signment of ω(N=O) vibrations in terminal nitroso compounds.

Also of interest is a strong IR absorption at approximately 1235 cm^{-1} for 4-nitroso-*N*,*N*-dimethylaniline. When the ligand complexes to Zn, this peak is no longer present at the previous frequency and appears to have either been eliminated or has shifted into the region of a broad absorption band centered at approximately 1150 cm^{-1}. A large shift to lower frequency or an annihilation of this peak upon complexing would be expected to happen to the ω(N=O) motion upon complexing through the nitroso oxygen.

At present it appears that no conclusive correlation between IR spectroscopy and arylnitroso ligand binding characteristics can be assumed.

Acknowledgment. We acknowledge the support of the Natural Sciences and Engineering Research Council of Canada for an operating grant to B.E.R. and R.J.B., the University of Regina for computing time, and H. Wang for assistance with computing.

Registry No. $ZnCl_2[(CH_3)_2NC_6H_4NO]_2$, 123265-48-5; $SnCl_2(CH_3)_2[(CH_3)_2NC_6H_4NO]_2$, 82286-88-2; $PdCl_2[(CH_3)_2NC_6H_4NO]_2$, 63527-61-7; $[(CH_3)_2NC_6H_4NO]_2$, 138-89-6.

Supplementary Material Available: Table B, listing crystal data and experimental parameters, Table C, listing anisotropic thermal parameters for the non-hydrogen atoms, Table D, listing bond lengths involving hydrogen atoms for dichlorobis(4-nitroso-*N*,*N*-dimethylaniline)zinc(II), Figure A, showing relevant IR spectra, and Figure B, showing bond length differences in the ligand (6 pages); Table A, listing observed and calculated structure amplitudes for dichlorobis(4-nitroso-*N*,*N*-dimethylaniline)zinc(II) (19 pages). Ordering information is given on any current masthead page.

Contribution No. 7787 from the Arthur Amos Noyes Laboratory, California Institute of Technology, Pasadena, California 91125

Q-Band Splitting in the Spectra of Heme Proteins

J. A. Cowan and Harry B. Gray*

Received June 22, 1988

The origin of Q-band splitting and line broadening in the electronic absorption spectra of certain types of heme proteins has remained an unsolved problem, despite having been documented for many years.[1] Recently, we have found that certain metalloporphyrin-modified myoglobins[2-4] exhibit Q-band splittings, and we have performed studies to elucidate the origin of this effect. In this paper we first focus attention on the details of the absorption spectra of modified sperm whale myoglobin (Mb), noting the possible contributors to Q-band perturbation and outlining experiments designed to select among these factors. We then attempt to explain the general observation of Q(0,0)-band splitting in hemes.

Consider the three major contributors to perturbations in a porphyrin absorption spectrum. First, minor changes in both the λ_{max} of the Soret and Q bands, and their relative intensities, can arise due to differences in the polarity of the porphyrin environment.[5] Second, aggregation of porphyrin units[6] can lead to shifts and line broadening of absorption bands due to dipolar coupling between the chromophores.[7] Third, larger perturbations in both the position of λ_{max}, and the relative intensities of each of the absorption bands, can arise from changes in the coordination geometry of the porphyrin (for example, a change from four- to five-coordination or a change in the σ- or π-bonding character of the axial ligand).[5] The perturbations observed in the shapes

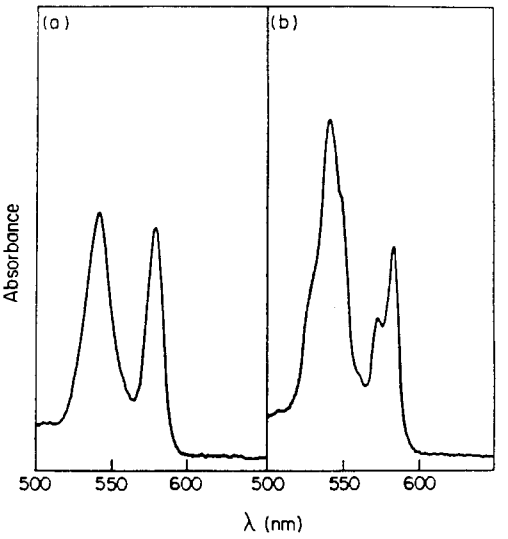

Figure 1. Q-band region in the electronic absorption spectra of (a) magnesium mesoporphyrin IX dimethyl ester (+ imidazole) in CH_2Cl_2 and (b) MgMb in μ = 0.1 M aqueous sodium phosphate buffer (pH 7.0, 22-24 °C). The absorbance scales for the spectra are similar but not identical.

Table I. Porphyrin Absorption Spectral Data[a]

	MP-DME		MP-DME + py[b]		MP-diacid/protein	
M	λ, nm	rel OD	λ, nm	rel OD	λ, nm	rel OD
Mg	397	1.0	411	1.0	409	1.0
	524	0.041	529	0.046	542	0.037
	561	0.070	562	0.032	584 (573 sh)	0.022
Zn	400	1.0	412	1.0	414	1.0
	531	0.046	514	0.052	541	0.047
	568	0.066	577	0.040	583 (573 sh)	0.024
Cd	406	1.0	419	1.0	421	1.0
	541	0.085	550	0.072	551	0.076
	576	0.066	585	0.031	591 (582 sh)	0.027
Pt	380	1.0	380	1.0	380	1.0
	498	0.078	499	0.079	499	0.052
	535	0.161	535	0.161	534	0.172
Pd	392	1.0	392	1.0	391	1.0
	511	0.073	511	0.074	510	0.083
	546	0.229	546	0.231	544	0.235
H_2	398	1.0	398	1.0	395	1.0
	497	0.084	497	0.084	496	0.080
	531	0.058	531	0.063	532	0.058
	567	0.039	567	0.042	562	0.042
	620	0.028	620	0.030	614	0.030
Sn	404	1.0	407	1.0	404	1.0
	537	0.081	538	0.084	537	0.050
	574	0.071	575	0.077	577 (569 sh)	0.031

[a] Reference 4. P = mesoporphyrin IX; MP-DME spectra were measured in CH_2Cl_2 solution; protein spectra were measured in μ = 0.1 M sodium phosphate buffer (22-24 °C). For each metal derivative, the peak intensities for the absorption bands are given as a ratio relative to the Soret (OD = 1.0). This is to facilitate comparison of data for each metal derivative. The ratios are not intended to be used between metal derivatives; that is, the Soret bands for the metal Mb complexes do not have exactly the same extinction coefficients. [b] Magnesium mesoporphyrin IX was titrated with imidazole in order to give a five-coordinate species.[10]

(1) Bartsch, R. G. *Methods Enzymol.* **1971**, *23*, 344. Hagihara, B.; Sato, N.; Yamanaka, T. In *The Enzymes*; Boyer, P. D., Ed.; Academic Press: New York, 1975; Vol. 11, p 549. Meyer, T. E.; Kamen, M. *Adv. Protein Chem.* **1982**, *35*, 105. Pettigrew, G. W.; Moore, G. R. *Cytochromes c*; Springer-Verlag: New York, 1987.
(2) Axup, A. W.; Albin, M. A.; Mayo, S. L.; Crutchley, R. J.; Gray, H. B. *J. Am. Chem. Soc.* **1988**, *110*, 435.
(3) Cowan, J. A.; Gray, H. B. *Chem. Scr.* **1988**, *28A*, 21.
(4) Cowan, J. A.; Gray, H. B. *Inorg. Chem.* **1989**, *28*, 2074.
(5) Gouterman, M. In *The Porphyrins*; Dolphin, D., Ed.; Academic Press: New York, 1978; Vol. III, pp 15-16.
(6) White, W. I. In *The Porphyrins*; Dolphin, D., Ed.; Academic Press: New York, 1978; Vol. V, p 303.
(7) Kasha, M.; Rawls, H. R.; El-Bayoumi, M. A. *Pure Appl. Chem.* **1965**, *11*, 371.

0020-1669/89/1328-4554$01.50/0

Table II. Correlation of Absorption Band Intensity [Q(0,0)][a] with CD Signal Intensity (Soret)[b]

Zn porphyrin	porphyrin IX DME + py[c] A[Q(0,0)]/A(Soret) = [A(py)]	porphyrin IX diacid + protein[d] A[Q(0,0)]/A(Soret) = [A(protein)]	[A(protein)]/[A(py)]	porphyrin IX diacid + protein I^{CD}(Soret)
deuteroporphyrin	0.030	0.020	0.67	67
mesoporphyrin	0.038	0.025	0.66	72
protoporphyrin	0.057	0.046	0.81	138

[a] Q(0,0) bands at 575, 577, and 586 nm and 579, 583, and 596 nm for zinc deuteroporphyrin, mesoporphyrin, and protoporphyrin in free solution or protein bound, respectively. [b] Soret bands at 413, 414, and 427 nm for protein-bound zinc deuteroporphyrin, mesoporphyrin, and protoporphyrin, respectively. [c] DME = dimethyl ester. Solutions in 1 mL CH_2Cl_2 (22–24 °C) with 50 mL of pyridine added. [d] Protein spectra were obtained in μ = 0.1 M sodium phosphate buffer (22–24 °C).

of the Q bands in some of the modified myoglobins described here (and in certain heme proteins reported elsewhere)[1] are illustrated in Figure 1. In particular, line broadening in Q(1,0) and broadening and band splitting in Q(0,0) have been observed.[3,4,8] In an attempt to rationalize these observations, consideration of the spectra of coordinatively saturated porphyrins[9] in a selection of organic solvents of differing polarity indicates that the first effect is small and need not be considered further. Aggregation effects also can be discounted, since each protein-bound porphyrin is held in isolation. We therefore will focus on the third factor and attempt to explain Q-band splitting as a manifestation of superimposed spectra arising from orientational isomerism of the porphyrin in the protein pocket.

The λ_{max} values and relative peak intensities of the absorption bands of porphyrins in dichloromethane solution are compared with those in a protein environment in Table I. The most obvious change arises in the Soret region of the absorption spectra, where a distinct red shift in λ_{max} is observed for those metalloporphyrins capable of coordinating the proximal His-93 residue. This can be modeled by titrating a solution of the metalloporphyrin with an aromatic nitrogenous base such as imidazole or pyridine.[5,10] Accompanying the red-shifted Soret there is often a characteristic change in λ_{max}[5] and inversion of the intensities of the Q bands (Table I). In the case of coordinatively saturated porphyrins, however, there are minimal changes in the porphyrin spectra following either titration with pyridine or insertion into a protein environment, reflecting the absence of binding to either the added ligand or the proximal histidine.

In a comparison of the relative intensities of bands for coordinatively unsaturated metalloporphyrins with axially bound pyridine (Table I) with those for the same porphyrin in the protein environment, the overall decrease in the peak intensities of these bands relative to the corresponding Soret is due to the broadening or splitting of the Q(0,0) bands; however, *the integrated intensity of each Q(0,0) band relative to that of the Soret remains relatively unchanged.* Small variations do arise in the absorption characteristics following insertion into the protein, although these would be expected, since after insertion the porphyrins are bound to the protein in strict isolation, whereas in general most metalloporphyrins form aggregates that affect their relative absorption characteristics.[6] Of interest are the origins of the Q-band broadening and splitting that we have observed for several metal systems (Figure 1). Two features are clear. In the case of myoglobin, line broadening and splitting are limited to coordinatively unsaturated metalloporphyrins and are particularly evident in those porphyrins containing closed-shell metal ions (Mg, Zn, Cd, Sn) where charge-transfer transitions are absent and cannot lead to other line-broadening influences. The effect was absent in noncoordinating (Pt, Pd, H_2) systems.

In the explanation of this behavior, two possibilities must be considered. It is known that the coordinating proximal histidine of Mb adopts a fixed orientation relative to the porphyrin unit.[11]

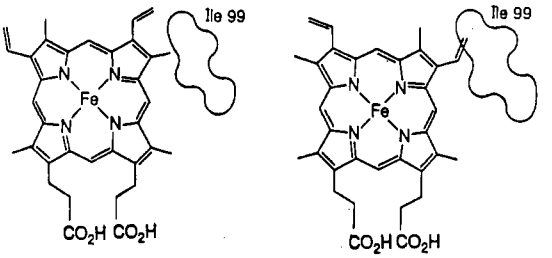

Figure 2. Heme orientational disorder. The difference in the steric interactions between the porphyrin pyrrole substituents and protein residues can result in slightly different heme geometries for the two orientational forms relative to the proximal histidine, resulting in the superposition of distinct electronic absorption spectra.

Since each of the Q bands in the porphyrin absorption spectrum corresponds to a degenerate transition [Q(0,0) (lower energy) and Q(1,0) (higher energy)],[12] the histidine-induced breakdown of axial symmetry could lead to band splitting. An alternative possibility is suggested from the NMR studies of La Mar and co-workers,[13] who found that the porphyrin moiety can adopt two distinct orientations in the Mb pocket (Figure 2) due to asymmetry in the steric interactions between inner protein residues and the pyrrole substituents on the porphyrin ring. As a result, the proximal histidine can adopt two different coordination modes, depending on the orientation of the porphyrin, and so the observed perturbations in the appearance of these bands may arise from the superposition of the absorption spectra of the two structurally distinct species.

It already has been demonstrated[13] that the ratio of the two orientational isomers is dependent on the nature of the porphyrin ring substituents. Heme reconstitution of apomyoglobin with a series of ferric porphyrins, in which the porphyrin ring is either meso-, proto-, or deuteroporphyrin, was found to alter the ratios in the approximate order 5.5:1, 11:1, and 12:1,[13,14] as determined from the relative intensities of the paramagnetically shifted pyrrole methyl resonances in the NMR spectrum of metmyoglobin.[13] A series of studies using metallomeso-, proto-, and deuteroporphyrins, therefore, offered a means of discriminating between the two possible sources for the effects that we have observed. Only if the observed Q-band perturbation were due to an orientational equilibrium should the nature of the spectra change, whereas if loss of orbital degeneracy were responsible, a minimal change would be expected. We have been unable to determine the precise ratio by NMR spectroscopy, since distinct resonances from each of the diamagnetic isomeric forms were not resolved. Following reconstitution with tin, magnesium, and zinc meso-, deutero-, and protoporphyrins, the perturbations to the Q bands were found to change with the nature of the ligand. Table II summarizes absorption and CD data obtained for a series of zinc porphyrin myoglobins. Recent work has shown that the latter technique is sensitive to the ratio of isomeric forms in native Mb and gives results in agreement with data from paramagnetic NMR spectroscopy.[14] In each case the relative ratio of Soret to Q(0,0) band [A(protein)]/[A(py)] follows the relative intensity of the CD band.

(8) Axup, A. W. Ph.D. Thesis, California Institute of Technology, 1986.
(9) Coordinatively saturated metalloporphyrins were used in order to minimize the influence of solvent binding.
(10) As determined from absorption spectra, solutions of magnesium porphyrin give mixtures of four-, five-, and six-coordinate Mg when titrated with pyridine but only five-coordinate Mg when imidazole is used.
(11) Bertini, I.; Luchinat, C. In *NMR of Paramagnetic Molecules in Biological Systems*; Lever, A. B. P., Gray, H. B., Eds.; Physical Bioinorganic Chemistry Series, Vol. 3; Benjamin/Cummings: Menlo Park, CA, 1986; p 182.
(12) Gouterman, M. In *The Porphyrins*; Dolphin, D., Eds.; Academic Press: New York, 1978; Vol. III, pp 12–17.
(13) La Mar, G. N.; Emerson, S. D.; Lecomte, J. T. J.; Pande, U.; Smith, K. M.; Craig, G. W.; Kehres, L. A. *J. Am. Chem. Soc.* **1986**, *108*, 5568.
(14) Aojula, H. S.; Wilson, M. T.; Moore, G. R.; Williamson, D. J. *Biochem. J.* **1988**, *250*, 853.

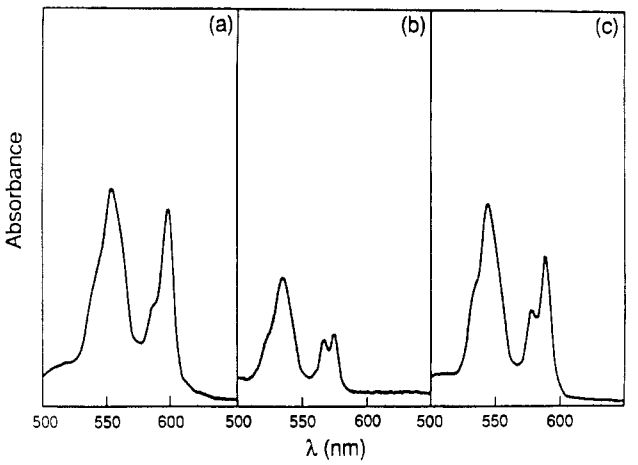

Figure 3. Q-band region in the electronic absorption spectra of Mb's modified with (a) magnesium protoporphyrin IX (this should be compared with Figure 1b), (b) tin mesoporphyrin IX, and (c) tin protoporphyrin IX diacids. All spectra were obtained in μ = 0.1 M aqueous sodium phosphate buffer (pH 7.0, 22–24 °C). The absorbance scales for the spectra are similar but not identical.

(Solutions of free porphyrin gave no CD signal.) We therefore attribute the CD signal to a net isomeric ratio in favor of one orientation. It is clear that the CD data support the conclusion derived from the electronic absorption spectra that the splitting/line broadening is due to the superposition of spectra from two orientational forms. The CD data in column 5 reflect an isomeric ratio zinc protoporphyrin > zinc mesoporphyrin ~ zinc deuteroporphyrin. This is also the trend displayed by column 4, a measure of the reduction in band intensity owing to the nonsuperposition of absorption bands from the two orientational isomers. The more uniform distribution of the split α-components in the SnMb spectrum (Figure 3) may be a consequence of six-coordination,[4] generating a more uniform distribution of porphyrin orientational isomers in the protein pocket.

We conclude that the manifestation of Q(0,0)-band splitting and broadening depends on distinct axial ligand geometries for each isomeric form. These two elements explain the presence (or absence) of a Q(0,0)-band perturbation in a heme protein. In some cases, such as deoxy Mb, a splitting could be obscured by the excess of one orientational isomer of the native protoporphyrin unit over the other, although more likely by the intrinsically broad absorbances that result from the charge-transfer character of the excited states in question.[15] It is significant that in the CO complex, where the charge-transfer character is reduced and individual Q bands can be resolved, there is a shoulder on the Q(0,0) absorbance that is similar to features observed with zinc and magnesium protoporphyrin IX modified Mb (Figure 3). The same behavior is exhibited by RuMb, which displays a broadened band in the Q region in Ru^{II}Mb, again attributable to significant charge-transfer character, but a split Q(0,0) band in the Ru^{II}COMb complex.[16] The more dramatic demonstration of splitting in this case is due to reconstitution with ruthenium mesoporphyrin, which gives a more uniform distribution of each orientational isomer.

Some evidence exists for heme orientational disorder[1,17,18] in cytochromes *b*, *c*, and *f*, where Q(0,0)-band splitting, or line-broadening with a corresponding decrease in overall peak intensity, is frequently observed. In those instances where no perturbations are readily seen, we would predict that either one orientational isomer of the heme is greatly preferred over the other or a similar coordination geometry for each isomer leads to equivalent overlapping absorption spectra.

In conclusion, we believe that the principles described above can be used to understand some of the unusual spectral features displayed by both native and modified heme proteins. It would appear that heme orientational disorder may be a general phenomenon, as originally postulated by La Mar,[17,18] and that absorption spectroscopy could be the simplest method for detecting its existence in various protein environments.

Acknowledgment. We thank Dr. K. Kalyanasundaram for early contributions to our work on metalloporphyrin-substituted myoglobins and for helpful discussions. This research was supported by the National Science Foundation (Grants CHE85-18793 and CHE88-22988). J.A.C. thanks the Science and Engineering Research Council (United Kingdom) for the award of a NATO Postdoctoral Fellowship.

Registry No. Heme, 14875-96-8; cytochrome *b*, 9035-37-4; cytochrome *c*, 9007-43-6; cytochrome *f*, 9035-46-5.

(15) Makinen, M. W.; Churg, A. K. In *Iron Porphyrins*; Lever, A. B. P., Gray, H. B., Eds.; Physical Bioinorganic Chemistry Series, Vol. 1; Addison-Wesley: Reading, MA, 1983; pp 179, 197.
(16) Paulson, D. R.; Addison, A. W.; Dolphin, D.; James, B. R. *J. Biol. Chem.* **1979**, *254*, 7002.
(17) La Mar, G. N.; Davis, N. L.; Parish, D. W.; Smith, K. M. *J. Mol. Biol.* **1983**, *168*, 887.
(18) La Mar, G. N.; Toi, H.; Krishnamoorthi, R. *J. Am. Chem. Soc.* **1984**, *106*, 6395.