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Model System of Cobalt–Cytochrome *c*

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The EPR spectra of the acidic conformational state of cobaltocytochrome *c* [Co(cyt *c*)] can be closely simulated by a 1:1 complex of cobaltoprotoporphyrin IX dimethyl ester (CoPPIX) and 2-(methylthio)ethanol (MTE). Mercaptoethanol (ME) also forms a 1:1 complex with CoPPIX but its EPR parameters are somewhat different from those of the first two cases. The EPR spectra of the basic conformational state of Co(cyt *c*) can be simulated by a 1:1 complex of CoPPIX with either pyridine or imidazole. MTE and ME are readily displaced by either pyridine or imidazole. MTE and ME do not form a 2:1 complex with CoPPIX, nor would they form a six-coordinated complex with the nitrogenous base as the fifth axial ligand. This behavior is consistent with the fact that MTE and ME are σ and π donors but are not π acceptors. EPR spectra of several oxygenated complexes are also described. The EPR spectra were analyzed according to a third-order perturbation theory to give energy separation between the ground $^2A_1$ state and the excited $^2B_1$ and $^4B_1$ states. The values of $\Delta(^2B_1)$ for various nitrogen and sulfur complexes increase in the order $N_4S < N_5 < N_5S \sim N_6$ due to destabilization of the ground state by the introduction of axial ligands. The more polarizable sulfur causes less destabilization than nitrogen. The values of $\Delta(^4B_1)$ increase in the order $N_6 < N_5S < N_5 < N_4S$ due to stabilization of the quartet state by octahedral ligand field. The EPR spectra of the pH-dependent conformational states of Co(cyt *c*) have been assigned.

Introduction

The understanding of functional properties of a metalloenzyme can often be furthered by the study of the prosthetic group itself. Thus, Wang[1] incorporated iron porphyrins into a nonpolar polymeric matrix and showed that it resisted autoxidation and was capable of reversible oxygen binding. Synthesis of iron porphyrin derivatives with picket fence side chains by Collman and coworkers [2] produces similar properties. EPR[3] measurements on cobalt porphyrins with nitrogenous ligands[4] and on single crystals of cobalt hemoglobin and myoglobin[5,6] complement each other. Comparison of the spectral parameters should show the influence of the protein moiety on the coordination chemistry of the metal and reciprocally reveal information about the effect of the metal on the conformation of the protein.

Recently, we synthesized cobaltocytochrome *c*.[7-10] The molecule exists in three pH-dependent conformational states I, II, and III at pH <4, 4 < pH < 12, and pH >12, respectively. Proton NMR and EPR results suggest that II has His-18 and Met-80 as axial ligands and III has only His-18 as the axial ligand. The EPR spectrum of I is unlike any other cobalt porphyrin derivative reported in the literature or observed in our laboratories. A study of the interaction of cobalt porphyrins with thioethers was undertaken to improve understanding of the spectrum of I, the affinity of cobalt porphyrins for sulfur ligands, and their ability to bind oxygen reversibly.

Experimental Section

CoPPIX was synthesized by a previously described procedure.[5] Pyridine, imidazole, 2-(methylthio)ethanol (MTE), and mercaptoethanol (ME) were obtained from Aldrich. The preparation of Co(cyt *c*) has been given elsewhere.[7,9] Because of its sensitivity to autoxidation, especially at high and low pH, all samples were handled under vacuum.

Toluene solutions of CoPPIX were mixed with various ligands in EPR sample tubes and degassed on a vacuum line. EPR spectra were obtained at 77 K with a Varian E-9 spectrometer. The spectra for oxygenated complexes were obtained with 1 atm of air present above the liquid samples.

Results

A 0.23 mM solution of CoPPIX in toluene at 77 K affords no EPR spectrum. This has been attributed by various workers[4a,11,12] to aggregation of CoPPIX causing excessive dipolar broadening. When MTE is added, an EPR spectrum emerges which is shown in Figure 1b. The spectrum remains the same except for small intensity differences for MTE:CoPPIX ratios of 0.78–200. At higher concentrations of MTE the spectrum begins to change. The $g_{\parallel}$ increases, $^{Co}A_{\parallel}$ decreases, and the perpendicular hyperfine splittings are obscured with the growth of new features at $g = 2.311$ and 2.274. At 8000-fold excess of MTE, the spectrum stabilizes which remains essentially unchanged in neat (18 M) MTE (Figure 2). These changes, though interesting and significant, are not believed to indicate the formation of a 2:1 complex. The changes, especially in $g_{\parallel}$ and $A_{\parallel}$, are too small and occur at concentrations of MTE which are too high compared to the dramatic changes associated with the formation of 2:1 complexes of amines.[4a] Instead, the spectral changes are attributed to the change of solvent environment from that of toluene to MTE. Thus, only a 1:1 complex is formed between CoPPIX and MTE.

A 1:1 complex was also formed between CoPPIX and ME; its EPR spectral parameters are listed in Table I. The CoPPIX·ME complex has slightly larger values of $g_{\perp}$ and 40% greater $^{Co}A_{\perp}$ than CoPPIX·MTE.

There have been several EPR studies of the interaction of cobalt porphyrins with nitrogenous ligands.[4,11,13] We extended these works to CoPPIX. The results for 1:1 complexes with pyridine and imidazole are given in Table I. CoPPIX forms a 1:2 complex with pyridine but not with imidazole, in agreement with the findings for other cobalt porphyrin derivatives.[4a]

All our efforts to establish the formation of a six-coordinated complex with both nitrogen and sulfur ligands, such as CoPPIX·py·MTE, were fruitless. For instance, a 0.23 mM solution of CoPPIX·py was titrated up to 450 mM in MTE without any discernible effect on the EPR spectrum. In two other series of experiments, increments of pyridine were added to a toluene solution of CoPPIX (1 mM) and MTE (450 mM) in vacuum. Pyridine at concentrations much less than 1 mM has no discernible effect on the EPR spectrum of CoPPIX·MTE; at the stoichiometric level, the spectral features began to change. At 50% excess of pyridine, the spectrum is indistinguishable from that of CoPPIX·py. There is no evidence for the formation of CoPPIX·py·MTE complex.

There is also no indication that imidazole can stablize the complexing of MTE by CoPPIX as a sixth ligand to CoPPIX. When imidazole is added to a toluene solution of CoPPIX·MTE, a spectrum very similar to that of CoPPIX·(imidazole) results. Titration of either CoPPIX·(imidazole) or CoPPIX·MTE with the opposite ligand gives EPR spectra which are simply linear combinations of the two five-coordinated complexes.

Introduction of oxygen to CoPPIX leads to a strong EPR signal characteristic of an oxycobalt porphyrin species (Table

Table I. EPR Parameters for Metal Compounds and Cobalt Enzymes

System[a]	$g_\perp$[b]	$g_\parallel$[b]	$10^4 \lvert {}^{Co}A_\perp \rvert$,[c] cm^{-1}	$10^4 \lvert {}^{Co}A_\parallel \rvert$,[c] cm^{-1}	$10^4 \lvert {}^{N}A_\parallel \rvert$,[c] cm^{-1}	Ref
(A) Five-Coordinated Complexes						
CoPPIX·py[d]	2.311	2.029	*e*	76.5	15.1	This work
CoPPIX·im[d]	2.306	2.034	*e*	73.4	15.5	This work
Co(cyt *c*), pH 12.9 (state III)	2.325	2.026	(11)[f]	75.7	16.6	10
CoMb[g]	2.327	2.028	5.5	83.4	18.5	5
CoHb[g]	2.310	2.037	<5[b]	79.9	17.5	6
Co(*p*-OCH_3)TPP·(amine)[d,h]	2.327–2.311	2.023–2.033	<11	76.4–81.2	13.1–18.2	3
CoPPIX·MTE	2.360	2.016	37.7	90.1	0	This work
CoPPIX·MTE + excess MTE[d,i]	2.311, 2.274[j]	2.026	*e*	79.1	0	This work
CoPPIX·ME[d]	2.384	2.018	54.4	93.2	0	This work
Co(cyt *c*), pH 0.5 (state I)	2.359	2.014	38.8	93.8	0	10
(B) Six-Coordinated Complexes						
CoPPIX·2 py[d]	2.247	2.067	53.6	64.7	*e*	This work
Co(*p*-OCH_3)TPP·2(amine)[d,h]	2.208–2.214	2.047–2.067	50–62	60–72	9.5–14.6	3
Co(cyt *c*) (state II)	2.223	2.035	51.7	58.3	15.2	10
(C) Oxygen Complexes						
CoPPIX·O_2[d]	1.985	2.052	25[f]	17	0	This work
CoPPIX·MTE·O_2[d]	2.002	2.072	21	12	0	This work
CoPPIX·ME·O_2[d]	2.003	2.059	19.2	14	0	This work
Co(*p*-OCH_3)TPP·(amine)·O_2[d]	1.998–2.004	2.073–2.081	15.8–18.4[k]	9.6–12.1[k]	*e*	3
CoMbO_2[g]	1.989, 2.006[j]	2.083	17.2, 6.4	10	*e*	5

[a] Abbreviations: CoPPIX, cobaltoprotoporphyrin IX dimethyl ester; py, pyridine; im, imidazole; Co(cyt *c*), cobaltocytochrome *c*; CoMb, deoxycobaltomyoglobin; CoHb, deoxycobaltohemoglobin; Co(*p*-OCH_3)TPP, mesotetra(*p*-methoxyphenyl)porphyrincobalt(II); MTE, 2-(methylthio)ethanol; ME, mercaptoethanol; CoMbO_2, oxycobaltomyoglobin. [b] *g* values are accurate to ±0.002. [c] *A* values are accurate to ±0.0001 cm^{-1}. [d] In toluene glass. [e] Not resolved. [f] Estimated; accurate to ±0.001 cm^{-1}. [g] Single-crystal data. [h] Range for 13 amines. [i] 1000-fold excess of MTE. [j] Rhombic tensor. [k] These values are reversed from the assignment given in ref 4a. Cf. ref 5 for discussion.

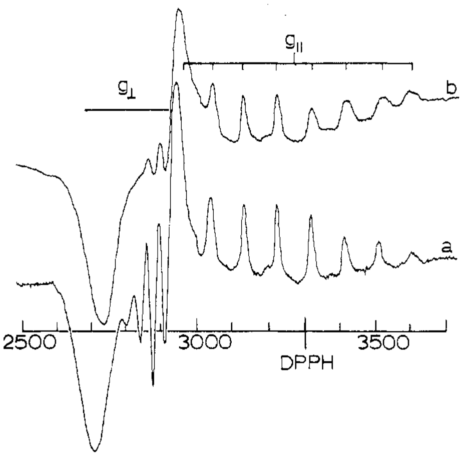

Figure 1. (a) EPR spectrum of Co(cyt *c*) at pH 0.5. Field in gauss; marker for DPPH; 20-mW microwave power at 9.260 GHz; 4-G modulation at 100 Hz. (b) EPR spectrum of 1 mM CoPPIX in toluene glass containing 0.78 mM MTE; same spectrometer settings as in Figure 1a.

I). The cobalt hyperfine splitting constants are larger than those of oxycobaltomyoglobin[5,11] and $O_2Co(p\text{-}OCH_3)TPP$-(amine).[4] Presumably, only solvent occupies the sixth coordination site in the absence of a free base. Oxygenation is reversible with no loss of EPR signal intensity over many cycles. CoPPIX is not easily autoxidized in nonpolar environments in contrast to the ease of autoxidation in aqueous pyridine.

Exposure of CoPPIX·MTE to air results in an EPR spectrum shown in Figure 3. This same spectrum is obtained at either low or high MTE concentration. A similar spectrum was also obtained with ME.

Discussion of Results

The unusual EPR spectrum of Co(cyt *c*) at low pH, shown in Figure 1a, is reproduced almost exactly by the spectrum of CoPPIX·MTE (Figure 1b). This agreement supports the assignment of a five-coordinate complex for state I of Co(cyt *c*) with Met-80 as the axial ligand. This assignment is also consistent with the known pK_a value of 2.5 for His-18 in native cytochrome *c*.[14] The unusually low pK_a has been attributed to the nonpolar environment for this residue. Protonation of His-18 severs its bond to the metal atom.

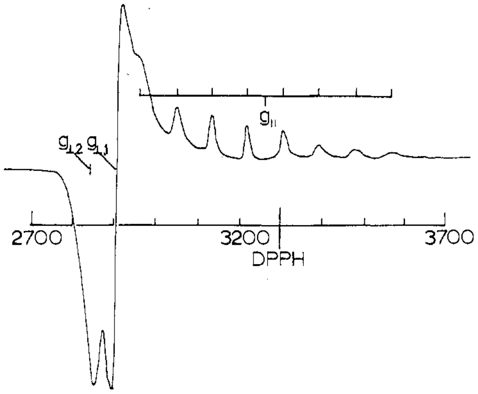

Figure 2. EPR spectrum of 0.23 mM CoPPIX in toluene with neat MTE (18 M); same spectrometer settings as in Figure 1.

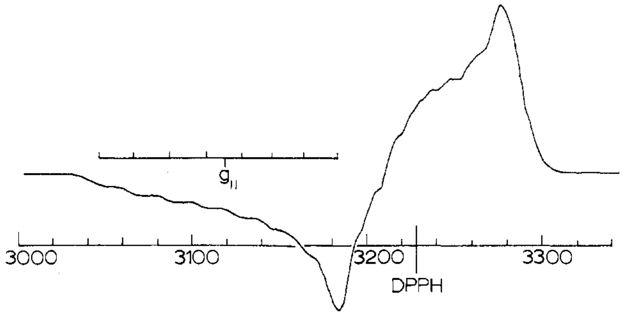

Figure 3. EPR spectrum of 1 mM CoPPIX in toluene containing 1.17 mM MTE and oxygen; same spectrometer settings as in Figure 1.

The EPR spectrum of Co(cyt *c*) at high pH, shown in Figure 4b, is nearly identical with that of CoPPIX·py (Figure 4a)

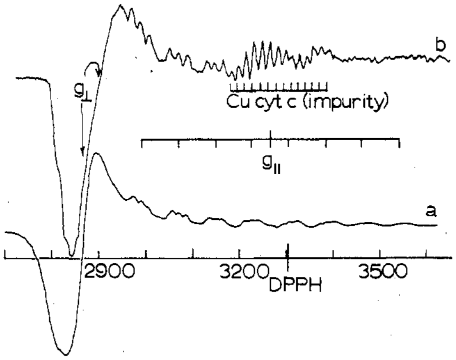

Figure 4. (a) EPR spectrum of Co(cyt *c*) at pH 12.9. (b) EPR spectrum of 0.23 mM CoPPIX in toluene with 0.23 mM pyridine; same spectrometer settings as in Figure 1.

except for the copper contamination in the former spectrum. Therefore, we can assign a five-coordinated structure for state III of Co(cyt *c*) with His-18 as the most probable axial ligand. The Co–Met-80 bond is apparently severed at high alkalinity.

The EPR spectrum of Co(cyt *c*) at physiological pH is clearly that of six-coordinated cobalt porphyrin complex (Table I). With proton NMR[10] the protein is shown to have Met-80 and His-18 as the axial ligands. By contrast, the prosthetic group alone does not complex with a nitrogen and a sulfur ligand simultaneously. Clearly, the protein places and constrains these ligands for complexation.

Recently, McGarvey[15] carried out the third-order perturbation treatment of low-spin cobalt complexes and included configuration interaction with the quartet excited state to give for the $^2A_1$ ground state

$$g_\parallel = 2.0023 + 2c_3^2 - 3c_1^2 \tag{1}$$

$$g_\perp = 2.0023 + {}^2\!/_3\, c_3^2 - 6c_1^2 + 6c_1 \tag{2}$$

where $c_1 = \xi/\Delta(^4B_1)$, $c_3 = \xi/\Delta(^2B_1)$, and ξ is the single electron spin–orbit coupling constant, and the Δ's represent energy separations between the ground state $^2A_1$ ($d^1_{z^2}$) orbital and the designated orbital ($^2B_1 \equiv d^1_{xz}$; $^4B_1 \equiv d^1_{yz}, d^1_{z^2}, d^1_{xy}$). The value of ξ for the free Co^{2+} ion is 530 cm^{-1}; however, covalency reduces this value by 20–40%.[12] Taking ξ = 400 cm^{-1} we obtained energy spacings for the various complexes which are given in Table II.

The $^2B_1$ state is derived from the d_{xz} and d_{yz} orbitals; its energy separation from the ground $^2A_1$ ($d_z{}^2$) state is $\Delta(^2B_1)$. Introduction of a base along the *z* axis destabilized the d_{z^2} orbital relative to the d_{xz} and d_{yz} orbitals thus increasing $\Delta(^2B_1)$. Therefore $\Delta(^2B_1)$ of six-coordinated complexes should be much larger than those of five-coordinated complexes as is shown in Table II. For the five-coordinates species, N_4S complexes have $\Delta(^2B_1)$ values which are more than 1000 cm^{-1} smaller than N_5 complexes. Apparently the more polarizable sulfur atom does not destabilize the d_{z^2} orbital as much as the nitrogen atom does. This difference is not seen for the six-coodinated complexes where N_5S and N_6 species have comparable values of $\Delta(^2B_1)$. A possible explanation is that Met-80 has strong bonding interaction with the metal atom in the native conformation.

The $^4B_1$ state is the ground state in an octahedral crystal field. Therefore, we would expect the energy separation of the quartet state and the $^2A_1$ state, $\Delta(^4B_1)$, to diminish as the crystal field approaches an octahedral field due to axial ligands. Results in Table II show that the values of $\Delta(^4B_1)$ are in the order of $N_6 < N_5S < N_5 < N_4S$ in agreement with this expectation. In both the five- and six-coordinate complexes, the substitution of a nitrogen ligand with a sulfur ligand increases $\Delta(^4B_1)$ by about 700 cm^{-1}.

Table II

Compd	$\Delta(^2B_1)$, cm^{-1}	$\Delta(^4B_1)$, cm^{-1}	10^4K, cm^{-1}	10^4P, cm^{-1}
A. Five-Coordinate N_5 Complexes				
CoPPIX·py	7 651	3030	−10.7	191.3[a]
CoPPIX·im	7 843	2834	−8.9	180.1[a]
Co(cyt *c*)-III (pH 12.9)	7 273	3110	−6.6	180.4
CoMb	7 246	3016	−13.5	189.6
CoHb	7 760	2728	−10.3	201.6
Co(*p*-OCH_3)TPP·(amine)[b]	7 443	3039	−6.1	188.6
B. Five-Coordinate N_4S Complexes				
CoPPIX·MTE	6 447	3540	+16	160.6
CoPPIX·ME	6 015	3305	+32.4	133.4
Co(cyt *c*)-I (pH 0.5)	6 447	3687	+16.7	165.6
C. Six-Coordinate Complexes				
CoPPIX·2py[c]	10 443	2147	−49.3	277.6
Co(*p*-OCH_3)TPP·2(amine)[b,c]	12 092	2343	−52.1	263.0
Co(cyt *c*)-II (pH 4–12)[c]	11 086	2945	−48.1	228.1

[a] With $A_\perp$ estimated from line width as 5×10^{-4} cm^{-1}.
[b] Computed from average of values in Table I. [c] As in ref 15 in order to achieve plausible *P* values; $A_\perp$ was assumed negative.

The isotropic Fermi hyperfine interaction parameter, *K*, and the dipolar splitting constant, *P*, can be calculated from the principal values of the hyperfine tensor, $A_\parallel$ and $A_\perp$

$$A_\parallel = K + (P/7)[4 - 4c_3 - 6c_1 + (2/9)c_3^2 + 15c_1^2] \tag{3}$$

$$A_\perp = K + (P/7)[-2 + 45c_1 + 2c_3 + (4/3)c_3^2 - (57/2)c_1^2] \tag{4}$$

where

$$P = 2\beta_e\beta_n\gamma_{Co}\langle 3d_{z^2}|r^{-3}|3d_{z^2}\rangle$$

β_e and β_n are the electronic and nuclear magnetons, respectively. c_1 and c_3 are as defined in eq 1 and 2, and p_0 for the free Co^{2+} ion has a value of 0.025 cm^{-1}.[16]

The values of *K* are positive for N_4S complexes but negative for the N_5 and the six-coordinate complexes. A positive value of *K* means an admixture of the 4s orbital with the $3d_{z^2}$ orbital. McGarvey[15] showed that there is a strong admixture of the two orbitals in the pure porphyrin or the porphyrin complexed with aromatic adducts, but this admixture is dramatically reduced when bases are complexed with the porphyrin systems. According to this interpretation, there is definite contribution from the 4s orbital to the ground-state electronic configuration of the N_4S complexes but not in those species with one or more axial nitrogenous ligands.

Since *P* is proportional to the electron density in the $3d_{z^2}$ orbital, the quantity $(P_0 - P)/P_0$ has been often used as a measure of electron delocalization to the ligands. The magnitude of the *P* values calculated from third-order perturbation theory (Table II) suggests appreciable delocalization in the N_5 and N_4S complexes but not for the six-coordinate complex, delocalization in the N_4S cases, and net gains in electron densities by the metal atom in the N_5 cases. It is apparent that the errors involved in the calculation of *P* values are large and not much significance can be attached to the degree of delocalization derived from them.

A more direct experimental measure of unpaired $3d_{z^2}$ electron density is the anisotropic cobalt hyperfine values. The values are much greater for N_4S than the N_5 complexes. This is consistent with the fact that MTE, ME, and Met are σ and π donors but not π acceptors. The differences between nitrogenous and sulfur ligands are greatly diminished in the six-coordinated species. Both N_6 and N_5S complexes have comparable values of ^{Co}A. It seems that pπ–dπ and pπ*–dπ interactions compensate one another such that the former interaction is diminished for the sulfur ligands because of the lack of $p\pi^*$–$d\pi$ interactions.

It has been established[5,6] that there is significant electron transfer from the cobalt atom to dioxygen in oxycobaltohemoglobin and oxycobaltomyoglobin. The comparison of ^{Co}A values in Table I for the dioxygen complexes is interesting. The values increase in the order of $CoMbO_2 \sim Co(p\text{-}OCH_3)TPP\cdot(amine); O_2 < CoPPIX\cdot ME, O_2, CoPPIX\cdot MTE; O_2 < CoPPIX\cdot O_2$. This is consistent with sulfur being a better donor than nitrogen and that there is $d\pi$–$p\pi^*$ back-donation from cobalt to nitrogen but not to sulfur.

In conclusion, this EPR study showed that N_6, N_5S, N_5, and N_4S have clearly distinguishable g and hyperfine values. The assignment of EPR spectra of the various pH-dependent conformational states of Co(cyt *c*)[10] has been verified as a result of this work.

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Registry No. CoPPIX·py, 36544-46-4; CoPPIX·im, 41188-38-9; CoPPIX·MTE, 58512-28-0; CoPPIX·ME, 58512-29-1; CoPPIX·2py, 58526-82-2; CoPPIX·O_2, 58503-65-4; CoPPIX·MTE·O_2, 58503-64-3; CoPPIX·ME·O_2, 58503-63-2.

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(3) Abbreviations: EPR, electron paramagnetic resonance; Co(cyt *c*), cobaltocytochrome *c*; NMR, nuclear magnetic resonance; CoPPIX, cobalt protoporphyrin IX dimethyl ester; MTE, 2-(methylthio)ethanol; ME, mercaptoethanol; py, pyridine; TPP, tetraphenylporphyrin.
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d-Orbital Energies and Low-Lying Excited States of Cobalt Porphyrins

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With assumed values of the Racah parameters, B and C, and the spin–orbit coupling parameter, ζ, it was found possible to deduce the relative d-orbital energies of cobalt porphyrin systems from their ESR data. The method consisted of diagonalizing the matrix of the combined ligand field, electrostatic, and spin–orbit coupling energies and using the ground-state eigenvectors thus obtained to calculate $g_\parallel$ and $g_\perp$. The sets of the d-orbital energies which satisfied the experimental $g_\parallel$ and $g_\perp$ were used to discuss the electronic structure of the various systems. Besides the ^2E state which was the only one considered in earlier works, a ^4E state and a $^4A_2$ state were found to be important in determining the nature of the $^2A_1$ ground state. For uncoordinated cobalt porphyrins, a $^4B_2$ state was further found to be very important and the experimental $g_\parallel$ could not be accounted for without it.

Introduction

Recently, an attempt was made to calculate the d-orbital energies in cobalt porphyrins from ESR data.[1] It was found that reasonable values of the relative energies of $d_{x^2-y^2}$, d_{z^2}, and (d_{xz}, d_{yz}) could be obtained through the use of the expressions for $g_\perp$, $A_\parallel$, and $A_\perp$, derived from the first-order perturbation treatment of the spin–orbit interaction. Although the energy of d_{xy} could also be calculated from the second-order equation for $g_\parallel$, the values obtained were always too high. It was suggested that low-lying quartet states which mixed in through spin–orbit coupling might not justify the use of the perturbation technique. In the present work, the spin–orbit interaction matrix is diagonalized together with the ligand field and the electrostatic energy. The ground state (a Kramers doublet) thus derived can be used to calculate $g_\parallel$ and $g_\perp$. It is hoped that by varying the orbital energies by trial and error one may find one set of energies which will yield calculated $g_\parallel$ and $g_\perp$ in agreement with the experimental ones.

Preliminary Considerations

Griffith's original designation[2] which was used in the previous work[1] is used again here, viz.

$$|\epsilon\rangle = (1/\sqrt{2})(|2\rangle + |-2\rangle) = d_{x^2-y^2}$$

$$|\theta\rangle = |0\rangle = d_{z^2}$$

$$|\mu\rangle = (1/\sqrt{2})(|2\rangle - |-2\rangle) = id_{xy}$$

$$|1\rangle = (1/\sqrt{2})(d_{xz} + id_{yz})$$

$$|-1\rangle = (1/\sqrt{2})(d_{xz} - id_{yz})$$

In C_{4v} or D_4 symmetry, $|\epsilon\rangle$ belongs to b_1, $|\theta\rangle$ to a_1, $|\mu\rangle$ to b_2, and $|1\rangle$ and $|-1\rangle$ to e. In a truly square-planar complex such as an uncoordinated metalloporphyrin, the b_1 orbital is very much higher than all of the others which are closely spaced among themselves. Molecular orbital theory as exemplified by the extended Hückel calculations of Zerner and Gouterman[3] placed a_1 slightly above e, which in turn, was placed slightly above b_2. Simple crystal field theory, on the other hand, would place b_2 above both a_1 and e. In the presence of solvent molecules, coordinated to the fifth and/or the sixth position, a_1 would be raised above both b_2 and e. How much so would depend upon the particular case. A recent work by Walker[4] showed the gradual increase of a_1 above e in cobalt tetraphenylporphyrin systems by a series of ligands, the effect being reflected in the decrease of $g_\perp$ from 3.2 to 2.2 with the simultaneous increase of $g_\parallel$ from 1.7 to 2.05. No quantitative interpretation was offered.