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Time-resolved fluorescence of hemoglobin species [1]

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Abstract

We used time-resolved fluorescence in the pico- to nanosecond time range to monitor the presence of tetramers, dimers and monomers in carbonmonoxyhemoglobin (COHb) solutions and to investigate how their distributions change under different experimental conditions. Comparison of fluorescence lifetime computed from the atomic coordinates of COHb (Vasquez et al., 1996) with those experimentally measured allowed identification of molecular species present in the hemoglobin solution. It was possible to observe modification of the distribution of tetramers, dimers, monomers and species with disordered hemes produced by different experimental conditions. Protein concentration affected the detectable lifetimes, indicating increasing amounts of dimers and monomers at low protein concentrations, while the amount of inverted hemes was not modified. Titration with up to 1 M NaCl modified only the extent of dissociation of hemoglobin into dimers, without affecting heme inversion and monomer formation. Hyperbaric pressure increased the amounts of dimers and monomers. This is the first time that monomeric subunits of hemoglobin have been detected at neutral pH in the normal system.

Keywords: Fluorescence lifetime; Hemoglobin species; Time-resolved fluorescence

1. Introduction

Time-resolved studies of fluorescence emission of hemoglobin and myoglobin reveal the presence of several lifetime components produced by tryptophan–heme interaction [1–8]. The lengths of these lifetimes span from single digit picoseconds to several nanoseconds.

Lifetimes approaching the nanosecond range were a puzzle because the small Trp–heme separation and the extensive overlap integral between the emission of tryptophan and absorption of heme imply a very efficient excitation energy transfer between the two chromophores, which should produce a massive quenching of tryptophan fluorescence. Thus the 'long' lifetimes were dismissed as being due to impurities containing non-quenched tryptophans. This hypothesis was supported by the observation that the amplitudes of the long lifetimes were very small, and the 'impurities' were only visible on the background of the weak hemoglobin fluorescence.

In this laboratory, any attempt to purify the hemoglobin solutions failed to eliminate the long lifetime components. Also, lifetimes of several hundred picoseconds, below 1.0 ns, appeared too short to

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[1] Paper dedicated to Professor Alfons Kawski on the occasion of his 70th birthday.

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originate from non-quenched tryptophans. This generated the hypothesis, already mentioned by several authors [1–3], that some peculiar spatial relationship between tryptophan and hemes was present which would reduce the quenching. Those authors, however, admitted that this hypothesis is not tenable as long as the heme is considered a symmetric molecule with transition moments equally distributed in all directions on the plane of the heme. This prompted a detailed investigation of the parameters which, in hemoglobin and myoglobin, regulate the excitation energy transfer between tryptophans and hemes.

Investigation of the linear dichroism of porphyrins and hemes of types IX and III embedded in stretched PVA films [9,10], and of their absorption in free solutions and inside the heme pocket of the hemoproteins, confirmed that heme is a non-symmetric light absorber. These studies allowed us to identify the single transition moment which, in the heme, is responsible for accepting excitation energy transfer from tryptophan, and to estimate its orientation on the plane of the porphyrin. On this basis, and using available atomic coordinates of myoglobin, it was possible to estimate the expected lifetimes of sperm whale [11] and horse heart [8] myoglobins.

Myoglobin is expected to be distributed in three molecular species: one where the heme is in normal position, as in the crystal; another where the heme is disordered, with a limiting case represented by its inversion, i.e. rotated 180° around the $\alpha-\gamma$–meso axis of the porphyrin [12–15]. As there are no covalent bonds between heme and protein, a third species is anticipated where heme-free myoglobin is in reversible dissociation equilibrium with the heme [16]. In our estimations, the long lifetime components of these systems are produced by small percentages of the last two species mentioned above.

Reported lifetime distributions for sperm whale myoglobin [1–5,11] were consistent with our hypothesis, so we started a detailed investigation of the time-resolved characteristics of the emission of horse heart myoglobin. In this system the computed lifetimes corresponded extremely well to the measured lifetimes, allowing assignment of the various emission lifetimes to the respective molecular species. The data confirmed the presence of the three molecular species mentioned and allowed their precise quantitization. This produced a complete resolution of the thermodynamics of the species distribution of horse heart myoglobin at various pH values from pH 7.0 to pH 4.5 [8].

In hemoglobin, species distribution is complicated by the polymeric nature of the molecule. Each tryptophan interacts with four hemes, one at intrasubunit distance (about 15 Å) and the other three at intersubunit distances (25–35 Å). The subunits in tetrameric hemoglobin are not held together by covalent bonds and are known to dissociate into dimers. Monomeric subunits of normal hemoglobin have never been detected in the native system; nevertheless, their presence cannot be excluded. Thus the number of molecular species present in solution potentially involves tetramers, dimers and monomers with hemes either in their normal position or disordered or dissociated. The resulting number of possible molecular species is very large. At this point we want to stress that our previous studies on isolated α subunits [17] show that in both their monomeric and dimeric forms the tertiary and secondary structures observed in the crystal of tetrameric hemoglobin are well preserved, regardless of the dissociation.

In order to analyze this complex system we have conducted measurements at high and low protein concentrations, at high and low ionic strength, and under hyperbaric conditions, procedures which are known to affect the quaternary structure of the protein without disturbing its secondary and tertiary structures [17–19]. The data allow identification of the species present in solution, opening the way to future investigations. For the first time it was possible to detect the presence in solution of monomeric hemoglobin subunits in equilibrium with dimers and tetramers and in reversible dissociation equilibrium with their hemes.

2. Materials and methods

A more detailed description of the methods used for collecting and analyzing data is presented in previous publications [7,8]. Here they are summarized.

2.1. Human carbonmonoxyhemoglobin (CO-Hb)

This was prepared and purified as previously described [6]. It was stored at −80°C, thawed and

repurified by free phase electrofocusing (Rotofor, Biorad) immediately before use. The carbonmonoxy derivatives were prepared by equilibrating both the buffers and the solutions of hemoglobin with carbon monoxide at atmospheric pressure. Hemoglobin concentration was measured using $E = 0.868\,cm^{-2}\,mg^{-1}$ at 540 nm. The solutions used for the measurements were in 0.05 M phosphate buffer at pH 7.0, at the indicated protein concentration at room temperature.

2.2. Fluorescence steady-state intensities

These were measured using an 8000 SLM photon-counting instrument.

2.3. Absorption spectra

Absorption spectra were measured using a double beam Aviv 14DS spectrophotometer.

2.4. High pressure measurements

Fluorescence measurements under hyperbaric pressure were performed in a high pressure cell designed as described by Paladini and Weber [19].

2.5. Fluorescence lifetimes

These were measured using a frequency domain 10 GHz fluorometer equipped with a Hamamatsu 6 μm microchannel plate detector (MCP-PMT), as previously described [20]. The instrument covered a wide frequency range between 15 and 5000 MHz, which allowed detection of lifetimes ranging from several nanoseconds to a few picoseconds. Samples were placed in a 1 cm path 'shielded cuvette', previously described [21], which eliminates stray light from the emission with the help of light absorbing filters inside the cuvette. The use of internal filters, however, was not possible with the small cylindrical bottle inside the pressure bomb. The only remedy was to search for a proper alignment of the laser beam through the windows of the bomb. The exciting light was tuned at 294 nm. Sample emission was filtered through an Oriel interference filter centered at 340 nm and a Corning 7–60 broad band filter. For reference, we used the scatter of the sample solution filtered through an Oriel interference filter at 289 nm together with neutral density filters. The filters used for the emission and the reference were calibrated so as to obtain identical optical length at 294 and 340 nm [21].

2.6. Data analysis

Time-resolved intensity decays were assumed to be a sum of discrete exponentials as in [22,23]

$$I(t) = I_0 \Sigma_i \alpha_i e^{t/\tau_i} \quad (1)$$

where α_i is the amplitude (pre-exponential factor) and τ_i the lifetime of the ith discrete component. The fractional steady-state intensity f_i of the ith lifetime component is related to the amplitude α_i by

$$f_i = \frac{\alpha_i \tau_i}{\Sigma_i \alpha_i \tau_i} \quad (2)$$

In frequency domain the measured quantities, at each frequency ω, are the phase shift (ϕ_ω) and the demodulation factors (m_ω) of the emitted light versus the reference light. Fractional intensity, amplitude and lifetime parameters were recovered by nonlinear least-squares procedures using either the Global Unlimited software [22] or software developed at the Center of Fluorescence Spectroscopy [23].

2.7. Lifetime computations

For these computations it is necessary to evaluate the parameters that regulate excitation energy transfer between tryptophan and heme in hemoglobin. They are the overlap integral, the distance and the angular relationships between donor and acceptor.

The overlap integral was computed from the extinction coefficients and normalized emission of heme and heme-free hemoglobin respectively [11,24].

The distances and angular orientations for computing the Förster orientation factors κ_i^2 [30] were obtained from the atomic coordinates of CO-hemoglobin [29]. To compute the orientation parameter, the transition moment of tryptophan was assumed to be at $-38°$ from the main axis of the indole plane [25–28]. Our earlier studies of linear dichroism of different heme derivatives [9,10] indicated an orientation between 50° and 60° from the α–γ–meso axis of the porphyrin ring for the heme transition moment

responsible for radiationless tryptophan–heme interaction. For computing an average effective orientation factor $<\kappa^2>$ we averaged the orientation factors computed in the interval 50°–60° at each 1° step.

Residual tryptophan lifetimes (τ_c) in the presence of energy transfer to hemes were computed from [8,24,30]:

$$\tau_c = \frac{\tau_0}{1 + \sum_i (R_{0,i}/R_i)^6} = \frac{\tau_0}{1 + T} \qquad (3)$$

where the ratios $(R_{0,i}/R_i)^6$ are the transfer rate factors from the donor-tryptophan to each of the hemes in the hemoglobin subunits. A total transfer rate factor $T = \sum_i (R_{0,i}/R_i)^6$ represents a cumulative quenching of tryptophan by all hemes available in the polymeric hemoprotein structure; τ_0 is the lifetime of the tryptophan in the absence of the heme acceptors. $R_{0,i}$ is the Förster distance, a separation between tryptophan and the ith heme, at which 50% of the excitation energy is transferred from donor to acceptor [30]. It is important to stress that $R_{0,i}$ is different for each of the Trp–heme pairs due to the different orientation factors (κ_i^2). Heme disorder may also strongly modify the orientation factor (κ_i^2) and change the $R_{0,i}$ value, limiting the efficiency of radiationless Trp–heme interaction.

3. Results

3.1. Computed lifetimes

The expected lifetimes of TRP α14, β15 and β37 in CO-hemoglobin were computed assuming that, in the absence of transfer to heme, tryptophan lifetime is 5 ns. The lifetimes estimated for the various tryptophans in the presence of normal, disordered and reversibly dissociated hemes are shown in Table 1. The lifetimes are computed for tetrameric, dimeric and monomeric species.

When the intrasubunit heme is in normal (N) position the rate of excitation energy transfer is very high and produces residual lifetimes in the short ps range. The shortest residual lifetime is that of TRP β37 which is simultaneously positioned at approximately 15 Å from both its intrasubunit heme and that of the α chains of the opposite dimer.

Table 1
Computed lifetimes of the various tryptophans in monomeric (τ_m), dimeric (τ_d) and tetrameric (τ_t) carbonmonoxyhemoglobin when the respective intrasubunit heme is either in its normal position (N), or disordered (D), or reversibly dissociated (RD). The lifetime of non-quenched tryptophan is assumed to be 5000 ps

CO-Hb	Heme	τ_m (ps)	τ_d (ps)	τ_t (ps)
α14	N	36	36	36
	D	1064	887	753
	RD	5000	2586	1700
β15	N	49	49	49
	D	1056	850	725
	RD	5000	2333	1582
β37	N	4	4	4
	D	110	105	54
	RD	5000	1631	601

Computations of the orientation factors show that disordered hemes (D) are poor acceptors of excitation energy transfer from the tryptophan of the same subunit. Fig. 1 shows the cumulative transfer rate factor T of the excitation energy transfer in the presence of normal and inverted hemes, respectively, for TRP α14, β15 and β37, in monomeric, dimeric and tetrameric hemoglobin. The lower value of T for the inverted hemes results from a greatly reduced excitation energy transfer from tryptophans, which decreases from over 100 times, in the presence of normal hemes, to only 3–6 times in the presence of disordered hemes. Therefore the residual lifetimes of hemoglobin tryptophans in the presence of disordered intrasubunit hemes are much longer.

It should be stressed that when the *intrasubunit* heme is either disordered or dissociated, excitation energy transfer to *intersubunit* hemes plays an important role, and the resulting lifetimes become sensitive to dimer formation. In fact, as shown in Fig. 2, intersubunit excitation energy transfer occurs to a single heme in dimeric subunits and to three hemes in tetrameric molecules. Using Eq. (3), it can be estimated that intersubunit excitation energy transfer decreases the lifetime of tryptophans by a factor near 2–3 within dimers and by a factor near 4–6 within tetramers. Therefore, even in the presence of dissociated intrasubunit hemes, the lifetime of free tryptophan (near 5 ns) cannot be produced by dimers and tetramers unless more than one heme is dissociated from the same hemoglobin molecule. The very low

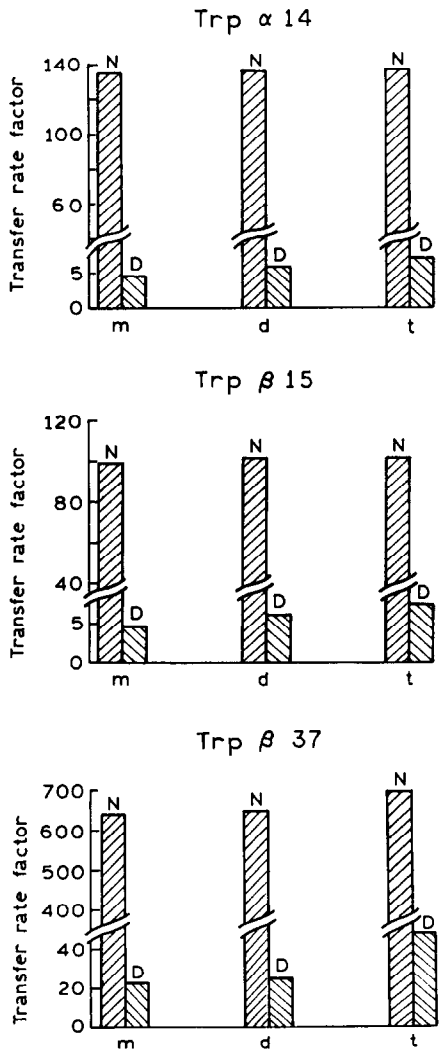

Fig. 1. Total transfer rate factors of tryptophans α14, β15 and β37 to all of the hemes in the molecule when the respective intrasubunit heme is either in normal (N) or inverted (D) position, in monomeric (m), dimeric (d) and tetrameric (t) carbonmonoxy-hemoglobin. Note the drastic decrease of the transfer rate when the respective intrasubunit heme is disordered, and how this decreased rate decreases further in the dimers and monomers because of the lower number of intersubunit hemes.

amount of dissociated hemes makes this situation very improbable. We can safely assume that the free tryptophan lifetime can originate only from *monomeric subunits with dissociated hemes*, where intersubunit transfer is not present.

Intersubunit transfer does not appreciably affect the shortest lifetimes in the ps range, produced by interaction with normally oriented hemes, because of the overwhelming intrasubunit excitation energy transfer.

3.2. Lifetimes of CO-hemoglobin at high and low protein concentrations

These data were obtained using the GB cuvette in front face optics [21] at protein concentrations of 15 mg/ml, and the 'shielded' cuvette in square geometry optics [21] at protein concentrations near 0.2 mg/ml, a 75-fold difference. The different concentrations affected the range of frequencies available to the measurements. Using front face optics at high protein concentrations, the stronger emission signal allowed the use of frequencies up to 10 GHz. Lifetime data obtained under these conditions in the GB cuvette have already been reported [7] and are used here for comparisons in Table 2. At low protein concentrations the lower intensity of the signal limited the frequency range to 5 GHz. The experimental data for low protein concentration are included in Fig. 3 (data before addition of NaCl, see Section 3.3), and the recovered lifetimes are shown in Table 2. The table shows that at both high and low protein concentrations three lifetimes were detected. However, they were somewhat different, indicating a dependence on protein concentration. The higher frequency range at high protein concentrations allowed the detection of an ultra-short ps lifetime. Conversely, at low protein concentration a very long lifetime of 3865 ps was resolved.

3.3. Effect of ionic strength on the lifetimes of CO-hemoglobin

These measurements were conducted at low protein concentration (0.2 mg/ml) to maximize dimer formation. The data in Fig. 3 show that the tracing of the frequency-dependent phase shifts and demodulations is well outside the range of experimental error. The recovered lifetimes are listed in Table 3. Three lifetimes were detected at all ionic strengths. The effect of increasing ionic strength was detectable only on the lifetime near 300 ps, which increased to near 700 ps in 1 M NaCl. The other two lifetimes

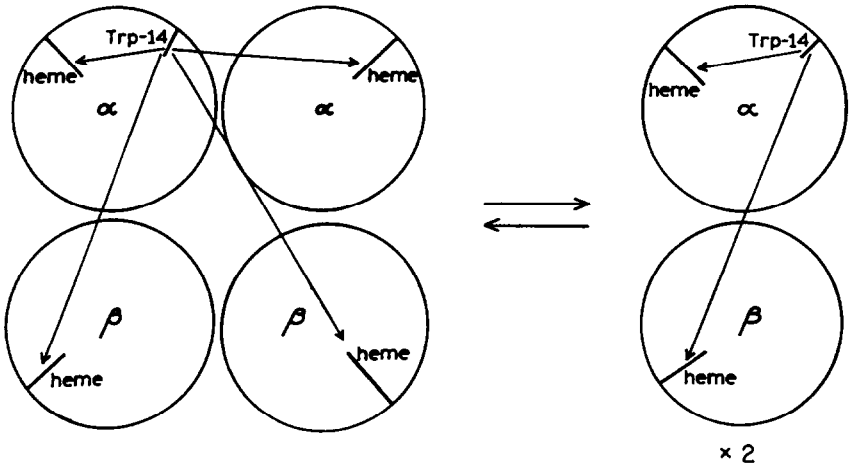

Fig. 2. Schematics illustrating the intra- and intersubunit excitation energy transfer from tryptophan α14 to the various hemes in tetrameric and dimeric hemoglobin.

were not appreciably sensitive to ionic strength. The amplitudes of the three lifetimes were also not modified by addition of NaCl.

3.4. Steady-state fluorescence intensity at normal and under hyperbaric pressure

As shown in Fig. 4, exposure to hyperbaric pressure more than doubled the intensity of the emission of CO-hemoglobin and produced a red shift of the emission spectrum. It also shows that this phenomenon was entirely reversible from 1.8 kbar. Above that pressure the fluorescence intensity further increased; however, the system lost reversibility, suggesting a non-reversible unfolding of the protein. For this reason we limited our observations to this maximum pressure.

3.5. Hyperbaric pressure effects on fluorescence lifetimes

The pressure dependence data up to 1800 bar in the frequency range 15–5000 MHz are shown in Fig. 5. The measurements were performed at low protein concentrations (0.5 mg/ml). The respective recovered parameters for discrete lifetime components are shown in Table 4 which shows that, as usual, three lifetime components were detected. At normal pres-

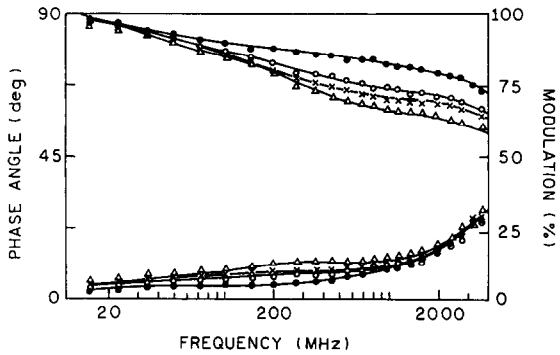

Fig. 3. Frequency dependence of phase shift (lower lines) and demodulation of carbonmonoxyhemoglobin titrated with increasing amounts of NaCl. In 0.05 M phosphate buffer at pH 7.0, at room temperature. Protein concentration 0.2 mg/ml. The lines through the experimental data points were computed with the recovered parameters.

Table 2
Lifetimes of carbonmonoxyhemoglobin at low and high protein concentration. In 0.05 M phosphate buffer at pH 7.0, at room temperature

HbA0	τ_1	α_1	f_1	τ_2	α_2	f_2	τ_3	α_3	f_3
0.2 mg/ml	21	0.992	0.779	293	0.007	0.077	3865	0.001	0.144
15 mg/ml	6	0.725	0.310	35	0.272	0.540	1100	0.003	0.150

Table 3
Lifetimes of carbonmonoxyhemoglobin in the presence of increasing amounts of NaCl. In 0.05 M phosphate buffer at pH 7.0, at room temperature, protein concentration 0.2 mg/ml. Both local (upper lines) and global analyses are presented. Global analyses were performed by linking the amplitudes of the lifetimes. Note the excellent correspondence of the two kinds of analysis. In the text only the data of the global analyses are discussed

Cl	τ_1	α_1	f_1	τ_2	α_2	f_2	τ_3	α_3	f_3
0 M	21	0.992	0.779	293	0.007	0.077	3865	0.001	0.144
0.2 M	20	0.992	0.691	561	0.007	0.137	4913	0.001	0.172
0.5 M	20	0.993	0.649	747	0.007	0.171	5522	0.001	0.180
1.0 M	22	0.988	0.642	662	0.010	0.196	3921	0.001	0.162

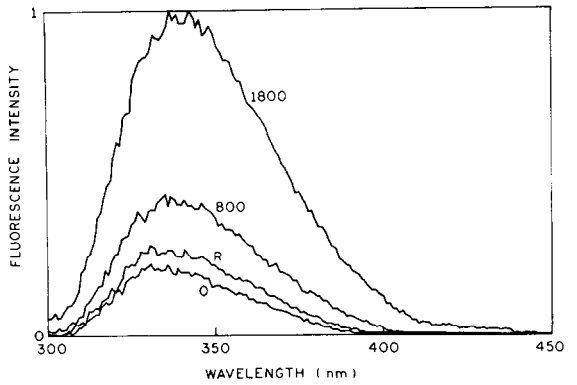

Fig. 4. Emission spectra of carbonmonoxyhemoglobin at normal pressure (O) and exposed to pressures of 800 and 1800 bar. The spectrum obtained upon pressure release is indicated by R. In 0.05 M phosphate buffer at pH 7.0, at room temperature. Protein concentration 0.5 mg/ml.

sure the two longer lifetimes appeared slightly shorter than those reported in Table 2. Otherwise the two sets of data are very consistent with each other. The slight discrepancy may result from the impossibility of using the 'shielded' cuvette inside the bomb. Even a meticulous alignment probably failed to completely eliminate reflections. Also, the small cuvette inside

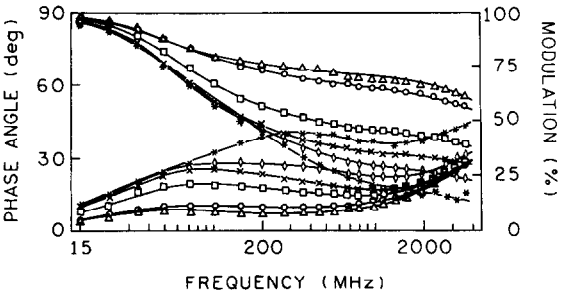

Fig. 5. Frequency dependence of phase shift (lower lines) and demodulation of the emission of carbonmonoxyhemoglobin exposed to increasing hyperbaric pressure from normal pressure (1.0 bar) to 1800 bar. The data at normal pressure are the top line for the demodulation and the bottom line for the phase shift. In 0.05 M phosphate buffer at pH 7.0, at room temperature. Protein concentration 0.5 mg/ml.

Table 4
Lifetimes (τ), fractional amplitudes (α) and fractional steady-state intensities (f) of carbonmonoxyhemoglobin in the presence of increasing hyperbaric pressure. In 0.05 M phosphate buffer at pH 7.0, protein concentration 0.5 mg/ml

Pressure	τ_1 (ps)	α_1 (ppt)	f_1	τ_2 (ps)	α_2 (ppt)	f_2	τ_3 (ps)	α_3 (ppt)	f_2
1 bar	21	990	0.700	276	8	0.071	3558	2	0.229
R 1 bar	21	987	0.628	313	10	0.085	3045	3	0.277
500 bar	20	981	0.429	409	12	0.107	3026	7	0.463
700 bar	20	976	0.332	464	14	0.110	3281	10	0.558
850 bar	23	971	0.349	471	18	0.130	3125	11	0.527
1000 bar	21	959	0.260	474	28	0.172	3381	13	0.568
1200 bar	24	949	0.264	521	37	0.224	3154	14	0.512
1400 bar	29	933	0.230	608	51	0.263	3733	16	0.507
1600 bar	29	911	0.180	671	69	0.316	3687	20	0.503
1800 bar	33	892	0.160	748	83	0.337	3706	25	0.503

the bomb prevented stirring of the solution, thereby leaving the same solution volume exposed to the exciting beam for a long time.

Hyperbaric conditions increased both the lengths and the amplitudes of the 'intermediate' lifetimes. The amplitudes of the longest lifetimes near 3000 ps also increased with pressure. All of these data returned to the initial values upon release of the pressure [2].

4. Discussion

4.1. Fluorescence lifetime assignment

Table 1 shows the expected lifetimes of the tryptophans in the tetrameric, dimeric and monomeric forms of human hemoglobin, when their respective intrasubunit heme is either in normal and disordered position or dissociated. To compute these values we assumed that intersubunit excitation energy transfer occurs only to normally oriented hemes, because in this case chromophore separation is the most relevant regulatory parameter of excitation energy transfer. We also assumed that the lifetime of nonquenched tryptophan in the hemoglobin system is 5 ns. The choice was based on the observation that 4.8 ns is the longest lifetime component previously detected in horse heart myoglobin and now present in hemoglobin. It is also consistent with the dominant lifetime component exhibited by tryptophan in a water environment and embedded in other proteins [31–33]. Another assumption was that the atomic coordinates of tetrameric hemoglobin are still valid for the dissociated subunits. This hypothesis is supported by observations of Paladini and Weber [18,19], that pressures up to 2000 bar do not alter the tertiary and secondary structures of various proteins. It is consistent with the reversibility of the increase of steady-state fluorescence intensity within the chosen range of hyperbaric conditions. The assumption is also supported by the observation that the measured lifetimes of isolated α subunits of hemoglobin are very similar to those anticipated on the basis of the crystal coordinates of tetrameric hemoglobin [17]. In Table 1, we also disregard the contributions of monomeric units with either normal or disordered hemes to all of the other lifetime components because of their very small fraction.

Table 1 instructs that in the emission of hemoglobin we may expect to resolve only three average lifetimes: (1) the short ps lifetimes which originate from tryptophans in the presence of normal intrasubunit hemes; (2) intermediate lifetimes of several hundred to 1000 ps which originate from tryptophans in the presence of disordered or dissociated intrasubunit hemes in tetramers and dimers; and (3) very long lifetimes of several ns originating from tryptophans in monomeric subunits with dissociated hemes.

As expected, three average lifetimes are found experimentally. The shortest lifetime components, between 6 and 15 ps, reported in Tables 2–4, correspond very well to the shortest computed lifetimes, confirming that they are an expression of the massive excitation energy transfer between tryptophans and their normal intrasubunit hemes.

The 'intermediate' lifetimes, near 300–1000 ps, detected in all of our measurements, correspond to the lifetimes computed for tryptophans interacting with either disordered or dissociated intrasubunit hemes. The lifetimes anticipated in Table 1 for these two events appear slightly longer than those experimentally detected. It should be kept in mind that computed lifetimes may not be exact and that the excitation energy transfer is sensitive to the 6th power of the distance. A slight wobbling in solution of the A helix, which contains tryptophans α14 and β15, may explain that small discrepancy. A displacement of the order of 1 Å is more than sufficient to explain the difference.

We also detected very long lifetimes of several ns, which correspond to the emission of monomeric subunits with dissociated hemes. Very good evidence that these long lifetimes originate from natural hemoglobin species is given by the experiments per-

[2] When pressure was released there was a sudden transitory fluorescence intensity increase which decreased in a few minutes to the new equilibrium value. We were not able to collect meaningful time-resolved data under these changing conditions, and we do not have any reasonable explanation of this phenomenon. It may suggest an intermediate protein state occurring during equilibration time.

formed at different protein concentrations and under hyperbaric conditions.

It should be stressed that the increased length of the intermediate and longest lifetimes implies a large contribution to the total steady-state emission intensity of the system. As shown in Table 3, a 3700 ps lifetime with an amplitude of only 25 ppt (parts per thousand) contributes 50% of the emission intensity of the system. This is why the amplitudes of these lifetimes can be estimated with great precision, even if they are very small.

4.2. Effect of protein concentration

The effect of a 75-fold increase of protein concentration is evident in Table 2. As mentioned, the intensity of the emission at high concentration allowed us to extend the frequency range for the analyses to 10 GHz. This, in turn, allowed the detection of an extra single-digit lifetime component in time and anisotropy decay, probably due to the $^1L_b \rightarrow {}^1L_a$ interconversion of the excited state transition moments of the indole moiety [7]. At low protein concentration, frequency probing could not exceed 5 GHz. Thus, the 25 ps lifetime is probably the average of the 6 and 35 ps lifetimes detectable at high protein concentration. It may be noted that the sum of the amplitudes of these two lifetimes correspond to the amplitude of the single 25 ps lifetime detected at low protein concentration. The other difference between the two experiments is that, at low protein concentration, two longer lifetimes are detectable at 293 and 3865 ps rather than only one at 1100 ps. As discussed above, the 3865 ps lifetime is probably produced by monomeric hemoglobin subunits in equilibrium with dimers, tetramers and dissociated hemes. Because of equilibrium conditions, at high protein concentration their relative fraction decreases below 1 ppt, which is the limit of resolution of the technique. The result is an average into a single 1100 ps lifetime component at the 293 and 3865 ps lifetimes, resolvable at low protein concentration.

This is a first indication that we can detect the presence of monomeric subunits of hemoglobin in equilibrium with the other molecular species. Additional evidence is provided by the data obtained under hydrostatic pressure.

4.3. Effect of hyperbaric conditions

These were the most difficult measurements because inside the bomb we could not use the 'shielded' cuvette. The steady-state data showed an increasing intensity and a red shift produced by hyperbaric conditions. This is consistent with previous data obtained by Hirsch et al. for *Lumbricus terrestris* hemoglobin [35], and was expected. In fact, hydrostatic pressure has been found to dissociate hemoglobin into dimeric and, possibly, monomeric subunits [36]. We should add that the collapse of the heme pocket upon heme release [37], by decreasing the volume of the protein, would also favor heme dissociation under hyperbaric conditions. According to the data in Table 1, both these events produce longer lifetimes and consequently a higher emission intensity.

In time resolution, it was very encouraging to observe that in spite of the optical difficulties we could resolve the lifetimes of CO-hemoglobin into the usual three components detectable in the other measurements. The intermediate and longest lifetimes, although in general consistent with those found under normal pressure, were somewhat shorter. We believe this was due to the imperfections of the sample cell inside the pressure bomb.

The measurements show that the amplitudes of the short picosecond lifetimes decrease with pressure, while those of the intermediate and very long lifetimes increase monotonically. The increasing amplitude of the intermediate lifetimes implies an increased fraction of either disordered or dissociated heme, or both, in dimeric and tetrameric species. At present we cannot distinguish between the two. Heme disorder does not decrease the volume of the system and should be insensitive to increasing pressure. Therefore the increasing amplitude of these lifetimes probably reflects an increase of the fraction of dissociated heme.

The experiments also show that the intermediate lifetimes become longer with pressure, indicating dissociation of subunits. The dependence of the amplitudes on hydrostatic pressure has a hyperbolic shape, which indicates cooperative behavior (Fig. 6) and suggests that heme dissociation and dimer formation are mutually supporting phenomena.

The reversible increase of the amplitude of the

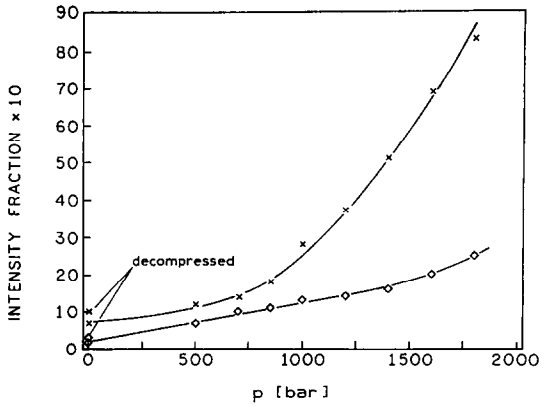

Fig. 6. Pressure dependence of the amplitudes of the intermediate (×) and longest lifetimes (◇) of the emission of carbonmonoxyhemoglobin. In 0.05 M phosphate buffer at pH 7.0, at room temperature, protein concentration 0.5 mg/ml.

lifetime above 3000 ps immediately implies that the system includes a fraction of heme dissociated monomeric subunits in reversible equilibrium with the rest of the system.

4.4. Effect of ionic strength

The only modification introduced by increasing ionic strength is the increasing length of the intermediate lifetime, consistent with the formation of dimers. It is relevant to note that the amplitudes of the various lifetimes were not modified. This implies that ionic strength did not modify the fractions of dissociated and inverted hemes. It is also important to note that the amount of monomers was not appreciably increased by ionic strength. It is possible that the increase was too small to be detected. These measurements prove that the only effect of ionic strength is a dimerization of hemoglobin with minimal modification of tertiary and secondary structures. Hirsch et al. [38,39] reported an increased steady-state intensity and red spectral shift of the emission of hemoglobin solutions in NaCl. In the absence of time-resolved data this was referred to exposure of TRP β37 to the solvent upon dimer formation. Time-resolved data show that the increased emission is due to the decrease of intersubunit quenching of tryptophans poorly quenched by disordered intrasubunit hemes. Experiments under hyperbaric conditions may clarify whether high ionic strength favors monomer formation.

4.5. Monomeric subunits

It is the first time that monomeric subunits of hemoglobin have been detected at neutral pH for the natural system. The finding is not totally surprising, especially in view of the extremely small amplitude of this component. Whether these monomers have relevance to the energetics of the conformational attitudes of the system remains to be seen. We can detect only that portion of the monomeric subunits which is in equilibrium with dissociated hemes. This implies the presence of a larger fraction of monomeric subunits in the system.

5. Conclusions

The data presented here are a qualitative overview of the information which can be obtained from time-resolved fluorescence spectroscopy of hemoglobin systems. Regarding the resolution of the species which originate the 'intermediate' lifetimes, spectrophotometry and spectropolarimetry under normal pressure and under hyperbaric conditions will help in resolving the constituent species. Under hyperbaric conditions, which favor heme dissociation, spectrophotometry will measure only the dissociated hemes, while spectropolarimetry will be sensitive to both heme dissociation and inversion. Spectropolarimetry will also monitor modifications of the secondary structure which may result from heme dissociation. These additional data will help in defining the thermodynamic parameters that regulate the species distribution in hemoglobin systems.

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