Facile Lactonization and Inversion of Vicinal Diols in Heme *d*-Type Chlorins: A Spectroscopic Study

Laura A. Andersson,[1a] Chariklia Sotiriou,[1b] Chi K. Chang,*[1b] and Thomas M. Loehr*[1a]

Contribution from the Department of Chemical and Biological Sciences, Oregon Graduate Center, Beaverton, Oregon 97006-1999, and the Department of Chemistry, Michigan State University, East Lansing, Michigan 48824. *Received June 18, 1986*

Abstract: As models for the heme *d* prosthetic group of the *Escherichia coli* terminal oxidase, *cis*-3′,4′-dihydroxy-2,4-dimethyldeuterochlorin IX dimethyl ester (diol chlorin) and the 5′-hydroxy-6,6′-*trans*-γ-spirolactone of 2,4-dimethyldeuterochlorin IX dimethyl ester (lactone chlorin) have been studied. The spirolactone ring was formed by a general base-catalyzed cyclization between the geminal OH and propionic ester groups. Such lactonization was found to take place on silica gel during chromatography, which also catalyzes an inversion of configuration at the ternary carbon, yielding a trans configuration for the two C–O bonds. The NMR and IR spectra of our free-base chlorin lactone strongly resemble those of the free-base "lactochlorin" recently isolated from *E. coli* [Timkovich et al. *J. Am. Chem. Soc.* **1985**, *107*, 6069–6075], supporting the trans configuration for the lactone. These observations clearly have implications for the structure of in vivo heme *d*. Resonance Raman (RR) (457.9-, 514.5-, and 647.1-nm excitation) and IR spectral properties of the Cu(II) chlorin complexes are in good agreement with the general characteristics of metallochlorin spectra previously reported by us. Of particular interest, RR spectroscopy reveals distinct frequency differences between the Cu-diol and Cu-lactone chlorins, which vary structurally only in the sites of reduction and the substituents on the reduced rings. A comparison of Cu-diol with Cu-$(Me)_7$-chlorin (copper(II) 3′-hydro-4′-methyl-2,4-dimethyldeuterochlorin IX dimethyl ester) also reveals differences in their RR frequencies, although the only structural change is from two methyl and two hydroxy substituents to three methyl groups and a hydrogen on their respective pyrroline rings. These observations indicate that nonconjugated substituents on the reduced ring of metallochlorins may significantly influence the structural, electronic, and vibrational properties of the macrocycle. The spectral properties of the model complexes reported herein are of relevance in understanding the properties of the in vivo macrocycle. On the basis of the observed RR spectral differences between the diol and lactone chlorin models, it may be possible to utilize selective RR enhancement to determine the actual substituent pattern on the pyrroline ring of the purified *E. coli* heme *d* terminal oxidase without prosthetic group extraction.

Escherichia coli, like many other microbes, have a branched respiratory system with two terminal oxidases which catalyze the reduction of O_2 to H_2O.[2] Under conditions of oxygen limitation, the cytochrome *o* system yields to the alternative terminal oxidase complex, cytochrome *d*.[3,4] This oxidase has been shown to have both a remarkably high O_2 affinity[5] and a green heme *d* prosthetic group.[6] The heme *d* chromophore of *E. coli* terminal oxidase was first observed in 1928,[7] as an unusual ~630-nm band in electronic absorption spectra. In 1933, Keilin[8] proposed the name "a_2" for cytochromes absorbing in the red region. This name was later changed to heme *d* to avoid confusion with the aa_3 hemes of other cytochromes oxidase.

The first structural study of microbial green hemes was performed in 1956 by Barrett,[6] who determined that the extracted prosthetic groups of *Azotobacter aerogenes* and *E. coli* were iron chlorins structurally related to protoporphyrin IX. Barrett's findings indicated the presence of at least one vinyl substituent, a hydroxyethyl or possibly ethyl moeity, and most likely two carboxylic acid substituents. Subsequently, Gennis and coworkers[9,10] and Kita et al.[2] isolated and purified the cytochrome *d* complex from *E. coli*. The former group reported finding two polypeptides; however, no evidence for non-heme iron, copper, or flavins was obtained, and only the *d* heme and a *b* heme were extractable.

Recently, Timkovich et al.[11] utilized ^1H NMR, IR, and mass spectroscopies to determine the structure of the metal-free, esterified *d* prosthetic group extracted from purified *E. coli* terminal oxidase. Their data indicated that the free-base heme *d* chromophore had two vinyl moieties, one propionic acid group, and a hydroxyl group trans to a γ-spirolactone substituent on the pyrroline ring C of the macrocycle. This species was suggested to arise in vivo from protoporphyrin IX via a vicinal dihydroxychlorin intermediate.[12]

As models for the heme *d* prosthetic group of *E. coli* terminal oxidase, we present herein the electronic absorption, NMR, IR, and resonance Raman (RR) spectral properties of Cu-diol, **1b** (copper(II) *cis*-3′,4′-dihydroxy-2,4-dimethyldeuterochlorin IX dimethyl ester), and Cu-lactone, **4b** (copper(II) 5′-hydroxy-6,6′-*trans*-γ-spirolactone-2,4-dimethyldeuterochlorin IX monomethyl ester). For the cytochrome *d* of *E. coli*, the only previous RR study was limited to the ~900–1200-cm^{-1} region to locate a putative $\nu(O_2^-)$ vibrational mode of an oxygenated heme *d*.[18] Spectra of the model chlorin complexes are compared with those of meso-deuteriated d_4-Cu-diol, Cu-porphyrin (copper(II) 2,4-dimethyldeuteroporphyrin IX dimethyl ester), and Cu-$(Me)_7$, **5** (copper(II) 3′-hydro-4′-methyl-2,4-dimethyldeuterochlorin IX dimethyl ester).

* To whom correspondence should be addressed.

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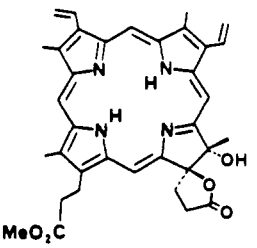
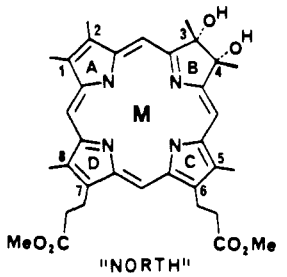
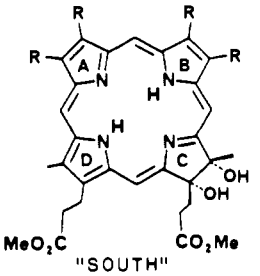
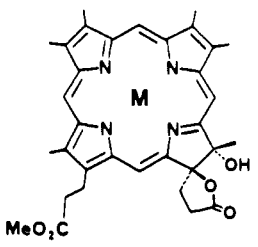




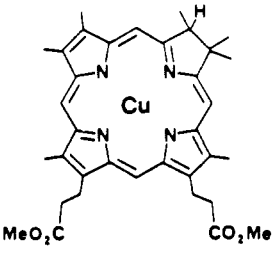
1a M = 2H
1b M = Cu



2 R = Me
3 R = Et



4a M = 2H
4b M = Cu



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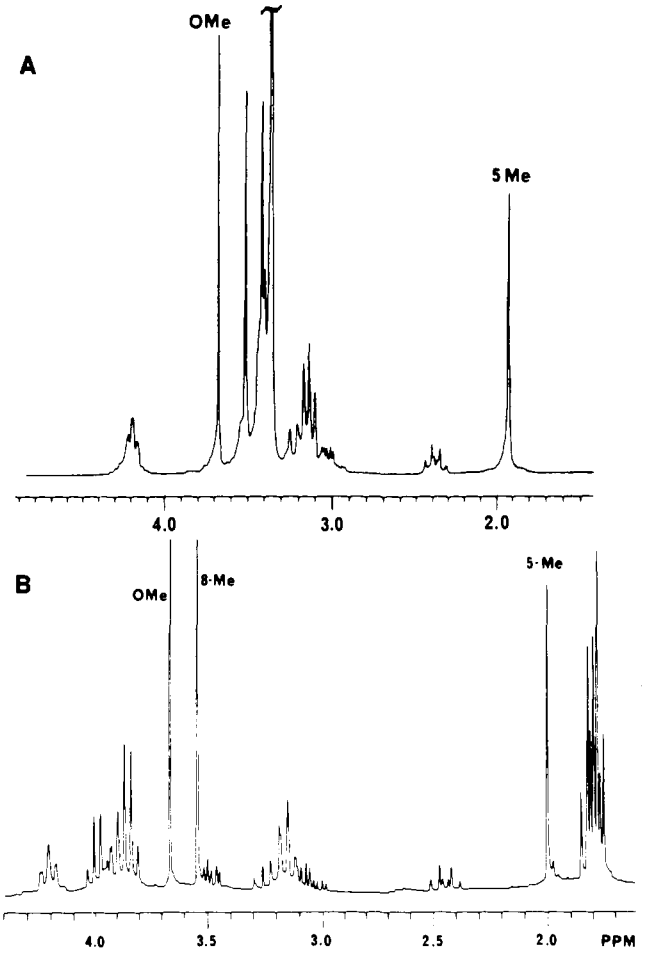

Figure 1. ^{1}H NMR (250 MHz, $CDCl_3$) partial spectra: (A) The trans lactone **4a**. This spectrum is to be compared to Figure 3 in ref 11. (B) Silica gel induced lactone of **3**, trans configuration; the lactone methylene protons are at 2.44, 3.05, 3.22, and 3.50 ppm.

Apart from the differences due to lactone cyclization, the general vibrational properties of the two heme *d* models (**1b** and **4b**) are in good agreement with our previous studies of metallochlorins.[19-21] However, the RR spectra of the two complexes, while similar, are not identical. The RR spectra of the Cu-diol and Cu-$(Me)_7$ chlorins are also distinguishable. These observations indicate that the nonconjugated substituents on pyrroline rings are "spectroscopically active". Resonance Raman spectroscopy may, therefore, provide a means to determine the in vivo prosthetic group structure of the purified *E. coli* heme *d* without necessitating a potentially deleterious prosthetic group extraction.

Experimental Section

Metal-free "north" (ring A or B reduced) and "south" (ring C or D reduced) vicinal dihydroxychlorin dimethyl esters, **1a** and **2**, respectively, were obtained from osmium tetraoxide oxidation of porphyrins as reported.[22] Similarly, copper(II)*meso*-tetradeuterio-*vic*-dihydroxy-2,4-dimethyldeuterochlorin IX dimethyl ester (d_4-Cu-diol) was synthesized from *meso*-tetradeuterio-2,4-dimethyldeuteroporphyrin IX dimethyl ester, prepared by dissolving the porphyrin[23] in concentrated sulfuric acid-d_2 overnight and repeating this exchange one more time to obtain ~96% isotope content. Cupric ion was inserted by heating the chlorins in CH_2Cl_2 with methanolic copper acetate containing a small amount of sodium acetate or collidine. The presence of a mild base was necessary to control the proton concentration and to prevent any possible rearrangements of the vicinal diols. However, such a base also efficiently catalyzed an intramolecular lactonization in the south dihydroxychlorin (**2**), yielding the γ-spirolactone (**4b**), which was characterized by IR and mass spectra ($C_{33}H_{34}N_4O_5Cu$, *m/e* 629, 631). Copper-free lactone chlorin, **4a**, was prepared either by heating the dihydroxychlorin, **2**, in methanol with sodium acetate under reflux for 20 min or by repeated chromatography on preparative TLC plates (Analtech, 1500 μm, silica gel). The spirolactone structure was characterized by ^{1}H NMR, IR (1785 cm^{-1}), and mass spectra ($C_{33}H_{36}N_4O_5$, *m/e* 568). Cu-$(Me)_7$, **5**, was prepared by copper insertion into the methylated chlorin prepared as reported.[22] Cu-porphyrin (copper(II) 2,4-dimethyldeuteroporphyrin IX dimethyl ester) was prepared by copper insertion into the parent porphyrin.[23]

IR spectra of the Cu(II) complexes were obtained from KBr pellets on a Perkin-Elmer Model 1800 FT-IR instrument. Raman spectra were obtained with a computer-controlled Jarrell-Ash scanning spectrophotometer[24] using Spectra-Physics 164-01 and 164-05 Kr and Ar ion lasers.[19-21] Spectra were collected in backscattering geometry on anaerobic solution samples[21] (CH_2Cl_2 or CS_2, ~1mg/mL) or on polycrystalline samples dispersed in KBr (~1-mg sample/100 mg of KBr); in both cases the samples were held in glass melting point capillaries maintained at ~2 °C in a Dewar.[25] Alternatively, room temperature spectra were recorded on the KBr samples pressed into the annular groove of a spinning sample holder (25-mm diameter). The presence of KBr served to greatly reduce fluorescence from trace amounts of free-base chlorins but had no significant effect on the RR peak positions and intensities relative to the solution spectra. Electronic absorption spectra of the samples, recorded on a Perkin-Elmer Lambda 9 spectrophotometer, indicated that no change had occurred as a result of laser irradiation. ^{1}H NMR spectra ($CDCl_3$, Me_4Si internal standard) were obtained with a Bruker WM-250

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Table I. Assignments of Cu-Diol and Cu-Lactone Optical Transitions[a]

	$Q_y(0,0)$	$Q_y(0,1)$	$Q_y(0,2)$	$Q_x(0,0)$	$Q_x(0,1)$	B_y	B_x
Cu-diol	609.8	564	532	494	460	398.0	388
	(16400)	(17730)	(18800)	(20240)	(21740)	(25125)	(25770)
Cu-lactone	614.4	568	532	494	460	397.2	384
	(16275)	(17600)	(18800)	(20240)	(21740)	(25175)	(26040)
Cu-$(Me)_7$	611.0	568	531	494	460	398.2	388
	(16370)	(17600)	(18830)	(20240)	(21740)	(25100)	(25770)

[a] (0,0), (0,1), and (0,2) refer to electronic transitions involving zero, one, and two simultaneous vibrational excitations, respectively. Absorption maxima in nm (cm^{-1}); samples in CH_2Cl_2 solution.

instrument. Nuclear Overhauser enhancements (NOE) were measured as previously described.[22] Mass spectra were obtained with a Varian MAT-CH-5 mass spectrometer equipped with an Ion Tech FAB gun.

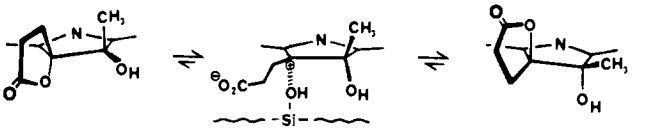
Results and Discussion

A. Chemical and Structural Characterization of the Complexes. Dihydroxylation of synthetic porphyrins possessing biological heme substituents has been achieved by using osmium tetraoxide followed by decomposing the osmate ester with H_2S. The preparative method results in the formation of isomeric vicinal dihydroxychlorin dimethyl esters referred to herein as north and south diols, **1a** and **2**, respectively, corresponding to the location of the reduced pyrrole ring.[22] The north and south diols have similar visible absorption and IR spectra and are shown by ^1H NMR to have the two methyl propionate ester groups present. Following insertion of Cu(II) using the copper acetate method, we began to probe the vibrational properties of the presumed north and south Cu(II)-diols and observed that the IR and RR spectra of the two complexes differed from each other. For example, the presumed south diol complex had a strong new band at 1783 cm^{-1} (see below) that was characteristic of a γ-spirolactone.[26,27] Mass spectral analyses of the two Cu(II) complexes confirmed that, whereas Cu-north (**1b**) was still a diol, the supposed south diol Cu(II) complex of **2** had lost a methanol and had become a spirolactone (**4b**) with the propionic acid connected to the nearest hydroxy group.

Further study revealed that this intramolecular lactonization is a general base-catalyzed reaction and is brought about during the copper insertion by the sodium acetate buffer present in the solution. Other bases, such as amines and pyridines, are also effective. Even during routine purifications, if the diols (**2** and **3**) are left on silica gel or basic alumina over an extended period of time, such as is often the case with preparative TLC plates, the lactone cyclization can proceed to completion. In fact, the TLC method is a convenient way to prepare small amounts of a chlorin lactone. On the other hand, uncyclized diols (**2** and **3**) will survive if they are rapidly eluted from a silica gel column.

Our lactone chlorin model compound, **4a** (prepared by extensive chromatography of **2**) exhibits a ^1H NMR spectrum (Figure 1A) almost indistinguishable from that of Timkovich's lactochlorin[11] for those structural elements that are directly comparable to one another. Particularly interesting is the region between 2.3 and 3.4 ppm where the methylene protons of the rigid spirolactone should appear. On the basis of the absence of a measurable NOE between the 5-Me group (2.0 ppm) and any lactone ring protons, as well as on analyses of the lactone methylene peaks, Timkovich et al. concluded that the two oxygen substituents have a trans configuration.[11] In our model chlorin lactone **4a**, we also could not detect any NOE between the 5-Me peak (1.92 ppm) and the lactone protons. The remarkable similarity of the overall pattern between the model complex and the heme *d* derived lactones would thus argue that the model should likewise have a trans configuration about the pyrroline substituents. If this is true, it means that an inversion of the diol configuration has taken place, because the osmium tetraoxide oxidation only affords cis diols.

Scheme I



In an effort to elucidate this possible inversion process, we have examined the lactonization of a number of south diol chlorins. The tetraethylchlorin complex **3**,[22] by virtue of its superior chromatographic mobility on silica gel, proves to be a more informative system for delineating the reactions involved. When a sample of freshly prepared diol **3** (from the H_2S treatment of the osmate) is heated briefly in MeOH with sodium acetate on a steam bath, TLC (silica gel, 10% ethyl acetate in CH_2Cl_2) indicates that the slow-moving diol (R_f 0.3) is cleanly converted into a fast-moving spot (R_f 0.8). Both mass spectral (diol **3** minus 32) and IR (1782 cm^{-1}) analyses suggest that this compound is a lactone, but ^1H NMR shows the lactone methylene protons merged together between 3.3 and 3.7 ppm, distinctively different from that of **4a** or the lactochlorin methyl ester.[11] If this green compound is rechromatographed on TLC, an even faster moving spot (R_f 0.88) emerges when the major spot is halfway up on the plate. If the plate is sufficiently long or the chromatography is repeated, the new pigment will eventually replace the original one and become the major spot. This new compound, as shown by mass spectroscopy and IR after isolation, is still a lactone and has ^1H NMR features (Figure 1B) very similar to those of the trans lactones observed earlier. This compound can also be shown to be identical with the lactone prepared by repeated chromatography of the diol **3**.

On the basis of these observations, we have assigned the NaOAc-cyclized product to be the cis lactone, while the silica gel induced lactone is trans.[28] The cis–trans isomerization is illustrated in Scheme I, with the key step being a unimolecular alkyl–oxygen fission process. The north diol chlorins (**1a** and **1b**), lacking the propensity to lactonize, apparently can resist inversion upon repeated chromatography on silica gel; we have not observed another isomeric diol during chromatography or base-catalyzed conditions. However, differentiation of cis and trans chlorin diols may not be obvious if they happen to be chromatographically inseparable. Even NMR would not be reliable since the cis diol as well as the cis lactone exhibits barely recognizable NOE's (≤2%) between the methylene protons and the adjacent methyl group, presumably because of twisting and staggering relative to the saturated pyrroline ring.

The cis–trans inversion also occurs with the methylated complexes **4a** and **4b**, although the isomers are harder to separate. However, the trans lactones, such as **4b**, can always be obtained in high purity simply by repeated chromatography on preparative TLC plates.

B. Electronic Absorption Spectra. The optical absorption spectra of Cu-diol and Cu-lactone are shown in Figure 2, and the electronic transitions of these complexes, together with those of Cu-$(Me)_7$, are assigned in Table I. The $Q_y(0,0)$ and $Q_y(0,1)$ transitions of Cu-lactone are both red-shifted by ~4 nm (~130 cm^{-1}) relative to those of Cu-diol. In the Soret region, the shoulder

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(27) The possibility of a 6-membered lactone fused across the 5,6-position (as suggested by a reviewer) is unlikely because the typical IR absorption for ν(C=O) of a δ-lactone is below 1760 cm^{-1}.[26] In addition, NOE results and analyses of coupling constants of the methylene protons further support the γ-lactone formulation (Chang, C. K.; Sotiriou, C., manuscript in preparation).

(28) The NMR assignments for the cis and trans chlorin lactones are discussed in detail in a forthcoming manuscript; see ref 27.

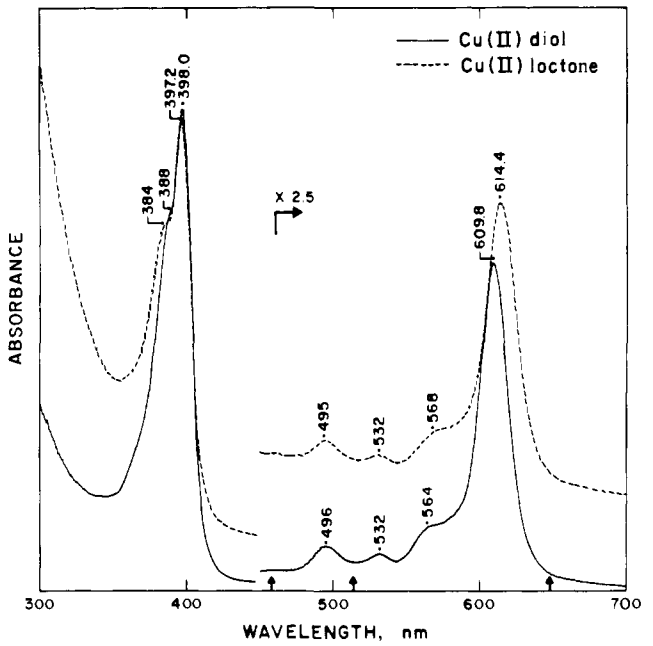

Figure 2. Electronic absorption spectra of Cu-diol (solid line) and Cu-lactone (dashed line), in CH_2Cl_2 solutions.

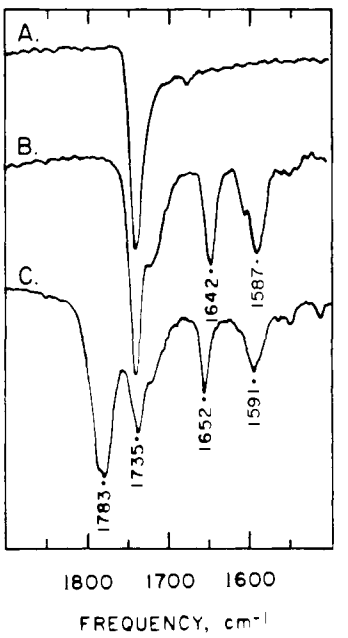

Figure 3. 1900–1500-cm^{-1} IR spectra of: (A) Cu-porphyrin; (B) Cu-diol; (C) Cu-lactone.

of the Cu-lactone is more clearly resolved and is ~4 nm (270 cm^{-1}) blue-shifted from that of Cu-diol. An analogous pattern was observed in the electronic spectra of the two chlorins in CS_2 solution (data not shown), although all of the absorption bands of each complex were red-shifted ~5 nm relative to those in CH_2Cl_2 solution. The electronic transitions of Cu-$(Me)_7$ (Table I) are similar to those of Cu-diol, with very slight red shifts of the $Q_y(0,0)$ and $Q_y(0,1)$ bands.

C. Vibrational Spectra of Chlorins. The infrared and resonance Raman spectral properties of metallochlorins are considerably different from those of the corresponding metalloporphyrins.[19-21] Comparison of the two sets of molecules readily indicates the profound physical and chemical changes which arise as a consequence of the 2-equiv reduction at one of the porphyrin pyrrole rings. These range from a decrease in effective molecular symmetry to a greater structural flexibility, which may include a pronounced S_4 ruffling.[29-32] We have previously established a generalized set of vibrational spectroscopic properties for metallochlorins: (1) an increase in the total number of IR and RR bands; (2) an increase in the number of polarized RR bands; (3) the notable presence of two or more polarized bands in the region of ν_4, $\nu(C_a-N)$, the so-called oxidation-state marker of metalloporphyrins; (4) a strong, polarized C_a-C_m mode > 1600 cm^{-1} (ν_{10} in porphyrins); (5) altered polarization properties for porphyrin A_{2g} and B_{1g} modes; (6) RR-active, split porphyrin E_u modes; (7) the predominance of polarized vibrational modes with both Soret and visible excitation; and (8) a close correspondence in frequency for IR and RR features.[19-21] Thus, we can confidently predict the basic spectral pattern which should occur for the heme *d*-type models and focus on the analysis of distinguishing characteristics between the spectra of the two complexes.

(1) Infrared Absorption Spectra. The IR spectra of metallochlorins are predictably different from those of substitutionally related metalloporphyrins.[21] For Cu-porphyrin, few similarities exist between the IR and RR spectra due to mutual exclusion based on an effective D_{4h} symmetry. In contrast, the IR spectra of the heme *d*-type chlorins generally match their respective RR spectra in frequency for all regions studied (not all data shown).[33,35] A general comparison of the Cu-lactone and Cu-diol IR spectra over the 4000–400-cm^{-1} range reveals that many of the more intense Cu-lactone bands appear ~5–10 cm^{-1} higher in frequency than the corresponding Cu-diol features.

Infrared spectra in the 1900–1500-cm^{-1} region of Cu-porphyrin, Cu-diol (**1b**), and Cu-lactone (**4b**) are shown in Figure 3. Each of the Cu(II) chlorins exhibits the characteristic IR chlorin mode,[36,37] as an intense band near ~1645 cm^{-1}, and an additional strong band at ~1590 cm^{-1}, which is absent from the spectrum of Cu-porphyrin. The presence of these bands is typical of chlorins and reflects the lowered symmetry of the macrocycle relative to the porphyrin.[21] We previously assigned the >1600-cm^{-1} band as $\nu_{37(1)}$, a C_a-C_m mode.[21] The assignment as a C_a-C_m mode is confirmed by the shift from 1642 to 1632 cm^{-1} in the d_4-Cu-diol IR spectrum (not shown). In the Ni(II) tetramethylchlorin, the corresponding frequency is observed at ~1620 cm^{-1}, indicating the sensitivity of the mode to the mass of the C_m substituents and to the metal ion.[21] Both Cu-porphyrin and Cu-diol have a strong

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(33) In the 4000–2000-cm^{-1} IR spectral region, both Cu-diol and Cu-lactone have a sharp feature at 3504 cm^{-1}, generally indicative of hydrogen-bonded hydroxyl group(s). A weak band at ~3056 cm^{-1} in the spectra of Cu-porphyrin, Cu-diol, and Cu-lactone is assigned as $\nu(C_m-H)$, since it is absent in the d_4-Cu-diol spectrum. This observation is in good agreement with $\nu(C_m-H)$ of Ni-OEP (nickel octaethylporphyrin).[34] The $\nu(C-H)$ of the β-pyrrole methyl groups occur at ~2920 and ~2850 cm^{-1} for all four complexes, and the $\nu(C-H)$ of the methyl ester(s) is observed at ~2953 cm^{-1}; these frequencies also concur with literature assignments.[34] In the 1800–900-cm^{-1} region of the IR, splitting of porphyrin E_u modes is evident in the Cu-diol and Cu-lactone spectra. This pattern was previously observed by us for other metallochlorins.[21] Comparison of Cu-diol and d_4-Cu-diol spectra indicates that bands at 1642, 1603, 1588, ~1350, 1315, 1269, 1248, 1221, ~1140, and 946 cm^{-1} in the Cu-diol IR spectrum arise in part from vibrational motions of the meso-carbons.
(34) Alben, J. O. In *The Porphyrins*; Dolphin, D., Ed.; Academic: New York, 1978; Vol. 3, Chapter 7.
(35) A new observation, interesting primarily due to the additional problems it raises, is that RR spectra of chlorins consistently contain more bands than expected, even allowing for the predicted splitting and Raman activation of the formerly E_u modes. A possible explanation is that the RR spectra of metallochlorins are not limited strictly to vibrational modes of the macrocycle but may also include local modes of peripheral groups. For porphyrins such local modes are only IR active; for metallochlorins we apparently observe many of these features in both IR and RR spectra.
(36) Scheer, H.; Inhoffen, H. H. In *The Porphyrins*; Dolphin, D., Ed.; Academic: New York, 1978; Vol. 2, pp 45-90.
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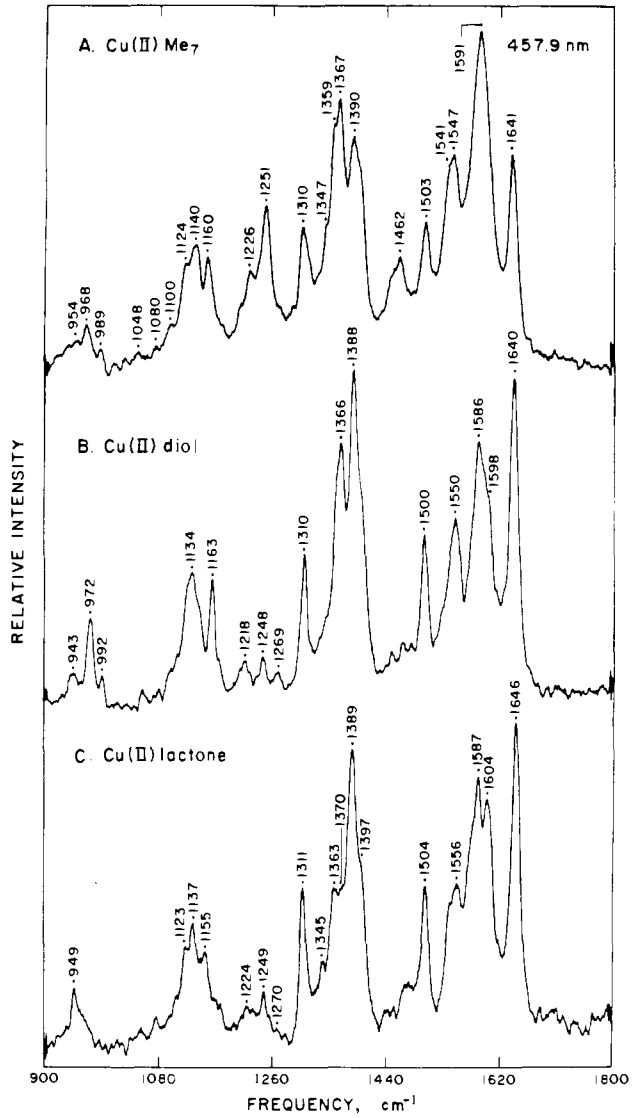

Figure 4. 457.9-nm excitation resonance Raman spectra, high-frequency region: (A) Cu-$(Me)_7$ chlorin; (B) Cu-diol; (C) Cu-lactone. All samples in a KBr matrix. Conditions: backscattering geometry; laser power, 50 mW at sample Dewar; slit width, 5 cm^{-1}; scanning speed, 2 cm^{-1}/s with repetitive scanning.

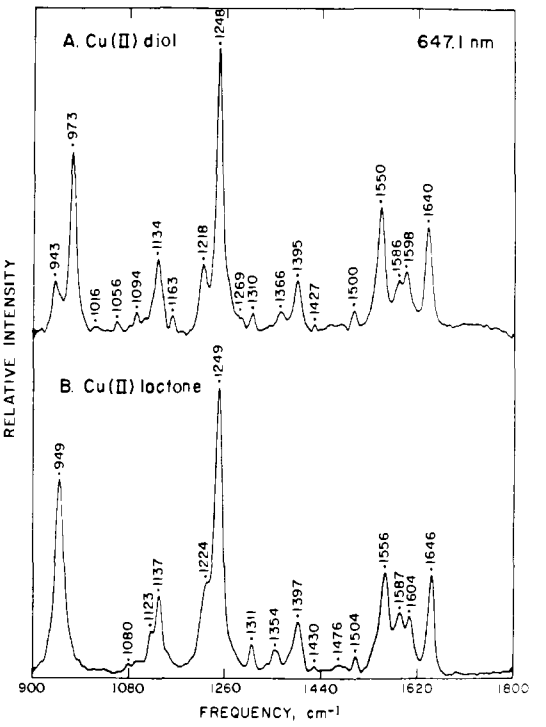

Figure 5. 647.1-nm excitation resonance Raman spectra, high-frequency region: (A) Cu-diol; (B) Cu-lactone. Both samples in a KBr matrix. Conditions: backscattering geometry; laser power, 150 mW at sample Dewar; slit width, 5 cm^{-1}; scanning speed, 2 cm^{-1}/s with repetitive scanning.

absorption at 1735 cm^{-1} arising from ν(C=O) of the propionate dimethyl ester. Cu-lactone also exhibits a band at 1735 cm^{-1}, but its calculated absorbance is only half that of the two former species, consistent with its formulation as a monomethyl ester. A similar intensity dependence of ν(C=O) was observed for mono- and dimethyl esters of iron pheophorbides.[38] A distinguishing feature of the Cu-lactone IR spectrum is the band at 1783 cm^{-1}, which we assign as ν(C=O) of the γ-spirolactone moiety.[26] The IR spectrum of the free-base esterified heme *d* macrocycle extracted from *E. coli*[11] also has bands at 1782 and 1736 cm^{-1}, analogous to those of the Cu-lactone, an observation supporting the validity of this species as a model for the extracted *E. coli* prosthetic group.

(2) Resonance Raman Spectroscopy of Cu(II) Chlorins. Because the *E. coli* heme *d* terminal oxidase has previously been studied by resonance Raman spectroscopy only in a very narrow spectral region,[18] we have a unique opportunity to examine the vibrational properties of model complexes and predict those properties which should be observed if a given model were structurally analogous to the in vivo prosthetic group. Four-coordinate Cu(II) complexes were chosen for this study to focus on the spectral properties of the macrocycles without the additional complexities arising from variations in coordination number, spin state, and oxidation state.

The major structural difference between Cu-diol and Cu-lactone rests in the site of pyrrole ring reduction and the type of substituents on the reduced ring. For metalloporphyrins, it has generally been assumed that as long as the substituents are connected to the conjugated macrocycle via saturated carbon atoms, the RR spectrum would be expected to be quite similar to that of octaethylporphyrin (OEP). Electronic coupling would be insignificant, and mechanical coupling would be largely limited to interaction with C_b–S (substituent) stretching and bending vibrations.[39] This assertion has consistently been shown to hold for a variety of metalloporphyrins with different nonconjugated substituents. However, in the case of metallochlorins, we find that substituents on the reduced pyrrole ring may have a profound effect on the vibrational properties of the macrocycle. The origins of this effect are not yet clear, although they may arise both from the chemical and electronic properties of the substituent and from the substituent stereochemistry and local chirality.

Resonance Raman spectra of the two heme *d*-type models and of Cu-$(Me)_7$ are shown in Figures 4–6. The general spectral patterns observed for Cu-diol and Cu-lactone concur with our previously defined characteristics of metallochlorins.[19-21] The presumed oxidation-state marker, ν_4, is observed at a lower frequency for the chlorins relative to a substitutionally related porphyrin: Cu-diol, 1366 cm^{-1}; Cu-lactone, 1363 cm^{-1}; and Cu-$(Me)_7$, 1367 cm^{-1}; vs. Cu-porphyrin, 1373 cm^{-1} (not shown). Since this phenomenon was formerly noted for a variety of other metallochlorins,[19-21] we suggest that it may be another characteristic property of the chlorins.[40]

Based on spectral shifts observed for d_4-Cu-diol, more specific assignments of some of the vibrational modes of Cu-diol are now

(38) Andersson, L. A.; Loehr, T. M.; Simpson, D.; Smith, K. M., manuscript in preparation.

(39) Spiro, T. G. In *Iron Porphyrins, Part II*; Lever, A. B. P., Gray, H. B., Eds.; Addison-Wesley: Reading, MA, 1983; Chapter 3.

(40) Exact assignment of the modes in the 1340–1390-cm^{-1} region of the metallochlorin spectra is not yet clear-cut given the multitude of bands. We have chosen to assign as ν_4 the band closest in frequency to that of the structurally related porphyrin. Generally, this polarized band is dominant with blue excitation but diminishes in intensity with longer wavelength excitation, as is true in the case of porphyrins.

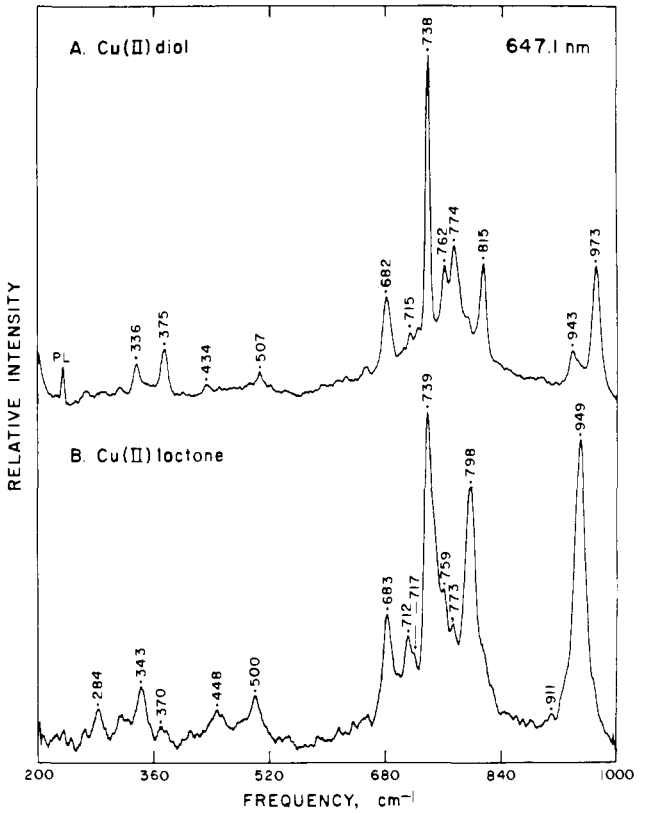

Figure 6. 647.1-nm excitation resonance Raman spectra, low-frequency region: (A) Cu-diol; (B) Cu-lactone. Both samples and conditions as in Figure 5.

Table II. Selected Resonance Raman Frequencies (cm^{-1}) for Cu(II)-Chlorins[a]

Cu-diol	d_4-Cu-diol	frequency shifts[b] obsd	frequency shifts[b] lit.	chlorin assignments[c]
1640	1631	9	10	ν_{10}, $\nu(C_a-C_m)$, A
1598	1584	14	16	ν_{19}, $\nu(C_a-C_m)$, B
1586	1570	16	16	ν_{37}, $\nu(C_a-C_m)$, A
1541[d]	1520	21	15	ν_{38},[e] $\nu(C_b-C_b)$, A
1500	1489	11	7	ν_3, $\nu(C_a-C_m)$, A
1310	896	414	418	ν_{21}, $\delta(C_m-H)$, B
1268	*f*	*f*		ν_{42b}, $\delta(C_m-H)$, B
1249	1198	50	93	ν_{42a}, $\delta(C_m-H)$, A
1218	945	273	270	ν_{13}, $\delta(C_m-H)$, A
738	673	65	72	ν_{16},[e] $\delta(C_a-N-C_a)$, A

[a] RR data obtained with 457.9-, 514.5-, and 647.1-nm excitation lines; the table entries do not reflect the dramatic intensity dependencies upon the excitation lines. [b] Raman downshifts observed for d_4 meso-substitution of Cu-diol; lit. is downshifts reported for d_4-Ni(II)-OEP, ref 43. [c] Based on C_2 symmetry, the assignments listed are tentative, particularly with respect to mode contributions since no normal-coordinate calculations for metallochlorins have yet been reported; the given mode contributions are adapted from those reported for metalloporphyrins (ref 43). [d] Resolved with 514.5-nm excitation. [e] This mode apparently includes some meso-position contribution. [f] Uncertain.

possible (Table II).[41] The RR spectra of Cu-diol, Cu-lactone, and Cu-$(Me)_7$ have a band at ~1249 cm^{-1}, which increases dramatically in intensity with longer wavelength excitation (Figure 5). Similar observations were made previously,[19,20] and an assignment of the frequency as $\nu_{42(a)}$, a $\delta(C_m-H)$ mode, had been proposed. This assignment is confirmed by RR spectra of d_4-Cu-diol, in which the strong 1249-cm^{-1} band is apparently replaced by a new feature at ~1198 cm^{-1}. Other likely $\delta(C_m-H)$ modes are the Cu-diol bands at 1310 (ν_{21}, d_4: 896 cm^{-1}) and 1218 cm^{-1} (ν_{13}, d_4: 945 cm^{-1}); both of these bands exhibit large isotopic shifts which parallel those reported for Ni(II)-OEP.[43] In the low-frequency spectra (Figure 6), the apparent 65-cm^{-1} isotopic shift of the dominant 738-cm^{-1} band of Cu-diol parallels a 72-cm^{-1} downshift for the 750-cm^{-1} band of Ni(II)-OEP[43] and a 68-cm^{-1} downshift for iron protoporphyrin IX complexes.[44] This mode was previously assigned as ν_{16}, a $\delta(C_a-N-C_a)$ mode, on the basis of the normal coordinate analyses of Abe et al.[43] but apparently also includes a significant C_m contribution as evidenced by its large downshift upon meso-deuteriation (Table II).

Table III. Key Spectral Differences in the RR Spectra of Cu(II)-Chlorins with Different Pyrroline Ring Substituents[a]

Cu-$(Me)_7$[b]	*cis*-Cu-diol	*trans*-Cu-lactone
	d	285
	336	343
	375	370
	738 vs	739 m
	815	798
954	943	*d*
d	*d*	949
968	973	*d*, *c*
989	992[b]	*d*
1160	1163	1155[b]
1226	1218	1224
1367	1366	1363[b]
1462	*d*	*d*
1503	1500	1504
1547	1550	1556
d	1598	1604
1641	1640	1646

[a] Frequencies, cm^{-1}; data < 900 cm^{-1} obtained with 647.1-nm excitation; data > 900 cm^{-1} obtained with 457.9- and/or 647.1-nm excitation. [b] 457.9-nm excitation. [c] See ref 46. [d] Absent or low-intensity feature.

(3) Nonconjugated Substituent Effects. Although the general spectral patterns of Cu-diol and Cu-lactone are similar, a closer examination reveals higher frequencies for many of the Cu-lactone modes. For example, bands at 1646, 1604, 1556, 1504, 1224, and 1137 cm^{-1} in the Cu-lactone spectrum are 4–7 cm^{-1} above those of the Cu-diol (Figures 4 and 5; Table III). These observations suggest higher force constants or different potential energy distributions in the normal modes for a given vibrational feature of Cu-lactone. Perhaps the most obvious spectral difference between the two heme *d*-type models is in the 650–1000-cm^{-1} region of the red excitation spectra (Figures 5 and 6).[45] Whereas Cu-diol has two bands at 943 and 973 cm^{-1}, the Cu-lactone spectrum has only a single band at 949 cm^{-1}.[46] Other low-frequency modes are also shifted between the two complexes, for example, 815 vs. 798 cm^{-1} for Cu-diol and Cu-lactone, respectively. It is indeed interesting that the lactone substituent appears to affect the entire macrocyclic vibrational pattern even though this moiety is not in conjugation.

To confirm the hypothesis that the observed spectral differences between the Cu-diol and Cu-lactone complexes are a function of

(41) The observed frequencies for Cu-diol, d_4-Cu-diol, and Cu-lactone are roughly comparable to those of Cu-OEC and d_2-Cu-OEC reported by Ozaki et al.[42] Specific assignments may vary due to our use of a $C_2(x)$ effective symmetry and the addition of new A and B chlorin modes arising from the former IR-active E_u modes. While the apparent downshifts for each of these modes correlates well with those reported by Abe et al.[43] for Ni(II)-OEP, we cannot be totally certain of any assignments for a metallochlorin without more extensive isotopic studies. Furthermore, it is entirely possible that the normal mode contributions for a metallochlorin might differ from those of a metalloporphyrin, thus altering predicted isotopic effects.

(42) Ozaki, Y.; Kitagawa, T.; Ogoshi, H. *Inorg. Chem.* **1979**, *18*, 1772–1776.

(43) Abe, M.; Kitagawa, T.; Kyogoku, Y. *J. Chem. Phys.* **1978**, *69*, 4526–4534.

(44) Choi, S.; Spiro, T. G. *J. Am. Chem. Soc.* **1983**, *105*, 3683–3692.

(45) The low-frequency RR spectral region of metallochlorins is very strongly enhanced with red excitation (into the $Q_y(0,0)$ electronic transition), in contrast with spectra of metalloporphyrin complexes where the low-frequency region is generally best resolved with blue (Soret and/or near-Soret) excitation.

(46) A shoulder at 974 cm^{-1} is, however, observed in the 514.5-nm excitation spectrum of Cu-lactone.

the substituents on the pyrroline ring, and do not arise solely from the rigidity of the lactone substituent, we compared Cu-diol with Cu-$(Me)_7$ (Figure 4). The structures of these two complexes vary only at the reduced ring: two methyl and two hydroxy moieties for Cu-diol vs. three methyl groups and one hydrogen substituent for Cu-$(Me)_7$. With near-Soret excitation, several differences are immediately obvious with respect to both frequencies and relative intensities (see also Table III). For example, the Cu-$(Me)_7$ spectrum has a medium-intensity feature at 1462 cm^{-1} that is absent, or very weak, in the Cu-diol spectrum. Whereas features at 1640 and 1388 cm^{-1} (ν_{10} and ν_{41a}, respectively) are dominant for Cu-diol, for Cu-$(Me)_7$ the dominant bands are at 1591 and 1367 cm^{-1} (ν_{37} and ν_4). Also, the intensities of the 1226- and 1251-cm^{-1} bands of Cu-$(Me)_7$ with blue excitation are very different from those of Cu-diol. Furthermore, the frequency of ν_4 for both Cu-$(Me)_7$ and Cu-diol is $\sim$3 cm^{-1} above that of Cu-lactone. Differences are also obvious between the spectra of Cu-lactone and Cu-$(Me)_7$. Aggregation effects (e.g., π-stacking or substituent hydrogen bonding) can be ruled out as the origin of the observed spectral differences between the chlorin complexes, since their respective RR spectra from samples in solution (CS_2; wet or dry CH_2Cl_2) are essentially identical with those shown here for samples dispersed in KBr. Hence, these observations are clear evidence that the substituents on the reduced pyrrole ring of a metallochlorin have a significant effect on both the frequencies and intensities of many vibrations of the macrocycle.[47]

(4) Relevance of Model Complexes to Heme *d*. Is it possible to define those properties which distinguish Cu-lactone from Cu-diol and hence might differentiate between a lactone and a diol structure for in vivo heme *d*? At this point, we offer a few inferences, which may be of relevance to this question (Table III). First, for Cu-lactone, the frequency of ν_{10} is $\sim$6 cm^{-1} above that of both Cu-diol and Cu-$(Me)_7$ and is independent of excitation wavelength. A second characteristic difference between the three complexes occurs in the 940–990-cm^{-1} spectral region which is most notable with red excitation: Cu-lactone has a single strong band at 949 cm^{-1}, whereas Cu-diol has two bands at 973 and 943 cm^{-1} of strong and medium intensity, respectively. Vibrational modes in this region contain contributions from the C_b substituents,[43,48] which differ between Cu-lactone and the other two chlorins.[49]

A third major difference is apparent in the low-frequency region of the two chlorin spectra (Figure 6). For Cu-diol, the most intense feature in the 200–1800-cm^{-1} region is the sharp peak at 738 cm^{-1} that is well separated from a doublet at 762/774 cm^{-1} and a sharp peak at 815 cm^{-1}. In contrast, for the Cu-lactone, a peak of only moderate intensity occurs at 739 cm^{-1}, the $\sim$760/770-cm^{-1} bands are not resolved, and the third component is at 798 cm^{-1}, significantly below that of the Cu-diol. The most notable spectral differences between the Cu-diol and Cu-lactone complexes are summarized in Table III.

Since in vivo heme *d* contains an iron, an important consideration is the transferability of our observations from the copper model chlorins. The frequency of ν_{10} is known to vary with both oxidation state and spin state in iron porphyrins,[39,48] and we have shown that these marker bands are generally similar in frequency for the analogous iron chlorin complexes.[19,20] Thus, we can predict that if in vivo heme *d* were a diol chlorin, its ν_{10} frequency should be observed in the "normal" frequency region for the given spin and oxidation state, whereas the ν_{10} of a lactone chlorin would presumably be slightly elevated in frequency. Furthermore, since the $\sim$940–990-cm^{-1} region of the diol and lactone chlorins differs markedly (*vide supra*), we would also predict the observation of two bands in this region if in vivo heme *d* were a diol chlorin, vs. a single band at $\sim$950 cm^{-1} for a lactone chlorin. A preliminary RR spectral comparison of the ClFe(III)-diol and ClFe(III)-lactone chlorin complexes does indeed reveal the predicted properties for the iron heme *d*-type models; i.e., for the iron lactone chlorin we observed an elevated ν_{10} frequency and a single band in the $\sim$940–990-cm^{-1} region.[50]

Concluding Remarks

The observation that the south dihydroxychlorin propionate esters will easily cyclize under a variety of conditions has led us to infer that the lactochlorin structure obtained by Timkovich et al.[11] may not be the true structure for in vivo heme *d* but could have arisen from a parent diol during isolation and purification. The possibility for such a rearrangement was also recognized by those authors. The stereochemistry of the diol also remains uncertain. Our studies indicate that the trans lactone will always prevail because chromatographic purifications catalyze inversions. The identification of the trans configuration of the lactochlorin,[11] therefore, may have no bearing on the configuration of the diol precursor. It is even possible that an epoxychlorin is the structure of in vivo heme *d*, which is attacked by the propionate to give the trans lactochlorin during isolation. Further work is needed to clarify this question.

We have presented evidence indicating that nonconjugated substituents on the pyrroline ring of a metallochlorin may have a significant influence on the vibrational spectroscopic properties of the macrocycle. The demonstration that the resonance Raman spectra of Cu-diol and Cu-lactone are distinguishable from one another may provide a means for identification of the true structure of the in vivo heme *d* terminal oxidase of *E. coli*, without extraction of the prosthetic group from the purified protein. Since recent studies indicate that only *b* and *d* hemes are present in this enzyme,[51] the use of selective RR enhancement, particularly with red excitation, should permit one to study the green chlorin *d* prosthetic group relatively free from interferences of the *b* heme.

Acknowledgment. This research was supported by the National Institutes of Health (GM 34468). We wish to thank Linda Schmidlin for excellent technical assistance.

(47) A comparative spectral study of *cis*- and *trans*-Cu(II)-OEC complexes reveals $\sim$5-nm differences in their absorption maxima and RR frequency shifts of up to 10 cm^{-1}, as well as many significant intensity differences: Andersson, L. A.; Loehr, T. M.; Stershic, M.; Stolzenberg, A. M., manuscript in preparation.

(48) Choi, S.; Spiro, T. G.; Langry, K. C.; Smith, K. N.; Budd, D. L.; La Mar, G. N. *J. Am. Chem. Soc.* **1982**, *104*, 4345–4351.

(49) An obvious structural difference between the Cu-lactone chlorin and the Cu-diol or Cu-$(Me)_7$ species is that the former has only one propionate ester, whereas the latter both have two. However, this structural distinction may not be the sole origin of differences in the 940–990-cm^{-1} region of the chlorin RR spectra, since iron pheophorbide dimethyl esters as well as the pyropheophorbide monomethyl esters exhibit similar spectral patterns to one another in this region.[38]

(50) Andersson, L. A.; Loehr, T. M.; Chang, C. K., unpublished results.

(51) Lorence, R. M.; Koland, J. G.; Gennis, R. B. *Biochemistry* **1986**, *25*, 2314–2321.