for larger temperature rises (20–100°) with simple alternative configurations is great.

Acknowledgment. The financial support of the National Institutes of Health (Grant GM-17190 (B.D.S.) and Training Grant Fellowship (J.H.B.)) and the Alfred P. Sloan Foundation (Fellowship to B.D.S.) is gratefully acknowledged.

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Triplet State Electron Paramagnetic Resonance Studies of Zinc Porphyrins and Zinc-Substituted Hemoglobins and Myoglobins

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Abstract: Electron paramagnetic resonance studies of photoexcited, five-coordinate zinc mesoporphyrin IX (ZnMesoPor) and protoporphyrin IX (ZnPor) complexes have demonstrated the importance of vinyl-group conjugation and axial-ligand π bonding in determining the properties of the lowest metalloporphyrin triplet state. The conformational properties of that state have also been discussed. In addition, ZnPor and ZnMesoPor have been incorporated into the heme crevice of apohemoglobin and apomyoglobin, and the triplet state properties of these zinc-substituted hemoproteins have been studied. Differences between the porphyrin–protein interaction in T-state hemoglobin and myoglobin (an analog for R-state hemoglobin), and chain differences within the T state, are observed and discussed.

Zinc porphyrins are diamagnetic in their ground state, with a tendency to bind a single additional ligand to the zinc atom.[1] The lowest lying porphyrin triplet state is sensitive to perturbations of the zinc porphin core and in this work electron paramagnetic resonance (epr) studies of photoexcited, five-coordinate zinc mesoporphyrin IX (ZnMesoPor) and protoporphyrin IX (ZnPor) complexes are used to examine the effects of lateral substituents and of coordinating nitrogenous bases to the metal. In addition, ZnPor and ZnMesoPor have been incorporated into the heme crevice of apohemoglobin and apomyoglobin, and the triplet state properties of these zinc-substituted hemoproteins have been studied. The influences of the protein environments of myoglobins (Mb) and hemoglobins (Hb) are compared, the 1-methylimidazole complexes of the zinc porphyrin–dimethyl esters serving as reference model compounds.

Porphyrin Triplet State

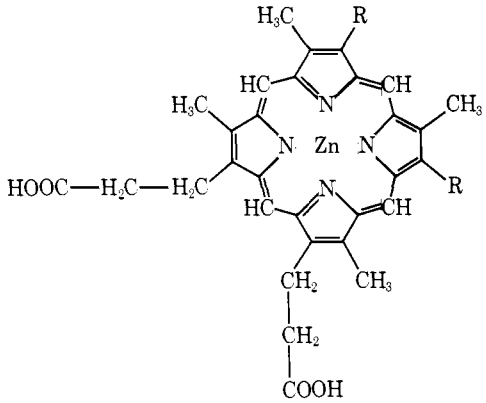
Closed-shell metalloporphyrins exhibit a metastable photoexcited triplet state which can be studied by epr.[2,3] In the "four-orbital" model of porphyrin excited states, the lowest triplet state of a fourfold symmetric porphyrin is largely independent of the metal atom and is spatially doubly-degenerate (^3E_u). An excited "square" porphin skeleton is Jahn-Teller unstable and is subject to a symmetry-breaking distortion into two equivalent, $S = 1$, vibronic states. These states may interconvert and, in the absence of further effects, are still of equal energy, but the orbital angular momentum and spin–orbit coupling expected of an ^3E state are quenched. Interactions with substituents on the porphin core, with axial ligands, as well as nonbonded interactions with the environment can lift the vibronic degeneracy and selectively stabilize one vibronic state by an energy, δ.[4,5]

The epr transitions of a triplet-state molecule can generally be described by the spin Hamiltonian: $H = g\beta\mathbf{S}\cdot\mathbf{H} + DS_z^2 + E(S_x^2 - S_y^2)$. Considering the zero-field splitting (ZFS) parameters, D is a measure of the spatial distribution of the triplet-state wave function, $D \propto \langle(3z^2 - r^2)/r^5\rangle$; E is a measure of the tetragonal distortion, $E \propto \langle x^2 - y^2\rangle$.[6] If symmetry-breaking perturbations split the vibronic states of the photoexcited ^3E level by an energy $\delta \gg kT$, the porphyrin undergoes a static distortion, E is nonzero,[4,5] and adjacent x and y transitions are split by $\delta H_{x,y} = 3E/g_e\beta_e$.[6]

In many of the spectra reported here the nature of the peaks in the region of the x–y transitions is, however, determined by a kind of dynamic Jahn-Teller effect. When kT approaches or exceeds δ, both vibronic states become populated. As the porphyrin interconverts between vibronic

states, the ZFS tensor x and y axes interchange, a process comparable to a physical rotation of a distorted porphyrin about the z axis.[4,5,7] Complete coalescence of x and y peaks of a "powder" sample[6] will occur when the vibronic states become equally populated ($\delta < kT$) *and* when the interchange frequency, ω_i, is greater than the static separation of adjacent x-y peaks (in frequency units): $\omega_i > g_e\beta_e\delta H_{x,y}/\hbar = E/\hbar$. Nonequal populations ($\delta > kT$) preclude coalescence, and slower exchange causes line broadening and a decrease in the observed x-y splitting which takes the form of an apparent decrease in E. In this work, the study of these dynamic processes for a variety of zinc porphyrin systems, at both 4.2 and 77°K, permits an observation of the dependence of δ on lateral substituents, axial ligands, and nonbonded interactions.

Zinc mesoporphyrin IX is an unsymmetrically-substituted metalloporphin in which the hydrogens of positions 2 and 4 of deuteroporphyrin (R = H) are replaced by ethyl groups (R = CH_2CH_3). Zinc protoporphyrin has vinyl substituents at positions 2 and 4 (R = $CH{=}CH_2$). In this work



the excited-state interactions between porphin core and unsaturated vinyl substituents are examined by comparing results for these two zinc porphyrins. Zinc tetraphenylporphin (ZnTPP) is also examined. The effects of the bonding to a fifth ligand are explored by employing several different nitrogenous bases. In particular, 1-methylimidazole (MeIm) is used to model the protein environment, since in Hb and Mb the metal is bonded to the imidazole of the "proximal" histidine.[8] The nature of porphyrin deformations in the excited state is also discussed.

Zinc-Substituted Hemoglobin and Myoglobin

The preparation of zinc hemoglobin (ZnHb) and myoglobin (ZnMb) by the incorporation of ZnPor into the apoproteins has previously been reported[9-10] and the optical and fluorescence properties have been reported.[10] Zinc hemoglobins and myoglobins do not undergo the ligand-binding reaction of hemoglobin itself. However, the conformational state of ZnHb has been determined.[11] Deoxy-Hb has a unique quaternary (and tertiary) structure, often called the "T" state, which differs from that of ligated and oxidized forms ("R" state). The Hb allosteric properties arise from a reversible transition between the two structures.[8] Recent functional studies of cobalt hemoglobin (coboglobin)[12] and manganese hemoglobin[13] show that both proteins adopt the T conformation when unliganded. Through the use of a variety of structural probes, we have recently shown that ZnHb also exhibits the T-state structure.[11]

The five-coordinate, high-spin, ferrous ions in Hb are unobservable in epr, but the properties of the metal atom in myoglobin and in T-state hemoglobins have been observed for cobalt[12a,14] and manganese substituted proteins.[15] These properties, although quite sensitive to the nature of out-of-plane coordination to the metal, are largely insensitive to perturbations of the porphyrin.[16] In contrast, the triplet-state epr studies of zinc-substituted hemoglobins and myoglobins reported here provide an opportunity to examine the properties of the porphin macrocycle as they are influenced by a protein environment.

Experimental Section

Protoporphyrin IX (Por) and its dimethyl ester (Por(DME)), mesoporphyrin (IX) dimethyl ester (MesoPor(DME)), and tetraphenylporphyrin (TPP) were commercially available and used as received. Mesoporphyrin IX (MesoPor) was prepared by the acid hydrolysis of the dimethyl ester. The zinc derivatives were prepared by the method of Adler et al. and, except for the free acid zinc porphyrins, were purified by dry-column chromatography on alumina.[17] Nitrogenous bases were distilled from CaH_2 before use.

Hemoglobin was prepared from pooled fresh human blood; horse heart and sperm whale skeletal muscle myoglobins were used as received (Miles Laboratories). Zinc-substituted hemoglobin (ZnHb) and myoglobin (ZnMb) were prepared as previously described by combining apoprotein with ZnPor; similar reconstitution with ZnMesoPor gave ZnMesoHb and ZnMesoMb.[11] Optical and epr studies of zinc porphyrins were performed in toluene or in the presence of 10% by volume of a liquid nitrogenous base. Room temperature optical studies of zinc proteins employed solutions of pH 6.7 using 0.01 M Bis-Tris buffer; low-temperature studies employed 0.5 M Bis-Tris, pH 6.6-glycerol in a 1:2.5 (v/v) ratio. No attempts were made to remove oxygen. Emission spectra were taken at room temperature or 77°K in an Hitachi (Perkin-Elmer) spectrofluorimeter. Epr spectra were taken at 77° or 4.2°K in a Varian Associates E-4 spectrometer using irradiation from a Hanovia 200 W Xe-Hg arc equipped with water filter. The microwave frequency was measured with a modified Sage Corp. tunable coherent synchronizer, a prescaler, and a digital frequency counter.

Irradiation of each zinc porphyrin system studied produced a metalloporphyrin triplet state spectrum. The samples were frozen solutions and exhibited the characteristic $\Delta M = \pm 1$ transitions centered at $g = 2.00$ (Figure 1) as well as the low-field "$\Delta M_s = \pm 2$" transition at $g \sim 4.0$.[6] From the spectrometer frequency and the canonical $\Delta M = \pm 1$ line positions, D, E, and g values were calculated by an iterative procedure as previously described; calculation was terminated when D and E remained constant to within ± 0.0001 cm^{-1}.[18] Because of the small values of D (Table II) compared to the x-band microwave quantum (~0.303 cm^{-1}) the first-order solutions for the fine-structure splitting along the principal axes of the ZFS tensor (ΔH_i, $i = x, y, z$) gave equivalent results: $g_e\beta_e\Delta H_z = 2D$; $g_e\beta_e\Delta H_{x,y} = D \pm 3E$.[6] However, because of the small values of D, coupled in many cases with appreciable values for E/D, the x transitions frequently overlapped with free-radical signals produced by the radiation and were not considered. Values of D and E for zinc porphyrin systems are listed in Table II.

Results

I. Optical Properties. Table I lists Soret absorption and fluorescence emission maxima for some zinc porphyrin complexes. Both maxima, as well as the α, β absorption bands, are shifted in the same direction by changes in lateral substituents and axial ligation.[19] Protein excitation spectra show that energy transfer from apoprotein to porphyrin is relatively unimportant.

As seen in Table I, for a given axial ligand, λ(ZnMesoPor) < λ(ZnPor) < λ(ZnTPP), indicating progressive red shifting by conjugating substituents. For a given porphyrin dissolved in toluene, coordination by a nitrogenous base causes a modest red shift. In addition, there is a substantial increase in the Soret peak extinction coefficient, and the ratio of α to β extinctions changes from greater than to less than unity. Similar differences seen between ZnPor in aqueous and in pyridine solutions[10] are therefore not due to changes in solvent polarity.

Binding of 1-methylimidazole should mimic the influence of the proximal histidine in the proteins, and the absorption and emission maxima for ZnMb do match those of

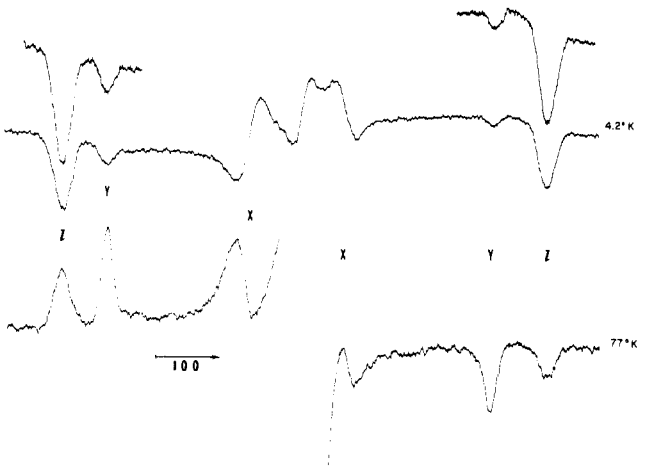


Figure 1. Triplet state epr spectrum of ZnMb. Upper spectrum (4.2°K) has reversed overall phase from lower spectrum (77°K). The three $\Delta M = \pm 1$ transition pairs are indicated. Inset in 4.2°K spectrum is taken at higher gain.

Table I. Soret Absorption and Fluorescence Emission Maxima (nm) for Zinc Porphyrin Complexes

	Zinc mesoporphyrin		Zinc protoporphyrin		Zinc tetraphenylporphin	
	Soret	Fluorescence	Soret	Fluorescence	Soret	Fluorescence
Toluene[a]	405	572	415	582	422	592
+ Piperidine[b]	417	581	427	591	430	609
+ Pyridine[b]	415	581	425	591	430	609
+ 1-Methylimidazole[b]	417	582	427	596	428	605
Protein[c]						
Myoglobin	415	583	428	596		
Hemoglobin	414	581	424	591		

[a] Dimethyl esters of zinc mesoporphyrin and zinc protoporphyrin. [b] Nitrogenous bases, 10% by volume. [c] See text for conditions.

ZnPor(DME)(MeIm). ZnHb appears to exhibit small, but significant, wavelength shifts from ZnMb. Differences between ZnMesoMb and ZnMesoHb are not pronounced.

II. Triplet State Epr. At 4.2°K, all the zinc porphyrin systems studied show $\Delta M_s = \pm 1$ transitions in which the high-field components of the triplet spectrum are inverted with respect to the free-radical signal at $g = 2$ and to their low-field counterparts (Figure 1). This inversion corresponds to stimulated microwave emission. The outermost (z) transitions are enhanced in magnitude relative to the x and y transitions and in some spectra the high-field y peak is unobservable; in the case of ZnTPP neither x nor y transitions are detectable. The $\Delta M_s = \pm 2$ transition is absorptive, but not intense.

These phenomena are caused by a highly nonequilibrium steady-state population of the triplet-state Zeeman sublevels, and indicate that the metalloporphyrin state is primarily, if not exclusively, populated by intersystem crossing into the zero-field $|z>$ sublevel. This behavior was previously observed in magnetic resonance studies of zinc porphin (ZnP) at zero applied field[4] and analogous behavior is well known in aromatic hydrocarbons.[20]

Observation of nonequilibrium popultations requires that the triplet state spin-lattice relaxation time, T_1, be long compared to the triplet state lifetime, τ. The triplet resonances saturate at incident microwave power levels above about 1-2 μW. For an upper limit to the value of T_1,[21a] we assume that $H_1^2 \approx 1(G^2/W)$(microwave power), that the intrinsic triplet lineshape is Lorentzian with width at half-maximum (obtained from the z peaks) of ~35 G; then T_1 at 4.2°K is of the order of 1 sec. On the other hand, preliminary observations[16] of the modulation-frequency dependence of rapid adiabatic passage signals[21b] indicate that $T_1 \gg 10$ msec. From attempts to determine τ by monitoring the epr decay upon interrupting the exciting light we find in the several systems examined $\tau \ll 20$ msec, even at 4.2°K. Triplet decay studies with higher time resolution are in progress.

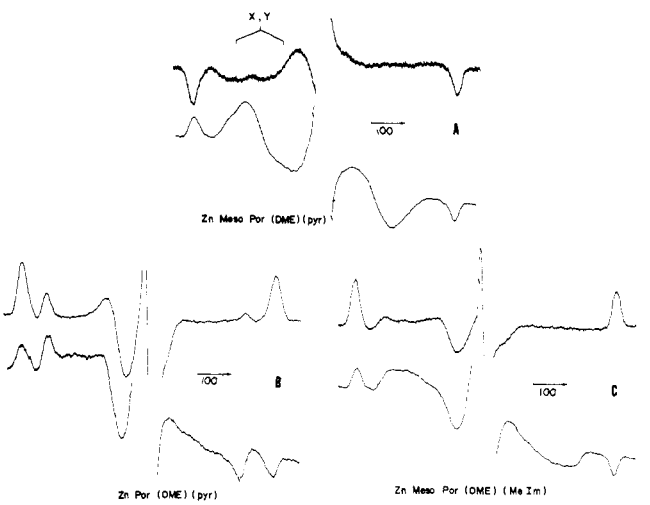


Figure 2. Triplet state epr spectra at 4.2°K (upper) and 77°K (lower): (A) ZnMesoPor(DME)(pyr). The 4.2°K spectrum has reversed phase and the lines indicate the x-y splitting $\delta H_{x,y}$; (B) ZnPor(DME)(pyr); (C) ZnMesoPor(DME)(MeIm). Arrows indicate 100 G.

At 77°K the emissive character of the $\Delta M_s = \pm 1$ transitions disappears (Figures 1-3), indicating a normal steady-state population distribution within the triplet sublevels. Although $\tau < 20$ msec at 77°K, saturation is not reached at 100-200 mW incident microwave power. An estimate similar to that given above shows that at 77°K, T_1 decreases to less than 10^{-5} sec.

ZnMesoPor(DME). Triplet state epr spectra of ZnMesoPor(DME) adducts are shown in Figure 2. At 4.2°K the high-field y transition of the MeIm adduct is unobservable (Figure 2B), as are both x- and y-high-field transitions of the pyridine complex (Figure 2A). Values of D are similar to the single reported value for ZnP (Table II).

The low-field x- and y-axis peaks of the ZnMesoPor(DME)(pyr) spectra at 4.2°K are quite broad and overlapped (Figure 2A) and the apparent value of E is small. These effects result from a dynamic Jahn-Teller effect, indicating that the upper vibronic state is partially populated and interconversion occurs. Thus δ must be greater than, but of the same order as, kT at 4.2°K (3 cm^{-1}). The apparent E for ZnMesoPor(DME)(MeIm) at 4.2°K is larger than that for the pyridine adduct and motional effects, although visible, are less pronounced (Figure 2C). Thus δ is larger with MeIm as axial base than with pyridine.

Spectra of ZnMesoPor(DME) at 77°K are dramatically changed from those at 4.2°K (Figures 2A and 2C). The value of E for the pyridine adduct has apparently gone to zero (Figure 2A), even though D changes only slightly. The g values at 77°K are still equal to 2.00, indicating that vibronic quenching of the orbital angular momentum is still effective. Apparent axial symmetry at 77°K occurs because the vibronic states are equally populated and rapid x-y axis interchange causes the x and y peaks to completely coalesce. Equal populations means that $\delta \ll kT$ at 77°K (50 cm^{-1}), consistent with the results at 4.2°K. Using the low-temperature value of E, coalescence requires that $\omega_i \gg 4 \times 10^9$ sec^{-1}.

Table II. Zero Field Splitting Parameters ($cm^{-1} \times 10^4$) for Zinc Porphyrin Complexes[a]

	Zinc protoporphyrin		Zinc mesoporphyrin		Zinc tetraphenylporphin		Zinc porphin		Zinc etioporphin	
	D	*E*	*D*	*E*	*D*	*E*	*D*	*E*	*D*	*E*
				4.2°K						
Nitrogenous base[b]							360	92[g]	350	50[h]
Methylimidazole	347	65	360	57						
Pyridine	350	66	364	42	306	[c]				
Piperidine	340	63								
Protein										
Hemoglobin	365	57	383	37[d]						
				12[d]						
Myoglobin	349	69[e]	372	64[f]						
				77°K						
Nitrogeneous base[b]										
Methylimidazole	345	63	357	≲53			350	0[j]	360	0[j]
Pyridine	349	62	360	0	304	~17[i]				
Piperidine	340	62								
Protein										
Hemoglobin[d]	358	~47	360	0						
	364	~50	378	0						
Myoglobin (whale)	353	67	367	62						
$+C_3H_6$	352	64	366	59						
Myoglobin (horse)	349	70								

[a] All *g* values 2.00; typical uncertainties, $\pm 1 \times 10^{-4}$ cm^{-1}. [b] 10% base in toluene; dimethyl esters of zinc protoporphyrin and zinc mesoporphyrin. [c] *x*–*y* transitions unobservable. [d] Resolved α–β chain contributions. [e] Horse heart myoglobin. [f] Whale muscle myoglobin. [g] *n*-Octane, ref 4. [h] Poly(methyl methacrylate).[5] [i] Spectrum indicates that a spread of *E* values exists. [j] Reference 2a.

At 77°K the MeIm adduct also shows substantial evidence of interconversion between states (Figure 2C). Unlike the pyridine adduct, complete averaging is not observed, and the apparent value of *E* is reduced from that at low temperature, but not to zero. These 77°K results further show that δ (MeIm) > δ(pyr).

ZnPor(DME). The values of *D* for ZnPor(DME) adducts are less than for those of ZnMesoPor(DME) and vary slightly with base (Table II). The smallest value occurs with a secondary amine, piperidine. The high-field *y* transition is observed with pyridine or piperidine as base, but not with methylimidazole. *D* does not change with temperature.

At 4.2°K, ZnPor(DME)(base) adducts exhibit the six-line $\Delta M = \pm 1$ pattern of transitions characteristic of a nondegenerate triplet state with rhombic symmetry and a well-defined value of *E*. The lineshapes give no evidence for interconversion between vibrational states of the ^3E level, or of a distribution of distorted configurations (Figure 2B). Thus, the 2,4-vinyl-substituted zinc protoporphyrin exhibits a static reduction from fourfold symmetry, with the lower-lying vibronic state stabilized by an energy much greater than *kT* at 4.2°K (3 cm^{-1}), in contrast with results for the alkyl-substituted zinc mesoporphyrin. In addition, values of *E* for ZnPor(DME) are substantially larger than for ZnMesoPor(DME) (Table II).

The contrast between zinc proto- and mesoporphyrin adducts observed at 4.2°K is even more dramatic at 77°K (Table II, Figure 2), clearly indicating the substantial role played by the vinyl substituents in determining the properties of the porphyrin triplet state. The *x*–*y* regions of the ZnPor(DME)(pyr) spectrum at 77°K are somewhat broadened (Figure 2B) and *E* is reduced from 4.2°K (Table II), but the complete coalescence that occurs for ZnMesoPor(DME)(pyr) (Figure 2A) is noticeably absent. Similarly, the MeIm adduct of ZnPor(DME) shows slight broadening, in the *x*–*y* region, and no change in *E*, as opposed to the extreme broadening and reduction in *E* for ZnMesoPor(MeIm) at 77°K.

The effects of vinyl substitution on δ may be semiquantitatively estimated from these two-temperature results for the pyridine adducts: δ[ZnPor(DME)] > 50 cm^{-1}, δ[ZnMesoPor(DME)] ≳ 3 cm^{-1}. More accurate estimates await variable-temperature studies.

Zinc Myoglobin. Incorporation of either ZnPor or ZnMesoPor into apomyoglobin produces pronounced effects on their triplet state epr properties. ZnMb and ZnMesoMb exhibit extremely well-resolved triplet-state spectra both at 4.2°K and at 77°K (Figure 1) with the $\Delta M = \pm 1$ pattern of a nonaxial ZFS Hamiltonian. In the 4.2°K, partially emissive, spectrum all six lines are observed. At neither temperature do the lineshapes suggest the dynamic Jahn-Teller effect found in adducts of ZnMesoPor(DME) and also observed in ZnPor(DME). Indeed the 77°K zinc myoglobin spectra are the only ones obtained here with the typical resonance envelope of a randomly oriented triplet with $E > 0$. ZFS parameters are given in Table II.

The value of *D* for ZnMb (horse) is the same as that for ZnPor(DME)(MeIm), while *E* is the largest observed for a zinc protoporphyrin IX system; *D* for ZnMb (whale) is slightly larger than for the horse protein, while the observed *E* is slightly smaller. The ZFS parameters are temperature independent.

At 4.2°K, *D* for ZnMesoMb (whale) is larger than that for the model compound, and *E* is the largest observed for a zinc mesoporphyrin. The changes of the ZnMesoMb zero-field splitting parameters with temperature are small, but outside experimental error.

The large values of *E*, the absence of appreciable temperature dependences of the ZFS, and the absence of motional broadening effects indicate a large stabilization energy for zinc myoglobins: $\delta \gg 50$ cm^{-1}. This value is greatly increased over those observed for the model compounds. Thus interactions between the myoglobin heme crevice and porphyrin greatly stabilize a single ^3E vibronic state. The effect is most noticeable in ZnMesoMb, but occurs for both ethyl and vinyl-substituted deuteroporphyrins.

It has been shown that cyclopropane reversibly binds to myoglobin, the binding site being in a cavity in the interior of the molecule, about equidistant from the proximal imidazole and one of the porphinato-pyrrole groups.[22] If a spectrum of ZnMb or ZnMesoMb (whole) is obtained and the sample is then warmed, equilibrated with one atmosphere of cyclopropane, and immediately reexamined, a small but significant decrease occurs in the observed value of *E*; *D* is

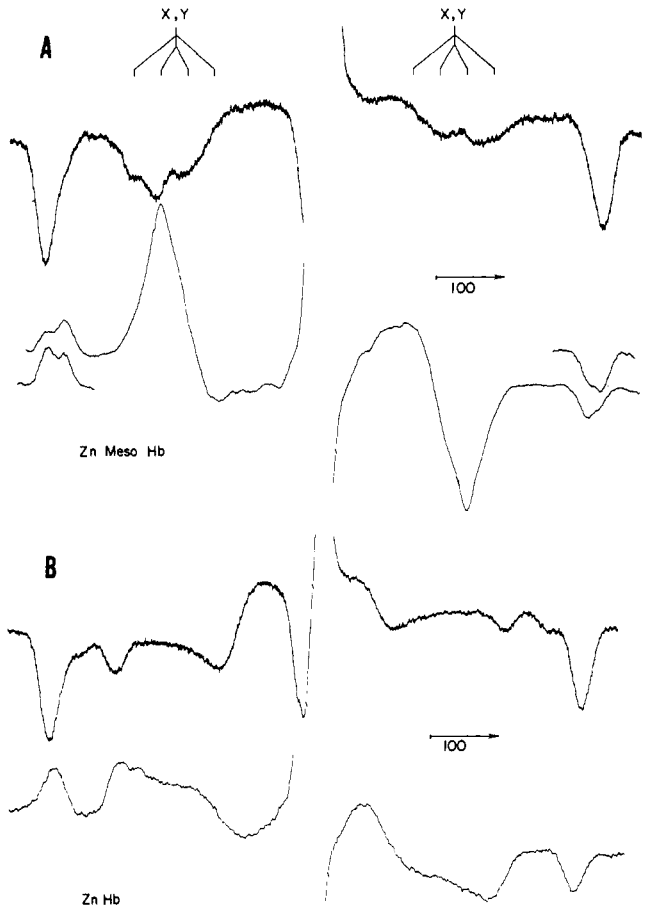


Figure 3. Triplet state epr spectra of zinc-substituted hemoglobins at 4.2°K (upper spectra, phases reversed) and at 77°K (lower spectra): (A) ZnMesoHb. Solid lines (4.2°K) indicate the two assigned x-y splittings. The full 77°K spectrum (lower) taken at ~50 mW incident microwave power; inserts taken at ~1 mW. (B) ZnHb. Arrows indicate 100 G.

probably the same within experimental error (Table II).

ZnMesoHb. The spectrum of ZnMesoHb (Figure 3A) at 4.2°K contains a single pair of z peaks; D is substantially greater than that for ZnMesoMb, which is in turn larger than for the imidazole-bound porphyrin in solution. At 77°K the z transitions are a superposition of doublets from two species of comparable concentration but with distinctly different values for D; both values are smaller than D at 4.2°K (Table II). The two species respond differently to microwave power variations (Figure 3A). The z doublet with the larger D saturates more readily, and thus has a longer spin-lattice relaxation time.

In sharp contrast to the results for the zinc myoglobins, incorporation of a zinc porphyrin into apohemoglobin *reduces* the splitting of the ^3E state, causing the vibronic states to be more nearly degenerate than in the model compounds. At 77°K (Figure 3A) the spectrum exhibits axial symmetry ($E \sim 0$), sharply different from spectra of ZnMesoMb (similar to Figure 1) and also ZnMesoPor(DME)(MeIm) (Figure 2C). At this temperature the two vibronic components of the ZnMesoHb ^3E state are therefore rapidly interconverting ($\omega_i \gg 4 \times 10^9$ sec^{-1}) and equally populated ($\delta \ll 50$ cm^{-1}).

At 4.2°K the x-y region of the spectrum (Figure 3A) is broadened by dynamic processes, but shows splitting, indicating unequal population of the vibronic sublevels ($\delta \gtrsim 3$ cm^{-1}). However, closer inspection, particularly of the lower-field x-y region, indicates that these transitions also arise from the overlapping absorptions of two ZnMesoPor triplets, one with $E \sim 40 \times 10^{-4}$ cm^{-1}, the other with $E \sim 13 \times 10^{-4}$ cm^{-1}. Both values are considerably less than for the model compound or for ZnMesoMb.

ZnHb. The z transitions of ZnHb at 77°K do not show the well-resolved doubling of ZnMesoHb (Figure 3). However, the measured values of D and E at high incident microwave power appear to be somewhat smaller than at low power (Table II). This effect is consistent with there being two overlapping and unresolved spectra with different spin-lattice relaxation times and slightly different ZFS. As with ZnMesoHb the longer T_1 would be associated with the species with the larger D. At 4.2°K the z transitions show no evidence of doubling. D is equal to the largest value measured at 77°K, and is larger than that for ZnMb and the appropriate model compound (Table II).

At 4.2°K, the E for ZnHb is considerably smaller than for ZnMb and the MeIm model compound; motional effects are not observed. At 77°K the x-y region of ZnHb shows substantial broadening (Figure 3B) and the smallest value of E (~50×10^{-4} cm^{-1}) of a Zn protoporphyrin system. The reduction of E with temperature, which arises through partial population of the upper vibronic state, is not observed with ZnPor(DME)(MeIm), and the 77°K broadening of the ZnHb spectrum is more pronounced. Thus δ for ZnHb is smaller than for the model compound, and must be larger than, but of the order of, 50 cm^{-1}. The reduction in δ by the hemoglobin environment is thus similar for both porphyrins examined.

The binding of organic phosphates to T-state hemoglobins acts as a mechanism for controlling the affinity for ligands, and ZnHb has been shown to exhibit such binding.[23] Addition of inositol hexaphosphate to ZnHb produced no resolvable change in spectra taken at 77 or 4.2°K. Spectra of ZnMesoHb at 77°K also showed no change. However, the pH of its buffer–glycerol mixture at its freezing point may have increased sufficiently to prevent binding.

If the exciting light is polarized (magnetophotoselection),[24] when E is parallel to the external magnetic field, H_0, the z peaks are decreased relative to the x-y peaks. When $E \perp H_0$, a reverse effect is observed. This behavior has been reported in studies of ZnEtio and MgEtio,[2] and arises because the transition moments of the porphyrin absorption bands are in-plane polarized. Occurrence of such polarization effects in the triplet epr spectra implies that energy transfer between magnetically nonequivalent ZnPor moieties of ZnHb is not substantial. This supports a similar conclusion based on fluorescence decay measurements.[10]

Discussion

With the exception of the work in ref 4 and 5, previous epr studies of metalloporphyrin triplet states were performed at liquid nitrogen temperatures or above. As a consequence, they focussed on D as a measure of the triplet state properties, either because $\Delta M_s = \pm 1$ transitions were not observed or because E was apparently zero. D was found to be insensitive to the diamagnetic metal Mg or Zn and to aliphatic porphyrin substituents,[2,3] and our results for ZnMesoPor(DME) are consistent with this observation.

The phenyl substituents of ZnTPP greatly reduce D from that of ZnP or zinc octaalkylporphyrins, presumably through partial spin delocalization. Comparing our results for complexes of ZnMesoPor with those of ZnPor, both free acids and dimethyl esters, shows that in all cases D for the protoporphyrin complex is lower, suggesting spin delocalization by excited state conjugation with the vinyl groups of positions 2 and 4 on the porphin core.

The vibronic properties of the porphin ^3E state make the lineshape of the x-y transitions, the observed value of E, and their temperature dependences a more sensitive indica-

tor of symmetry-breaking perturbations. From comparing the results for similar complexes of zinc protoporphyrin and mesoporphyrin, it can be seen that the vinyl side chains of zinc protoporphyrin IX have considerable power to stabilize a single vibronic state of ^3E; Figure 2 graphically demonstrates the effect. These results appear to be strong evidence for side chain conjugation and spin delocalization in the excited state. Results for the pyridine adducts indicate that replacing ethyl groups by vinyl groups at the 2 and 4 positions increase δ to $\gtrsim 50$ cm^{-1}.

Although the value of D somewhat reflects the effects of bonding of different axial bases to zinc (Table II), observation of the x-y transitions also provides a particularly sensitive index of these interactions, as Figure 2 clearly shows. The present study shows that δ for ZnMesoPor(DME) is considerably larger when the metal fifth ligand is an imidazole than when it is pyridine; a similar, but less pronounced, effect is seen with ZnPor(DME). It is reasonable to attribute these effects to a greater degree of π bonding by imidazole. Treating ZnMesoPor(DME) as a symmetrical octaalkylporphyrin,[19a] then for a five-coordinate metal the maximum (approximate) symmetry is C_{4v}. π-Bonding by an added ligand would reduce the symmetry to C_{2v} or less, depending on the orientation of the ligand, and would tend to lift a vibronic degeneracy and stabilize a particular vibronic state.

Incorporation of substituted zinc deuteroporphyrins gives an opportunity to probe the porphyrin–apoprotein interactions in myoglobin and T-state hemoglobin. The properties of myoglobin are known to be similar to those of the subunits of R-state hemoglobin and to isolated chains. The subunits of T-state hemoglobin show different spectral and functional properties from R-state subunits or chains.[8]

The enhanced stabilization of one vibronic state of the ^3E level in zinc myoglobin is consistent with the simple expectation of strong, stereochemically specific interactions between the porphyrin and the well-defined environment of the heme crevice. Because of the similarity in functional properties it is probable that the interactions in zinc myoglobin are similar to those in R-state hemoglobin and in chains.

ZnHb has been shown to adopt the T-state quaternary conformation.[11] The ^3E state of hemoglobin-incorporated zinc porphyrins shows quite unexpected behavior. Whereas the zinc myoglobins show increased E and splitting (δ) compared to the imidazole-complexed model compounds, the zinc hemoglobins show decreases in E and δ; the T-state hemoglobin environment appears to decrease the susceptibility to distortion of the triplet porphyrins.

It is possible that the stereochemistry of the proximal histidine–metal interaction in myoglobin enhances imidazole π bonding over that in the model compound, thus increasing the splitting, δ, of the zinc porphyrin ^3E state. However, it seems less satisfactory to attribute the reduced ^3E splittings in zinc hemoglobins to the converse of this mechanism. Pyridine is a poor π-bonding ligand, as shown by its complex with ZnMesoPor(DME). Nevertheless, E and δ for the pyridine complex of ZnPor(DME) are greater than the same parameters for ZnHb. Thus, minimized π bonding in zinc hemoglobin is unlikely to be solely or even primarily responsible for decreased values of E and δ.

An alternative source of the effects of the globin environments is stresses produced by van der Waals contacts between porphyrin and amino acid side chains. The metalloporphyrins lie in nonpolar pockets and experience about 60 interactions in which atoms of the globin come to within ~4 Å.[25] Contacts with phenylalanine CD1,[26] valine E11,[27] and leucine F7[28] in particular have all been implicated in the allosteric mechanism of hemoglobin.

There is no possibility of specific interactions between cyclopropane and zinc porphyrins. Thus the observation of ZFS parameter changes upon binding cyclopropane to zinc myoglobins further implicates nonbonded interactions as a way by which the porphyrin properties can be modified by the protein environment. The X-ray study shows that the cyclopropane is on the opposite side of the porphyrin from valine E11 and is in contact with the porphyrin, proximal histidine, and several other groups which in turn contact the porphyrin.[22]

In the previous discussions of vibronic effects in the ^3E state of four-coordinate zinc porphyrins, the ground-state geometry was idealized as D_{4h} and only the Jahn-Teller active distortions to D_{2h} were discussed.[4,5] The addition of a fifth ligand necessarily reduces the symmetry, but in the absence of π bonding (see above) does not split an E state. However, when considering the effects of environmental stresses, it must be noted that the porphin skeleton is easily deformed normal to the mean plane.[29] As one example, nickel octaethylporphyrin crystallizes in both a triclinic and a tetragonal form. In one case the porphin core is effectively planar, in the other it is markedly nonplanar.[30] Indeed, the pyridine adduct of zinc tetrapyridylporphin exhibits a pronounced quasi-D_{2d} ruffling of the porphin core. Although a D_{2d} ruffle would not split an E state, the observed core distortion has components of lower symmetry, quasi-C_{2v} which would act to lift an E degeneracy.[31]

Since in this study the effects of porphyrin substituents, of bonding to an axial ligand, and of nonbonded interactions with the environment are primarily responsible for the stabilization of a single vibronic state, degeneracy-lifting vibrations other than the Jahn-Teller modes, producing even lower symmetries, should be considered. In particular the tendency to out-of-plane deformations will be magnified in the ^3E state.[32] Stresses from van der Waals contacts in myoglobin, and thus presumably R-state hemoglobin chains, which favor a folding or ruffling of the porphin core whereby a nonplanar quasi-C_{2v} conformation is adopted would increase the splitting of the ^3E state of five-coordinate zinc porphyrin in ZnMb and ZnMesoMb.

Stresses in T-state hemoglobin which, for example, tend to stabilize the quasi-D_{2d} type of ruffling seen in zinc tetrapyridylporphin (pyr) would stabilize the ^3E state against degeneracy-breaking distortions. It may be the presence of such stresses which is responsible for the unique properties of T-state hemoglobin; indeed, changes in the ruffling of the porphin core upon ligation may be an important component in the mechanism of cooperative ligand binding.

These considerations of van der Waals contacts *appear* analogous to recent discussions of porphyrin deformations in T-state hemoglobin. However, its vibronic properties make the ^3E state particularly susceptible to distortion. Thus, it is possible that functionally important *stresses* which do not produce perceptible *strains* in a ground-state porphyrin may do so when the porphyrin is photoexcited.[34]

Since the environments of the α- and β-heme crevices are necessarily different,[25] it is logical to assume that the superposition of spectra in ZnMesoHb and ZnHb occurs because the spectra of individual chains can be resolved. The spectral differences reflect slightly different porphyrin–apoprotein interactions in the subunits of T-state hemoglobin; the slight differences between ZnMb (horse) and ZnMb (whale) are of analogous origin. Such α-β differences in T-state hemoglobin have previously been observed by pmr,[35] optically,[8] and in ligand-binding studies.[36]

Summary

The present study has demonstrated the importance of vinyl-group conjugation and axial-ligand π bonding in de-

termining the properties of the lowest metalloporphyrin triplet state, and the conformational properties of that state have been discussed. These observations are also relevant in studies of the triplet states of the chlorophylls.[37] Differences between the porphyrin–protein interaction in T-state hemoglobin and myoglobin, an analog for R-state hemoglobin, have been observed and discussed, and chain differences within the T state have been observed.[38] Present studies of the details of the triplet state population, depopulation, and spin relaxation and the use of other porphyrins will further explore these phenomena.

Acknowledgment. Thanks are due to Anne Griffin and James Anderson for expert technical assistance, to Professor R. G. Pearson, and to Drs. R. G. Little and C. Bull for many illuminating discussions. This work was supported by the National Institutes of Health through Grant HL-13531 and through a Career Development Award, and by the donors of the Petroleum Research Fund, administered by the American Chemical Society.

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