Effect of Disordered Hemes on Energy Transfer Rates between Tryptophans and Heme in Myoglobin

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ABSTRACT Our recent linear dichroism study of heme transitions (Gryczynski, Z., E. Bucci, and J. Kusba. 1993. *Photochem. Photobiology.* in press) indicate that heme cannot be considered a planar oscillator when it acts as an acceptor of radiationless excitation energy transfer from tryptophan. The linear nature of the heme absorption transition moment in the near-UV region implies a strong dependence of the transfer rate factors on the relative angular position of the heme and tryptophan, i.e., on the κ^2 orientation parameter of the Förster equation. Using the atomic coordinates of SW myoglobin we have estimated the variation of κ^2 parameter as a function of the heme absorption transition moment direction. The simulations proved that transfer is very efficient and anticipates lifetimes in the picosecond range. Also, they showed that transfer is very sensitive to rotations of the heme around its α-γ-meso-axis, which may reduce the efficiency of transfer to almost zero values, producing lifetimes very similar to those of free tryptophan, in the nanosecond range. Comparisons between the lifetime values reported in the literature and those here estimated suggest that natural heme disorder, in which heme is rotated 180° around its meso axis, is at the origin of the nanosecond lifetimes found in myoglobin systems.

INTRODUCTION

Almost half a century ago Förster developed an exact quantum mechanical theory of resonance energy transfer for "weak" dipole-dipole interactions (Förster, 1948, 1965). The inverse six-power distance dependence of the transfer rate makes of this phenomenon a powerful means for determining intermolecular separations and conformational dynamics of biological systems. The use of this technique has produced an extensive and rapidly growing literature for many different applications on various systems (Stryer, 1978; Dale and Esinger 1974, 1976, 1979; Steinberg, 1971; Haas et al., 1975; Katchalski-Katzir and Steinberg, 1981; Shiller, 1985; Cheung, 1991). The main difficulty in evaluating energy transfer rates is a precise estimation of the reciprocal angular orientation of donor and acceptor transition moments. The strong dependence of transfer rates on the orientation parameter κ^2, may reduce the transfer to zero values even for very short distances, or make it very large also at long distances (Dale and Eisinger, 1974, 1976, 1979).

For some systems total averaging of the reciprocal orientation of donor and acceptor chromophores occurs on a time scale shorter than the transfer time, allowing the use of $\kappa^2 = 2/3$ (Dale and Eisinger, 1976, 1979). Instead, in heavily quenched systems like hemoproteins, the resulting lifetimes in the picosecond time range are too short for allowing dynamic averaging of the relative donor-acceptor positions, which may also be restricted by the rigidity and stereochemistry of the matrix where they are embedded. This implies the necessity of estimating as accurately as possible the reciprocal orientations of donor and acceptor transition moments.

In a recent paper we published measurements of energy transfer from tryptophan to heme in hemoglobin, based on the atomic coordinates of the protein and on the assumption that heme is a planar oscillator with its transition moments equally distributed on the plane of the porphyrin (Gryczynski et al., 1992). Those measurements anticipated lifetimes between 10 and 30 ps, which were confirmed by experimental measurements (Bucci et al., 1988, 1992a,b; Szabo et al., 1989). However, our experiments showed also the presence of longer lifetimes, of several hundred picoseconds, with very small amplitudes, 1–3% of the total emitting fluorophores. These lifetimes were too long for the prevailing transfer rates, however too short for originating from non-hemoglobin impurities containing nonquenched tryptophans. We observed that they could be justified by the failure of some of the hemes in hemoglobin to accept transfer from the tryptophans of the same subunit, therefore allowing the tryptophans to be quenched only by hemes at intersubunit distance. Data present in the literature show that a similar situation prevails in myoglobin systems, where long lifetimes of small amplitude have been reported, similar to lifetimes of free tryptophan (Hochstrasser and Negus, 1984; Janes et al., 1987; Bismuto et al., 1989). We argued that, in monomeric myoglobin, in the absence of transfer to the heme, tryptophans do not find other quenching moieties and should appear as free tryptophans. Therefore we speculated that even in these systems some of the hemes do not accept transfer, either because they are absent, or because they acquire peculiar positions inside the heme pocket. Hochstrasser

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Abbreviations used: Mb, myoglobin; CO-Mb, carbon monoxy myoglobin; deoxy-Mb, unliganded myoglobin; Met-Mb, ferric myoglobin; TrP, tryptophan; PVA, polyvinyl alcohol; SW, sperm whale.

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and Negus (1984) and Janes et al. (1987) had already formulated this hypothesis; however, they acknowledged its inconsistency with a planar model of heme absorption.

Using NMR spectroscopy, La Mar et al. (1983, 1984) have shown that, in myoglobin solutions, 3–8% of the molecules carry a "disordered" heme, rotated 180° around the α-γ-meso axis of the porphyrin ring. The challenge was to find whether the orientation parameters of disordered hemes could affect energy transfer from tryptophans.

This prompted us to reinvestigate the assumption that heme is a planar oscillator. Linear dichroism measurements conducted on iron and noniron hemes, embedded in stretched PVA films, indicated that radiationless energy transfer from tryptophan is regulated by the overlap of its emission with a single absorption band of the heme in the near-UV region of the spectrum (between 300 and 380 nm), whose transition moment is oriented at about 60° from the α-γ-meso axis of the porphyrin ring (Gryczynski et al., 1993). These findings make the radiationless interaction between tryptophan and heme very sensitive to rotations of the heme around its α-γ-meso axis (Fig. 1).

The tridimensional structure of sperm whale (SW) myoglobin has been extensively characterized by crystallographic analyses up to 1.6-Å resolution. This enabled us to use its atomic coordinates for estimating the rates of energy transfer from each of the two tryptophan to the heme, for various liganded derivatives of SW myoglobin (met-, deoxy-, CO-Mb) (Takano, 1977a,b; Philips, 1978, 1980). The Förster equations were applied to these systems, in the assumption that heme is a linear and not a circular oscillator. We analyzed the resonance energy transfer process as function of the orientation of the transition moments of the heme on the porphyrin plane, as shown by the angle Θ in Fig. 1. Also, as shown in Fig. 1, the analyses were done for two positions of the heme in the heme pocket, normal as present in the crystal (position A), and disordered, i.e., rotated 180° around the α-γ-meso axis of the porphyrin (position B). Here we present evidences that in both positions the orientation of the transition moment of the heme in heme moiety is extremely relevant, and it can reduce the rate of radiationless energy transfer to near zero values. This suggests that the long lifetimes reported for myoglobin originate from the natural heme disorder of the system.

MATERIALS AND METHODS

Crystallin SW myoglobin was obtained from Sigma Chemical Co., St. Louis, MO. Treatments based on reduction with dithionite in the presence of CO or N_2 were used to reduce the iron to its ferrous form and obtain the met-, deoxy-, and CO-Mb derivatives. Myoglobin concentration was measured using extinction coefficients as reported by Antonini and Brunori (1971).

Absorption spectra were measured with a Cary 14 dual beam spectrophotometer.

Deconvolutions of the spectra in terms of Gaussian components were performed, assuming that the band envelope of the *i*th component had the form of a Gaussian error function distribution as in (Kawski et al., 1992; Gryczynski et al., 1993)

$$A(\tilde{\nu}) = \sum_i A_{i,\max} \exp\left[-\frac{(\tilde{\nu} - \tilde{\nu}_{i,\max})^2}{2\sigma_i^2}\right], \quad (1)$$

where $\tilde{\nu}_{i,\max}$ is the wavenumber of the *i*th peak position, $A_{i,\max}$ is the maximum absorbance, and σ_i is the standard deviation of the wavenumber of the *i*th band, respectively.

The orientation angles between tryptophans and heme were computed from the atomic coordinates of the respective derivatives of myoglobin using a Silicon Graphics computer and the SYBYL software. The atomic coordinates were obtained from the Pittsburgh Supercomputing Center, derived from the Protein Data Bank at Brookhaven National Laboratories (Phillips, 1978; Takano, 1977).

For evaluating the overlap integral we used the corrected emission spectrum of heme-free myoglobin, which proved to be practically identical to that of heme-free hemoglobin (unpublished).

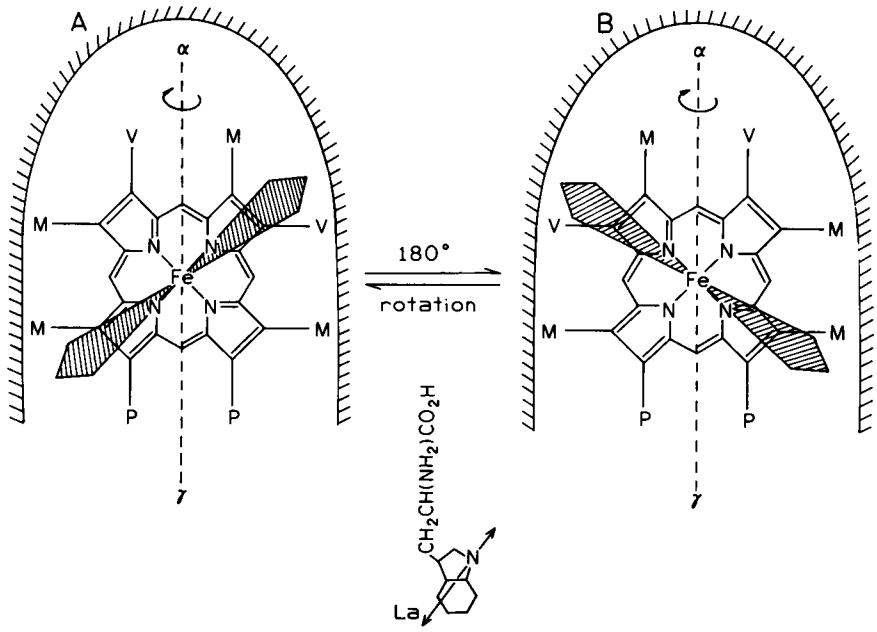

FIGURE 1 Schematics of heme before and after 180° rotation around its α-γ-meso axis. Note the mirror image position of the vynyl (*V*) and methyl (*M*) groups in position A, normal, and position B, disordered. The shaded arrows show the range of orientations (angle Θ) of the transition moment of the heme absorption band in the 300–380-nm range. It is assumed that in the normal position of the heme the angle is positive. The tryptophan moiety with its transition moment is arbitrarily positioned in the figure, for emphasizing the changes of the angular relationships between donor and acceptor vectors when the heme rotates from normal to disordered position. (Adopted from Aojula et al. 1986)

RESULTS

Deconvolution of the absorption spectra of myoglobin derivatives and evaluation of the overlap integrals

Fig. 2 shows the deconvolved absorption spectra of met-, deoxy-, and CO-Mb in the Soret (380–450 nm) and near-UV (300–380 nm) regions of the spectrum. As previously reported for isolated hemes and hemoglobin, the spectra in the Soret region are an envelope of several absorption bands, while in the near-UV absorption area they show the presence of a single band, solely responsible for the overlap between tryptophan and heme, as shown in Fig. 3.

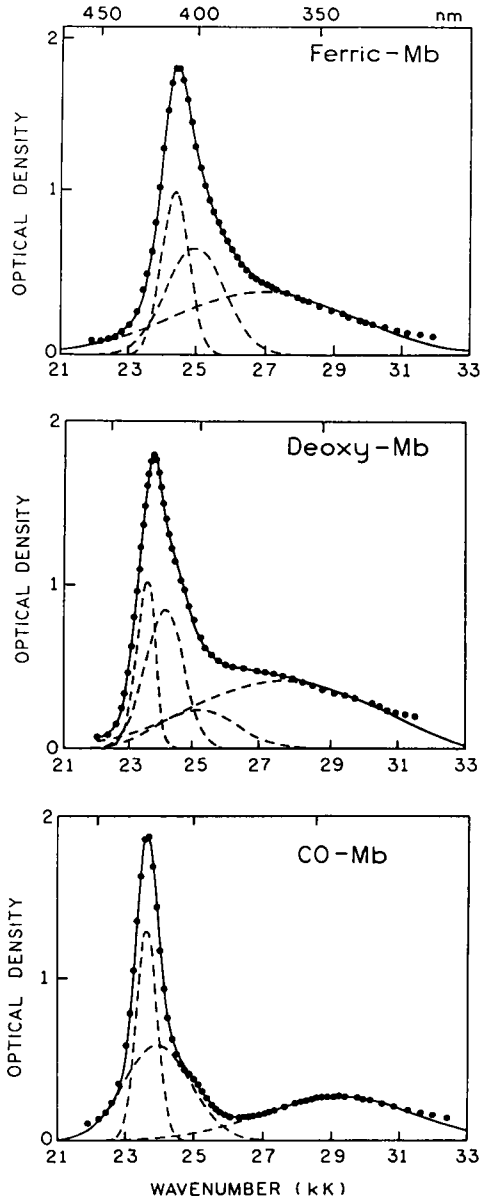

FIGURE 2 Deconvolution of the absorption spectrum of met-, deoxy-, and CO-Mb between 300 and 450 nm into Gaussian components. Note the multiple bands under the major absorption peak in the 380–450-nm range and the single band present in the 300–380-nm range.

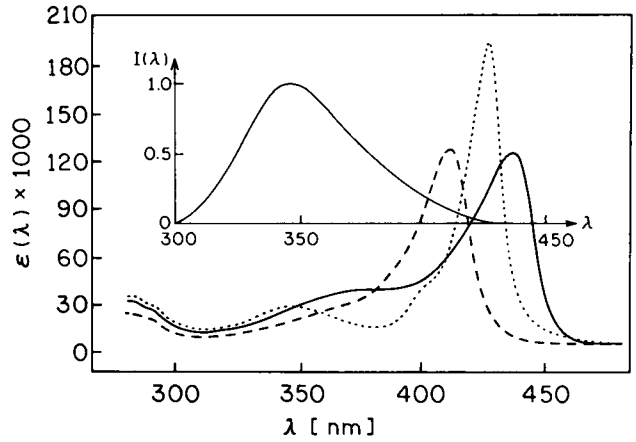

FIGURE 3 Comparison between the emission spectrum of tryptophan and the absorption spectra of met- (*dashed line*), deoxy (*full line*), CO-Mb (*dotted line*), showing the extensive overlap.

The overlap integral, J, was computed from the normalized corrected spectrum of heme-free myoglobin, which was identical to that of heme-free hemoglobin (Gryczynski et al., 1992), and the absorption spectrum of myoglobin between 300 and 450 nm (Förster, 1965).

The computed integrals are listed in Table 1. They appear to be very similar in spite of the substantial difference of the absorption of the Soret band above 380 nm for the various derivatives of myoglobin (Fig. 3). This confirms that also in this case the energy transfer from tryptophan to heme is almost completely regulated by the single absorption band of the heme in the near-UV region (300–380 nm).

Dependence of the orientation parameter κ^2 from the direction of the heme absorption transition moment

According to the Förster theory the orientation factor κ^2 is a function of the angle (α_{DA}), defined by the direction of the transition moments **D** and **A**, for donor and acceptor, respectively, and of the angles (α_D, α_A), that the two vectors form, respectively, with the translation vector **T** connecting the centers of the two oscillators (see Fig. 1 in Gryczynski et al. (1992) and Dale and Eisinger (1974)).

$$\kappa^2 = (\cos \alpha_{DA} - 3 \cos \alpha_D \cos \alpha_A)^2. \quad (2)$$

TABLE 1 Values of the overlap integrals between corrected emission spectrum of heme-free myoglobin and heme absorption for met-, deoxy-, and CO-Mb

Mb	Overlap integral
	cm/M × 10^{14}
Met	5.16
Deoxy	5.28
CO	4.70

Commuted with Eq. 2.

Tryptophan is endowed with two electronic transitions ^1L_a and ^1L_b in the range 270–300 nm, whose transition moments are oriented in orthogonal fashion (Albinsson et al., 1989, 1992; Rugiero et al., 1990; Hense et al., 1992). The most often used excitation wavelength for tryptophan fluorescence is close to 300 nm, and preferentially populates the ^1L_a state of the chromophore. The internal conversion rate between ^1L_b and ^1L_a states, in free tryptophan and tryptophan embedded in proteins, has been recently estimated to be near 2 ps from upconversion measurements (Rugiero et al., 1990; Hensen et al., 1992). Consistently with these data, we have recently shown that the anisotropy decay of tryptophan fluorescence in various hemoglobins includes a correlation time component of 2–4 ps, which probably reflects the interconversion rate of the two transitions (Bucci et al., 1992). The low population and extremely fast decay of the ^1L_b state, in practice, limits the interactions of tryptophan with heme to the ^1L_a transition.

For computing the values of κ^2 we assumed a fixed value for the orientation of the ^1L_a transition moment in the plane of tryptophan at 38° from the major axis of the tryptophan rings (Albinsson et al., 1989, 1992). In fact the very short picosecond lifetime and the rigidity of the protein matrix limit the orientational freedom of the tryptophan ring.

From measurements of linear dichroism of heme embedded in stretched PVA films we estimated a range of 50–70° for the angle Θ in the protoporphyrin plane, i.e., between α-γ-meso axis of the heme moiety and the transition moment of its absorption band in the near-UV (300–380 nm) (Gryczynski et al., 1993). We examined the dependence of κ^2 on the orientation of the transition moment of the heme by changing the angle Θ from 0° to 90° arbitrarily chosen as for normal hemes, and from 0° to −90° arbitrarily chosen as for disordered hemes, as shown in Fig. 1.

The curves in Fig. 4 show the dependence of κ^2 on Θ for normal (position A) and disordered (position B) hemes in ferric-, deoxy-, and CO-Mb. The computations were performed for the two tryptophans in position 7 (*dashed lines*) and 14 (*solid lines*), respectively. The dashed horizontal lines define the value $\kappa^2 = 2/3$, for the complete dynamic averaging of the relative donor-acceptor orientations.

As expected, the curves are very similar for the three derivatives and show a very strong dependence of κ^2 on the angle Θ. Also, the computed values of κ^2 are very different from the dynamic average value of 2/3 for most orientations of the heme transition moment. For Trp-14, normal orientation of the heme (position A, *solid lines*) produces large values of κ^2, while disordered orientation (position B) shows a wide range of Θ, between 50° and 70°, where κ^2 is near zero. For Trp-7, normal heme orientation (position A, *dashed lines*) produces curves with minima near zero in the range 35–40°, while very large values of κ^2 are produced by the disordered heme (position B). The shaded areas in Fig. 4 correspond to the variability of the angle Θ as estimated from the linear dichroism of hemes embedded in stretched PVA films (Gryczynski et al., 1993). It should be stressed that the range of Θ for which κ^2 is near 0 for Trp-14 is practically coincident with the width of the shaded areas in Fig. 4.

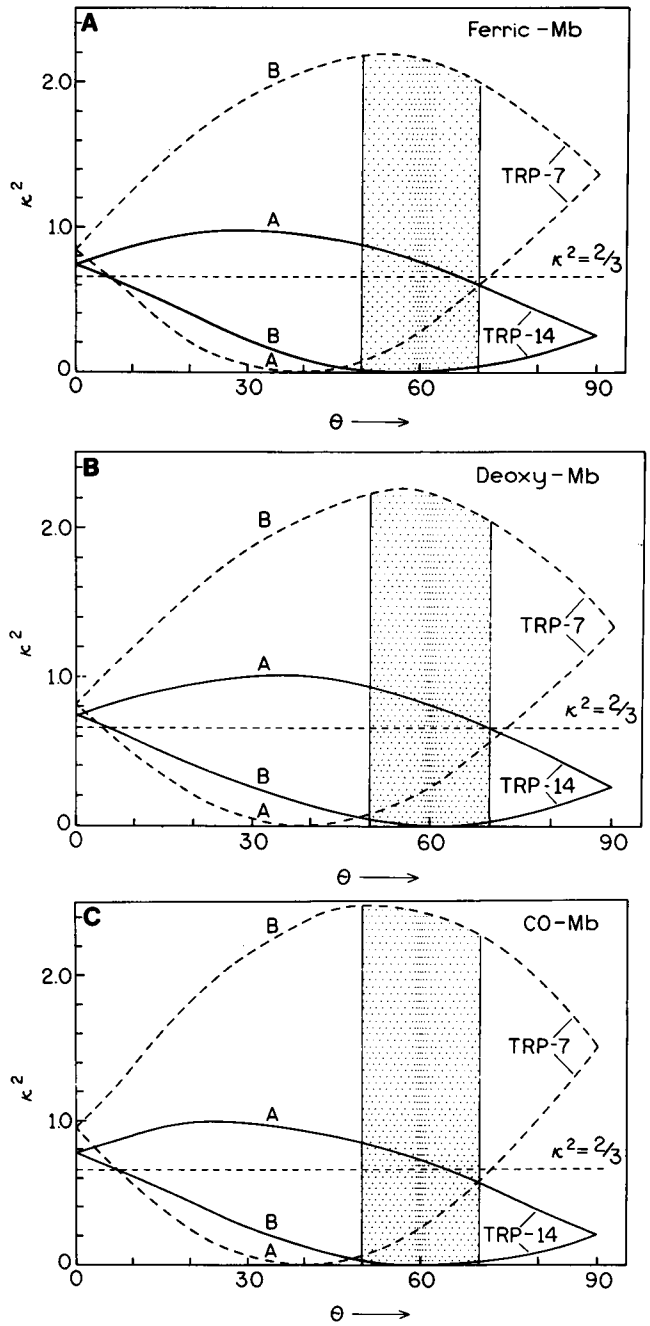

FIGURE 4 Dependence of the orientation factor κ^2 on the angle Θ between the direction of the transition moment of the heme absorption and the α-γ-meso axis of the porphyrin ring. Solid lines are for TrP-14 and dashed lines for TrP-7. Position A is for normal hemes and position B for disordered hemes. The horizontal dashed lines are for $\kappa^2 = 2/3$. The shaded areas show the range estimated for the angle Θ by linear dichroism measurements.

In Table 2 we present values of the orientation factors κ^2 of the two tryptophans assuming that the orientation of the absorption transition moment of the heme is either at +60° (normal heme) or at −60° (disordered heme) over the α-γ-meso-axis. It clearly shows that the value of κ^2 approaches zero for Trp-14 and disordered heme.

TABLE 2 Distances, orientation factors and transfer rate factors computed with equation (3) and (4) for Trp 7 and 14 for normal and disordered hemes respectively

Mb	Trp	Distance Trp-Heme	Heme orientation	Orientation factor	Transfer rate factors
		Å			
Met	7	20.56	Normal (A)	0.28	5.1
			Disordered (B)	2.13	39.1
	14	15.06	Normal (A)	0.76	71.9
			Disordered (B)	0.00001	0.0009
Deoxy	7	20.44	Normal (A)	0.27	5.2
			Disordered (B)	2.20	41.6
	14	14.92	Normal (A)	0.77	80.3
			Disordered (B)	0.00004	0.004
CO	7	20.35	Normal (A)	0.28	5.3
			Disordered (B)	2.33	44.4
	14	14.95	Normal (A)	0.73	65.8
			Disordered (B)	0.0023	0.2

It is assumed that the angle Θ, between the orientation of the transition moment of the heme absorption and the α-γ-meso axis of the porphyrin ring, is 60°.

Transfer rates and expected lifetimes

Lifetimes of donors in the presence of energy transfer can be computed using:

$$\tau_c = \tau_0/[1 + (R_0/R)^6], \quad (3)$$

where

$$R_0^6 = 8.785\ 10^{-25}\ \kappa^2\ n^{-4}\ \Phi J, \quad (4)$$

is the characteristic Förster distance (Förster, 1965; Gryczynski et al., 1992), τ_0 is the lifetime of the donor in the absence of the acceptor, n is the refractive index of the solution, Φ is the quantum yield of tryptophan, J the overlap integral, and R is the distance between the center of tryptophan and heme, respectively, along the translation vector. Note the dependence of R_0 on the orientational factor κ^2.

We have computed the transfer rate factors $(R_0/R)^6$ and the resulting lifetimes assuming the transition moment of the heme absorption oriented at 60° either in normal or disordered heme.

Besides the orientation factors as mentioned above, Table 2 shows the distances between hemes and tryptophans, and the resulting transfer rate factors for normal and disordered heme positions. Based on the parameters listed in Table 2, and on the assumption that free tryptophan has a lifetime of 2 ns, we computed the lifetimes of myoglobin listed in Table 3. It is clear that the transfer from Trp-14 to disordered heme produces lifetimes very similar to those of free tryptophan.

DISCUSSION

In a previous work (Gryczynski et al., 1992) we computed transfer rates between tryptophans and hemes in hemoglobin, assuming that the heme was a planar oscillator, in the sense that its transition moments were equally distributed on the surface of the porphyrin ring. That assumption must be revised. As shown by Gaussian deconvolutions (Gryczynski et al., 1993), the absorption spectrum of myoglobin and hemoglobin between 380 and 450 nm is a complex envelope of three absorption bands. They probably have individual transition moments with different directions, difficult to resolve, so as to mimic a planar oscillator (Eaton and Hofrichter, 1981). Nevertheless, as proven by the CD signal observed at these wavelengths, the resulting ellipsoid has a principal axis, whose direction was estimated by Hsu and Woody (1971) to be at 45–65° over the main axis of the porphyrin ring. Below 380 nm, in the region between 300–380 nm, the absorption of myoglobin and hemoglobin is due to a single absorption band (Gryczynski et al., 1993). Evidence obtained from measurements of linear dichroism of hemes embedded in stretched PVA films, indicates that the transition moment of this single band is oriented about 60° over the α-γ-meso axis on the porphyrin plane (Gryczynski et al., 1993). The absorption of the heme above 380 nm is not relevant to the transfer between tryptophan and heme, because in this region of the spectrum the overlap between emission and absorption is minimal. This was confirmed by the independence of the overlap integral from the state of ligation of myoglobin, shown in Table 1. Instead, the orientation of the single transition moment of the heme absorption in the 300–380-nm region is of paramount importance for the transfer from tryptophan. As shown in Fig. 4 its direction produces large variations of the orientation parameter κ^2.

TABLE 3 Lifetimes of met-, deoxy-, and CO-Mb, computed with Eq. 6 from the parameters in Table 2, and the assumption that free tryptophan has a lifetime of 2 ns

Mb	Heme orientation	Calculated lifetimes	
		Trp-7	Trp-14
		ps	ps
Ferric-	Normal (A)	328	27
	Disordered (B)	50	1998
Deoxy-	Normal (A)	322	25
	Disordered (B)	47	1992
CO-	Normal (A)	317	30
	Disordered (B)	44	1667

Values for normal and disordered hemes are presented.

The probability distribution of κ^2 value for luminophores attached to macromolecule was discussed in detail by Dale and Eisinger (1974, 1976, 1979). It was shown that there is approximately a 60% probability that κ^2 has a value outside the range 1/3–4/3, and an almost 20% probability that it is less than 0.1. It is not uncommon to find values of κ^2 much below 0.1, especially when the system is characterized by short lifetimes and low mobility inside the protein matrix. In myoglobin, Fig. 4 shows that only for a few discrete values of the angle Θ the value of κ^2 approaches 2/3.

In our previous work (Gryczynski et al., 1993), because of the low symmetry of protoporphyrin IX, we could not assign a precise value to the orientation angle Θ, and were able only to estimate a range for its position, between 50° and 70°, as

TABLE 4 Expected lifetimes and amplitudes of met-, deoxy-, and CO-Mb computed from the values in Table 3 assuming that 8% of the hemes are disordered

Mb	τ_1	A_1	τ_2	A_2	τ_3	A_3	τ_4	A_4
	ps		ps		ps		ps	
met	27	0.44	50	0.04	327	0.44	1998	0.04
deoxy	25	0.44	47	0.04	322	0.44	1992	0.04
CO	30	0.44	44	0.04	317	0.44	1667	0.04

TABLE 5 Experimental values of myoglobin lifetimes reported in the literature by various authors

Reference	Mb	τ_1	A_1	τ_2	A_2	τ_3	A_3
		ps		ps		ps	
Hochstrasser and Negus (1984)	met*	16	0.53	135	0.39	2220	0.08
	dox*	14	0.57	106	0.40	2680	0.03
	CO*	26	0.60	132	0.33	2170	0.02
Janes et al. (1987)	met‡	96	0.85	222	0.13 8	2830	0.00 5
	met§	28	0.96	790	0.01 6	2900	0.02 4
Willis et al. (1990)	met¶	21.5	0.45	112.5	0.55		
	dox¶	18	0.45	105.8	0.55		
	CO¶	23.4	0.45	125.4	0.55		

* Sperm Whale Mb (excitation at 290 nm).
‡ Sperm Whale Mb (excitation at 283 nm).
§ Aplysia Mb (excitation at 292).
¶ Sperm Whale Mb (excitation at 292 nm).

indicated by the shaded areas in Fig. 4. Nevertheless that range provides extensive information.

It is interesting to note that for Trp-14 a 180° rotation of the heme around its α-γ-meso axis, as shown in Fig. 1, produces a variation of κ^2 from near 0.8 in the normal position to about zero in the disordered position, for a very wide range of the angle Θ, from 50° to 70° (Fig. 4). This is due to the relative orientation of the transition moment of Trp-14 and the plane of the heme, which are positioned in an almost parallel fashion. It should also be stressed that the range where κ^2 is much smaller than 0.1, is wider than the range of uncertainty experimentally found by us for the angle Θ (Gryczynski et al., 1993). Also, Trp-7 produces very low transfer rates to heme in the normal position. However this occurs for values of Θ between 30° and 45°, incompatible with our estimates. Therefore it appears that the interaction of Trp-14 and disordered heme produces long lifetimes in myoglobin, suggesting that the arbitrarily chosen "normal" and "disordered" positions of the heme are the real alternatives in natural myoglobin.

Table 4 shows the expected lifetimes components of myoglobin obtained from the values listed in Table 3 on the assumption, after La Mar at al. (1983, 1984), that about 8% of the hemes are disordered in the natural system. They are very consistent with the lifetimes reported in the literature for sperm whale myoglobin, as follows.

Our simulations anticipate the presence of four distinct lifetimes in myoglobin, two produced by Trp-14 at 25–30 ps for normal hemes and at 1.7–2.0 ns for disordered heme, and two produced by Trp-7 at 45–50 ps for disordered hemes and at 310–330 ps for normal hemes. Consistent with this proposition the reported experiments detected three components. The shortest and longest components are practically identical to those listed in Table 5. The intermediate lifetime is probably an average of the lifetimes produced by Trp-7, difficult to resolve numerically. The longer values predicted by our simulations probably result from the approximations used in the computations regarding the value of Θ and the fixed positions assumed for the transition moments of the two tryptophans. These approximations are much less relevant for the very short and very long lifetimes computed for Trp-14. It can be estimated that a small 10% change in distance and/or orientation factor for the Trp-7-heme pair would reduce the computed lifetime to values below 40 and 200 ps for the disordered and normal heme position, respectively.

We stress that our simulations show that fluorescence decay in myoglobin is a very complex phenomenon, that its measurement implies the use of very advanced instrumentations pushed to the limits of their performance, and that its numerical resolution into four components, demanding seven or eight floating parameters, is a very difficult endeavor. On these considerations, the consistence between experimental and simulated lifetimes components is exceptional.

It should be stressed that spectroscopically we cannot distinguish between the nonquenching activity of missing and disordered hemes. The only difference is that disordered hemes fail to quench the fluorescence of only Trp-14. Therefore the amplitude of the nanosecond lifetimes would double in the case of missing hemes. The good correspondence between the fraction of disordered hemes seen by NMR, and the amplitudes of the nanosecond lifetimes reported in the

literature (Hochstrasser and Negus, 1984; Janes et al., 1987; Bismuto et al., 1989) support our hypothesis that the long lifetimes in myoglobin are produced by heme disorder. The hypothesis is further supported by the observation that heme free myoglobin is easily resolved by purification procedures and that an impurity of 3–5% (resulting in long lifetimes amplitudes of 6–10%) is easily detectable in isoelectrofocusing.

As shown in Table 4, Willis et al. (1990) did not find long components in the nanosecond time scale. They claim this to be the result of extensive purification of their sample, which did not contain impurities of non quenched tryptophans. Also the samples of Hochstrasser and Negus (1984), and Janes et al. (1987), were extensively purified and homogeneous in isoelectric focusing. We are not in the position of determining whether the purifications of Willis et al (1990) were better or worse than those of Hochstrasser and Negus (1984) and Janes et al (1987). We can only stress that if we accept the presence of disordered hemes in myoglobin, and the amounts of their fractions as reported by La Mar et al. (1983, 1984), there is an extremely good consistence among data emerging from entirely different and independent approaches, namely: linear dichroism (Gryczynski et al., 1993), fluorescence (Hochstrasser and Negus, 1984; Janes et al., 1987), crystallography (Phillips, 1980; Takano, 1977), and NMR (La Mar et al., 1983, 1984). Therefore, while the two components detected by Willis et al. (1990) are very consistent with the other data reported in Table 4, the missing long component is puzzling, because it would negate the presence of disordered hemes and their spectroscopic effect based on linear dichroism, crystallographic and NMR data. It is also possible that the analytical high-performance liquid chromatography used by Willis et al., (1990) resolved the myoglobin with disordered hemes from the main fraction of normal heme myoglobin. They report on chromatographic fractions with longer lifetimes, which were not characterized.

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