The Photoexcited Triplet State as a Probe of Chromophore-Protein Interaction in Myoglobin

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ABSTRACT The photoexcited metastable triplet state of Mg^{2+}-mesoporphyrin IX (MgMPIX) or Mg^{2+}-protoporphyrin IX (MgPPIX) located in the heme pocket of horse myoglobin (Mb) was investigated by optical and electron paramagnetic resonance (EPR) spectroscopy, and its properties were compared with the model complexes, MgMPIX, MgPPIX, and Mg^{2+} etioporphyrin I (MgETIOI), in noncoordinating and coordinating organic glasses. Zero-field splitting parameters, line shape, and Jahn-Teller distortion in the temperature range of 3.8–110 K are discussed in terms of porphyrin-protein interactions. The triplet line shapes for MgMPIXMb and MgPPIXMb show no temperature-dependent spectral line shape changes suggestive of Jahn-Teller dynamics, and it is concluded that the energy splitting is $\gg$150 cm^{-1}, suggesting symmetry breaking from the anisotropy of internal electric fields of the protein, and consistent with previous predictions (Geissinger et al. 1995. *J. Phys. Chem.* 99:16527–16529). Both MgMPIXMb and MgPPIXMb demonstrate electron spin polarization at low temperature, and from the polarization pattern it can be concluded that intersystem crossing occurs predominantly into in-plane spin sublevels of the triplet state. The splitting in the $Q_{0,0}$ absorption band and the temperature dependence and splitting of the photoexcited triplet state of myoglobin in which the iron was replaced by Mg^{2+} are interpreted in terms of effects produced by electric field asymmetry in the heme pocket.

INTRODUCTION

The unraveling of the mechanisms through which the polypeptide chain modulates the chemical and physical properties of the prosthetic group is central to the understanding of protein function. The situation is complicated by the rich physics that proteins possess by virtue of being mesoscopic particles with length scales on the order of tens of Ångstroms. Proteins are highly organized structures, i.e., their building blocks, amino acids, are spatially correlated and most proteins can be crystallized. However, in the crystalline state and in frozen glassy solution, they possess physical properties that point to the presence of two-level system (TLS)-like disorder modes and consequently behave in a glass-like manner (Anderson et al., 1972; Phillips, 1972). It is postulated that proteins must exhibit this level of complexity (possessing organization and a degree of randomness) to be capable of the multivariate functionality required in biological systems (Frauenfelder et al., 1988).

The glassy properties of proteins are most directly reflected by a linear temperature dependence of the specific heat at temperatures below 3 K (Miyazaki et al., 1993; Schulte and Murray, 1987; Singh et al., 1984; Yang and Anderson, 1986) and by their inhomogeneously broadened optical line shape at low temperatures (Friedrich, 1995; Vanderkooi et al., 1997). Nevertheless, it can be demonstrated that space, as seen from the chromophore imbedded in protein, is not homogeneous, as would be the case for a chromophore embedded alone in a glassy solvent. Fluorescence line narrowing and hole burning spectroscopies have yielded results that indicate that the glassy models of proteins are not entirely successful, and that there is a correlation between the structures of the chromophore (and hence its physical and chemical properties) and the structure of the surrounding protein matrix (Friedrich, 1995; Kaposi et al., 1993; Koehler et al., 1996; Vanderkooi et al., 1997).

It is becoming increasingly evident that local structure surrounding the porphyrin moiety imparted by the polypeptide matrix and the electric fields they generate are important in controlling metalloporphyrin chemical and physical properties. In particular for myoglobin and cytochrome *c*, the magnitudes of the electric fields within their respective heme pockets differ significantly, with those of myoglobin being a factor of 10 larger than those of cytochrome *c* (Geissinger et al., 1995; Koehler et al., 1996)

The photoexcited triplet state has been demonstrated in numerous studies to be an informative probe for the study of structure and dynamics in a variety of matrices (Budil and Thurnauer, 1991; Gonen and Levanon, 1985, 1986). To explore the chromophore-polypeptide interaction, we examined the electron paramagnetic resonance (EPR) spectroscopy of the photoexcited triplet state of Mg^{2+} protoporphyrin IX (MgPPIX) and Mg^{2+} mesoporphyrin IX (MgMPIX) derivatives of myoglobin (Mb) and of pertinent Mg^{2+} porphyrin model complexes in a variety of coordinating and

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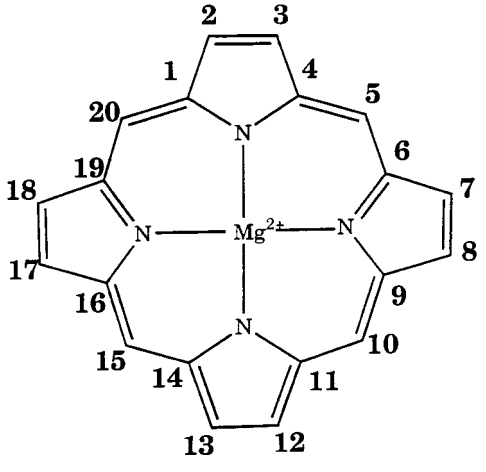
noncoordinating glassy solvents. Fig. 1 shows the structures of the Mg^{2+} porphyrins used in this study.

Myoglobin, a heme-containing globular protein composed of a single polypeptide chain of 23 kDa molecular mass and one of the first proteins with a determined structure (Kendrew et al., 1961), has long served as a model for addressing the unique physics of proteins, and the issue of prosthetic group/polypeptide chain interactions is of paramount importance for both the electron transfer and the small molecule transport functions of heme proteins. The S = 1 triplet state is an exquisite probe, because it reports directly on local symmetry via the spin-spin interaction. Moreover, at temperatures where the excited state lifetime is short compared to the spin lattice relaxation time between the triplet spin sublevels, electron spin polarization effects provide information on $S_1 \rightarrow T_1$ intersystem crossing dynamics and spin level selectivity. Spectral line shapes as a function of temperature are interpreted with the model of Jahn-Teller crystal field effects on the degenerate triplet manifold. Qualitative arguments and limiting "crystal field" energy splittings reveal that the Mg^{2+} porphyrin embedded in myoglobin experiences a local symmetry reduction greater than that experienced by the porphyrin in a solvent that provides axial ligation and far greater than that in a noncoordinating glassy solvent, and that the extent of the triplet state splitting appears to correlate with internal electric field strength and anisotropy.



	MgPP IX	MgMP IX	MgETIO I
2	$-CH_3$	$-CH_3$	$-CH_3$
3	$-CH=CH2$	$-CH_2CH_3$	$-CH_2CH_3$
7	$-CH_3$	$-CH_3$	$-CH_3$
8	$-CH=CH_2$	$-CH_2CH_3$	$-CH_2CH_3$
12	$-CH_3$	$-CH_3$	$-CH_3$
13	$-CH_2CH_2COOH$	$-CH_2CH_2COOH$	$-CH_2CH_3$
17	$-CH_2CH_2COOH$	$-CH_2CH_2COOH$	$-CH_3$
18	$-CH_3$	$-CH_3$	$-CH_2CH_3$

FIGURE 1 Metalloporphyrin structures.

METALLOPORPHYRIN SPECTROSCOPY

Optical spectroscopy

The spectral properties of heme proteins and model compounds—arising from porphyrin transitions—have been thoroughly reviewed (Adar, 1978; Owens and O'Connor, 1988). The four-orbital model proposed for the interpretation of metalloporphyrin spectra (Gouterman, 1978) describes transitions allowed under D_{4h} symmetry, between the two highest occupied molecular orbitals, $a_{1u}(\pi)$ and $a_{2u}(\pi)$, and the two degenerate lowest unoccupied orbitals, $e_g(\pi^*)$. The degenerate nature of these latter orbitals results in strong electron interaction between them and accounts for the relatively pure π-π^* transition, the B or Soret band, and for the weak $Q_{0,0}$ band or α. An additional band, the $Q_{1,0}$ or β, is attributed to vibronic coupling. Most theoretical calculations have been performed on square planar D_{4h} models. Asymmetrical substituents on the porphyrin ring, metal movement out of the porphyrin plane, and insertion of the heme into a protein matrix effectively lower this symmetry to C_{4h} (Adar, 1978), and in some cases even to C_s, where the only remaining element of symmetry is the planarity of the porphyrin macrocycle (Valance and Strekas, 1982).

Porphyrin triplet photophysics: the EPR line shape

Mg^{2+} porphyrins are diamagnetic in the ground state (S = 0). With visible wavelength excitation into the first excited singlet manifold and subsequent intersystem crossing (ISC), a metastable triplet state is formed. The spin Hamiltonian is governed mainly by the Zeeman interaction and the dipolar spin-spin interaction of the two electrons in the triplet molecular orbital. Within the molecular axis system, the total spin Hamiltonian describing these two interactions is

$$H_T = \beta_e \vec{H} \cdot \bar{\bar{g}} \cdot \vec{S} + \vec{S} \cdot \bar{\bar{D}} \cdot \vec{S} \qquad (1)$$

Here $\vec{H}$ is the applied magnetic field, $\vec{S}$ the total spin, $\bar{\bar{g}}$ the g-value tensor, and $\bar{\bar{D}}$ the zero field splitting (ZFS) tensor, which contains contributions from the spin-spin dipolar and spin-orbit interactions. In the molecular axis system, selected to diagonalize the ZFS tensor, the Hamiltonian can be recast using two independent parameters, D and E, giving the familiar phenomenological spin Hamiltonian:

$$H_T = g_e \beta_e \vec{H} \cdot \vec{S} + D(S_z^2 - \tfrac{1}{3}S^2) + E(S_x^2 - S_y^2) \qquad (2)$$

The magnitude of the ZFS parameter, $|D|$, is a measure of the electronic spatial distribution of the triplet molecular orbital and is proportional to $\langle r^3 \rangle^{-1}$. The magnitude of $|E|$ is related to the degree of distortion from tetragonal symmetry. The quotient $3E/D$ lies in the range $-1 \leq 3E/D \leq 0$, where the two extremes represent axial symmetry ($E = 0$) and orthorhombic symmetry ($3|E|/|D| = 1$) (Poole and Farach, 1974).

The EPR line shape for randomly oriented triplets has previously been described (Kottis and Lefrebvre, 1964; Wasserman et al., 1964). The anisotropy of the zero field splittings, in general, leads to six observable lines or turning points in the first derivative spectrum. Assuming that D is positive, as is commonly observed for planar aromatics, and $E < 0$ (an arbitrary assignment) the $|0\rangle \leftrightarrow |+1\rangle$ transition has Z, X, and Y components at field positions displaced from that of a free electron ($h\nu/g_e\beta_e$) by $-D$, $+ (D - 3E)/2$, and $+(D + 3E)/2$, corresponding to Z_I, X_I, and Y_I field positions, respectively. Likewise, the $|0\rangle \leftrightarrow |-1\rangle$ transition has lines at field positions displaced from g_e by $+D$, $-(D - 3E)/2$, and $-(D + 3E)/2$ that are defined as the Z_{II}, X_{II}, and Y_{II} transitions, respectively, following the convention of Thurnauer (1979). Thus, from the resulting randomly oriented spectrum, ZFS parameters are readily extractable, with the separations in field units between pairs of transitions defined as $\Delta H_z = 2|D|$, $\Delta H_y = |D| + 3|E|$, and $\Delta H_x = |D| - 3|E|$.

Electron spin polarization

Spin state dynamics can be obtained from the EPR excited triplet spectrum under steady-state illumination conditions. Entry into the lowest triplet state is governed primarily by spin-orbit coupling. Consequently, at temperatures (typically in the vicinity of liquid helium) where the $S_0 \leftarrow T_1$ lifetime is short compared to the spin lattice relaxation time between two spin state sublevels (T_1), a non-Boltzmann occupation (electron spin polarization or, more appropriately, electron spin alignment) of the triplet manifold results (Hausser and Wolf, 1976). Hence, some transitions will be emissive (e) in nature, whereas those that are absorptive (a) will be enhanced. The resulting polarization pattern of absorption and emission under conditions of steady-state illumination can thus be used to ascertain information concerning ISC, spin dynamics, and relaxation (Budil and Thurnauer, 1991; Thurnauer, 1979; Thurnauer et al., 1975).

The dynamic Jahn-Teller effect in a "crystal field"

For square planar central metal porphyrins, the lowest triplet should be spatially degenerate and in the D_{4h} point group, and would have a representation of ^3E_u. The special characteristics of porphyrin excited triplet states are reviewed by van der Waals et al. (1979). Because a porphyrin with C_4 or S_4 symmetry is Jahn-Teller (JT) unstable, it is subject to symmetry-relieving interactions resulting in two energy equivalent triplet states. Jahn-Teller instability alone is not enough to relieve the energy degeneracy of the triplet state, but interactions with axial ligands, porphyrin substituents, or environmental asymmetries may stabilize one state with respect to the other by an energy of δ_{JT}. One theory proposed to account for the dynamic Jahn-Teller effect involves coupling to the in-plane porphyrin vibrational modes, giving rise to two vibronic triplets separated in energy by δ_{JT} (de Groot et al., 1969; van der Waals et al., 1979). A result of JT coupling will manifest itself as a splitting in the optical absorption spectrum. This has been well documented for porphyrins in crystals (Canters, 1981; Canters et al., 1972, 1973; Kielman-van Luijt et al., 1976). Splitting in the optical absorption bands of heme proteins has been attributed to this phenomenon (Reddy et al., 1996). For the EPR triplet spectrum, the resulting "powder" pattern, when $\delta_{JT} \gg k_BT$, will show a static distortion with a nonzero $|E|$, with a splitting between the Y and X transitions (ΔH_{xy}) equal to $3|E|$. However, at temperatures that are comparable to or exceed the Jahn-Teller splitting energy, the X and Y transitions will merge, with complete coalescence occurring when both states are equally populated and when the exchange frequency, ν_{JT}, is greater than the separation of the X and Y transitions, i.e., when $\nu_{JT} \gg g_e\beta_e\Delta H_{xy}/\hbar$ (Carrington and McLachlan, 1967). The populations of the vibronic triplet states are governed by the Boltzmann distribution law, and the in-plane anisotropy, as measured by the zero field splitting parameter, $|E|$, will be temperature dependent. For square planar porphyrins of D_{4h} symmetry, coupling to either b_{1g} or b_{2g} vibrational modes has the effect of merely changing the sign of E, with $|E|$ unchanged. The distortions are equivalent to an interchange of the x and y molecular axes. The ramification is that as $k_BT \gg \delta_{JT}$, the $|E|$ value should approach zero and a coalescence of the Y and X transitions should occur. The in-plane anisotropy, as measured by the $|E|$ ZFS parameter, is

$$E = E_o \tanh\left(\frac{\delta_{JT}}{2k_BT}\right) \quad (3)$$

where E_o is the value E when $k_BT \ll \delta_{JT}$ (van der Waals et al., 1979).

MATERIALS AND METHODS

Metal-substituted myoglobin (Mb) derivatives were prepared following the methodology of Teale, with the modifications described (Teale, 1959). Horse Mb was purchased from Sigma Chemical Co. (St. Louis, MO) and used without prior purification. Fifty milligrams of Mb was dissolved in 2 cm^3 of chilled buffer (0.01 M Tris, pH 7.0). The pH was lowered to 3.8–4.0 with 1 M HCl under gentle stirring. The solution was added to a separatory funnel and gently extracted (1.5–2× the volume) with prechilled 2-butanone (methylethylketone). The extraction process was repeated several times, with the pH adjusted each time, until a colorless layer was obtained. The apoMb was dialyzed against water (two changes, 1.5 h/run), and the resulting product had an absorbance at 280 nm (tryptophan), which was 7–10× the absorbance at 410 (Fe heme Soret band). The pH was raised to 7.0 with 1 M ammonium hydroxide. Incorporation of the desired porphyrin substitute was carried out by dissolving the porphyrin in dimethylformamide or dimethylsulfoxide (~3 mM) and added dropwise with gentle stirring, maintaining the pH at 7.0. The sample was then incubated on ice for 45 min while stirring was maintained. Reaction progress was assessed spectrophotometrically. The final preparation was performed by washing the substituted Mb with 100 mM Tris buffer at pH 7.0 (in a cold room) in a concentration vessel and was brought to a final concentration of ~1–2 mM. Visible absorption spectra were obtained with a Hitachi U-3000 UV-visible spectrophotometer interfaced with a Packard Bell 386 PC.

Electron paramagnetic spectroscopy

Electron paramagnetic resonance spectroscopy was performed on a Bruker ESP300E spectrometer. The sample was irradiated directly through the front louvers with fiber optics and a 150-W Kuda quartz-halogen illuminator. Temperatures in the range from 3.8 K upward were obtained with an Oxford ESR 900 continuous-flow cryostat controlled with an Oxford ITC4 temperature controller. Frequency was measured with a Hewlett-Packard 5350B microwave frequency counter. All experiments were conducted at microwave powers that ensured there was no saturation of resonances. The spectra are presented as the resultants of light minus dark spectra; this procedure removed the contribution of a paramagnetic contamination present in the cavity. In most cases the irradiation procedure caused no permanent changes in the sample as monitored by absorption and emission spectra before and after intracavity irradiation. In some cases irradiation led to the appearance of increased free radical intensity, which allowed for internal field calibration.

RESULTS

Optical spectroscopy

Incorporation of Mg^{2+} porphyrin into apomyoglobin was confirmed through optical spectroscopy. Fig. 2 *A* is the absorption spectrum of MgMPIXMb at 298 K. Of note is the definitive split of the Q(0−0) band into two bands at 583.5 nm and 574 nm. The intense B-band (Soret), which represents the $S_0 \rightarrow S_2$ electronic transition, is at 410 nm (8.5 nm FWHM), and this band likewise shows splitting; the small band to the high side of the B-band is at 390 nm. The band with a λ_{max} at 543 nm is attributed to a vibronic envelope (Q(0−1)), and the 338-nm band is likewise attributed to a porphyrin transition (Storm et al., 1966). The band at 281 nm arises from tryptophan and tyrosine residues in the protein.

The absorption spectrum of MgPPIXMb at 298 K is shown in Fig. 2 *B*. It shows an intense B-band at 423.5 nm (27 nm FWHM) with a shoulder to the high energy side at ~400 nm. The Q(0−0) band is again split, with bands at 596.5 nm and 584.5 nm. The vibronic Q(0−1) band has a λ_{max} located at 552 nm. There is a broad band located at 351 nm attributed to the porphyrin. The Q(0−0) band and B-band are broader in the MgPPMb derivative than in the MgMPMb derivative.

The Q(0,0) band splitting, 284 cm^{-1} for MgMPIXMb and ~315 cm^{-1} for MgPPIXMb, has not been observed for any Mg^{2+} porphyrins in solution. We also saw no Q band splitting for these porphyrins in *N,N′*-dimethylformamide/glycerol (DMF/glycerol) glass at room temperature (data not shown).

EPR spectroscopy: myoglobin derivatives

The EPR spectra as a function of temperature of MgMPIXMb and MgPPMb in the photoactivated triplet state are shown in Figs. 3 and 4, respectively, and the zero-field splitting (ZFS) parameters $|D|$ and $|E|$ obtained from these data are given in Table 1. At temperatures less than or equal to 5 K, both samples show non-Boltzmann

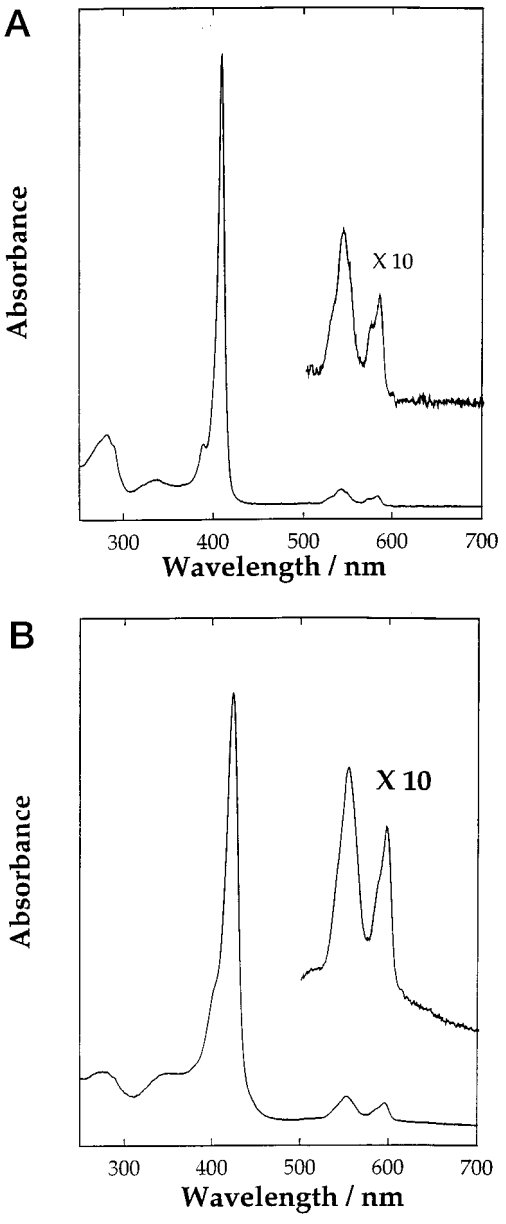


FIGURE 2 Absorption spectra of MgMPIXMb (*A*) and MgPPIXMb (*B*) at 25°C in 50 mM potassium phosphate buffer, pH 6.5.

entry into the triplet state. Although the spectrum of MgMPIXMb only shows Z_I to be in true emission, it can been seen from the spectrum at 5 K that the intensity of $Y_{II} > Y_I$ and $X_{II} < X_I$ (Fig. 3). From this it can be inferred that in the absence of spin lattice relaxation, the polarization pattern would be **eae-aea** (**a**, enhanced absorption; **e**, emission), indicating that ISC into the triplet manifold occurs through the $|T_x\rangle$ spin sublevel. At 3.6 K, MgPPIXMb shows a polarization pattern of **eea-eaa** (Fig. 4), indicating that entry into the triplet state from the first excited singlet state occurs principally through the in-plane spin sublevel $|T_y\rangle$, (given our sublevel convention). The $|D|$ value for MgMPIXMb decreases from 0.0375 cm^{-1} at 5 K to 0.0370 at 100 K. This small but significant change signals that the

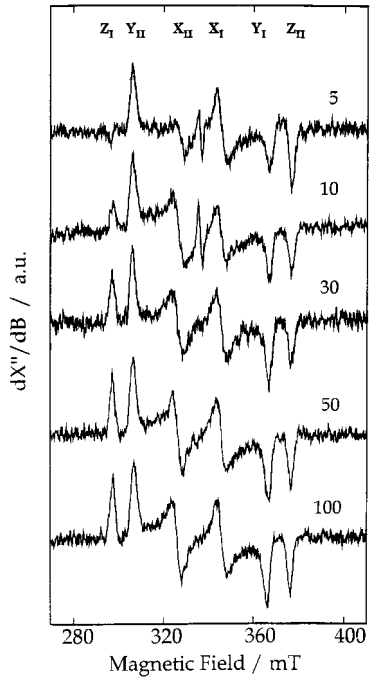


FIGURE 3 EPR spectra of the excited triplet state of MgMPIXMb. Experimental conditions: modulation amplitude 2 mT (100 kHz modulation frequency); microwave power 5 μW (5 K), 10 μW (10 K), 20 μW (30 K), 100 μW (50 K), 500 μW (100 K); sample concentration ~1 mM in a 1:1 (v/v) glycerol:aqueous (buffer 50 mM phosphate, pH 6.5) glass. Temperatures are in kelvins.

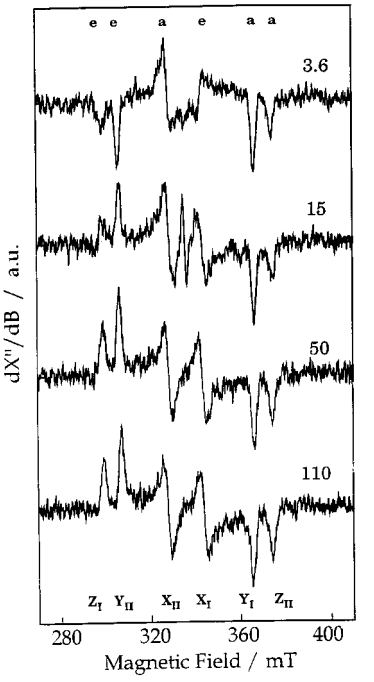


FIGURE 4 EPR spectra of the photoactivated triplet state of MgPPIXMb. Experimental conditions: modulation amplitude 2 mT (100 kHz modulation frequency); microwave power 2 μW (3.6 K), 50 μW (15 K), 100 μW (50 K), 500 μW (110 K); sample concentration ~1 mM in a 1:1 (v/v) glycerol:aqueous (buffer 50 mM phosphate, pH 6.5) glass. Temperatures are in kelvins.

spatial extent of the triplet molecular orbital is larger at 100 K. The $|E|$ value of MgMPIXMb also decreases from 0.0064 cm^{-1} at 5 K to 0.0062 cm^{-1} at 100 K. This large value of $|E|$ indicates that the triplet orbital is experiencing a perturbation that lifts the in-plane degeneracy expected of a porphyrin macrocycle. Furthermore, there is no evidence of a resonance at field values corresponding to $|E| \approx 0$, indicating that the triplet state occupies the lower vibronic state of the triplet manifold.

In contrast, the $|D|$ and $|E|$ values for MgPPIXMb show no change over the temperature range 3.6–100 K. The temperature dependence of the EPR triplet spectrum (Fig. 4) shows no evidence of a resonance corresponding to $|E| \approx 0$ over the same temperature range.

EPR spectroscopy: Mg^{2+} porphyrins in glassy matrices

The EPR spectra of the photoactivated triplet state as a function of temperature in the range 4–100 K for MgMPIX in DMF/glycerol glass 1:1 (v/v) and in pyridine/toluene 1:1 (v/v) are shown in Fig. 5, *A* and *B*, respectively. The distinguishing ZFS parameters are given in Table 1. For MgMPIX in DMF/glycerol (Fig. 5 *A*), a noncoordinating solvent system, the spectrum at the lowest temperature studied (10 K) shows no evidence of non-Boltzmann entry into the triplet state. The spectrum at 10 K shows multiple *X* and *Y* resonances on top of a broad resonance that is centered approximately at field values corresponding to $|E| \approx 0$. With increasing temperature, the triplet spectrum attains an axial line shape, clearly revealing that in the limit of high temperatures, an apparent $|E| \approx 0$ is reached. In contrast, MgMPIX in a strongly coordinating solvent (10% pyridine/toluene, v/v) shows no resonances at fields corresponding to $|E| \approx 0$ at low temperatures (4–50 K), but the spectrum does reveal a small resonance corresponding to triplet species possessing axial symmetry ($|E| \approx 0$) at 100 K (Fig. 5 *B*; see *arrows*). Although there is no clear polarization pattern from the *Z* transitions, it is clear that the low-field *Y* transition and the high-field *X* transition would be in emission in the absence of spin lattice relaxation. This is similar to the MgPPIXMb derivative.

The triplet state EPR spectra of MgPPIX in DMF/glycerol glass as a function of temperature are shown in Fig. 6. This series of spectra shows that by 70 K, a resonance at stationary field positions (*arrows*) indicative of axial symmetry ($E \approx 0$) emerges and thus demonstrates the dynamic Jahn-Teller effect. The signals associated with the $E \approx 0$ ZFS parameter increases with increasing temperature. At 3.6 K, MgPPIX shows evidence of electron spin polarization. The polarization pattern can be inferred from the spectrum and is the same as that of MgPPIXMb (Fig. 4).

Fig. 7 shows the triplet EPR spectra as a function of temperature for Mg^{2+} etioporphyrin I (MgETIOI) in DMF/glycerol 1:1 (v/v) glass. The spectrum at 3.6 K exhibits

TABLE 1 Zero field splittings of the Mg porphyrin myoglobin derivatives and model compounds

Compound	T (K)	$\|D\|$ (cm^{-1})#	$\|E\|$ (cm^{-1})#	Solvent	Ref.
MgMPIXMb	5	0.0375	0.0064	glyc./H_2O	1
MgMPIXMb	100	0.0370	0.0062	glyc./H_2O	1
MgPPIXMb	3.6	0.0352	0.0058	glyc./H_2O	1
MgPPIXMb	110	0.0349	0.0057	glyc./H_2O	1
MgMPIX	10	0.0363	0.0059	DMF/glyc. (1:1)	1
MgMPIX	100	0.0359	≈0	DMF/glyc. (1:1)	1
MgMPIX	5	0.0350	0.0077	pyr/tol(1:1)	1
MgMPIX	100	0.0350	0.0075,≈0	pyr/tol(1:1)	1
MgMPIX	77	0.0360	≈0	EtOH/pyr	2
MgPPIX	3.6	0.0344	0.0058	DMF	1
MgPPIX	77	0.0331	<0.0005	EtOH	2
MgPPIX	90	0.0341	0.0057,≈0	DMF	1
MgETIOI	3.6	0.0350	0.0074,≈0	EtOH/Et_2O	1
MgETIOI	100	0.0349	0.0070,≈0	EtOH/Et_2O	1
MgETIOI	77	0.0370	≈37	EtOH/PMA	2
MgETIOI	320	0.0330	≈0	EtOH/PMA	2
MgP	1.3	0.0333 0.0345	0.0099	*n*-octane	2
MgTPP	77	0.0298	<0.0005	EtOH	2
MgTPP	85	0.0380	0.0075	*n*-octane (polycryst.)	3
MgTPP	85	0.0299	≈0	tol/EtOH	3
MgTPP	100	0.0310	≈0	tol	4
MgTPP	116	0.0295	0.0075	Liq. cryst.	7
MgTPP	120	0.0294	0.0076	tol	8
MgTPP	120	0.0288	0.0028	EtOH	8
MgTPP	120	0.0304	0.0084	Liq. cryst.	5
MgTPP	10–80	0.0311	0.0092	tol/EtOH	9
MgTBP	5	0.0334	0.0065	pyr/tol(1:1)	6
MgTBP	10,80	0.0339	—	MTHF	2
MgOEP	140	0.0300	0.0075	CH_2Cl_2/EtOH	7
MgOEP	10–80	0.0378	0.0035	tol/EtOH	9
MgDP	77	0.0350	<0.0005	EtOH	2

Abbreviations: MPIX, Mesoporphyrin IX dimethylester; Et_2OH, diethylether; PPIX, protoporphyrin IX dimethylester; PMA, polymethylmethacrylate; ETIOI, etioporphyrin I; TPP, 5,15,15,20-tetraphenylporphyrin (were both *n*-octane samples and could have had some crystalline character); TBP, tetrabenzoporphyrin; OEP, octaethylporphyrin; P, porphin; glyc, glycerol; DMF, *N,N′*-dimethylformamide; tol, toluene; EtOH, ethanol (neat).

± 0.0002 cm^{-1}.

(1) This work.
(2) van der Waals et al. (1979).
(3) Levanon and Scherz (1975).
(4) Nissani et al. (1977).
(5) Grebel and Levanon (1980).
(6) Levanon and Norris (1978).
(7) Levanon et al. (1993).
(8) Scherz and Levanon (1980).
(9) Yamauchi et al. (1996).

electron spin polarization with a polarization pattern of **eea-eaa**, indicative of ISC into an in-plane spin sublevel ($|T_x\rangle$). The spectrum thermalizes to a Boltzmann distribution among the three spin sublevels by 30 K. Surprisingly, the spectrum at 70 K, although possessing an $E \approx 0$ component, does not differ much proportionately from the signal strength at 30 K, indicating that the $E \approx 0$ signal is not coming from dynamic averaging between the two split triplet manifolds, but is a manifestation of the inherent fourfold symmetry of the MgETIOI porphyrin (Fig. 1). There is also a ZFS $|E|$ value of 0.0070 cm^{-1}, which probably represents a small fraction of the MgETIOI molecules that underwent symmetry breaking, possibly because of the formation of microcrystals, producing local symmetry-breaking conditions.

DISCUSSION

It is increasingly being recognized that the asymmetrical environment of the protein heme pocket plays a role in modifying the intrinsic properties of the heme cofactor of hemoproteins (Angiolillo and Vanderkooi, 1995; Reddy et al., 1996; Shelnutt et al., 1977). The data presented here lead directly to information on the role of the polypeptide chain of myoglobin in modifying the properties of the heme.

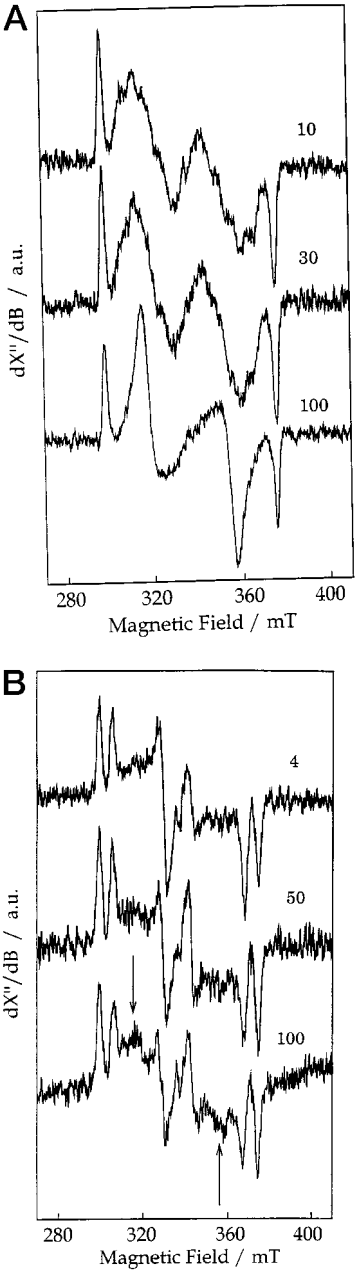


FIGURE 5 EPR spectra of the photoactivated triplet state of MgMPIX. (*A*) 1:1 (v/v) DMF/glycerol glass. Experimental conditions: modulation amplitude 2 mT (100 kHz modulation frequency); microwave power 50 μW (10 K), 20 μW (30 K), 500 μW (100 K); sample concentration ~1 mM. (*B*) 1:1 (v/v) pyridine:toluene glass. Experimental conditions: modulation amplitude 2 mT (100 kHz modulation frequency); microwave power 5 μW (4 K), 100 μW (50 K), 500 μW (100 K); sample concentration ~1 mM. Temperatures are in kelvins.

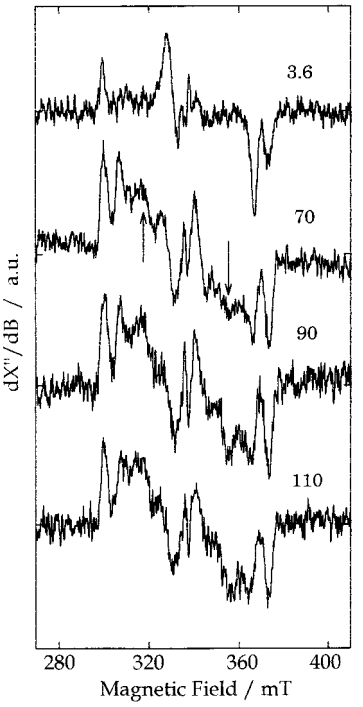


FIGURE 6 EPR spectra of the photoactivated triplet state of MgPPIX in 1:1 (v/v) DMF/glycerol glass. Experimental conditions: modulation amplitude 2 mT (100 kHz modulation frequency); microwave power 50 μW (3.6 K), 500 μW (70 K), 500 μW (90 K), 1000 μW (110 K); sample concentration ~1 mM. Temperatures are in kelvins.

The photoexcited triplet state as a measure of symmetry breaking in protein structures: ZFS parameters and electron spin polarization

The spin-spin dipolar interaction is extremely sensitive to local symmetry. For porphyrins especially, the $|E|$ ZFS parameter is a measure of in-plane anisotropy, $E = (3\mu_o/16\pi)(g\beta_e)^2\langle(Y^2 - X^2)/r^5\rangle$, where Y and X are the components of the interelectron vector expressed in the principal axial system of the molecule, r is the magnitude of the interelectron distance, μ_o is the permeability of free space, and g and β_e are the free-electron g-value and Bohr magneton, respectively. As a consequence of the vibronic properties of the triplet manifold (^3E_u in the D_{4h} point group), the value of $|E|$ and its dependence on temperature are sensitive indicators of symmetry breaking (Angiolillo and Vanderkooi, 1995; Hoffman, 1975; Hoffman and Ratner, 1978).

Several features emerge from Figs. 3 and 4. Values of $3|E|/|D|$, which is a measure of "rhombicity," are ~0.5 for both MgMPIXMb and MgPPIXMb and therefore suggest symmetries significantly lower than that expected for the porphyrin molecule. These rhombicity values compare well with those obtained by Hoffman on Zn^{2+} mesoporphyrin IX and Zn^{2+} protoporphyrin IX derivatives of myoglobin (Hoffman, 1975). In that study, $3|E|/|D|$ values of 0.59 and 0.52 were obtained for the Zn^{2+} mesoporphyrin IX and Zn^{2+} protoporphyrin IX derivative, respectively.

For both Mg^{2+} porphyrin myoglobin derivatives, there is no emergence of a resonance corresponding to an $|E|$ value of zero in the temperature range 4–100 K. These data permit an estimate of the energy splitting between the two split triplet states to be $\gg$150 cm^{-1} ($2k_BT$ at 100 K). This splitting of the vibronic triplet states is interpreted to be the result of symmetry breaking at the level of the porphyrin due to perturbations generated by the protein matrix, and we

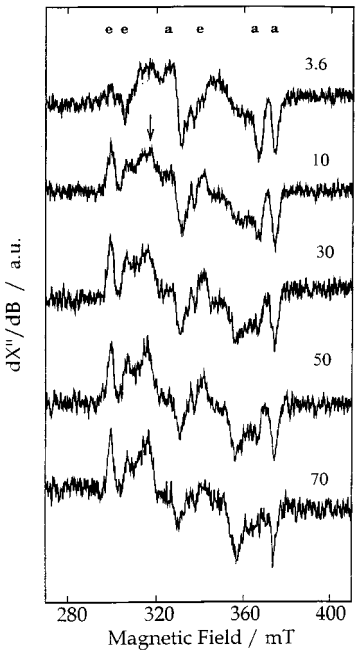


FIGURE 7 EPR spectra of the photoactivated triplet state of magnesium etioporphyrin in 1:1 (v/v) diethylether:ethanol. Experimental conditions: modulation amplitude 2 mT (100 kHz modulation frequency); microwave power 2 μW (3.6 K), 10 μW (10 K), 50 μW (30 K), 100 μW (50 K), 100 μW (70 K); sample concentration ~1 mM. Temperatures are in kelvins.

conclude that in both derivatives the triplet state occupies the lower vibronic triplet state.

Factors possibly contributing to the reduction of symmetry of the triplet state of the porphyrin macrocycle have been put forth earlier (Angiolillo and Vanderkooi, 1995; Hoffman, 1975). Among them are lateral substitution on the heme ring, axial ligation, and nonbonding interactions due to electrostatic effects within the heme crevice. Resolving the overall interaction is a difficult task that has only received qualitative investigation for Zn^{2+} porphyrins in heme proteins (Angiolillo and Vanderkooi, 1995; Hoffman, 1975). The situation is even more complex in Mg^{2+} porphyrins, because Mg^{2+} displays complex coordination chemistry, including five- and six-ligated species, in addition to the possible formation of dimers and aggregates when free in solution (Scherz and Levanon, 1980). Our data show that for MgMPIX in solvent, the presence of an axial nitrogenous ligand serves to stabilize one vibronic state (Figs. 5 and 6). It is striking, however, that even in the presence of an axial nitrogenous ligand (in this case, pyridine-), the triplet state splitting, determined from the temperature dependence of the EPR spectra, is lower than in the protein matrix. In MgPPIX the evolution of an $E \approx 0$ signal occurs at higher temperatures relative to MgMPIX in a similar solvent, thus indicating that the lateral substitution in the PPIX macrocycle produces a greater departure from fourfold symmetry. This is due to the presence of vinyl groups in PPIX that are known to enter into conjugation with the porphyrin macrocycle (Spiro, 1975).

Comparisons with Mg porphyrins that possess a rigorous C_4 rotation axis (MgETIOI, Fig. 7) demonstrate that in a glassy host, even at the lowest temperature used in this study (~4 K), the $|E|$ ZFS parameter reflects an environment that can be considered to be isotropic. If the triplet state is split because of local electric field perturbations, it can be estimated that the splitting for a large proportion of the molecules is $\ll$100 cm^{-1}. In fact, even at 3.6 K, a significant proportion of the molecules already exhibit an axial EPR spectrum ($|E| \approx 0$) (*arrow*, Fig. 7), indicating a δ_{JT} on the order of 10 cm^{-1}. Attempts thus far to put a metalloetioporphyrin I in a protein environment have failed. However, it would be interesting to investigate the effect of the protein environment on a porphyrin with known high symmetry. This question is under investigation.

Electric field asymmetry in the heme pocket

Our data support the notion that the polypeptide chain induces a large asymmetry on the heme in myoglobin. This is substantiated by a variety of other experiments. In oxymyoglobin, Mössbauer spectroscopy has established the electric field gradient tensor at the Fe atom. These data reveal that the direction of largest electric field gradient component is most likely in the heme plane, with an asymmetry parameter value η in the range of 0.3–0.4 (Maeda et al., 1981). More recently, in the iron-free PPIXMb, it has been shown that with reference to the center of the porphyrin, the internal electric field components are directed orthogonally along the pyrollic nitrogens of the porphyrin ring with values of E_x = 29.9 MV/cm and E_y = 38.6 MV/cm (Geissinger et al., 1995). These values, in addition to exceeding by more than an order of magnitude the internal field of those obtained in *n*-alkane matrices (Kohler and Woehl, 1995), show a clear asymmetry of the electric field at the porphyrin center. From this study it has been postulated that in the hydrophobic environment of the heme crevice, internal electric fields of this strength mandate the existence of charges within a few Ångstroms of the porphyrin macrocycle, due to deprotonated propionic acid groups on the PPIX ring.

The lack of spectral dynamics of the EPR triplet spectrum in the temperature range 4–110 K supports the view of an environment that is quite asymmetrical with respect to the porphyrin plane. Comparable data in the Zn^{2+} porphyrin myoglobin derivatives has also demonstrated that in addition to porphyrin side-chain interactions, the protein's heme crevice provides a large stabilization energy for the lowest vibronic state (Hoffman, 1975). Spectral hole-burning experiments, involving the Stark effect for the free-base PPIX-substituted horse myoglobin compared with the chromophore directly dissolved in a glassy matrix, show some intriguing physics of the protein state (Gafert et al., 1993, 1995a,b). These experiments show that there is a well-defined internal electric field at the level of the chromophore in the protein that breaks the inversion symmetry

of the porphyrin π-electron system, leading to a very characteristic splitting of the hole profile when probed in external electric fields. This splitting of the hole profile is not in evidence with identical chromophores embedded in an isotropic glassy matrix (Gafert et al., 1995b).

Hole burning and triplet state EPR spectroscopy of another metallo-substituted protein, horse cytochrome *c*, reinforce the conclusions obtained from the myoglobin studies. Stark-effect hole-burning experiments coupled with electric field calculations at the heme site have clearly demonstrated that for the Zn(II) derivative of cytochrome *c*, there exists a definite in-plane electric field anisotropy (Iben et al., 1989). Corresponding triplet state EPR spectroscopy of Zn(II) cytochrome *c* has clearly demonstrated that in the low-temperature regime (~4 K), a rhombic spectrum is obtained with $|D|$ and $|E|$ values of 0.00342 cm^{-1} and 0.0073 cm^{-1}, respectively (Angiolillo and Vanderkooi, 1995). Analysis of the temperature-dependent line shape changes in the *XY* region of the spectrum over the temperature range 4–150 K showed that there was a coalescence of the *XY* canonical transitions, in accordance with the dynamic Jahn-Teller effect (Angiolillo and Vanderkooi, 1995; Hoffman and Ratner, 1978). The upper limit of the energy splitting between the two vibronic triplet states was estimated in the case of cytochrome *c* to be ~180 cm^{-1} (Angiolillo and Vanderkooi, 1995).

A summary of all of these data leads to the conclusion that the polypeptide chains of both myoglobin and cytochrome *c* impose an asymmetrical field on the heme, and that the effect is larger in myoglobin.

Optical transitions and spin delocalization measured by $|D|$ values for porphyrin in protein or solvents

The line widths of the optical transitions of MgMPIX Mb and MgPPIXMb are of particular note. The Soret band of MgMPIXMb shows a FWHM of 8.5 nm, whereas the Soret band of MgPPIXMb has a FWHM of 27 nm (Fig. 2). Storm et al. have shown that electron-withdrawing groups placed on the periphery of the porphyrin nucleus markedly increase the width of the optical transitions (Storm et al., 1966). The vinyl groups of the PPIX derivative thus follow this trend relative to the electron-promoting ethyl groups of MPIX.

The $|D|$ ZFS parameter for MgPPIXMb is significantly smaller than for MgMPIXMb (Table 1); these data suggest an increased spin delocalization in the excited triplet state onto the vinyl groups at positions 3 and 8 of the porphyrin ring (Fig. 1). This conclusion is supported by the data of Hoffman on Zn(II) porphyrin complexes, where it was noticed that in both the free acids and the dimethyl esters, the $|D|$ ZFS values were lower in the PPIX complexes as compared to MPIX complexes (Hoffman, 1975) .

The $|D|$ ZFS parameters for the protein species are also larger than those for chromophore dissolved in host glassy matrix. When compared at low temperature, MgMPIXMb has a $|D|$ value of 0.0375 cm^{-1}, and MgMPIX in DMF/glycerol has a $|D|$ value of 0.0363 cm^{-1}. For MgPPIXMb, the values are 0.0352 cm^{-1} in the protein, 0.0344 cm^{-1} in DMF, and 0.0331 cm^{-1} in EtOH glasses. These data represent differences in the $|D|$ values of 3.3% for MgMPIX and 2.3% (DMF) and 6.3% (EtOH) for MgPPIX in protein and glassy matrix, respectively. Similar results were obtained for chlorophyll *a* in frozen phosphatidylcholine vesicles as compared to chlorophyll *a* in glassy matrices, where $|D|$ was determined to be larger in a frozen vesicle environment. This suggested that spectral diffusion via triplet migration took place in glassy matrices that contained high concentrations of chromophore (Hiromitsu and Kevan, 1988). A similar effect was seen in solutions of metalloporphyrins (Scherz and Levanon, 1980). One of the benefits of embedding a chromophore within a protein is that the spectroscopic system can be considered infinitely dilute. In myoglobin, the chromophore is buried deep in a hydrophobic core, with only the propionic acid groups exposed to the solvent. Consequently, both optical and EPR spectroscopic details are not complicated by aggregation effects, and the EPR line shape and any changes can thus be interpreted to be due solely to individual chromophore dynamics and chromophore-protein interactions. In fact, triplet EPR studies in the Zn(II) derivative of horse cytochrome *c* revealed no changes in the ZFS parameters or in the spectral line shape over nearly three orders of magnitude in concentration, suggesting no chromophore-chromophore interaction (Angiolillo, 1996).

Analysis of the Mg myoglobin derivatives is placed in perspective when compared to pertinent model complexes in a variety of coordinating and noncoordinating solvent systems (Table 1). In general, the $|D|$ ZFS parameter appears to be bracketed by the $|D|$ values of Mg(II) porphine and Mg(II) octaethylporphyrin, with the exception of the results obtained by Levanon and Scherz (Scherz and Levanon, 1980) on Mg tetraphenylporphyrin in an *n*-octane polycrystalline sample (Table 1). Similarly, as with the Zn(II) porphyrins, *meso*-position phenyl substitution (Mg(II) tetraphenylporphyrin) significantly reduces $|D|$ as compared to Mg(II) porphine. As in the Zn(II) porphyrins, it is tempting to interpret the decrease in $|D|$ due to *meso*-phenyl substitution as increased delocalization onto the phenyl groups. Time-resolved resonance Raman spectroscopy, however, fails to provide any evidence in support of this supposition, as indicated by the lack of a frequency shift between the first excited triplet and ground states of the $C_{meso}C_{phenyl}$ and C_{phenyl} C_{phenyl} stretches (de Paula et al., 1992).

Electron spin polarization and spin-level specific entry into the triplet state in MPIXMb and PPIXMb

It is interesting that MPIX and PPIX differ in their specificities of intersystem crossing, which for MgMPIXMb

occurs through the $|T_x\rangle$ spin sublevel, and for MgPPIXMb is in the $|T_y\rangle$ sublevel (Figs. 3 and 4). This shows the rather exquisite sensitivity the substitutents have in determining specificity, which should also be reflected in determining direction-dependent reaction rates for the triplet state.

Further information on porphyrin/protein interactions comes from observing the relaxation of the spin polarization. Both MgMPIX and MgPPIX in the protein environment at low temperature show electron spin polarization up to temperatures of ~10 K (Figs. 3 and 4). This result would also seem to suggest that the protein provides a structurally more ordered environment relative to the organic glasses (Kaiser and Friedrich, 1991; Kaiser et al., 1993). In an ordered lattice the electron spin relaxation is due to magnetic interactions between the electronic spin system and the environment modulated by phonons. At low temperatures the phonon density of states in typical crystals resonant with the zero field splitting energies is rather small. Consequently, at low temperatures spin relaxation is inefficient, and electron spin polarization is observed. In disordered solids, in addition to electron spin-phonon interactions, structural transitions modeled as transitions in localized double wells (two-level systems, TLS; vide infra) also provide possible spin relaxation pathways (Kaiser and Friedrich, 1991; Kaiser et al., 1993).

Comparison of the triplet state and the singlet state data

The EPR data can be compared with the optical absorption spectra. The absorption spectra of MgPPIXMb and MgMPIXMb show some resolved details, including a split in the $Q_{0,0}$ band at room temperature (Fig. 2). It has been postulated previously for Zn-Mb derivatives, where the Q(0,0) band is also split, that the mechanism responsible for the splitting is due to axial ligation resulting from the two possible orientational isomers (Cowan and Gray, 1989). In cytochrome *c*, where the heme is covalently attached, the Q(0,0) splitting is consistent with an electrostatic-dependent (crystal field) splitting of the Jahn-Teller susceptible first excited singlet state (Reddy et al., 1996; Shelnutt, 1980). The splitting in horse Fe(II) cytochrome *c* and its Zn(II) derivative has been determined to be on the order of 100 cm^{-1}. Recent data on the lowest excited triplet state of Zn cytochrome *c* demonstrates quite clearly the dynamic Jahn-Teller effect and that the maximum splitting of the two vibronic triplet states is on the order of 180 cm^{-1} (Angiolillo and Vanderkooi, 1995). In other words, the two techniques for the two excited states (optical spectroscopy for the singlet state and EPR for the triplet state) are both indicators of the asymmetry of the environment imposed by the protein.

In the Mg^{2+} porphyrin Mb derivatives investigated in this study, the Q(0,0) band splittings are ~300 cm^{-1}. Even for the Mg porphyrin-ethanol complex in an *n*-octane single crystal, the ^1E_u splitting between the $|y\rangle$ and $|x\rangle$ components of the 0,0 has been determined to be 195 cm^{-1} (Platenkamp, 1982). Consequently, it is speculated that if there is a correlation between the splitting of the singlet manifold and the triplet manifold, then the splitting of the triplet state, as manifested by line shape changes in the triplet EPR spectrum, should indicate energy splitting much greater than the 180 cm^{-1} obtained for Zn cytochrome *c*. This turns out to be the case, because in the temperature range studied there is no temperature dependence of the line shapes for both Mg^{2+} myoglobin derivatives, indicating that the energy splitting between the vibronically split triplet states is $\gg$150 cm^{-1} ($\approx 2k_BT$ at 100 K).

The photoexcited triplet state in proteins as it relates to the complex nature of the protein state of matter

Over the past decade it has become increasingly evident that the physics describing the protein's "state of matter" is not as straightforward as once thought. Proteins possess properties reminiscent of both crystalline solids, as shown by their highly regular x-ray diffraction patterns, and amorphous or glassy structures, as documented by low-temperature specific heat measurements (linear in *T* below 1 K) (Miyazaki et al., 1993), anomalous thermal conductivity and dielectric response (Narashimhan et al., 1990), and large inhomogeneously broadened optical transitions (Vanderkooi et al., 1997). Even at temperatures of 2 K and below, it has recently been demonstrated by spectral hole burning and photon echo spectroscopies that proteins undergo relaxation processes that are on the order of days (Gafert et al., 1995b; Leeson et al., 1994). In this respect, proteins exhibit behavior consonant with glasses. These apparent glass-like properties of proteins can be successfully explained using the two-level system (TLS) model (Anderson et al., 1972; Phillips, 1972). Fluorescence line narrowing spectroscopy has been an especially powerful technique in studying the distribution of conformations within the ensemble. As the positions of the neighboring atoms change, the chromophores experience different electric fields (Langsetmo et al., 1991; Northrup et al., 1990; Wendoloski and Matthew, 1989), which change the 0,0 transition energy. It follows that the observed spectral dispersion in the transition frequency should be sensitive to the protein electrostatic properties (Anni et al., 1994; Zollfrank et al., 1991a). The inhomogeneous width of the optical transition for various chromophores in crystalline, amorphous, and protein matrices has been studied in detail over the past few decades (Vanderkooi et al., 1997). It is striking that proteins have thus far consistently yielded distributions of ground-state electronic levels that straddle distributions found in crystalline ($\leq$1 cm^{-1}) and completely amorphous materials ($\gg$100 cm^{-1}) (Vanderkooi et al., 1997). Moreover, the distributions found in many proteins can rarely be fit using a mono-Gaussian distribution, and in most cases require two or more Gaussians—unlike what is found for chromophores in amorphous materials. The inhomogeneous

width data suggest that, although glass-like by some experimental criteria, proteins are very different and thus cannot be completely described by the physics used to model the glassy state. Hole burning studies are in agreement with this notion and seem to indicate that, in addition to possessing some glass-like properties at low temperature, proteins possess properties indicative of a more structured nature. Thermally induced spectral diffusion broadening in horseradish peroxidase substituted with mesoporphyrin IX demonstrates a discrete step around 12 K, suggesting a correlated structural change of the protein (Zollfrank et al., 1991b).

Results obtained thus far on the triplet state in proteins have indicated that the triplet state is an excellent probe for assessing local electrostatic and symmetry considerations (Angiolillo and Vanderkooi, 1995; Hoffman, 1975). In the heme proteins studied thus far, it is clear that at least locally there is a high degree of order as seen from the heme group. This is reflected in the rather large and nonrandom E ZFS values and in the large splittings inferred from the temperature profiles of the triplet state EPR as seen in this study and other protein systems (Angiolillo and Vanderkooi, 1995; Hoffman, 1975).

CONCLUSIONS

It is shown in this study that the protein matrix in horse myoglobin exerts a very strong asymmetrical perturbation on the inherent symmetry of the porphyrin plane. This is most strongly indicated by the complete lack of dynamics in the photoexcited triplet state of probe molecules placed within the heme crevice, as contrasted to identical chromophores in glassy coordinating and noncoordinating matrices. For both chromophores that were placed in the heme site, it was determined that the crystal field splitting of the triplet state is in excess of 150 cm^{-1}. When compared to model complexes, it can be concluded that axial ligation, although partly responsible for symmetry breaking of the porphyrin triplet electronic system, cannot completely account for the extent of splitting in evidence for the protein system. These results are in accord with recent electric field calculations on myoglobin, which have clearly shown that the internal electric field strength at the porphyrin center is $\sim$10 times that of a chromophore in an alkane matrix. This can only be the result of large, well-ordered electric fields within the protein heme crevice.

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REFERENCES

Adar, F. 1978. Electronic absorption spectra of hemes and hemoproteins. *In* The Porphyrins. Academic Press, San Diego. 167–209.

Anderson, P. W., B. I. Halperin, and C. M. Varma. 1972. Anomalous low-temperature thermal properties of glasses and spin glasses. *Philos. Magazine.* 25:1–9.

Angiolillo, P. J. 1996. The Photoactivated Triplet State of Metal-Free and Metal Substituted Hemeproteins and Model Compounds. Ph.D. thesis, University of Pennsylvania, Philadelphia.

Angiolillo, P. J., and J. M. Vanderkooi. 1995. Electron paramagnetic resonance of the excited triplet state of metal-free and metal substituted cytochrome *c*. *Biophys. J.* 68:2505–2518.

Anni, H., J. M. Vanderkooi, K. A. Sharp, T. Yonetani, S. C. Hopkins, L. Herenyi, and J. Fidy. 1994. Electric field and conformational effects of cytochrome *c* and solvent on cytochrome *c* peroxidase studied by high-resolution fluorescence spectroscopy. *Biochemistry.* 33:3475–3486.

Budil, D. E., and M. C. Thurnauer. 1991. The chlorophyll triplet state as a probe of structure and function in photosynthesis. *Biochim. Biophys. Acta.* 1057:1–41.

Canters, G. W. 1981. Crystal field effects on the vibronic properties of the Jahn-Teller unstable S_1 state of zinc porphin. *J. Chem. Phys.* 74: 157–162.

Canters, G. W., J. van Egmond, T. J. Schaafsma, I. Y. Chan, W. G. van Dorp, and J. H. van der Waals. 1973. The study of Zn porphin in an *n*-octane crystal by optical spectroscopy and magnetic resonance. *Ann. N.Y. Acad. Sci.* 206:711–721.

Canters, G. W., J. van Egmond, T. J. Schaafsma, and J. H. van der Waals. 1972. Optical and Zeeman studies of the first excited singlet state of zinc porphin in a single crystal of *n*-octane: evidence for Jahn-Teller instability. *Mol. Phys.* 24:1203–1215.

Carrington, A., and A. D. McLachlan. 1967. Introduction to Magnetic Resonance. Chapman and Hall, London. 115–131, 204–220.

Cowan, J. A., and H. B. Gray. 1989. Q-band splitting in the spectra of heme proteins. *Inorg. Chem.* 28:4554–4556.

de Groot, M. S., I. A. M. Hesselmann, and J. H. van der Waals. 1969. Paramagnetic resonance in phosphorescent aromatic hydrocarbons. VI. Mesitylene in B-trimethylborazole. *Mol. Phys.* 16:61–68.

de Paula, J. C., V. A. Walters, C. Nutaitis, J. Lind, and K. Hall. 1992. Transient resonance Raman spectrum of meso-tetrapheylporphine: an analysis of chemical factors that influence the dynamics of the excited triplet states of metalloporphyrins. *J. Phys. Chem.* 96:10591–10594.

Frauenfelder, H., F. Parak, and R. D. Young. 1988. Conformational substates in proteins. *Annu. Rev. Biophys. Biophys. Chem.* 17:451–479.

Friedrich, J. 1995. Hole burning spectroscopy and physics of proteins. *Methods Enzymol.* 246:226–259.

Gafert, J., J. Friedrich, and F. Parak. 1993. A comparative pressure tuning hole burning study of protoporphyrin IX in myoglobin and in a glassy host. *J. Chem. Phys.* 99:2478–2486.

Gafert, J., J. Friedrich, and F. Parak. 1995a. Stark-effect experiments on photochemical holes in chromoproteins—protoporphyrin IX-substituted myoglobin. *Proc. Natl. Acad. Sci. USA.* 92:2116–2120.

Gafert, J., J. Friedrich, J. M. Vanderkooi, and J. Fidy. 1995b. Structural changes and internal fields in proteins: a hole-burning Stark effect study of horseradish peroxidase. *J. Phys. Chem.* 99:5223–5227.

Geissinger, P., B. E. Kohler, and J. C. Woehl. 1995. Electric field and structure in the myoglobin heme pocket. *J. Phys. Chem.* 99: 16527–16529.

Gonen, O., and H. Levanon. 1985. Time-resolved EPR spectroscopy of electron spin polarized ZnTPP triplets oriented in a liquid crystal. *J. Phys. Chem.* 89:1637–1643.

Gonen, O., and H. Levanon. 1986. Energy transfer and fine structure axes determination in a hybrid porphyrin dimer oriented in a liquid crystal. Time resolved triplet EPR spectroscopy. *J. Chem. Phys.* 84:4132–4141.

Gouterman, M. 1978. Optical spectra and electronic structure of porphyrins and related rings. *In* The Porphyrins, Vol. III. Academic Press, San Diego. 1–156.

Grebel, V., and H. Levanon. 1980. EPR study of oriented photoexcited triplets of porphyrins and chlorophylls in a liquid crystal. *Chem. Phys. Lett.* 72:218–224.

Hausser, K. H., and H. C. Wolf. 1976. Optical spin polarization in molecular crystals. *In* Advances in Magnetic Research. Academic Press, San Diego. 85–121.

Hiromitsu, I., and L. Kevan. 1988. Chlorophyll: a triplet-state ESR in frozen phosphatidylcholine vesicles. *J. Phys. Chem.* 92:2770–2773.

Hoffman, B. M. 1975. Triplet state electron paramagnetic resonance studies of zinc porphyrins and zinc-substituted hemoglobins and myoglobins. *J. Am. Chem. Soc.* 97:1688–1694.

Hoffman, B. M., and M. A. Ratner. 1978. Jahn-Teller effects in metalloporphyrins and other four-fold symmetric systems. *Mol. Phys.* 35: 901–925.

Iben, I. E., D. Braunstein, W. Doster, H. Frauenfelder, M. K. Hong, J. B. Johnson, S. Luck, P. Ormos, A. Schulte, P. J. Steinbach, A. H. Xiem, and R. D. Young. 1989. Glassy behavior of proteins. *Phys. Rev. Lett.* 62:1916–1919.

Kaiser, G., and J. Friedrich. 1991. Relaxation phenomena of a triplet spin probe in glassy and crystalline *o*-terphenyl. *J. Phys. Chem.* 95: 1053–1057.

Kaiser, G., H. Pierron, and J. Friedrich. 1993. Structural disorder and spin-lattice relaxation. *J. Chem. Phys.* 99:605–609.

Kaposi, A. D., J. Fidy, S. S. Stavrov, and J. M. Vanderkooi. 1993. Optical fine-structure investigation of chromophore/protein interactions: Mg protoporphyrin myoglobin. *J. Phys. Chem.* 97:6317–6327.

Kendrew, J. C., H. C. Watson, B. E. Strandberg, R. E. Dickerson, D. C. Phillips, and V. C. Shore. 1961. A partial determination by x-ray methods and its correlation with chemical data. *Nature.* 190:161–163.

Kielman-van Luijt, E. C. M., P. J. M. Dekkers, and G. W. Canters. 1976. MCD of transitions to Jahn-Teller unstable states in metalloporphins of Mg, Zn, Cu, Pd and Pt. *Mol. Phys.* 32:899–919.

Koehler, M., J. Gafert, J. Friedrich, J. M. Vanderkooi, and M. Laberge. 1996. Stark effect experiments in cytochrme *c*-type proteins: structural hierarchies. *Biophys. J.* 71:77–85.

Kohler, B. E., and J. C. Woehl. 1995. Measuring internal electric fields with atomic resolution. *J. Chem. Phys.* 102:7773–7781.

Kottis, P., and R. Lefrebvre. 1964. Calculation of the electron spin resonance line shape of randomly oriented molecules in a triplet state. II. Correlation of the spectrum with the zero field splittings. Introduction of an orientation-dependent linewidth. *J. Chem. Phys.* 41:379–393.

Langsetmo, K., J. A. Fuch, C. Woodward, and K. A. Sharp. 1991. Linkage of thioredoxin stability to titration of ionizable groups with perturbed pKa. *Biochemistry.* 30:7609–7614.

Leeson, D. T., O. Berg, and D. A. Wiersma. 1994. Low-temperature protein dynamics studied by the long-lived stimulated photon echo. *J. Phys. Chem.* 98:3913–3916.

Levanon, H., and J. R. Norris. 1978. The photoexcited triplet state and photosynthesis. *Chem. Rev.* 78:185–198.

Levanon, H., A. Regev, T. Galili, M. Hugerat, C. K. Chang, and J. Fajer. 1963. Photoelectron transfer between a magnesium-free base porphyrin heterodimer and duroquinone. Selective excitation and time-resolved EPR studies. *J. Chem. Phys.* 97:13198–13205.

Levanon, H., and A. Scherz. 1975. EPR study of electron spin polarization in the photoexcited triplet state of chlorophyll a and b. *Chem. Phys. Lett.* 31:119–124.

Maeda, Y., T. Harami, Y. Morita, A. Trautwein, and U. Gonser. 1981. Mossbauer studies on O_2 and CO binding to the heme iron in myoglobin. *J. Chem. Phys.* 75:37–43.

Miyazaki, Y., T. Matsuo, and H. Suga. 1993. Glass transition of myoglobin crystal. *Chem. Phys. Lett.* 213:303–308.

Narashimhan, L. R., K. A. Littau, D. W. Pack, Y. S. Bai, A. Elscher, and M. D. Fayer. 1990. Probing organic glasses at low temperature with variable time scale optical dephasing measurements. *Chem. Rev.* 90: 439–457.

Nissani, E., A. Scherz, and H. Levanon. 1977. The photoexcited triplet state of tetraphenyl chlorin, magnesium tetraphenyl porphyrin and whole cells of Chlamydomonas Reinhardi. A light modulation-EPR study. *Photochem. Photobiol.* 25:93–101.

Northrup, S., T. G. Wensel, C. F. Meares, J. J. Wendoloski, and J. B. Matthew. 1990. Electrostatic field around cytochrome *c*: theory and energy transfer experiment. *Proc. Natl. Acad. Sci. USA.* 87:9503–9507.

Owens, J. W., and C. J. O'Connor. 1988. Comparison of the electronic and vibrational spectra of complexes of protoporphyrin-IX, hemeoctapeptide, and heme proteins. *Coordination Chem. Rev.* 84:1–45.

Phillips, W. A. 1972. Tunneling states in amorphous solids. *J. Low Temp. Phys.* 7:351–360.

Platenkamp, R. J. 1982. Site selection and Zeeman spectroscopy of the $S_1 \leftrightarrow S_0$ transition of magnesium porphin in *n*-octane at 4.2 K. II. The magnesium porphin-ethanol complex. *Mol. Phys.* 45:113–127.

Poole, C. P., and H. A. Farach. 1974. Standardization of convention for zero field splitting parameters. *J. Chem. Phys.* 61:2220–2221.

Reddy, K. S., P. J. Angiolillo, W. W. Wright, and J. M. Vanderkooi. 1996. Spectral splitting in the α (Q0,0) absorption band of ferrous cytochrome *c* and other heme proteins. *Biochemistry.* 35:12820–12830.

Scherz, A., and H. Levanon. 1980. Photoexcited triplet state and chemical dynamics in frozen solutions of metalloporphyrins. An EPR-optical study. *J. Phys. Chem.* 84:324–336.

Schulte, A., and R. Murray. 1987. Specific heat of hemoglobin solutions at temperatures between 1.3 and 10 K. *Phys. Rev. B.* 36:1772–1774.

Shelnutt, J. A. 1980. The Raman excitation spectra and absorption spectrum of a metalloporphyrin in an environment of low symmetry. *J. Chem Phys.* 72:3948–3958.

Shelnutt, J. A., L. D. Cheung, R. C. C. Chang, N.-T. Yu, and R. H. Felton. 1977. Resonance Raman spectra of metalloporphyrins. Effects of Jahn-Teller instability and nuclear distortion on excitation profiles of Stokes fundamentals. *J. Chem. Phys.* 66:3387–3398.

Singh, G. P., H. J. Schink, H. v. Lohneysen, F. Parak, and S. Hunklinger. 1984. Excitations in metmyoglobin crystals at low temperatures. *Z. Phys. B. Condensed Matter.* 55:23–26.

Spiro, T. G. 1975. Resonance Raman spectroscopic studies of heme proteins. *Biochim. Biophys. Acta.* 416:169–189.

Storm, C. B., A. H. Corwin, R. R. Arellano, M. Martz, and R. Weintraub. 1966. Stability constants of magnesium porphyrin-pyridine complexes. Solvent and substituent effects. *J. Am. Chem. Soc.* 88:2525–2532.

Teale, F. W. J. 1959. Cleavage of the haem-protein link by acid methylethylketone. *Biochim. Biophys. Acta.* 35:543.

Thurnauer, M. C. 1979. ESR study of the photoexcited triplet state in photosynthetic bacteria. *Rev. Chem. Intermed.* 3:197–230.

Thurnauer, M. C., J. J. Katz, and J. R. Norris. 1975. The triplet state in bacterial photosynthesis: possible mechanisms of the primary photo-act. *Proc. Natl. Acad. Sci. USA.* 72:3270–3274.

Valance, W. G., and T. C. Strekas. 1982. Low-frequency resonance Raman spectra of ferrocytochrome *c* and liganded derivatives. Evidence of symmetry lowering in native ferrocytochrome *c*. *J. Phys. Chem.* 86: 1804–1808.

van der Waals, J. H., W. G. van Dorp, and T. J. Schaafsma. 1979. Electron spin resonance of porphyrin excited states. *In* The Porphyrins. Academic Press, San Diego. 257–312.

Vanderkooi, J. M., P. J. Angiolillo, and M. Laberge. 1997. Fluorescence line narrowing spectroscopy: a tool for studying proteins. *Methods. Enzymol.* 278:71–94.

Wasserman, E., L. C. Snyder, and W. A. Yager. 1964. ESR of the triplet states of randomly oriented molecules. *J. Chem. Phys.* 41:1763–1772.

Wendoloski, J., and J. B. Matthew. 1989. Molecular dynamics effects on protein electrostatics. *Proteins.* 5:313–321.

Yamauchi, S., Y. Matsukawa, Y. Ohba, and M. Iwaizumi. 1996. State mixings in the excited triplet and singlet states of fluorine-substituted magnesium tetraphenylporphines studied by optical and time-resolved EPR spectroscopy. *Inorg. Chem.* 35:2910–2914.

Yang, I., and A. C. Anderson. 1986. Specific heat of melanin at temperatures below 3 K. *Phys. Rev. B.* 34:2942–2944.

Zollfrank, J., J. Friedrich, J. Fidy, and J. M. Vanderkooi. 1991a. Photochemical holes under pressure: compressibility and volume fluctuations of a protein. *J. Chem. Phys.* 94:8600–8603.

Zollfrank, J., J. Friedrich, J. M. Vanderkooi, and J. Fidy. 1991b. Conformational relaxation of a low temperature protein as probed by photochemical hole burning: horseradish peroxidase. *Biophys. J.* 59:305–312.