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A Fluorescence Study of Hybrid Hemoglobins Containing Free Base and Zinc Protoporphyrin IX†

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ABSTRACT: We have studied the fluorescence emission and excitation spectra and fluorescence lifetimes of the following hybrid hemoglobins containing protoporphyrin IX (P) or zinc protoporphyrin IX (ZnP): $\alpha(+)_2\beta(P)_2$, $\alpha(P_2)\beta(+)_2$, $\alpha(ZnP)_2\beta(+)_2$, $\alpha(+)_2\beta(ZnP)_2$, $\alpha(P)_2\beta(ZnP)_2$, and $\alpha(ZnP)_2\beta(P)_2$. The results clearly demonstrate energy transfer between unlike chains with α to β transfer more pronounced than β to α. Changes in the energy transfer rate constant observed on converting the hybrid hemoglobins from oxy to deoxy indicate a change in heme–heme orientation with the change in protein conformation. The change is more pronounced when the oxy to deoxy transition occurs in the β chains.

It has long-been known that free base porphyrins and metalloporphyrins other than heme may be incorporated into the heme binding sites of apohemoglobin (Hill and Holden, 1962; Gibson, 1964; Sebring and Steinhardt, 1970). Similar results have been reported for other heme proteins like myoglobin (Breslow and Koehler, 1965) and cytochrome *c* peroxidase (Yonetani and Asakura, 1968). 8-Anilino-1-naphthalenesulfonate and structurally similar molecules have been the preferred choice of fluorescent dyes for studying dye–protein conjugates because they have a very small fluorescence quantum yield in aqueous solution and quantum yields approaching unity when bound to a hydrophobic protein site. Their emission maxima are usually red shifted from the longest wavelength absorption maxima so there is usually little problem with self-absorption. This is particularly important in fluorescence depolarization studies.

Free base and closed shell metalloporphyrins have a distinct red fluorescence when excited at the Soret or other absorption maxima (Becker and Allison, 1963; Allison and Becker, 1960). The shortest wavelength emission maximum is approximately at the same wavelength as the longest wavelength absorption maximum. Quantum yields are typically 0.05–0.2. The lower quantum yield and approximate coincidence of emission and absorption maxima are disadvantages to the use of fluorescent porphyrins as dye labels for proteins. However, with those heme proteins in which the heme group may be replaced by a fluorescent porphyrin, the above disadvantages are offset by the greatly increased structural similarity between the porphyrin-labeled protein and the naturally occurring heme protein. We report here the results of our fluorescence study of protoporphyrin globin (PHb), zinc protoporphyrin globin (ZnPHb), and hemoglobin hybrids in which the α chains contain heme while the β chains contain protoporphyrin IX (P), or zinc protoporphyrin IX (ZnP). These will be referred to as $\alpha(+)_2\beta(P)_2$ and $\alpha(+)_2\beta(ZnP)_2$, respectively. We have also studied the corresponding hybrids $\alpha(P)_2\beta(+)_2$ and $\alpha(ZnP)_2\beta(+)_2$ in which substitution has occurred in the α chain. The hybrids $\alpha(ZnP)_2\beta(P)_2$ and $\alpha(P)_2\beta(ZnP)_2$ were also prepared and studied.

Experimental Section

Materials and Methods. Protoporphyrin IX dimethyl ester was obtained from Sigma. Free protoporphyrin IX was prepared from the dimethyl ester by acid hydrolysis in 6 N HCl (Falk, 1964). Zinc protoporphyrin IX was prepared from the free base by refluxing 100 mg of free base protoporphyrin and 456 mg of hydrated zinc acetate in 50 ml of dimethylformamide. The reaction was complete within 10 min. The reaction may be monitored by recording visible absorption spectra of aliquots withdrawn from the reaction flask. The materials were chromatographed on $CaCO_3$ and recrystallized.

Aqueous solutions of both porphyrins were prepared by dissolving crystalline material in a minimum volume of 0.1 N NaOH and diluting with water or buffer.

Crystalline human hemoglobin was prepared from citrated whole blood as described previously (Waterman and Yonetani, 1970). Apohemoglobin was prepared from hemoglobin by the acid–butanone method. Hemoglobin subunits ($\alpha(+)$ and $\beta(+)$), apo subunits ($\alpha(-)$ and $\beta(-)$) and semi-hemoglobins ($\alpha(+)_2\beta(-)_2$ and $\alpha(-)_2\beta(+)_2$) were all prepared as described previously (Waterman *et al.*, 1971).

Protoporphyrin IX or zinc protoporphyrin IX was incorporated into apoprotein or semi-hemoglobin by mixing the porphyrin and protein (porphyrin in slight excess) in 50 mM potassium phosphate buffer (pH 7.0) and allowing the solution to stand overnight at 0°.

The hybrids $\alpha(ZnP)_2\beta(P)_2$ and $\alpha(P)_2\beta(ZnP)_2$ were prepared by combining the porphyrins with the apo subunits as de-

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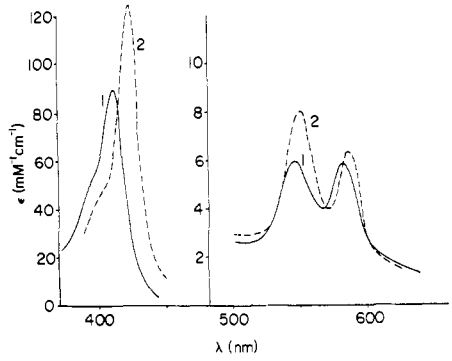

FIGURE 1: Absorption spectra of ZnP in H_2O (1) and bound to apohemoglobin (2) in 20 mM potassium phosphate buffer (pH 7).

scribed above, then mixing the appropriate subunits together with the β subunit in excess. The solutions were allowed to stand for 48 hr and then dialyzed against 10 mM potassium phosphate buffer (pH 6.0). The solutions were then added to CM-cellulose columns equilibrated with 10 mM potassium phosphate buffer (pH 6.0). Uncombined β subunit was eluted off with 10 mM phosphate buffer (pH 6.0). The hybrid hemoglobin was eluted with 100 mM potassium phosphate buffer (pH 7.0). All the hybrid hemoglobins prepared in this study bound to CM-cellulose with 10 mM phosphate buffer (pH 6.0) as eluent. Unreacted β subunit and non-protein-bound porphyrin were eluted off the column with this eluent.

All the hybrid hemoglobins prepared in this study were indistinguishable from human hemoglobin by electrophoresis on Gelman Sepraphere III paper using the Tris–borate EDTA buffer system (Bucci *et al.*, 1965).

Instruments. All absorption spectra were recorded on a Cary 15 recording spectrophotometer. Fluorescence emission and excitation spectra were recorded with a Perkin-Elmer Hitachi MPF-2A fluorescence spectrophotometer operating in the ratio mode with a R446 HTV photomultiplier tube (red sensitive tube). The fluorescence spectra are not corrected for phototube response.

Fluorescence radiative lifetimes, τ_f, were determined with an Ortec 7200 fluorescence lifetime spectrophotometer. The digitized output of the fluorometric system was stored on magnetic tape. The true fluorescence decay and the lamp signal were deconvoluted into sums of simple exponentials by computer.

Results

Spectra and Protein Binding. It is known that protoporphyrin IX binds to apohemoglobin with an increase in extinction coefficient and sharpness of all absorption bands (Rossi-Fanelli *et al.*, 1959; Sebring and Steinhardt, 1970). Absorption spectra of zinc protoporphyrin in aqueous solution and bound to apohemoglobin are shown in Figure 1. The increase in extinction coefficient and small red shift (7–10 nm) are both indicative of protein binding. All absorption spectral data are presented in Table I. A spectrophotometric titration of apohemoglobin with ZnP is shown in Figure 2. The heme-binding capacity of the apohemoglobin was also determined by spectrophotometric titration with hemin. The protein binds one ZnP molecule per heme molecule.

Fluorescence emission spectra of protoporphyrin and zinc protoporphyrin are shown in Figures 3 and 4. In protoporphyrin the 0–0 emission maximum shifts from 619 nm in aqueous solution to 633 nm in 0.1% Triton X-100. The protein-bound material has its maximum at 626 nm. Zinc

TABLE I: Extinction Coefficients.

Compound	Solvent	Wavelength (nm)	Extin Coef (mM^{-1} cm^{-1})
P	Water	365 (br)	36.6
		536	7.00
		585	4.25
		642	2.66
P	Pyridine	408	77.9
		505	13.9
		540	11.3
		576	8.72
		630	6.66
PHb	10 mM buffer (pH 7)	403	141
		505	10.8
		540	9.60
		568	6.20
		620	3.10
ZnP	Water	412	87.4
		544	6.17
		582	6.01
ZnP	Pyridine	425	126
		551	12.3
		588	10.0
ZnPHb	10 mM buffer (pH 7)	423	122
		550	8.37
		587	6.49

protoporphyrin has 0–0 emission maxima at 589 nm in aqueous solution, 595 nm in pyridine, and 593 nm when bound to protein. Thus there is a red spectral shift with decreasing polarity of the porphyrins' environment. The peaks appearing at about 640 nm for ZnP and about 680 nm for P are the 1–0 vibronic emission peaks.

The emission spectra of the hybrids $\alpha(P)_2\beta(+)_2$ and $\alpha(+)_2\beta(ZnP)_2$ are shown in Figure 5. Spectra of the respective corresponding hybrids were identical with those presented in the figure. In each case, the position of the emission maximum is indicative of porphyrin to protein binding. In each case, if the heme groups were converted from oxy to deoxy by adding a small amount of sodium dithionite, there was no detectable shift in the emission maximum. It is very difficult to correlate the small changes in fluorescence intensity which were observed since both the heme absorption spectrum changes and the dithionite itself absorb around 400 nm giving rise to complicated inner filter effects. In each of the

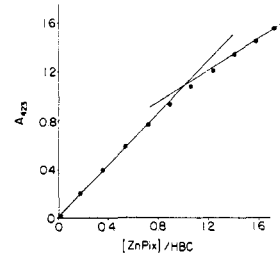

FIGURE 2: Titration of apohemoglobin with ZnP. The heme-binding capacity of the apohemoglobin was determined by titration with hemin. Experiments were done in 100 mM potassium phosphate buffer (pH 7).

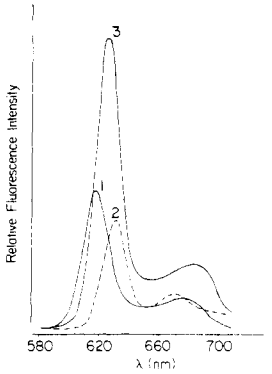

FIGURE 3: Emission spectra of protoporphyrin IX: (1) 5 μM P in 100 mM potassium phosphate buffer (pH 7), (2) same solution plus 0.1% Triton X-100, (3) 16 μM PHb solution in same buffer. Excitation was at 400 nm.

hybrids, the fluorescence excitation spectrum was identical with that of PHb or ZnPHb.

It is interesting to note that ZnP apparently bound to apoprotein immediately on mixture. That is, both the red shift in fluorescence and the red shift and increased absorbance could be immediately observed. However, when P and apoprotein are mixed, there is an immediate shift in fluorescence from 619 to 623 nm with little change in absorption spectrum. After several hours, the characteristic PHb absorption spectrum appears.

Fluorescence Depolarization. Other workers (Stryer, 1965; Anderson and Weber, 1970) have reported on the fluorescence depolarization of 8-anilino-1-naphthalenesulfonate bound to human apohemoglobin. Human apohemoglobin is predominantly dimeric (Rossi-Fanelli and Antonini, 1958), and a rotational relaxation time of 43 nsec was reported. We recently (Leonard and Yonetani, 1973) studied the fluorescence depolarization of 8-anilino-1-naphthalenesulfonate bound to human apohemoglobin subunits under conditions when both the α and β subunits are dimeric and calculated rotational relaxation times of 37 and 42 nsec. PHb has a tetrameric structure like hemoglobin (Rossi-Fanelli *et al.*, 1959), and we attempted to study the fluorescence depolarization of the porphyrin. At PHb concentrations above 10 μM, self-absorption of the porphyrin fluorescence becomes apparent and this contributes to the fluorescence depolarization. At 5 μM PHb, there is no self-absorption and a linear Perrin plot results (Perrin, 1926). The linear correlation coefficient is 0.9973. The cell compartment was thermostated at 25° and the viscosity was varied by sequential additions of sucrose. From these data, we calculate a rotational relaxation

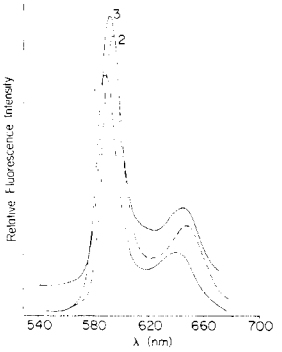

FIGURE 4: Emission spectra of zinc protoporphyrin (1) in 100 mM buffer, (2) in pyridine, (3) bound to apohemoglobin; concentration of ZnP was 12 μM; excitation at 425 nm.

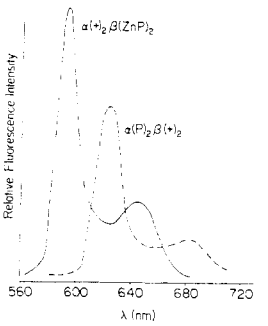

FIGURE 5: Emission spectra of hybrids in 100 mM (pH 7) potassium phosphate buffer (pH 7). Excitation at 420 nm for ZnP and 400 nm for P.

time of 59.5 nsec. For a spherical protein with $M = 64{,}000$, the rotational relaxation time is calculated to be 58.4 nsec.

Energy Transfer and Fluorescence Lifetime. Apohemoglobin and the semi-hemoglobins all exhibit protein fluorescence at about 340 nm when excited at 290 nm. This protein emission is partially quenched on binding of P or ZnP and there is a small peak at 290 nm in the P or ZnP fluorescence excitation spectrum, thus indicating protein to porphyrin energy transfer.

The fluorescence decay times of the hybrid hemoglobins are listed in Table II. Results of a typical experiment are shown in Figure 6. The ZnP lifetimes are all on the order of 3 nsec and with our reproducibility of ±0.5 nsec, there can be little quantitative discussion about them. The P lifetime increases from 14.1 to 18.1 nsec on binding to apohemoglobin in agreement with an observed increase in fluorescence intensity. In PHb, there is a possibility for P to P energy transfer either between like or unlike chains. In the iron–P hybrids, there is still the possibility for P–P transfer between like chains and also for P–heme transfer between unlike chains.

In oxy-$\alpha(+)_2\beta(P)_2$, the lifetime of 17.8 nsec indicates little α(P)–β(heme) energy transfer; 17.2 nsec for deoxy-$\alpha(+)_2$-$\beta(P)_2$ indicates little change on the oxy to deoxy transition. In the oxy-$\alpha(P)_2\beta(+)_2$ hybrid, the value of 13.4 nsec for oxy indicates more efficient α(P) → β(heme) energy transfer. In the deoxy form, the value of 7.1 nsec indicates a much greater energy transfer.

Energy transfer was also observed in the hybrids $\alpha(P)_2$-$\beta(ZnP)_2$ and $\alpha(ZnP)_2\beta(P)_2$ (Figure 7). The ZnPHb has a 595-nm fluorescence with an excitation peak at 550 nm. PHb has

TABLE II: Fluorescence Decay Times.

Sample	τ_f (nsec) ± 0.5
P in buffer	14.1
PHb	18.1
$\alpha(+)_2\beta(P)_2$ oxy	17.8
$\alpha(+)_2\beta(P)_2$ deoxy	17.2
$\alpha(P)_2\beta(+)_2$ oxy	13.4
$\alpha(P)_2\beta(+)_2$ deoxy	7.1
ZnP in buffer	2.9
ZnPHb	2.9
$\alpha(ZnP)_2\beta(+)_2$ oxy	2.8
$\alpha(ZnP)_2\beta(+)_2$ deoxy	2.7
$\alpha(+)_2\beta(ZnP)_2$ oxy	2.5
$\alpha(+)_2\beta(ZnP)_2$ deoxy	2.6
$\alpha(ZnP)_2\beta(P)_2$	3.5 (595-nm emission)
	18.3 (625-nm emission)

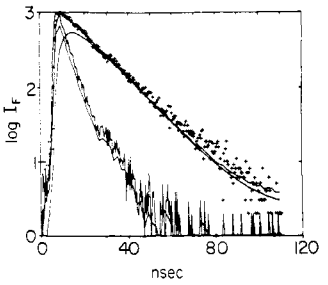

FIGURE 6: Typical results of a lifetime experiment. The upper decay (+) is the observed decay of sample $(\alpha(P)_2\beta(+)_2$oxy, 10 μM in 100 mM phosphate buffer, pH 7) and excitation lamp. The lamp decay is shown in the lower jagged trace. The solid lines through points are the results of the computer fit.

three 626-nm fluorescence excitation peaks at 506, 542, and 573 nm. (Each, of course, has a Soret band.) In these hybrids there may be ZnP to P energy transfer. Comparing the 625 fluorescence excitation spectra of $\alpha(P)_2\beta(ZnP)_2$ and $\alpha(ZnP)_2$-$\beta(P)_2$ and noting the intensity of the peak at 540–550 nm clearly shows this ZnP to P transfer. In $\alpha(P)_2\beta(ZnP)_2$ the peak is more intense relative to the 506 peak than in PHb. In $\alpha(ZnP)_2\beta(P)_2$ it is the most intense peak.

There is some ZnP fluorescence at 625 nm, and this may account for the increase in the 540–550-nm peak in the 625-nm fluorescence excitation spectrum of $\alpha(P)_2\beta(ZnP)_2$. However, the still larger increase in the 540–550-nm peak in the spectrum of $\alpha(ZnP)_2\beta(P)_2$ indicates energy transfer in this hybrid. If energy transfer is occurring in both hybrids, then it is more efficient in the latter.

Discussion

The red shift in fluorescence maxima and the changes in absorption spectrum on binding to protein are probably due to the decreased polarity of the protein cavity. This is especially likely when one compares spectra in water with those taken in Triton-X or pyridine. The measured rotational relaxation time of 59.5 nsec for PHb is in excellent agreement with the calculated value of 58.4 nsec for a spherical protein with M = 64,000. The true rotational relaxation time for PHb may be slightly larger since at a concentration of 5 μM, the PHb tetramer may be slightly dissociated. Also, energy transfer between porphyrin molecules in the same tetramer may contribute to the observed fluorescence depolarization.

From the fluorescence decay times (Table II) it is obvious that the $\alpha(P)_2\beta(+)_2$ hybrid is much different from the $\alpha(+)_2$-$\beta(P)_2$ hybrid. In the latter, the porphyrin fluorescence lifetime differs very little from the value in PHb indicating little energy transfer. There is a very slight decrease in τ_f on converting to deoxy. In the former, τ_f is significantly less than the PHb value and is cut almost by 50% on forming the deoxy derivative.

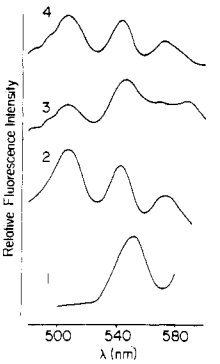

FIGURE 7: Excitation spectra of (1) the 595-nm emission of ZnPHb, (2) the 625-nm emission of PHb, (3) the 625-nm emission of $\alpha(ZnP)_2$-$\beta(P)_2$, and (4) the 625-nm emission of $\alpha(P)_2\beta(ZnP)_2$. All in 100 mM phosphate buffer (pH 7).

This type of energy transfer has been studied by Förster (1959). Its efficiency is known to depend on the distance between donor and acceptor molecules, the relative orientation of their transition dipoles, and the spectral overlap of the donor fluorescence spectrum with the acceptor's absorption spectrum. Förster gives the following expression for the "rate constant" for energy transfer

$$k_t = (8.940 \times 10^{-26})\left(\frac{X^2}{\tau_0 R^6}\right)\int_0^\infty f_s(\nu)\epsilon_a(\nu)\frac{d\nu}{\nu 4} \qquad (1)$$

$f_s(\nu)$ is the normalized and corrected donor fluorescence spectrum and $\epsilon_a(\nu)$ is the acceptor absorption spectrum in molar absorptivity both measured on a wave number scale. R is the distance between donor and acceptor. τ_0 is the natural radiative lifetime of the donor, and X^2 is an orientation factor.

Consider the following mechanism

$$P + h\nu \longrightarrow P^* \qquad \text{excitation} \qquad (2)$$

$$P^* \xrightarrow{k_f} P + h\nu \qquad \text{fluorescence} \qquad (3)$$

$$P_1^* + P_2 \xrightarrow{k_{tPP}} P_1 + P_2^* \qquad \text{P–P transfer} \qquad (4)$$

$$P^* + \text{heme} \xrightarrow{k_{tPh}} P + \text{heme} \qquad \text{P–heme transfer} \qquad (5)$$

$$P^* \xrightarrow{k_q} P \qquad \text{all other quenching processes} \qquad (6)$$

Step 4 does not change the concentration of excited porphyrin molecules but only introduces a time delay. Since no deviation from simple exponential decay was observed in the porphyrin hybrids, we neglect step 4. Now, in the absence of P–heme transfer (step 5), $\tau_f = 1/(k_f + k_q)$. This would be the case in PHb. In the hybrids, $\tau_f' = 1/(k_f + k_q + k_{tPh})$. Thus,

TABLE III

Sample	k_{tPh} (sec^{-1})	X^2/R^6	R^a (Å)	X^b $\alpha_1\beta_2$	X^b $\alpha_1\beta_1$
$\alpha(+)_2\beta(P)_2$ oxy	9.31×10^5	9.35×10^{37}	43.9	0.15	0.45
$\alpha(+)_2\beta(P)_2$ deoxy	28.9	25.3	37.2	0.24	0.74
$\alpha(P)_2\beta(+)_2$ oxy	194	195	26.4	0.689	2.06
$\alpha(P)_2\beta(+)_2$ deoxy	856	748	21.3	1.35	4.03

[a] Assumes a random orientation of transition moments, *i.e.*, $X^2 = {}^2/_3$. [b] Calculation of X using R values of 25 Å for $\alpha_1\beta_2$ and 36 Å for $\alpha_1\beta_1$.

$(\tau_f')^{-1} - (\tau_f)^{-1} = k_{tPh}$. From values for the spectral overlaps and the natural radiative lifetime, τ_0, of protein-bound protoporphyrin, one may calculate values of X^2/R^6 from eq 1. From the Strickler–Berg equation (Strickler and Berg, 1962) we calculate τ_0 for PHb to be 197 nsec. The overlap integrals were numerically evaluated and values for X^2/R^6 are given in Table III. There is less than a 15% change in spectral overlap integrals for P–oxyheme and P–deoxyheme so differences in observed lifetimes must reflect differences in the P–heme orientation and distance. The values of R (Table III) calculated on the basis of a random orientation ($X^2 = {}^2/_3$) are reasonable in light of the dimensions of the hemoglobin molecule and the heme–heme distances in horse hemoglobin (Perutz *et al.*, 1968). The α_1–β_2 heme–heme distance is 25 Å as in the α_2–β_1 distance in the oxy form. Both values change very little in going to the deoxy form (24.7 Å). The α_1–β_1 and α_2–β_2 distances are 35.0 Å in oxy and 36.9 Å in deoxy. R_0, the Forster critical transfer distance, for P and heme randomly orientated is 23 Å, so we should expect transfer in these hybrid hemoglobins. If we assume the Perutz distances of 25 Å for $\alpha_1\beta_2$ and $\alpha_2\beta_1$ and an average 36 Å for $\alpha_1\beta_1$ and $\alpha_2\beta_2$ in both oxy and deoxy hybrids, we can calculate values of X. These values are listed in Table III. There is a change in each orientation when the hybrids are converted from oxy to deoxy. In $\alpha(+)_2\beta(P)_2$ there is about a 60% change in each X, while for $\alpha(P)_2\beta(+)_2$ there is almost a 100% change. Thus, we observe a change in orientation in each case, but it is more pronounced when the oxy–deoxy transition occurs in the β chains. In all cases, a single exponential fluorescence decay is observed, so if there is a tetramer–dimer equilibrium, both must have very similar decay times. Thus, the observed decay time changes reflect orientation changes rather than a shift in equilibrium.

This result is in complete agreement with electron paramagnetic resonance experiments on spin-labeled hemoglobins selectively labeled in the α or β chains (Asakura and Drott, 1971) and nuclear magnetic resonance studies on valency-hybrid hemoglobins (Ogawa and Shulman, 1972). Both our results and those of Asakura and Drott and Ogawa and Shulman are consistent with Perutz's observations (Perutz, 1970) that the α-heme pockets in deoxyhemoglobin are large enough to accommodate an O_2 molecule with little interference by protein, whereas in the β heme pockets, the protein conformation would have to change for the β pockets to accommodate O_2 while it would not have to change for the α pockets. So, whether the probe is a spin label or a porphyrin, the greatest response would be observed when the oxy to deoxy transition occurs in the β chains.

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