experimental frequencies. Nevertheless, the qualitative trends in the observed intensity ratios are in good agreement with the theory.

The above example is really intermediate between intra- and intermolecular vibrational resonance coupling, since the coupled vibrations are localized on different ligands of the same molecular complex. However, coupling of fundamental vibrations of different ligands is allowed because $(\partial^2 V/\partial Q_A \partial Q_B)$ is not required to be zero. As expected, the weak coupling limit is not valid (and was not used), since the absolute values of the coupling parameter U are not small compared to those of the unperturbed frequency difference Δ. In ref 8 is reported an example of intermolecular Fermi resonance, which illustrates that intensity borrowing and frequency shifts can still be observed in more weakly coupled systems. As reported by Kincaid et al.,[8] in CH_2Cl_2 solutions containing pyridine, the ^{16}O-^{16}O stretch of Co(TPP-d_8)O_2 is observed at 1143 cm^{-1}, along with an enhanced solvent mode at 1155 cm^{-1}. In CD_2Cl_2 solution, the $^{16}O_2$ vibration was found at 1148 cm^{-1}, and the 1155-cm^{-1} mode disappeared. These numbers imply a coupling parameter $|U| = 5.9$ cm^{-1}, which is smaller than the values given in Table I for interligand coupling. However, the uncoupled frequencies are $\nu(^{16}O_2) = 1148$ cm^{-1} and $\nu(CH_2Cl_2) = 1150$ cm^{-1}, so $|U|$ is not small compared to $|\Delta| = 2$ cm^{-1}. If the frequency shift for the complex with $^{18}O_2$ is evaluated, it is found to be negligible, due to the much larger value of $|\Delta|$, 61 cm^{-1}. The intensity borrowing is correspondingly weak, since $(D - \Delta)/2U$ (see eq 23) is only about 0.07. Thus in the case of the $^{18}O_2$ complex, the theoretical prediction of negligible solvent enhancement or O_2 frequency shift is in agreement with the experimental results.

IV. Conclusion

A simple theory of intermolecular vibrational resonance coupling has been presented and has been shown to be valid for the strong coupling observed in a molecular complex. Frequency shifts and intensity perturbations may be observed for any general type of intermolecular interaction, provided there is a dependence of the intermolecular potential on the normal coordinates of the coupled vibrational modes. In the case of intermolecular coupling involving a solute surrounded by a solvation sphere, an experimental approach is suggested for determining solvation numbers from resonance Raman spectra in a mixed solvent. An important conclusion is that intensity perturbations can result even in the weak coupling case, where a frequency shift cannot be observed, especially in the case of resonance enhancement of the solute Raman mode.

One limitation of the derivation is that all the interacting solvent molecules are considered equivalent. The intermolecular coupling U is actually a function of distance and relative orientation. The distance dependence, incidently, was illustrated very nicely in ref 7, where the solvent intensity borrowing was shown to vanish for the very hindered picket-fence porphyrin adduct. However, if U is averaged over an equilibrium distribution of solvent coordinates, then it is the same for every solute-solvent pair, and the approximation of equivalent solvent molecules is valid. Further experiments to determine the utility of the proposed approach for determining solvation numbers are in progress.

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Proposed Structure for the Prosthetic Group of the Catalase HPII from *Escherichia coli*

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Abstract: *Escherichia coli* contains two catalases designated HPI and HPII. The heme prosthetic group of HPII is an unusual green chromophore that was believed to be a member of the family of *d*-type hemes. The heme was extracted and compared to the heme of the terminal oxidase cytochrome complex in *E. coli*. The two hemes were very similar by visible spectroscopy but were resolved by chromatography. The heme was converted to a free-base, esterified derivative that was characterized by ^1H NMR spectroscopy, infrared spectroscopy, and mass spectrometry. The proposed structure for the free base is 12-hydroxy-13-*cis*-spirolactone-2,7,12,18-tetramethyl-3,8-divinyl-17-propionylporphyrin.

The respiratory electron-transport chain of certain aeorobic bacteria contains an unusual prosthetic group for the terminal oxidase. This group, originally called heme a_2 but later renamed to heme *d*, is the oxygen, carbon monoxide binding site. It is spectroscopically characterized by a red shift of the α band in the visible spectrum. In 1956 Barrett[1] extensively investigated the properties of heme a_2 and the porphyrin derived by iron removal and tentatively proposed that it was a dihydroporphyrin. In 1985 it was shown by isolation and purification of the chlorin derived from the heme of the terminal oxidase in *E. coli* that the basic structure was analogous to that of protoporphyrin IX but that ring C contained extra oxygen functional groups.[2] The demetalized free base actually characterized was a hydroxyl, spirolactone form, **1** (Chart I). It was pointed out that this final product could have arisen from spontaneous cyclization of a diol **3**. Later infrared analysis of freshly prepared heme extracts indicated that a diol was directly obtainable from the haloenzyme.[3] The proposed structure for the natural product was confirmed by a total synthesis by Sotiriou and Chang.[4] The total synthesis also

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(1) Barrett, J. *Biochem. J.* **1956**, *64*, 626-639.
(2) Timkovich, R.; Cork, M. S.; Gennis, R. B.; Johnson, P. Y. *J. Am. Chem. Soc.* **1985**, *107*, 6069-6075.
(3) Vavra, M. R.; Timkovich, R.; Yap, F.; Gennis, R. B. *Arch. Biochem. Biophys.* **1986**, *250*, 461-468.

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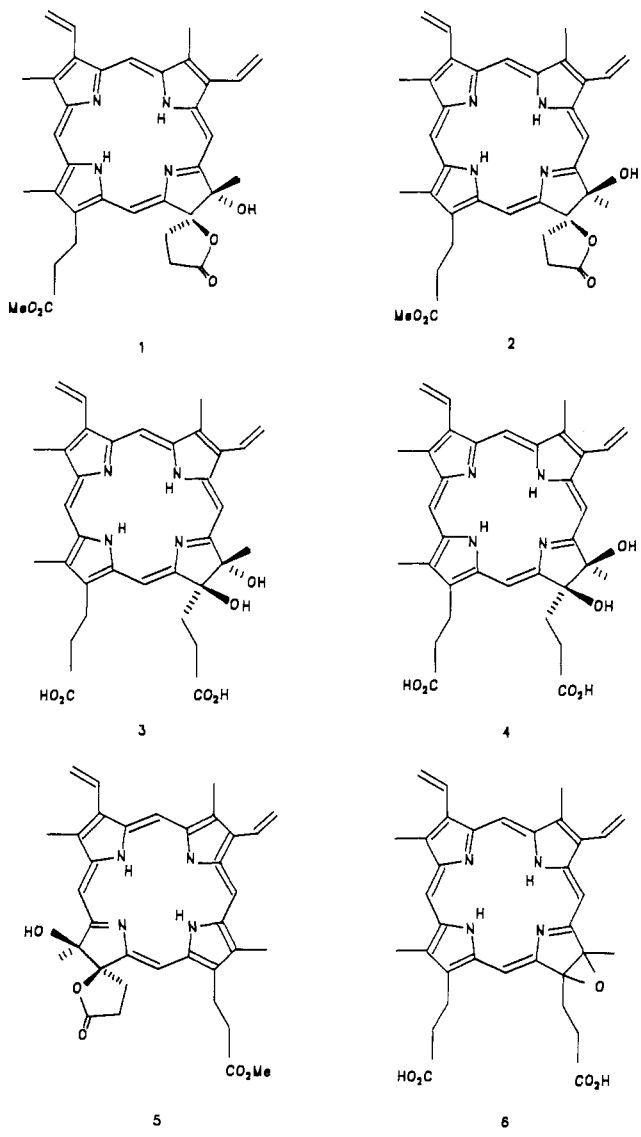
Chart I



confirmed that the natural product from the terminal oxidase was the trans isomer, that is, with the oxygens pointing on opposite sides of the pyrroline plane. Neither work on the natural product nor synthesis has yet established the absolute configuration at the chiral centers. The synthesis of Sotiriou and Chang also produced the cis-isomer **2**, which at that time was important, but only for establishing that there was a clear NMR spectroscopic difference between cis and trans forms.

E. coli also produces two catalases called HPI and HPII.[5] HPI is a tetrameric enzyme, 84 000 daltons per subunit, that also has peroxidase activity. The prosthetic group in each subunit is normal iron protoporphyrin IX. HPII functions only as a catalase and is a hexamer of 92 000-dalton subunits. By visible spectroscopy there is a single type of heme prosthetic group. In the oxidized state, the protein has a prominent visible band at 590 nm, compared to 645 nm for the equivalent state for the heme *d* chromophore in the terminal oxidase complex. However, initial extraction experiments indicated a reduced pyridine hemochromogene band at circa 615 nm and an oxidized acidic hemin band at 603 nm, which are very comparable to the bands of heme *d* extracted from the terminal oxidase. This suggested identity or similarity between the chromophores. Neither the catalase nor the heme *d* containing terminal complex are constitutive in *E. coli*. The catalase is produced at much higher levels when the culture

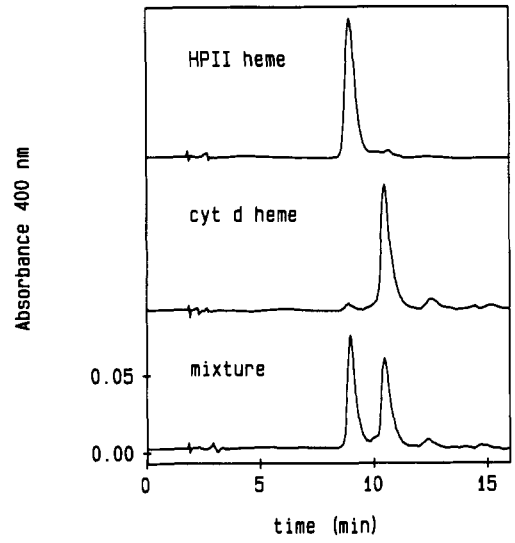


Figure 1. Reverse-phase HPLC chromatograms for the heme isolated from the HPII catalase and from the cytochrome complex of the terminal oxidase. The column was 4.6 × 250 mm packed with Whatman 5-μm ODS III (a C_{18} coating), eluted at 1 mL/min with water (50%)–acetonitrile(45%)–acetic acid(5%).

is grown with high aeration, while the cytochrome complex is produced when *E. coli* is grown under low aeration conditions when the oxygen supply becomes critical.[6] This could raise interesting biosynthetic questions concerning the occurrence of *d*-type hemes depending upon the actual heme structures. The purpose of the present report is to establish the structural relation between the chromophores of the terminal oxidase and catalase of *E. coli*.

Results

The heme was extracted from purified HPII catalase and in parallel experiments, from the terminal oxidase complex, by previously published methods[2,3] or modifications to be discussed. Heme *d* has distinctive visible spectra in acidic acetone and acidic methanol.[3] In acidic acetone, heme *d* as isolated from the oxidase complex has maxima at 750 (broad), 603, 555–560 (shoulder), 520–530 (shoulder), 475–480 (shoulder), and 387 nm. The Soret maximum at 387 nm is sensitive to precise acid concentration and is not a simple, reliable indicator of detailed structure. In acidic methanol, distinctive bands appears at 729 (broad), 595, 550, 520, 480, and 395 nm that to our knowledge are very unique to heme *d*. The heme extracted from HPII had spectra that showed no reliable, consistent difference from those of heme *d* in wavelength maxima or relative intensity. The reduced pyridine hemochromogen of both extracts showed the prominent visible band at 613 nm, and the metal-free esters showed a prominent visible band at 653 nm and a Soret band at 401 nm. Although the nominal spectrophotometer resolution (0.25 nm) was more than adequate, the band widths of even the sharpest peaks can lead to some apparent variation of at least 1–2 nm for routine spectra at room temperature. Therefore, subtle differences on this order would have required more elaborate spectroscopy than was performed.

Reverse-phase HPLC provided the first evidence that the two hemes possessed different structures to some degree. Figure 1 contrasts chromatograms for the heme obtained by extraction of the cytochrome terminal oxidase complex and by extraction of the HPII catalase. For the particular experiment shown, both hemes had been partially purified by passage through a low-resolution silica column as described previously,[2] which is especially necessary for the heme from the cytochrome complex in order to separate it from lipids and endogenous heme *b*. Subsequent control experiments on both extracts indicated that the retention time of the heme was not altered by the initial silica chromatography step. Following demetalization by ferrous sulfate in glacial acetic acid and esterification by diazomethane treatment,

(4) Sotiriou, C.; Chang, C. K. *J. Am. Chem. Soc.* **1988**, *110*, 2264–2270.
(5) Loewen, P. C.; Switala, J. *Biochem. Cell Biol.* **1986**, *64*, 638–646.
(6) Green, G. N.; Gennis, R. B. *J. Bacteriol.* **1983**, *154*, 1269–1275.

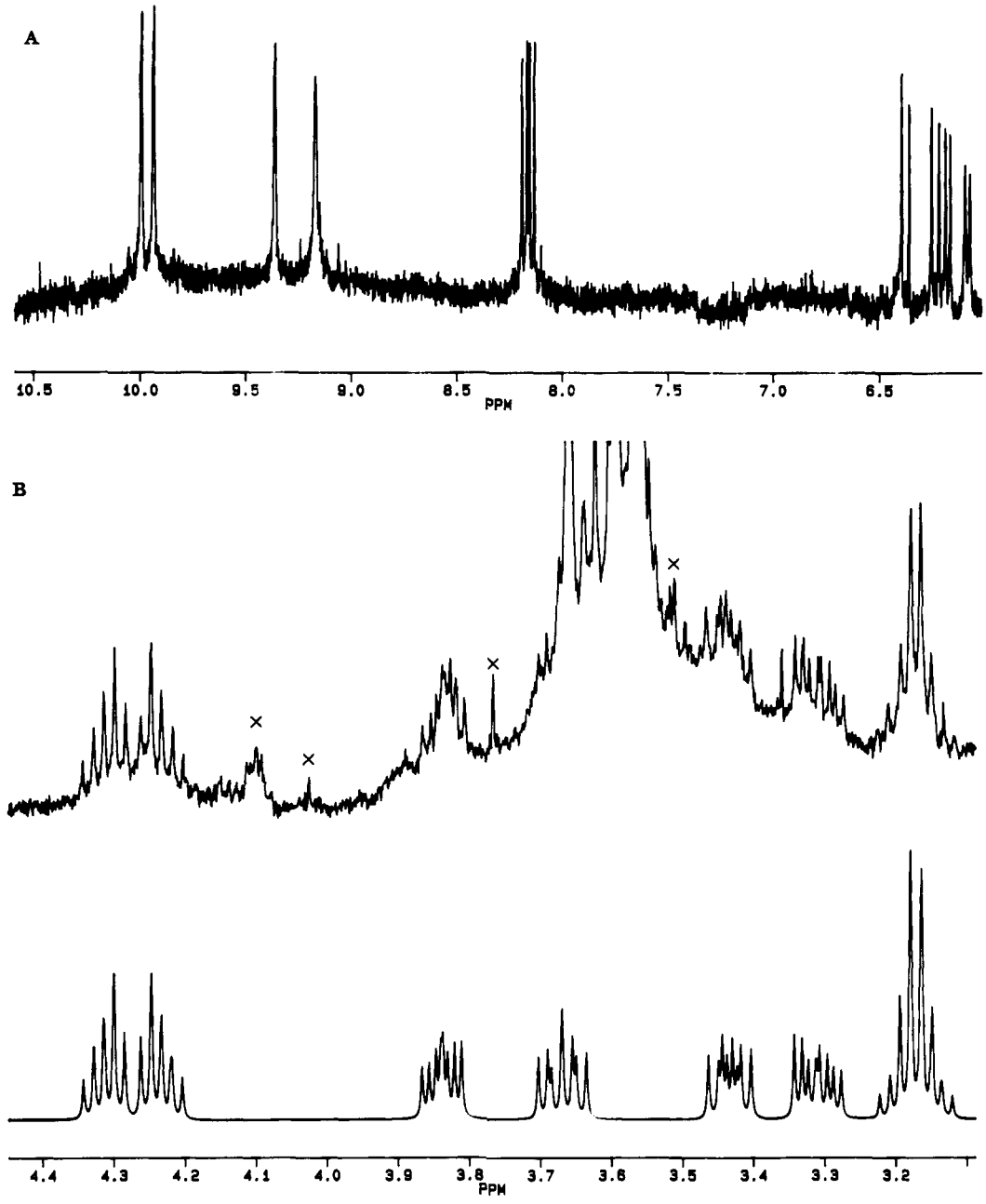


Figure 2. (A) High-frequency region of the ^1H NMR spectrum in deuterated methylene chloride of the free-base chlorin isolated from the HPII catalase, showing the four meso protons and the two vinyl ABX subspectra. The sample contained all available chlorin, except for portions used for chromatographic comparisons, visible and infrared spectroscopy, and mass spectrometry. The total amount was estimated to be 20–50 μg. The spectrum was signal averaged for 16 h. (B) Low-frequency region of the spectrum containing the spirolactone and propionate protons. Remaining impurity peaks are marked ×. The 13^2 H2 proton is badly obscured by overlap with a methyl resonance. For the simulation of the spirolactone protons shown beneath the experimental spectrum, the spin-coupling data from ref 4 were used directly without any adjustments. The chemical shifts used correspond to Table I, and a line width of 2 Hz was introduced to match the experimental average. The simulation of the propionate methylene protons used the shifts reported in Table I, geminal coupling constants of −14 Hz, and vicinal coupling constants of 7, 8, 7, and 8 Hz. Because of noise, more precise fitting was not attempted, but it should be noted that to match even approximately the general multiplet pattern, at least two unequal vicinal coupling constants were required.

HPLC on silica gel confirmed different chromatographic behavior for porphyrins derived from the cytochrome complex and from the catalase HPII.

The main peak corresponding to the porphyrin derived from HPII was collected after silica HPLC and pooled from multiple experiments to provide material for NMR spectroscopy. Chemical shifts recorded in $C^2H_2Cl_2$ and C^2HCl_3 are given in Table I, and portions of the spectra shown in Figure 2. The NMR data strongly support the conclusion that the final chlorin isolated from the HPII catalase is the cis-isomer **2**. Additional evidence was provided by FT-IR spectroscopy, which revealed a 1774-cm^{-1} mode for the spirolactone when the spectrum was recorded on a sample as a dried film on a polished aluminum surface. Fast-atom bombardment mass spectrometry gave the expected mass of 593 for the $(M + H)^+$ ion of the free base.

Experience with heme *d* in low amounts from the cytochrome complex showed that the diol form **3** readily closes to the spirolactone form **1**, especially under conditions that favor dehydrations, even such simple manipulations as removing solvents in vacuo. These observations have been confirmed on a larger scale and with greater control of conditions in the synthetic work of Sotiriou and Chang. In the case of the HPII heme, less starting enzyme was available than in the original work on the cytochrome complex, so no attempt was made to trap and manipulate the less stable diol form. Presumably the cis-diol **4** was the actual species in the enzyme-active site of HPII. This question will be readdressed

Table I. ^{1}H NMR Comparisons for the Chlorin Isolated from HPII

	chem shifts for compounds,[b] ppm				
		HPII chlorin in			
assngt[a]	**1**	$C^2H_2Cl_2$	C^2HCl_3	**2**	**5**
5	9.902	10.015	9.922	9.856	9.71
10	9.145	9.383	9.292	9.272	9.72
15	8.856	9.192	9.081	9.048	9.04
20	9.764	9.960	9.844	9.737	9.15
3^1 and 8^1	8.091	8.160	8.104	8.073	8.00
3^2 or 8^2 H_a	6.297	6.380	6.317	6.281	6.25
H_b	6.104	6.243	6.176	6.165	6.13
8^2 or 3^2 H_a	6.164	6.185	6.127	6.101	6.07
H_b	6.023	6.096	6.032	6.022	5.99
2^1	3.499	3.571	3.517	3.490	3.32
7^1	3.599	3.597	3.552	3.527	3.39
12^1	1.995	1.868	1.850	1.823	3.43
18^1	3.528	3.564	3.517	3.433	1.84
13^4					3.54
17^4	3.663	3.663	3.624	3.558	
17^1	4.188	4.311,[c] 4.236	4.285, 4.196	4.263	
17^2	3.137	3.189, 3.154	3.139, 3.104	3.087	
13^1					4.10
13^2					3.06
13^1 H_1	3.490	3.433	3.438	3.443	
13^1 H_4	2.431	3.838	3.840	3.846	
13^2 H_3	3.231	3.311	3.327	3.345	
13^2 H_2	3.032	3.669	3.728	3.731	
17^1, 17^2					3.27–3.88[d]
NH	−2.251	−2.6	−2.6	−2.618	−2.70

[a] IUPAC nomenclature for tetrapyrroles. [b] Data for compound **1** were taken from ref 2 and for compounds **2** and **5** from ref 4. All these were measured in C^2HCl_3. [c] As shown in the simulation in Figure 2, the geminal methylene protons are best described as nonequivalent. [d] Only a range was reported.

during some comments upon the possible biosynthesis of heme *d*.

When dealing with microgram quantities of natural porphyrins, we have found it prudent to use dichloromethane as the preferred NMR solvent. Commercial deuterated chloroform may contain traces of impurities and acids that have a deleterious effect on trace amounts of free-base porphyrins. In very dilute solutions, we have observed that photoporphyrin formation via attack on the vinyl substituents[7] may proceed faster in chloroform. This is why the majority of NMR spectroscopy was performed in a solvent different from the chloroform used by Sotiriou and Chang. Only at the end was a small portion of material rerun in chloroform to obtain the data in Table I. The chemical shifts of the porphyrin derived from HPII are not a perfect match to the literature report of synthetic *cis*-chlorin *d*. However, it is known that there is a chemical shift dependence on concentration due to aggregation and perhaps interaction with traces of residual water for porphyrins with a protoporphyrin IX like skeleton.[7] We have observed these before on previous samples of chlorin *d* from the cytochrome complex. The slight discrepancies in Table I are therefore attributed to different experimental concentrations.

The high-frequency region of the spectrum in Figure 2 establishes that the sample was a single porphyrin species (only four well-defined meso resonances) and that two vinyl groups were present (two characteristic ABX patterns of six protons). The most critical region for identification was the low-frequency portion shown in Figure 2. Sotiriou and Chang showed that the cis and trans isomers have different and distinctive chemical shift and coupling patterns for the four protons of the spirolactone ring. As shown in the simulation, the HPII-derived chlorin matches the literature spin system for the cis isomer extremely well and is very different from the trans system. Another characteristic difference between cis and trans isomers lies in the resolution of the two sets of methylene protons on the normal propionate ester at ca. 4.3 and 3.2 ppm. In most propionate-bearing porphyrins or chlorins, the geminal methylene protons appear equivalent. Presumably there is rapid rotation of the free substituent, and although the methylene protons are prochiral, they are sufficiently distant from possible chiral centers that their average environments become identical. They typically give rise to a high- and a low-frequency triplet, where the shift difference between the sets is due to ring currents and deshielding by the carbonyl. The geminal methylene protons in *cis*-chlorin *d* are atypical in showing pronounced inequivalency, as demonstrated by the simulation in Figure 2. Sotiriou and Chang did not elaborate upon this point for the cis and trans spirolactones, but it is evident in their experimental spectra (see especially their Figure 1A versus 1C).

In prior studies, configuration of substituents has been established for natural and synthetic chlorins by nuclear Overhauser enhancement determinations. In the present case, the amount of final purified chlorin was too small to observe these weak effects. Fortunately, because of the complete synthetic work of Sotiriou and Chang, the alternative cis-isomer **5** (Chart I) may be eliminated from consideration, again by chemical shift correlations. Table I recounts the reported shifts for **5**. Key differences between **5** and **2** lie in the spread of the meso resonances, and then the relatively low frequencies for **5** compared to **2** for the pyrrole methyls, propionate methylenes, and vinyl resonances. The observed spectrum for the HPII chlorin consistently is closer to that of **2**.

Discussion

Sotiriou and Chang showed that *cis*-chlorin *d* slowly converted to *trans*-chlorin *d* and that the trans isomer was the more stable form of the two. Therefore it is unlikely that *cis*-chlorin *d* from the HPII catalase arose from an earlier trans form. But could *trans*-chlorin *d* from the cytochrome complex of the terminal oxidase have arisen from an earlier cis form? Experiments based on parallel isolations from the two sources argue against this possibility. The heme from HPII was compared to the heme freshly isolated from the cytochrome complex by reverse-phase chromatography and found to be distinct. Extraction was by the same procedures, and experimental time was the same. Sotiriou and Chang reported that "when this lactone" (i.e., cis) "was chromatographed on silica gel repeatedly, the expected isomeric chlorin emerged". Elsewhere they reported that prolonged exposure on TLC plates favored the isomerization. In the original procedure for the isolation from the cytochrome complex, an initial low-resolution silica column was needed to separate heme *d* from

(7) Padavanija, P.; Timkovich, R. *J. Chem. Soc., Perkin Trans. 2* **1986**, 1785–1787.

high levels of heme *b* and lipid coextracted from the complex. But the heme from HPII passed through the same step and still came out cis. In modified procedures, the first silica column was omitted, and extracts demetalated, esterified by diazomethane, and separated by silica gel HPLC with a total contact time on silica of ca. 12 min. The material from HPII still came out cis, and that from the complex, trans.

Scrutiny of the chromatograms in Figure 1 reveals small components in each extract possibly coincident with the main component of the other. These were always less than 5% of the area of the main component. Recovered amounts were too small to characterize even by visible spectroscopy and their identity remains open. A small amount of chemical isomerization is thus possible, but, under present conditions the main fractions remained distinct.

Present data do not rule out the possibility that the two hemes share a common structure such as an epoxide **6** (Chart I) at some point in their lifetimes. It is not clear why the epoxide would open two ways with such high respective specificity, unless this pertains to an enzyme-catalyzed biosynthetic step or unless the heme pocket environment somehow directs specificity either in situ or during acid acetone extraction. In the absence of direct heme or porphyrin models, there are no precedents for whether the opening would be facile under extraction conditions. Simple alkyl epoxides would not open readily in cold, mildly acidic acetone.

It should be stressed that the absolute configuration at the chiral carbons is still unknown. The isomers refer only to the relative configuration at C12 and C13. Even with gene-amplified proteins, this question is not likely to be answered in the near future.

Experimental Section

Purification of the terminal oxidase from *E. coli* strain MR43L/F152, extraction of the heme, and conversion and isolation of the *trans*-chlorin *d* have been described previously.[2] The source of the HPII catalase was *E. coli* strain UM255. This is a *katE*::*TN10* derivative lacking HPII that has been transformed with *pAMkatE6* which encodes for HPII.[8] The advantage of this system is that more enzyme is produced because the cloned gene is on a high-copy plasmid. The procedure for the isolation of the catalase HPII from bacterial cells has been described.[5] Approximately 200 mg of protein, corresponding to about 2 μmol of heme, formed the starting material for this study. Heme extraction, demetalization, and chlorin purification from the catalase were performed just as for the prosthetic group from the terminal oxidase complex, and in some experiments the manipulations were done in parallel to ensure comparable experimental conditions. In some heme extraction experiments with the catalase, the protein adopted a gellike state unpon exposure to acidic acetone. Unfortunately, this tended to physically entrap heme and lowered the initial yield. In agreement with previous results, HPII was found to contain only a single type of heme group and was free of any detectable heme *b*.[5] In some extraction experiments, the initial low-resolution silica gel column chromatographic separation of the crude extract, which has been previously described,[2] was omitted. This step is not critical for the catalase heme but is quite useful for the heme from the cytochrome complex in order to free it from detergents that stabilize the native enzyme, lipid in the complex, and the endogenous heme *b* prosthetic groups. Chromatographic comparisons on preparations with and without this step indicated that the retention times of either heme were unaffected.

^{1}H NMR spectra were obtained at 500 MHz on a Bruker AM500 spectrometer. Spectral simulations were done with standard Bruker software packages. FT-IR spectra were obtained on dried films of the *cis*-chlorin *d* derived from HPII by using a Bruker IRS88 spectrometer equipped with a microscope accessory. In this mode, the sample was applied as a solution in dichloromethane to a highly polished aluminum mirror and dried, leaving a thin film. The polished surface allows multiple-pass reflection spectroscopy on very small samples. The technique was necessary because of sample limitations in this case. Mass spectra were obtained by using the fast-atom bombardment technique at the Midwest Center of Mass Spectrometry, Department of Chemistry, University of Nebraska-Lincoln.

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(8) Mulvey, M. R.; Sorby, P. A.; Triggs-Raine, B. L.; Loewen, P. C. *Gene* **1988**, *73*, 337-345.

Resonance Raman Spectra of Dioxygen Adducts of Cobalt Porphyrin-Imidazole Complexes. Remarkable Spectroscopic Consequences of Hydrogen Bonding of the Coordinated Imidazole and the Lack of an Effect on the Cobalt-Oxygen Linkage

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Abstract: Resonance Raman (RR) spectroscopic studies of O_2 adducts of the imidazole complexes of a highly protected cobalt-porphyrin are reported. Strategic isotopic labeling studies, employing dioxygen isotopomers and selectively deuteriated imidazole analogues, are used to document and interpret the complicated spectral patterns that emerge as a result of complex vibrational coupling of ν(O-O) with internal modes of the trans-coordinated imidazole ligand. In addition, the observed spectral patterns are shown to be dependent upon temperature, imidazole concentration, and the addition of a hydrogen-bond acceptor. These results indicate that hydrogen bonding of the ligated imidazole to other solution components does not lead to differences in the inherent frequency of ν(O-O) but does result in dramatic alterations of the observed spectral patterns as a consequence of differences in vibrational coupling parameters. The implications of these studies for the interpretation of RR spectra of O_2 adducts of cobalt-substituted hemeproteins are discussed.

The essential goal of hemeprotein research remains the elucidation of the structural and electronic factors that control the remarkably varied functional properties of the active-site heme group. The nature and number of axial ligands that the protein

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