200 reflections. In all, 1236 reflections were considered indistinguishable from the background on the basis that $I < 2\sigma(I)$. These reflections were assigned intensities equal to $T^{1/2}$ for least-squares refinement purposes (T = [P + 4(RH + LH)]). The intensity data were corrected for Lorentz and polarization factors, and for absorption effects, which was applied by a Gaussian method[18] using 216 sampling points. Each structure factor was assigned an individual weight.[19]

Structure Solution and Refinement. A sharpened Patterson synthesis was calculated, and revealed the positions of the Fe atom and the six O atoms surrounding it. Refinement of the octahedron was not encouraging. A difference Fourier synthesis did not reveal the atoms bonded to the octahedron, but did show a separate fragment of the molecule. This fragment, along with the iron and six oxygen atoms, was included in a weighted Fourier synthesis of the MULTAN program.[20] This gave the positions of all atoms in the hexapeptide ring. Another weighted Fourier synthesis including these atoms yielded the remaining nonhydrogen atoms of the molecule.

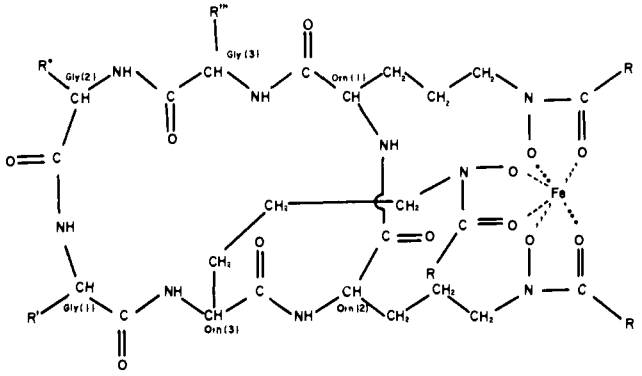


Figure 2. Schematic drawing of ferrichrome showing the molecular formula and labeling of amino acid residues: ferrichrome, R′ = R″ = R‴ = -H, R = -CH_3; ferrichrome A, R′ = -H, R″ = R‴ = -CH_2OH, R = -CH=C(CH_3)COOH (trans).

Initially, refinement of these atoms was performed by using a block-diagonal least-squares program. Subsequent difference Fourier syntheses showed 10 peaks of varied heights. Some of the peaks are within bonding distance of each other, but none of them are within van der Waals' distance from the ferrichrome molecule. These peaks were assumed to be disordered solvent molecules, and they were included in the least-squares refinement with full or partial occupancy. In addition, the contribution of the hydrogen atoms was included at their calculated positions. The final refinement was by full-matrix least-squares methods. It is believed that the large extinction effects observed in some low-order reflections and the disorder of the solvent molecules had a detrimental effect on the structure refinement. The final R factor was 0.141 for all reflections and 0.108 for 3067 observed reflections. Scattering factors for Fe, O, N, and C atoms were taken from the International Tables for X-ray Crystallography,[22] and those for H atoms from Stewart, Davidson, and Simpson.[21] The final positional parameters are listed in Table 1, while the thermal parameters and the structure factors are included in the supplementary material (see paragraph at end of paper).

Determination of Absolute Configuration. The absolute configuration of ferrichrome was determined by the Bijvoet method using the anomalous dispersion of Cu radiation by the Fe atom. The procedure for selecting the best Friedel pairs and subsequent measurements have been described previously.[18] The intensities of 15 reflections with the largest values of SF = $[|F^2(+) - F^2(-)|/\sigma(I)]$ were measured at −135 (2) °C.

Description of the Structure and Discussion

A stereoscopic view of the ferrichrome molecule is shown in Figure 1. The molecular structure confirmed the postulated chemical structure as the ferric chelate of the cyclic hexapeptide, (Gly)$_3$-(N^δ-acetyl-N^δ-hydroxy-L-ornithine)$_3$. The central iron atom is coordinated octahedrally by six oxygen donor atoms of the hydroxamic acid moieties of three ornithine residues, which, with the three glycine residues, make up the peptide backbone. The amino acid residues are labeled (Figure 2) using the scheme proposed originally by Zalkin, Forrester and Templeton[6] and subsequently adapted by other researchers. The general atom numbering of the residues is given in Figure 3.

Table I. Final Positional Parameters ($\times 10^4$) of Nonhydrogen Atoms[a]

Fe	943.0 (11)	2723.0 (6)	61 (3)
		Orn(1)	
C(1)	3539 (9)	3946 (5)	2209 (21)
C(2)	3338 (9)	3481 (6)	2741 (19)
C(3)	3499 (8)	3120 (5)	1542 (20)
C(4)	3480 (8)	2658 (5)	2171 (19)
C(5)	3324 (8)	2299 (5)	935 (18)
C(6)	2346 (9)	2333 (5)	−1254 (19)
C(7)	2938 (8)	2188 (7)	−2397 (18)
N(1)	2517 (6)	3437 (4)	3339 (15)
N(2)	2548 (7)	2376 (4)	176 (19)
O(1)	4096 (8)	4008 (4)	1345 (18)
O(2)	1925 (6)	2510 (3)	1178 (12)
O(3)	1610 (6)	2428 (3)	−1591 (12)
		Orn(2)	
C(1)	2367 (8)	3307 (4)	4787 (21)
C(2)	1468 (8)	3177 (4)	5171 (20)
C(3)	1366 (8)	2674 (5)	4841 (18)
C(4)	551 (8)	2443 (5)	5249 (18)
C(5)	−192 (9)	2521 (5)	4198 (16)
C(6)	−4 (9)	2124 (4)	1755 (18)
C(7)	−362 (9)	1677 (4)	2302 (21)
N(1)	873 (7)	3455 (4)	4249 (13)
N(2)	17 (7)	2479 (4)	2584 (15)
O(1)	2894 (6)	3256 (3)	5738 (13)
O(2)	346 (5)	2863 (3)	1973 (11)
O(3)	250 (6)	2155 (3)	402 (10)
		Orn(3)	
C(1)	828 (9)	3918 (5)	4514 (18)
C(2)	120 (9)	4139 (5)	3620 (18)
C(3)	165 (10)	4036 (5)	1889 (19)
C(4)	959 (11)	4201 (5)	1178 (18)
C(5)	1022 (12)	4111 (5)	−484 (18)
C(6)	224 (9)	3443 (5)	−1470 (17)
C(7)	−415 (10)	3716 (6)	−2342 (22)
N(1)	164 (7)	4625 (4)	3879 (16)
N(2)	845 (8)	3634 (4)	−774 (14)
O(1)	1252 (6)	4106 (3)	5450 (12)
O(2)	1382 (5)	3342 (3)	−38 (14)
O(3)	152 (6)	3008 (3)	−1438 (12)
		Gly(1)	
C(1)	−483 (11)	4906 (5)	3572 (27)
C(2)	−361 (11)	5407 (5)	3581 (25)
O(1)	−1178 (8)	4736 (4)	3225 (21)
N(1)	471 (8)	5557 (4)	3648 (18)
		Gly(2)	
C(1)	964 (11)	5545 (5)	2388 (25)
C(2)	1834 (11)	5746 (5)	2678 (24)
O(1)	749 (8)	5381 (4)	1199 (16)
N(1)	2455 (7)	5441 (4)	2172 (15)
		Gly(3)	
C(1)	2521 (11)	5044 (5)	2797 (17)
C(2)	3241 (9)	4755 (5)	2322 (19)
O(1)	2056 (6)	4897 (3)	3762 (13)
N(1)	3107 (9)	4280 (5)	2746 (16)

solvent				occupancy
P(1)	1800	1604	2361	1.0
P(2)	2597	1159	−94	1.0
P(3)	5914	3713	948	0.4
P(4)	5594	3510	1982	0.4
P(5)	2858	264	40	0.4
P(6)	3229	324	1064	0.4
P(7)	1758	1108	4833	0.5
P(8)	2943	1159	5679	0.3
P(9)	5290	3932	3920	0.3
P(10)	4772	29	1078	0.4

[a] Atoms in the disordered solvent molecules are listed as P(1), ... P(10), along with their occupancy factors. Estimated standard deviations are in parentheses.

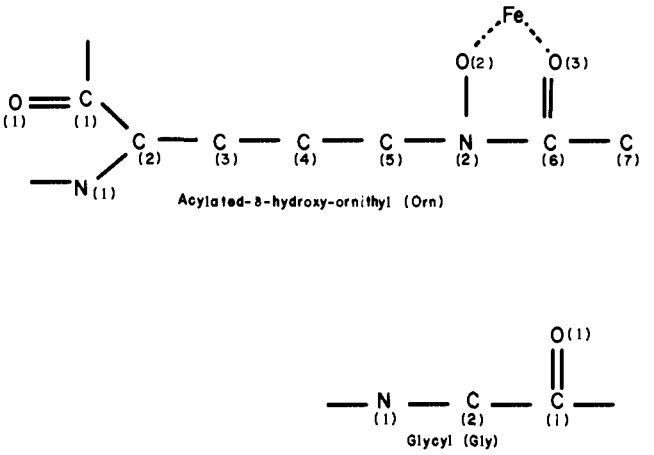


Figure 3. Schematic of the atom numbering followed in the text and the tables.

Table II. Bond Distances in Ferrichrome

bond	Orn(1)	Orn(2)	Orn(3)
Fe-O(2)	1.983 (11)	2.001 (10)	1.966 (9)
Fe-O(3)	2.023 (10)	2.044 (10)	2.036 (10)
N(2)-O(2)	1.405 (16)	1.368 (14)	1.393 (15)
C(6)-O(3)	1.266 (16)	1.276 (16)	1.291 (16)
N(2)-C(6)	1.319 (21)	1.283 (17)	1.313 (19)
C(6)-C(7)	1.464 (20)	1.524 (18)	1.528 (21)
C(5)-N(2)	1.448 (17)	1.480 (19)	1.460 (19)
C(4)-C(5)	1.550 (20)	1.544 (19)	1.506 (23)
C(3)-C(4)	1.477 (21)	1.536 (18)	1.518 (22)
C(2)-C(3)	1.531 (20)	1.524 (19)	1.571 (22)
N(1)-C(2)	1.444 (17)	1.511 (18)	1.458 (18)
C(2)-C(1)	1.489 (21)	1.549 (18)	1.544 (20)
C(1)-O(1)	1.202 (18)	1.213 (17)	1.215 (18)
C(1)-N′	1.302 (19)	1.366 (20)	1.391 (18)
bond	Gly(1)	Gly(2)	Gly(3)
N(1)-C(2)	1.426 (20)	1.425 (21)	1.470 (19)
C(2)-C(1)	1.496 (21)	1.555 (24)	1.509 (21)
C(1)-O(1)	1.274 (19)	1.214 (21)	1.224 (18)
C(1)-N′	1.369 (19)	1.378 (23)	1.305 (19)

The individual bond distances and bond angles are presented in Tables II and III. It should be noted that the accuracy of these values is not high due to experimental limitations. The average peptide bond distances of ferrichrome can be compared with those observed in other cyclic peptides[23] (in parentheses): C(1)-C(2), 1.524 (1.526) Å; C(1)-O(1), 1.224 (1.233) Å; C(1)-N, 1.352 (1.337) Å; and N(1)-C(1), 1.456 (1.452) Å. No significant differences are observed in these values. In addition, the average length of the C(5)-N(2) type bond is 1.463 Å as compared to 1.463 Å in ferrioxamine E,[15] and 1.470[6] and 1.466[12] in ferrichrome A. The bonds around atoms of type N(2) and C(6) are planar, as is observed in ferrioxamine E[24] and ferrichrome A, and the averages of the individual bond angles around N(2) and C(6) are within 2° of those observed in these compounds. In the ornithine groups, the average of the nine alkane distances is 1.529 Å. For the nine tetrahedral bond angles of C(3), C(4), and C(5) in the ornithine side chains, eight are larger than the normal tetrahedral value, having an average value of 113.4°. This indicates a certain amount of strain in the alkane portion of the ornithyl side chains, and we calculate this to be 5700 cal. A similar observation is made in the structure of ferrichrome A, where the average of the same nine bond angles is 113.1°.

The geometry of the iron coordination sphere is given in Figure 4, and is further described and compared to other iron-trihydroxamate complexes, i.e., ferrichrome A, ferriox-

Table III. Bond Angles in Ferrichrome

angle	Orn(1)	Orn(2)	Orn(3)
O(2)-Fe-O(3)	78.2	77.1	79.3
Fe-O(3)-C(6)	115	112	112
Fe-O(2)-N(2)	111	111	112
O(3)-C(6)-N(2)	116	118	119
O(2)-N(2)-C(6)	117	117	116
O(3)-C(6)-C(7)	121	119	118
N(2)-C(6)-C(7)	122	122	122
O(2)-N(2)-C(5)	112	114	113
C(6)-N(2)-C(5)	131	128	131
N(2)-C(5)-C(4)	111	113	109
C(5)-C(4)-C(3)	112	118	114
C(4)-C(3)-C(2)	112	119	113
C(3)-C(2)-C(1)	113	108	113
C(3)-C(2)-N(1)	111	111	110
N(1)-C(2)-C(1)	114	110	107
C(2)-C(1)-N′	118	116	112
C(2)-C(1)-O(1)	121	119	126
O(1)-C(1)-N′	122	124	122
angle	**Gly(1)**	**Gly(2)**	**Gly(3)**
N(1)-C(2)-C(1)	116	110	111
C(2)-C(1)-N′	120	113	117
C(2)-C(1)-O(1)	121	124	118
O(1)-C(1)-N′	119	123	125

[a] The standard deviations are between 0.4 and 0.7° for angles involving Fe, and between 1.0 and 1.5° for all other bond angles.

amine E, and tris(benzhydroxamato)iron(III),[25] in Table IV. The ligand bite (the ratio of the O-O distances to the Fe-O distances) and the twist angle (60° for an ideal octahedron and 0° for the trigonal prismatic arrangement) are used to describe the metal coordination.[26,27] The calculated twist angle was obtained from the ligand bite (b) using the formula: twist angle = $(-73.9 + 94.10b)$.[28] A difference in the observed and calculated twist angle is caused by the fact that the plane through carbonyl oxygen atoms is not parallel to the plane through the nitroso oxygen atoms. Llinás and Wuthrich[10j] suggested from their spectroscopic investigations of alumichrome that the Al-O distances for Orn(1) are longer than those for Orn(2) and Orn(3). The limited accuracy of the present structure determination and the resultant standard deviations do not allow assessment of any significant differences in ferrichrome. However, in our structure determination of alumichrome A and ferrichrome A,[12] both the Fe(Al)-O(2) and Fe(Al)-O(3) distances of Orn(1) are more than 4σ longer than the next longest distance of the same type, while the original structure determination of ferrichrome A[6] also indicated that the Fe-O distances of Orn(1) were the longest.

In ferrichrome, a distinction can be made between the Fe-O(2) distances, as a group, and the Fe-O(3) distances in that the latter are on the average 0.05 Å longer. This has been observed in all other iron(III) hydroxamate structures and reflects a difference in charge on the (N)-O and (C)-O atoms. Also, all other parameters of the chelate rings in ferrichrome are nearly the same as those observed in other iron(III) hydroxamate groups.

Leong and Raymond[29] showed that the coordination of the kinetically more inert Cr(III) complexes of desferriferrichrome and desferriferrichrysin was Λ-cis, as determined by a comparison with the Λ-cis optical isomer of tris(N-methyl-L-methoxyacethydroxamato)chromium(III). Also, a comparison[30] of the CD spectrum of dissolved ferrichrome A with the one of the resolved ferric tris(thiobenzohydroxamato) complexes indicated that only the Λ isomer was present in solution.

The absolute configuration of the structure was determined using a set of 15 reflections most sensitive[19] to the anomalous

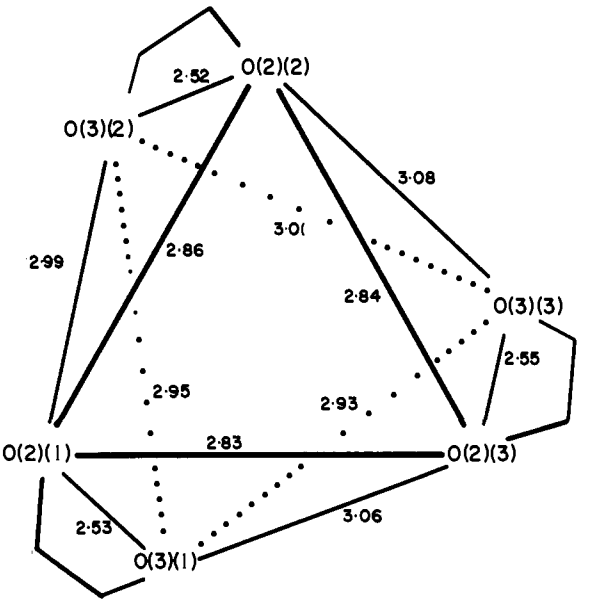


Figure 4. A view of the coordination geometry showing O-O distances outlining the octahedral edges.

Table IV. Comparison of Structural Parameters of Chelate Rings in Some Known Siderophores

	ferrichrome	ferrichrome A	ferrioxamine E	$Fe(benz)_3$
Fe-O(2)	1.983 (10)	1.980 (6)	1.953 (9)	1.980 (5)
Fe-O(3)	2.034 (6)	2.033 (6)	2.055 (2)	2.057 (10)
N-O(2)	1.389 (9)	1.372 (5)	1.381 (3)	1.373 (7)
C-O(3)	1.278 (6)	1.265 (5)	1.275 (3)	1.287 (7)
N-C	1.305 (9)	1.326 (3)	1.307 (3)	1.323 (7)
O···O	2.534 (10)	2.527 (7)	2.549 (3)	2.537 (3)
ligand bite	1.26	1.26	1.27	1.26
calcd twist	44.7	44.7	45.6	44.7
obsd twist	42.9 (5)	41.4 (6)	45.1 (7)	34.7 (15)
O-Fe-O	78.2 (5)	78.0 (1)	78.9 (1)	78.8 (3)

Table V. Absolute Configuration of Ferrichrome Using the 15 Pairs of Reflections Most Sensitive to the Anomalous Dispersion of Fe by Cu Kα Radiation

	$\lvert F\rvert^2_H/\lvert F\rvert^2_{\bar{H}}$ (calcd)	$I_H/I_{\bar{H}}$ (obsd)
1 8 2	<1	<1
4 2 1	<1	<1
6 11 3	>1	>1
3 8 3	>1	>1
7 11 2	<1	<1
1 10 2	<1	<1
1 10 1	<1	<1
5 6 2	<1	<1
4 1 3	<1	<1
2 3 2	<1	<1
1 12 4	>1	>1
4 3 1	>1	>1
4 4 3	<1	<1
4 6 1	<1	<1
4 5 3	<1	<1

dispersion of Fe with Cu radiation. These results, presented in Table V, indicate the same absolute configuration, as given in all figures. The absolute configuration, as determined, shows the coordination to be Λ-cis, while the hexapeptide contains L-ornithines. The occurrence of N^{δ}-acyl-N^{δ}-hydroxy-L-ornithine in ferrichrome and ferrichrome A was initially proven by Emery and Neilands.[4b] However, our observations[17] on the natural siderophore ferric N,N',N''-triacetylfusarinine showed

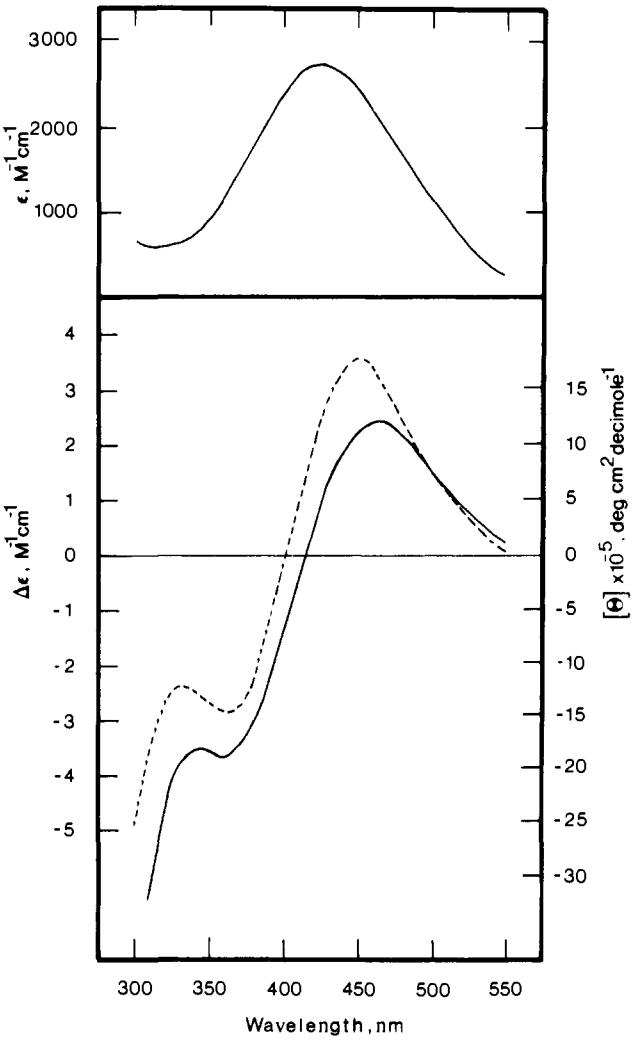


Figure 5. Visible (upper) and circular dichroism (lower) spectra of ferrichrome. Solid curves represent solution spectra, and the dashed curve is single crystals dispersed in KBr.

that the absolute configuration of the iron coordination sphere was different in the crystals, obtained from ethanol, compared to the predominant form in solution and crystals obtained from chloroform. This indicated clearly that it is always necessary for kinetically labile complexes to determine the absolute configuration of the crystalline material *itself* by circular dichroism. The CD spectra of powdered single crystals in a KBr pellet and of an aqueous solution of ferrichrome are shown in Figure 5. The solution spectrum is the same as observed previously,[8] and nearly the same as the spectrum of the powdered crystals, which shows that in the case of ferrichrome the iron coordination is Λ-cis both in the single crystals and in solution, and that this is most likely the biologically active form of the siderophore.

The conformational angles are shown in Figure 6 for ferrichrome, ferrichrome A, and ferrichrysin. The conformations of the ornithine side chains are similar for ferrichrome and ferrichrome A, although there are differences of up to 12° in individual conformational angles. Each ornithyl side chain has a different overall conformation from the other two. Using a potential energy barrier of 3000 cal for a rotation about the C-C bond, there is an estimated strain energy of 2300 cal in the nine C-C bonds of the ornithine side chains in ferrichrome. This with the 5700 cal of strain in the bond angles gives a minimum of 8000 cal of strain. This amount does not include the possible strain in the attachment of the hydroxamate groups, which is also different in each of the three ornithine

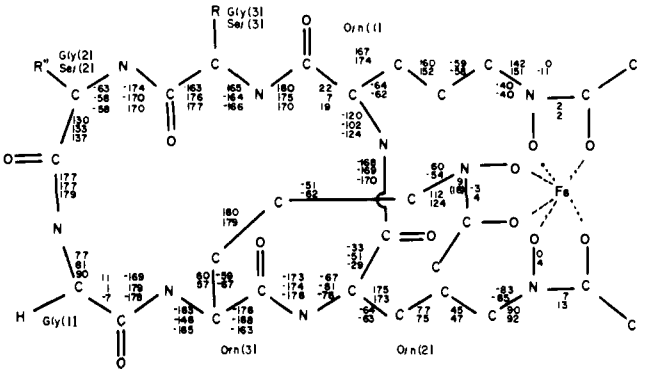


Figure 6. Conformational angles in ferrichrome (first line), ferrichrome A (second line), and ferrichrysin (third line, where applicable).

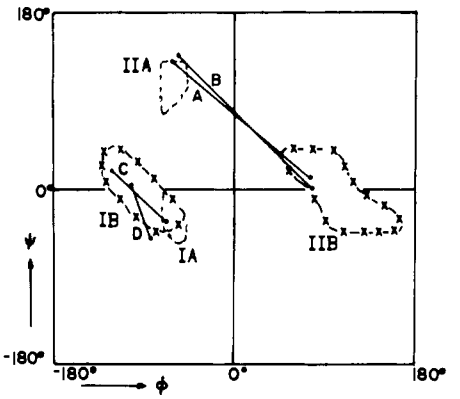


Figure 7. Conformational map showing the calculated[31] allowed region for β(I) and β(II) turns.

side chains (−40, 112, −83°) (Figure 6), or the strain in the cyclic hexapeptide portion of the molecule. It is clear, however, that on iron coordination of the desferrisiderophore, the ligand becomes strained to a certain extent.

The conformation of the hexapeptide ring is similar in ferrichrome, ferrichrome A, and ferrichrysin. The ϕ and ψ angles for the ornithines in ferrichrysin are closer to those in ferrichrome, while for the remaining three residues they are closer to ferrichrome A. The residues Gly(1) and -(2) in ferrichrome and Gly(1) and Ser(2) in ferrichrome A form a β(II) bend,[23,31] shown in the Venkatachalam plot (Figure 7) as vectors **A** and **B**. The result is a weak intramolecular hydrogen bond between N(1)(Orn(3)) and O(1)(Gly(3))(Ser(3)) (Table V), which is slightly stronger in ferrichrome A than in ferrichrome. The H···O distances are 2.14 and 2.35 Å, respectively (Table VI).

The ornithyl residues, Orn(1) and Orn(2), occupy the other corner of the hexapeptide. The conformational angles ϕ and ψ of these residues for ferrichrome fall within the allowed regions for a β(I) turn in the Venkatachalam plot (vector **C**). If a β(I) turn exists, one would expect a resulting hydrogen bond between N(1)(Gly(3)) and O(1)(Orn(3)). However, the N···O and H···O distances of 3.89 and 2.97 Å are significantly larger (0.6 Å) than the sum of the van der Waals' radii and preclude the existence of a hydrogen bond in the crystal structure. The corresponding N···O and H···O distances in ferrichrome A are still longer (4.48 and 3.67 Å). For ferrichrome A, the base of the vector (D) falls outside the allowed region in the Venkatachalam plot. For ferrichrome, however, both the base and tip of the vector (C) fall within allowed regions and it is remarkable that no hydrogen bond occurs as a result. The cause can only be sought in the nonplanarity of the peptide linkage between Orn(1) and Orn(2).

Earlier NMR experimental studies of ferrichrome[10a,b] indicated the existence of a hydrogen bond between N(1) of

Table VI. Contacts of the N-H and C=O Groups in the Hexapeptide Ring of Ferrichrome with Some Comparisons in Ferrichrome A in Parenthesis

N(1)Orn(1)···O(2)Orn(1): 3.48 Å			no H bond
N(1)Orn(1)···O(2)Orn(3): 3.53 Å			no H bond
N(1)Orn(2)···O(2)Orn(2): 2.81 Å	H···O: 1.99 Å	N-H···O: 145°	strong H bond
(N(1)Orn(2)···O(2)Orn(2): 2.77 Å	H···O: 1.94 Å	N-H···O: 146°)	
N(1)Orn(3)···O(1)Gly(3): 3.18 Å	H···O: 2.35 Å	N-H···O: 150°	weak H bond
(N(1)Orn(3)···O(1)Ser(3): 2.98 Å	H···O: 2.14 Å	N-H···O: 145°)	
N(1)Gly(3)···O(1)Orn(3): 3.89 Å	H···O: 2.97 Å	N-H···O: 168°	no H bond
(N(1)Ser(3)···O(1)Orn(3): 4.48 Å	H···O: 3.67 Å	N-H···O: 163°)	
N(1)Gly(2)···O(1)Orn(3):[a] 2.93 Å	H···O: 2.09 Å	N-H···O: 148°	H bond
N(1)Gly(1)···O(1)Orn(1):[b] 2.81 Å	H···O: 1.91 Å	N-H···O: 165°	strong H bond
O(1)Orn(2) no contacts less than 3.5 Å			
O(1)Gly(2)···P(10): 2.84 Å			H bond
O(1)Gly(1)···P(8): 3.16 Å			weak H bond

[a] $\frac{1}{2} - x, 1 - y, -\frac{1}{2} + z$. [b] $\frac{1}{2} - x, 1 - y, \frac{1}{2} + z$.

Gly(3) and O(1) of Orn(3) in the liquid state, but subsequent experiments[10i] indicated that this interaction was in reality either a very weak hydrogen bond or an electrostatic interaction. From the crystal-structure results of both ferrichrome and ferrichrome A, it is concluded that this hydrogen bond does not exist in the solid state. For an electrostatic interaction, it is significantly weaker in ferrichrome A than in ferrichrome, as is reflected from the corresponding N···O distance, which is 0.6 Å longer in ferrichrome A than in ferrichrome. The $\beta(11)$ bend hydrogen bond is slightly stronger in ferrichrome A than in ferrichrome. These observations are in partial agreement with the conclusions made from NMR data[10b,i] in that the seryl for glycyl substitution results in a general strengthening of the intramolecular hydrogen bonding. The X-ray data shows that in the seryl for glycyl substitution the $\beta(11)$ bend hydrogen bond is strengthened slightly, and the electrostatic interaction from the $\beta(1)$ bend decreases significantly.

The conformational angles, ϕ and ψ, of the ornithine residues in the hexapeptide ring for ferrichrome and ferrichrome A show an average difference of 16°, which certainly is significant and quite unexpected. It appears that the triornithyl part of hexapeptide is destabilized in ferrichrome A compared to ferrichrome.

An analysis of the interactions of all C=O and N—H groups in the cyclic peptide is given in Table VI. The N-H groups of Gly(1) and Gly(2), which are both exposed (Figure 7), form intermolecular H bonds with symmetry-related molecules of ferrichrome. The N-H group of Orn(1) is buried in the interior of the molecule and does not form an H bond, although it does interact weakly with the O(2) atoms of Orn(1) and Orn(3). The N-H group of Orn(2) forms a strong H bond with O(2) of Orn(2), as was observed in ferrichrome A. The N-H groups of Orn(3) and Gly(3) have already been described, but it should be noted as well (Figure 1) that the N-H group of Gly(3) is to some extent exposed. Emery observed[32] that in ferrichrome there were 1.8 to 3.8 slowly exchanging protons going from pH 7.0 to 3.0, where normally in small peptides all hydrogens are exchanged quite rapidly. This is in general agreement with the results from the structure determination in the solid state, which predict the following sequence in increasing rate of exchange for NH protons: Orn(2) < Orn(3) ≈ Orn(1) < Gly(3) < Gly(1) ≈ Gly(2), with the last two being exchanged at any pH and the first one being exchanged only very slowly. The NMR exchange experiments[10a] indicated that two glycyl N-H protons are exchanged very fast and that the Gly(3) proton is exchanged in minutes, while the three Orn N-H's exhibit half-lives of the order of days. These observations are also in general agreement with our interpretation of the solid-state results.

The contacts for the C=O groups (Table VI) for Orn(1), Orn(3), and Gly(3) have been discussed in the description of the N-H groups. The C=O of Orn(2), although exposed, does not form an H bond, while the C=O groups of Gly(2) and Gly(1) form H bonds with solvent molecules.

The relationship between molecular structure and biological activity (iron transport) of siderophores has been of considerable interest because of the findings of Emery[9,33] and others.[34,35] For example, relative to ferrichrome, ferrichrome A is taken up by *U. sphaerogena* at a rate of 8%, while for ferrichrysin, which has the peptide structure of ferrichrome A and the acyl moiety of ferrichrome, this value is 50%. On the other hand, ferrichrysin is taken up at a significantly greater rate than ferrichrome by a *Neurospora* mutant incapable of coprogen synthesis. In addition, the two molecules exhibit similar growth factor activities toward *Arthrobacter flavescens JG-9*, while ferrichrome A elicits a definite negative response. Such results indicated that: (1) *Arthrobacter* JG-9 is rather nonspecific in its recognition of siderophores; (2) ferrichrome A is definitely inactive as an ionophore; and (3) membrane receptors of fungi are able to recognize small changes in structure and conformation of the hexapeptide ring of siderophores (i.e., activities of ferrichrome vs. ferrichrysin).

It was hoped that the structure determination of ferrichrome and a comparison with ferrichrome A would yield a clue to the difference in their ability to actively transport iron. The differences in transport activities of the two molecules can result from either conformational differences in the cyclic hexapeptide and iron coordinate sphere, and/or chemical and steric differences resulting from acyl substitution of the ornithine residues. The observed differences, however, are at best circumstantial, and are: (1) a larger strain in the triornithyl portion of the hexapeptide ring in ferrichrome A, (2) a possible larger asymmetry in the Fe-O distances in ferrichrome A, and (3) differences in the conformation of the side chains of Orn(1) and Orn(3) (Figure 6). In the crystalline state, the ferrichrome molecules are packed in such a manner as to allow two short intermolecular Fe-Fe distances of 8.2 and 8.9 Å. The orientation of the first Fe-Fe vector is nearly parallel to the *a* axis, while that of the latter is in the direction of the *c* axis. In both cases, the Fe-Fe vectors intersect segments of the ornithyl side chains (Orn(1) for the vector along the *a* axis and Orn(2) for the one along the *c* axis). In ferrichrome A, the chelate molecules are packed to give two short Fe-Fe intermolecular distances of 8.0 and 11.0 Å. These values are in contrast to corresponding Fe-Fe distances of 12.1 and 13.4 Å observed in ferric *N,N',N''*-triacetylfusarinine.[17]

A comparison of the overall conformation of ferrichrome and ferrichrome A was made using a least-squares fit of all Fe, C, O, and N atoms of ferrichrome (with C(7)Orn(3) excluded because it is affected by disorder in ferrichrome A) to the same atoms in ferrichrome A. The distances between the atoms in both molecules are listed in Table VII; the least-squares fit of

Table VII. Distances between Equivalent Atoms in Ferrichrome and Ferrichrome A after a Least-Squares Fit to the Positional Coordinates Obtained from the Low-Temperature Single-Crystal X-ray Diffraction Structure Determination

Orn(1)		Orn(2)		Orn(3)		Gly(1)	
C(1)	0.27 Å	C(1)	0.31 Å	C(1)	0.27 Å	C(1)	0.11
C(2)	0.28	C(2)	0.32	C(2)	0.25	C(2)	0.33
C(3)	0.35	C(3)	0.22	C(3)	0.32	O(1)	0.31
C(4)	0.24	C(4)	0.32	C(4)	0.49	N(1)	0.49
C(5)	0.14	C(5)	0.30	C(5)	0.69	Gly(2)	
C(6)	0.16	C(6)	0.07	C(6)	0.50	C(1)	0.55
C(7)	0.29	C(7)	0.36	C(7)		C(2)	0.66
N(1)	0.22	N(1)	0.30	N(1)	0.23	O(1)	0.61
N(2)	0.15	N(2)	0.13	N(2)	0.45	N(1)	0.37
O(1)	0.62	O(1)	0.75	O(1)	0.47	Gly(3)	
O(2)	0.24	O(2)	0.19	O(2)	0.30	C(1)	0.28
O(3)	0.18	O(3)	0.24	O(3)	0.38	C(2)	0.33
Fe	0.17					O(1)	0.61
						N(1)	0.38

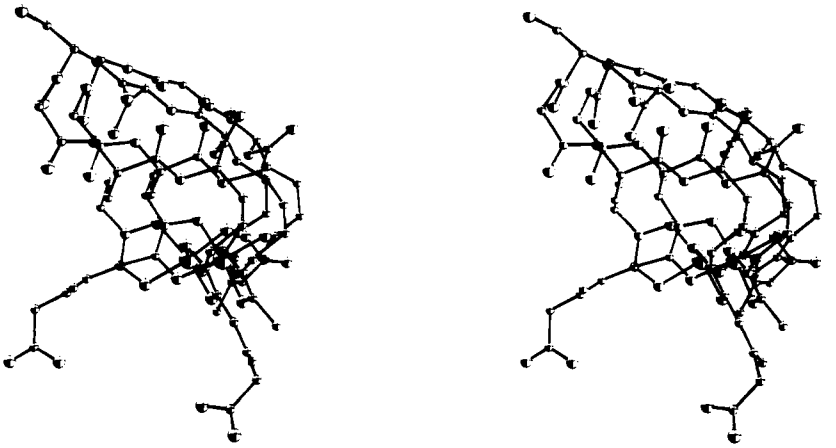


Figure 8. Stereoview of the superimposed molecules of ferrichrome and ferrichrome A separated from each other by a translation of 0.5 Å. Ferrichrome A is at left and ferrichrome at right.

the positional parameters of the two molecules is shown in Figure 8 after a 0.5-Å translation of one molecule. Differences of more than 0.5 Å are observed in the O(1) atoms of Orn(1), Orn(2), Gly(2), and Gly(3), C(5) of Orn(3), and C(1) and C(2) of Gly(2), and the stereoview shows that, indeed, most of the differences are in the cyclic hexapeptide ring. Although these differences may be important for the different chemical and biological properties of the two siderophores, it is apparent that the serine for glycine substitution in ferrichrome A has little effect on iron transport characteristics of the molecule, as witnessed by the similarity in iron uptake rates of ferrichrome and ferrichrysin.

More likely, differences in the acyl substitution of the ornithine residues in these compounds are responsible for the observed differences in biological activity of ferrichrome and ferrichrome A. One needs to consider two aspects, i.e., the acidic charged nature of the acyl groups in ferrichrome A and the bulkiness of the acyl groups.

With respect to the first, it should be noted that malonichrome, a ferrichrome type siderophore with malonic acid moieties as acyl groups, does not actively transport iron,[36] but neither does the trimethyl ester derivative of ferrichrome A.[33] Although this leaves open the question of the effect of the carboxylate groups on active iron transport in fungi, it is interesting to note that in *E. coli* K-12, ferrichrome A, as the free acid or the monomethyl ester, is inactive in competing with the phage T5 for the *Ton* A receptor site; however, further neutralization of the negatively charged carboxyl groups by esterfication (di- and trimethyl esters) does impart activity to ferrichrome A.[13e]

With respect to the second point, the iron atom in ferrichrome is rather open to close approach by a specific siderophore membrane binding protein, although the involvement of such a protein has not yet been demonstrated. On the other hand, in ferrichrome A, the *trans*-β-methylglutaconic acid groups may sterically and conformationally reduce chances of close approach by a membrane receptor for two reasons: the bulk of the acyl groups and acyl group chain branching. Emery's investigations,[33] although not conclusive, showed that the former reason may not be as important as the latter, inasmuch as propionyl and butyryl ferrichrome were highly active in iron transport. Conversely, the branched methyl groups in concert with the terminal carboxyl groups in ferrichrome A may offer significant "screening" of the central metal ion, as seen in Figure 8. It may well be necessary to further investigate structure-transport characteristics of other synthetic derivatives of these compounds in order to fully explain the biological activity of ferrichrome with respect to ferrichrome A.

Acknowledgment. This research was supported by Grant GM-21822 from the National Institutes of Health. One of the authors (D.v.d.H) expresses his gratitude to Dr. T. Emery, Utah State University, for the help and hospitality he received while working in his laboratory. We wish to extend our appreciation to Dr. Neil Purdie and John M. Bowen of Oklahoma State University, Stillwater, for their assistance in obtaining the CD data. We thank the University of Oklahoma for computing services.

Supplementary Material Available: Final anisotropic thermal parameters ($\times 10^4$) (Table 8) and structure factors (Table 9) (24 pages). Ordering information is given on any current masthead page.

References and Notes

(1) Neilands, J. B. "Inorganic Biochemistry"; Eichhorn, G. L., Ed.; Elsevier: New York, 1973; Vol. I, pp 167–202.
(2) Emery, T. "Metal Ions in Biological Systems"; Sigel, H., Ed.; Marcel Dekker: New York, 1978; Vol. 7, pp 77–126.

(3) (a) Neilands, J. B. *J. Am. Chem. Soc.* **1952**, *74*, 4846-4847. (b) Keller-Schierlein, W.; Prelog, V.; Zahner, H. *Fortschr. Chem. Org. Naturst.* **1964**, *22*, 279-322. (c) Diekman, H.; Krezdorn, E. *Arch. Microbiol.* **1975**, *106*, 191-194.
(4) (a) Emery, T. F.; Nielands, J. B. *J. Am. Chem. Soc.* **1960**, *82*, 3658-3662. (b) Emery, T. F.; Neilands, J. B. *Ibid.* **1961**, *83*, 1626-1628. (c) Rogers, S.; Warren, R. A. J.; Neilands, J. B. *Nature (London)* **1963**, *200*, 167. (d) Rogers, S.; Nielands, J. B. *Biochemistry* **1964**, *3*, 1850-1855.
(5) (a) Keller-Schierlein, W.; Maurer, B. *Helv. Chim. Acta* **1969**, *52*, 603-610. (b) Isowa, Y.; Ohmori, M.; Kurita, H. *Bull. Chem. Soc. Jpn.* **1974**, *47*, 215-220.
(6) Zalkin, A.; Forrester, J. D.; Templeton, D. H. *J. Am. Chem. Soc.* **1966**, *88*, 1810-1814.
(7) Norrestam, R.; Stensland, B.; Brändén, C.-I. *J. Mol. Biol.* **1975**, *99*, 501-506.
(8) Naegeli, H.-U.; Keller-Schierlein, W. *Helv. Chim. Acta* **1978**, *61*, 2088-2095.
(9) Emery, T. *Biochemistry* **1971**, *10*, 1483-1488.
(10) (a) Llinás, M.; Klein, M. P.; Neilands, J. B. *J. Mol. Biol.* **1970**, *52*, 399-414. (b) Llinás, M.; Klein, M. P.; Neilands, J. B. *Ibid.* **1972**, *68*, 265-284. (c) Llinás, M.; Klein, M. P.; Neilands, J. B. *J. Biol. Chem.* **1973**, *248*, 915-923. (d) Llinás, M.; Klein, M. P.; Neilands, J. B. *Ibid.* **1973**, *248*, 924-931. (e) Llinás, M.; Klein, M. P. *J. Am. Chem. Soc.* **1975**, *97*, 4731-4737. (f) Llinás, M.; Wilson, D. M.; Klein, M. P.; Neilands, J. B. *J. Mol. Biol.* **1976**, *104*, 853-864. (g) Llinás, M.; Horsley, W. J.; Klein, M. P. *J. Am. Chem. Soc.* **1976**, *98*, 7554-7558. (h) Llinás, M.; Wilson, D. M.; Neilands, J. B. *Ibid.* **1977**, *99*, 3631-3637. (i) Llinás, M.; Wilson, D. M.; Klein, M. P. *Ibid.* **1977**, *99*, 6846-6850. (j) Llinás, M.; Wüthrich, K. *Biochim. Biophys. Acta* **1978**, *532*, 29-40.
(11) Anderegg, G.; L'Eplattenier, F.; Schwarzenbach, G. *Helv. Chim. Acta* **1963**, *46*, 1400-1408.
(12) van der Helm, D.; Baker, J. R.; Loghry, R. A., submitted for publication in *Acta Crystallogr.*
(13) (a) Neilands, J. B. *Adv. Chem. Ser.* **1977**, *No. 162*, 3-32. (b) Hantke, K.; Braun, V. *FEBS Lett.* **1975**, *49*, 301-305. (c) Luckey, M. R.; Wayne, R.; Neilands, J. B. *J. Bacteriol.* **1972**, *111*, 731-738. (d) Wayne, R.; Neilands, J. B. *Ibid.* **1975**, *121*, 497-503. (e) Luckey, M.; Wayne, R.; Neilands, J. B. *Biochem. Biophys. Res. Commun.* **1975**, *64*, 687-693.
(14) Garibaldi, J. A.; Neilands, J. B. *J. Am. Chem. Soc.* **1955**, *77*, 2429-2430.
(15) Tadenum, M.; Sato, S. *Agric. Biol. Chem.* **1967**, *31*, 1482-1489.
(16) Ho, N.; Takagi, T. *Biochim. Biophys. Acta* **1970**, *221*, 430-441.
(17) Hossain, M. B.; Eng-Wilmot, D. L.; Loghry, R. A.; van der Helm, D., *J. Am. Chem. Soc.*, In press.
(18) Coppens, P.; Leiserowitz, L.; Rabinovich, D. *Acta Crystallogr.* **1965**, *18*, 1035-1038.
(19) Ealick, S. E.; van der Helm, D.; Weinheimer, A. J. *Acta Crystallogr., Sect. B* **1975**, *31*, 1618-1626.
(20) Germain, G.; Main, P.; Woolfson, M. M. *Acta Crystallogr., Sect. A* **1971**, *27*, 368-376.
(21) Stewart, R. F.; Davidson, E. R.; Simpson, W. T. *J. Chem. Phys.* **1965**, *42*, 3175-3187.
(22) "International Tables for X-ray Crystallography"; Kynoch Press: Birmingham, England, 1974; Vol. IV, p 73.
(23) Hossain, M. B.; van der Helm, D. *J. Am. Chem. Soc.* **1978**, *100*, 5191-5198.
(24) van der Helm, D.; Poling, M. *J. Am. Chem. Soc.* **1976**, *98*, 82-86.
(25) Lindner, H. J.; Göttlicher, S. *Acta Crystallogr., Sect. B*, **1969**, *25*, 832-842.
(26) Kepert, D. L. *Inorg. Chem.* **1972**, *11*, 1561-1563.
(27) Avdeef, A.; Fackler, J. P. *Inorg. Chem.* **1975**, *14*, 2002-2006.
(28) Raymond, K. N.; Isied, S. S.; Brown, L. D.; Fronczek, F. R.; Nibert, J. H. *J. Am. Chem. Soc.* **1976**, *98*, 1767-1774.
(29) Leong, J.; Raymond, K. N. *J. Am. Chem. Soc.* **1974**, *96*, 6628-6630.
(30) Abu-Dari, K.; Raymond, K. N. *J. Am. Chem. Soc.* **1977**, *99*, 2003-2005.
(31) Venkatachalam, C. M. *Biopolymers* **1968**, *6*, 1425-1436.
(32) Emery, T. F. *Biochemistry* **1967**, *6*, 3858-3866.
(33) Emery, T.; Emery, L. *Biochem. Biophys. Res. Commun.* **1973**, *50*, 670-675.
(34) Leong, J.; Neilands, J. B.; Raymond, K. N. *Biochem. Biophys. Res. Commun.* **1974**, *60*, 1066-1071.
(35) Winkelmann, G. *FEBS Lett.* **1979**, *97*, 43-46.
(36) Emery, T. F., *Biochim. Biophys. Acta*, in press.

Covalent Linkage of Glucose Oxidase on Modified Glassy Carbon Electrodes. Kinetic Phenomena

C. Bourdillon,* J. P. Bourgeois, and D. Thomas

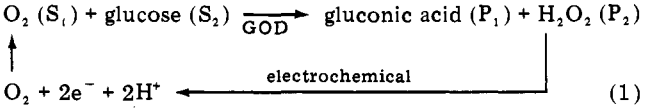
Contribution from the Laboratoire de Technologie Enzymatique, E.R.A. No. 338 du CNRS, Université de Compiègne, 60206-Compiegne, France.
Received November 13, 1979

Abstract: Direct grafting of glucose oxidase molecules onto glassy carbon electrodes was done to optimize molecular proximity between enzymatic and electrochemical sites. Peroxide molecules produced by the enzyme reaction are measured and transformed into O_2 by the use of the electrode. Kinetic phenomena arising from the coupling between enzyme and electrochemical reactions were experimentally and theoretically studied as a function of the working potential values.

Recent works dealing with chemically modified electrodes have made possible the permanent chemical modification of the surface of various electrode materials.[1,2] Several chemical functions, electroactive or not, have been fixed onto electrode surfaces, generally through covalent linkages. Simultaneously, the analytical possibilities of an association between electrochemical sensors and enzyme membranes have been developed by numerous authors.[3]

The covalent binding of enzyme molecules as a quasi-monomolecular layer on the surface of a carbon electrode will be described in the present paper. The method of immobilization gives optimum molecular proximity between enzyme active sites and the electrochemical surface.

Owing to the possible amperometric measurement of one of the products (H_2O_2), glucose oxidase (GOD) was chosen as a model system. One of the substrates (O_2) can be electrochemically regenerated according to eq 1. In such a system, the enzyme activity can be controlled by the local electrochemical oxygen regeneration. The overall behavior is ruled by mass transfer phenomena near the electrode. In order to work under well-defined hydrodynamic conditions, a rotating cylinder electrode was used. The rotation speed can be varied from 100 to 1400 rpm.



(1)

Carbon is not a very attractive material for enzyme immobilization. Its chemical inertia toward coupling reagents is high, and mechanical strength decreases when the specific area increases. Enzyme adsorptions on graphite or activated carbon followed by glutaraldehyde[4,5] or soluble carbodiimide[6] cross-linking were described. Quite recently this last method was used after superficially oxidizing the carbon.[7,8] Glucose oxidase was immobilized with glutaraldehyde and bovine serum albumin on a carbon paste electrode.[9]

In order to get a direct covalent binding of the enzyme molecules, electrochemical oxidation and carbodiimide activation of the carbon were performed. Carbon oxidation gives

0002-7863/80/1502-4231$01.00/0