Rates of energy transfer between tryptophans and hemes in hemoglobin, assuming that the heme is a planar oscillator

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ABSTRACT Using the Förster equations we have estimated the rate of energy transfer from tryptophans to hemes in hemoglobin. Assuming an isotropic distribution of the transition moments of the heme in the plane of the porphyrin, we computed the orientation factors and the consequent transfer rates from the crystallographic coordinates of human oxy- and deoxy-hemoglobin. It appears that the orientation factors do not play a limiting role in regulating the energy transfer and that the rates are controlled almost exclusively by the intrasubunit separations between tryptophans and hemes. In intact hemoglobin tetramers the intrasubunit separations are such as to reduce lifetimes to 5 and 15 ps/ns of tryptophan lifetime. Lifetimes of several hundred picoseconds would be allowed by the intersubunit separations, but intersubunits transfer becomes important only when one heme per tetramer is absent or does not accept transfer. If more than one heme per tetramer is absent lifetimes of more than 1 ns would appear.

INTRODUCTION

The theory of radiationless energy transfer between donors and acceptors was first developed for fluorophores by Perrin and Förster (1–5). The dependence of transfer rate on the sixth power of the distance makes this technique very suitable for estimating distances between donors and acceptors situated in the same molecule (6); increasingly, this procedure is used for investigating the topology of synthetic peptides and in biomolecules like proteins and nucleic acids (6–15).

In hemoglobin, energy transfer upon excitation with UV light is rich in information regarding the relative distance and position of hemes and tryptophans. Therefore, these measurements may constitute a very good means for monitoring structure-function relationships in the system. The very short lifetime of tryptophan due to quenching by energy transfer to the heme, can be used to reveal very fast processes occurring in the molecule. The anisotropy decay of the signal may be able to detect internal rearrangements of the electronic structure of tryptophan upon excitation (16).

The major difficulty in the interpretation of the data is the mutual orientation of donor and acceptor chromophores, which could push the transfer to zero values. When both donor and acceptor have ample degrees of freedom it can be assumed without great error that they have a random orientation so that, provided angular equilibration rates are fast compared with the transfer rates, the orientation factor acquires an isotropic dynamic value of two-thirds. For rigid systems like the tryptophans and hemes in hemoglobin, this assumption does not hold and the actual orientation must be evaluated in order to obtain an accurate interpretation of the data.

In hemoglobin there are six tryptophans and four hemes. Using the atomic coordinates of oxy- and deoxy-hemoglobin, the Förster equations and the overlap between tryptophan emission and hemes absorption spectra, we have charted the rates of energy transfer from each tryptophan to the four hemes and included the case where one of these four hemes in the tetramer is missing. In so doing, we have assumed that the transition moments of the heme are isotropically distributed in the plane of the porphyrin, to produce a planar oscillator, and that all of the tryptophans can be excited by the incident beam.

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MATERIALS AND METHODS

Human oxy-hemoglobin and its heme-free derivatives were prepared using standard procedures available in our laboratory (17, 18). Deoxygenation was obtained by flashing the solutions with nitrogen.

All solutions were in 0.03 M phosphate buffer at pH 7.0. For absorption measurements we used concentrations near 1 mg/ml, and the pathlength of the cuvette was adjusted as necessary. The steady-state emission of apohemoglobin was measured in square geometry optics at protein concentrations near 0.5 mg/ml.

The absorption was measured with an AVIV 14DS spectrophotometer and the emission with a SLM 8000 spectro-fluorimeter.

The separations and angular relationships of donor and acceptors in oxy- and deoxy-hemoglobin were estimated from the atomic coordinates (19, 20) obtained from Pittsburgh Supercomputing Center, derived from the Protein Data Bank at Brookhaven National Laboratories. The SYBYL software was used with a Silicon Graphics Computer.

THEORY

Radiationless energy transfer due to a dipole-dipole interaction between a donor (D) and an acceptor (A) is function of the spectroscopic properties of the two fluorophores and the physical characteristics of the solvent, as represented in the classic Förster formulation by:

$$R_0^6 = 8.785 \times 10^{-25} \times \kappa^2 \times n^{-4} \times \Phi \times J, \qquad (1)$$

which defines R_0, the distance at which transfer depopulates the donor excited state at the same rate as all other deexcitation processes, including emission of fluorescence. In Eq. 1, n is the refractive index of the solvent, Φ

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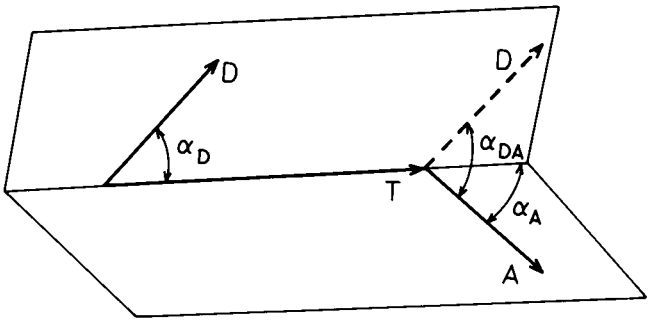

FIGURE 1 Schematic of energy transfer. $\vec{D}$ is the donor vector, $\vec{A}$ is the acceptor vector, and $\vec{T}$ is the translation vector.

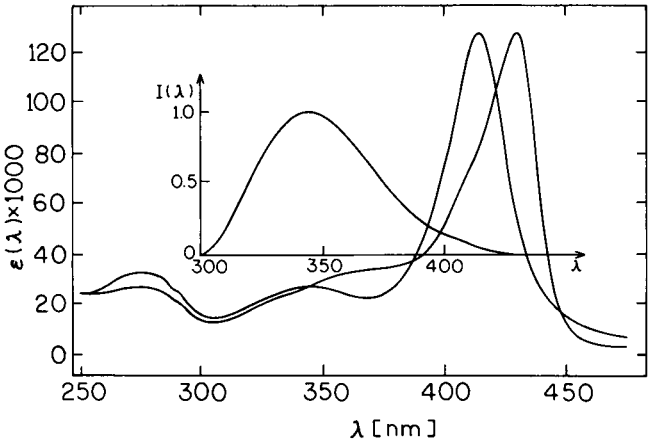

FIGURE 2 Comparison between the emission spectrum of tryptophan and the absorption spectrum of oxy- and deoxy-hemoglobin, showing the extensive overlap, which is one of the requirements for high efficiency of energy transfer.

is the quantum yield of the donor in the absence of acceptors, κ^2 is the orientation factor of the donor-acceptor system, and J is the overlap integral between the normalized emission spectrum of the donor and the absorption spectrum of the acceptor. The overlap integral is computed from

$$J = \int_0^\infty \frac{I(\tilde{\nu})\epsilon(\tilde{\nu})\,d\tilde{\nu}}{\tilde{\nu}^4}, \qquad (2)$$

where $\epsilon(\tilde{\nu})$ is the extinction coefficient of A, and $I(\tilde{\nu})$ is the normalized to unit fluorescence intensity of D.

The orientation factor κ^2 is a function of the angle (α_{DA}) defined by the direction of the transition moments $\vec{D}$ and $\vec{A}$ and of the angles (α_D, α_A), that the two vectors form with the translation vector $\vec{T}$ connecting the centers of the two oscillators (Fig. 1):

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

EVALUATION OF THE OVERLAP INTEGRALS

The overlap integral between tryptophan and heme was evaluated using Eq. 2 from the emission spectrum of heme-free hemoglobin (apohemoglobin) and the absorption spectrum of oxy- and deoxy-hemoglobin. Fig. 2 shows the absorption spectra of oxy- and deoxy-hemoglobin and the corrected emission spectrum of apohemoglobin. Considering that, in the presence of heme, the emission spectrum of tryptophans, as detected in apohemoglobin, may have been shifted by changes in the environment of the protein; we estimated the overlap integrals using apohemoglobin emissions as detected and also displaced 10 nm either toward the blue or toward the red, in a parallel fashion. Table 1 shows that the displacement did not produce significant modifications of the values of the overlap integrals and that they were practically insensitive to the difference in the absorption spectra of oxy- and deoxy-hemoglobin.

EVALUATION OF $\langle\kappa^2\rangle$ FOR A PLANAR OSCILLATOR

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 (21–23). Probably both transitions emit, and the excitation wavelength mostly used for tryptophan fluorescence, being close to 300 nm, preferentially populates the ^1L_a state of the chromophore. For this reason, in computing the values of κ^2, we assumed the ^1L_a direction of the transition moment of tryptophan, which lies in the plane of the ring and passes through the nitrogen atom making an angle 38° with the major axis of the molecule (22–23).

The absorption of the heme in the range 300–500 nm is due to a multiplicity of transitions located on the plane of the porphyrin. Following the suggestion of Eaton et al. (24–25) and of Case et al. (26), due to the essential symmetry of the porphyrin moiety, we assumed that the heme approximates a planar oscillator, i.e., in which the

TABLE 1 **Overlap integrals of the emission of tryptophan and absorption of hemoglobin**

	DeoxyHb			OxyHb		
Donor shift (nm)	None	+10	−10	None	+10	−10
Overlap (cm/M × 10^{14})	5.89	6.65	5.17	5.75	6.50	5.06

The corrected emission spectrum of heme-free hemoglobin was taken for the donor. The donor spectrum has been shifted in a parallel way toward the blue and toward the red for testing the effect of spectral shifts due to different environments.

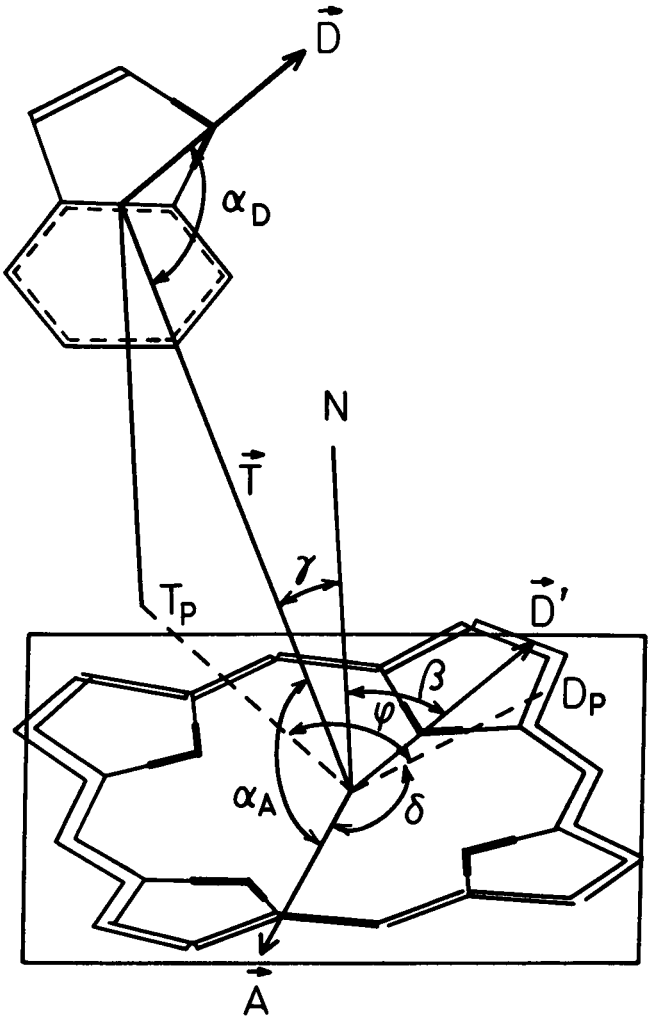

FIGURE 3 Schematics for energy transfer between tryptophan and heme in hemoglobin. $\vec{D}$ is the donor vector and $\vec{T}$ is the translation vector. The acceptor vector $\vec{A}$ is assumed to be symmetrically distributed in the plane of the heme. N is the normal to this plane. The dashed lines D_p and T_p are the projections on this plane of the donor vector (**D**) and the translation vector (**T**), respectively.

transition moments are symmetrically distributed about the center of the porphyrin ring where the iron is located. This requires definition of an average value $\langle \kappa^2 \rangle$ for the angular part of the interaction of the donor vector with the plane of the acceptor.

The planes of the tryptophan and of the heme were established, and the normal to the porphyrin plane also drawn (see Fig. 3). Using $\vec{D}$ for the donor vector, $\vec{A}$ for the acceptor vector, $\vec{T}$ for the translation vector, and N for the normal to the plane of the porphyrin; the software gave the angles α_D between $\vec{D}$ and $\vec{T}$, γ between $\vec{T}$ and N, and β between $\vec{D}$ and N, as shown in Fig. 3. Indicating by D_p and T_p, the projection on the plane of the porphyrin of $\vec{D}$ and $\vec{T}$, respectively, from the angles γ and β defined above, it is possible to define the angle φ between D_p and T_p using:

$$\cos \alpha_{DA} = \sin \beta \sin \gamma + \cos \beta \cos \gamma \cos \varphi, \quad (4)$$

where α_{AD} is the angle between $\vec{A}$ and $\vec{D}$ also given by:

$$\cos \alpha_{DA} = \sin \beta \cos \delta. \quad (5)$$

Similarly,

$$\cos \alpha_A = \sin \gamma \cos (\delta + \varphi), \quad (6)$$

where δ is the angle between $\vec{A}$ and D_p.
Substituting Eqs. 5 and 6 into Eq. 3 we have:

$$\kappa^2 = \sin^2 \beta \cos^2 \delta - 6 \sin \beta \sin \gamma \cos \alpha_D \cos \delta \cos (\delta + \varphi) + 9 \cos^2 \alpha_D \sin^2 \gamma \cos (\delta + \varphi). \quad (7)$$

For computing the average interaction of $\vec{D}$ with the plane of the porphyrin where $\vec{A}$ is isotropically distributed around the normal N, it is necessary to integrate over the angle δ. The isotropic nature of the distribution for a planar oscillator implies that $\langle \cos^2 \delta \rangle = 0.5$, $\langle \cos \delta \rangle = 0$, and $\langle \cos \delta \sin \delta \rangle = 0$. Therefore, we have:

$$\langle \kappa^2 \rangle = 1/2(\sin^2 \beta - 6 \sin \beta \sin \gamma \cos \alpha_D \cos \varphi + 9 \cos^2 \alpha_D \sin^2 \gamma), \quad (8a)$$

or using Eq. 4,

$$\langle \kappa^2 \rangle = 1/2(\sin^2 \beta + 6 \cos \alpha_D \cos \beta \cos \gamma - 3 \cos^2 \alpha_D (3 \cos 2\gamma - 1). \quad (8b)$$

Eq. 8 implies that the range of variability of $\langle \kappa^2 \rangle$ is from 0 to 2.0 rather than from 0 to 4 as for linear oscillators.

Table 2 presents the distances of the centers of tryptophans from the center of the various hemes in oxy- and deoxy-hemoglobin. Table 3 presents the corresponding values of the angles α_D, γ, and β. Table 4 shows the values of $\langle \kappa^2 \rangle$ computed with Eq. 8.

TABLE 2 Distances in Angstroms between tryptophans and hemes in human hemoglobin

	Heme			
TRP	α_1	α_2	β_1	β_2
$\alpha_1$14	15.5	36.1	34.8	38.6
	15.5	35.9	33.3	38.6
$\beta_1$15	34.1	37.4	16.3	40.5
	33.0	39.0	16.1	36.9
$\beta_1$37	25.8	14.3	16.3	34.5
	25.8	15.0	14.4	30.7

For each tryptophan the line in top shows the distances in deoxyhemoglobin, the line below those in oxyhemoglobin.

TABLE 3 **Values of the angles α_D, γ, and β for donor-acceptor pair between tryptophans and hemes in human oxy- and deoxy-hemoglobin**

	α_1			β_1			α_2			β_2		
Heme	α_D	γ	β	α_D	γ	β	α_D	γ	β	α_D	γ	β
TRP-Deoxy												
α-14	91.8	25.7	8.2	23.5	172.7	23.5	132.3	52.7	33.4	110.4	30.5	11.4
β-15	167.5	37.0	28.7	87.6	28.7	4.6	115.2	25.2	14.3	130.3	55.7	32.8
β-37	98.6	7.7	9.5	43.6	3.95	44.4	78.6	49.7	50.0	59.6	19.1	3.8
TRP-Oxy												
α-14	86.1	21.7	17.0	17.0	159.0	36.0	120.0	57.1	26.6	110.3	20.0	27.5
β-15	159.0	36.0	20.1	85.3	26.1	−19.1	110.1	25.5	25.0	116.8	56.0	30.4
β-37	107.9	9.7	20.1	30.9	7.9	37.4	102.3	39.2	36.0	66.7	14.1	24.6

EVALUATION OF TRANSFER RATES AND OF DONOR LIFETIMES

The values of donor lifetimes in the presence of energy transfer can be computed (τ_c) from the transfer rate factor, $(R_0/R)^6$, as:

$$\tau_c = \tau_0 \Big/ \left[1 + \sum_i (R_0/R_i)^6\right], \qquad (9)$$

where τ_0 is the lifetime of the donor in the absence of the acceptor and the sum extends over all i acceptors.

In order to estimate the effect of possible wobbling of both hemes and tryptophans within the protein matrix, for each tryptophan-heme pair, we computed $\langle \kappa^2 \rangle$ varying the angles α_D and γ by ±5°. The observed values of $\langle \kappa^2 \rangle$ did not change by more than 20%, which was essentially without effect on the computed lifetime.

From the table it appears that the transfer is largely dominated by intrasubunit interactions even allowing maximum wobbling of the donor-acceptor pairs. The least quenched residue is the β15 Trp, quenched ~50-fold, followed by the α14 Trp ~70 times and by the β37 Trp, which is quenched ~200 times. The exaggerated quenching of the β37 Trp is caused by its simultaneous proximity to two hemes, that of the same subunit, and that from the α subunit of the opposite dimer. For all tryptophans the presence or absence of ligand on the hemes does not modify the quenching in any substantial way. The different tilting of the heme and of the proximal histidine in oxy- and deoxy-hemoglobin (27) does not appreciably influence in intrasubunit transfer.

Table 5 also shows the expected lifetimes computed using the average transfer rate factors $(R_0/R_i)^6$ there listed, assuming a lifetime of 2 ns for tryptophan in the absence of acceptors. They range from the 10 ps lifetimes of the β37 Trp to the 30 and 40 ps estimated for the α14 Trp and β15 Trp, respectively. Even allowing a wobbling of 5° in the angles used for measuring the orientation factor; in intact hemoglobin tetramers no lifetime near or above 100 ps is expected.

In view of the multiplicity of lifetimes detectable in hemoglobin reported from various laboratories (28–30), it is of interest to investigate whether hemoglobin anomalies can produce longer lifetimes. Loss of hemes can in-

TABLE 4 **Values of the orientation factor $\langle \kappa^2 \rangle$ for the various donor acceptor pairs in human oxy- and deoxy-hemoglobin**

	Tryptophan		
Heme	α_1-14	β_1-15	β_1-37
α_1	0.49	1.10	0.53
	0.47	0.83	0.61
β_1	1.24	0.49	1.32
	1.03	0.42	1.43
α_2	0.62	0.73	0.53
	0.78	0.95	0.13
β_2	0.63	0.56	0.79
	0.34	1.02	0.48

The upper lines are for deoxy-hemoglobin.

TABLE 5 **Transfer rates factors $(R_0/R_i)^6$ and calculated lifetimes τ for the various tryptophans in oxy- and deoxy-hemoglobin, assuming that the lifetime of tryptophan in the absence of energy transferbefore is 2 ns**

TRP / HEME		α_1-14		β_1-15		β_1-37	
		$(R_0/R)^6$	τ_c [ns]	$(R_0/R)^6$	τ_c [ns]	$(R_0/R)^6$	τ_c [ns]
deoxy	α_1	70.4		0.62		3.31	
	α_2	0.44	0.028	0.36	0.038	114.2	0.012
	β_1	0.55		52.1		52.1	
	β_2	0.30		0.22		.58	
oxy	α_1	69.0		0.74		3.24	
	α_2	0.45	0.028	0.27	0.036	84.0	0.011
	β_1	0.70		54.9		107.3	
	β_2	0.29		0.38		1.14	

TABLE 6 **Rate factors $(R_0/R_i)^6$ and calculated lifetimes τ for human oxy- and deoxy-hemoglobin, when one heme per tetramer is lost**

TRP / HEME		α_1-14		β_1-15		β_1-37	
		$(R_0/R)^6$	τ_c[ns]	$(R_0/R)^6$	τ_c[ns]	$(R_0/R)^6$	τ_c[ns]
NO α_1	α_2	0.45		0.27		84.0	
	β_1	0.70	0.820	54.9	0.036	107.3	0.010
	β_2	0.29		0.38		1.14	
NO α_2	α_1	69.0		0.74		3.24	
	β_1	0.70	0.028	54.9	0.036	107.3	0.018
	β_2	0.29		0.38		1.14	
NO β_1	α_1	69.0		0.74		3.24	
	α_2	0.45	0.028	0.27	0.835	84.0	0.022
	β_2	0.29		0.38		1.14	
NO β_2	α_1	69.0		0.74		3.24	
	α_2	0.45	0.028	0.27	0.036	84.0	0.010
	β_1	0.70		54.9		107.3	
NO α_1	α_2	0.44		0.36		114.2	
	β_1	0.55	0.874	52.1	0.038	52.1	0.012
	β_2	0.30		0.22		0.58	
NO α_2	α_1	70.4		0.62		3.31	
	β_1	0.55	0.028	52.1	0.038	52.1	0.036
	β_2	0.30		0.22		0.58	
NO β_1	α_1	70.4		0.62		3.31	
	α_2	0.44	0.028	0.36	0.910	114.2	0.016
	β_2	0.30		0.22		0.58	
NO β_2	α_1	70.4		0.62		3.31	
	α_2	0.44	0.028	0.36	0.380	114.2	0.012
	β_1	0.55		52.1		52.1	

It is assumed that the lifetime of tryptophan before quenching is 2 ns.

deed produce such components. Table 6 shows the allowed lifetimes if one heme per tetramer is missing. Lifetimes of several hundred picoseconds are produced, and the tryptophans α14 and β15 have longer lifetimes in deoxy-hemoglobin than in oxy-hemoglobin. Losses of more than one heme per tetramer would produce slightly longer lifetimes, up in the nanosecond range. However, simultaneous loss of more than one heme is highly improbable, and loss of two or more hemes per tetramer would imply substantial damage to the structure of the molecule, which could hardly then be considered native hemoglobin.

It should be stressed that heme loss is only one of the possible causes for lifetimes longer than those presented in Table 5. In fact, energy transfer from tryptophan residues to heme can be inhibited either by modifications of the orientation parameter $\langle \kappa^2 \rangle$, or by simultaneous excitation of heme and tryptophan in the same subunit. Another possible cause is the presence of non-hemoglobin impurities whose tryptophans are not quenched by the heme (27). We are trying to give an answer to these questions.

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