Resolution of the Lifetimes and Correlation Times of the Intrinsic Tryptophan Fluorescence of Human Hemoglobin Solutions using 2 GHz Frequency-domain Fluorimetry*

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We used 2 GHz harmonic content frequency-domain fluorescence to measure the intensity and the anisotropy decays from the intrinsic tryptophan fluorescence from human hemoglobin (Hb). The tryptophan intensity decays are dominated by a short-lived component which accounts for 35–60% of the total steady state intensity. The decay time of this short component varies from 9 to 27 ps and this component is sensitive to the ligation state of Hb. Our error analyses indicate the uncertainty is about ±3 ps. The intensity decays also show two longer lived components near 0.7 and 8 ns, which are probably due either to impurities or to Hb molecules in conformations which do not permit energy transfer. The anisotropy decays indicate the tryptophan residues in Hb are highly mobile, with apparent correlation times near 55 ps.

There is a considerable interest in the structure and dynamics of hemoglobin which resulted in elucidation of its three-dimensional structure allostERIC properties and “static” models which try to explain the structure-function relationships of the system (1). There is now a desire to understand how the dynamics of the molecule contributes to its functional properties. This problem has been approached by computer simulations, probing the local motions of individual residues (in the picosecond and nanosecond range), and the correlation of these motions with protein conformation and ligand binding (2, 3).

Processes which occur on the picosecond-nanosecond timescale can be studied by fluorescence spectroscopy. While such measurements have always been possible in principle, the time resolution and sensitivity of fluorescence methods have increased considerably within the past several years. In hemoglobin, fluorescence spectroscopic studies have been limited because the emission of either extrinsic or intrinsic probes is strongly quenched by resonance energy transfer to the heme (4, 5). Nonetheless, several laboratories have published careful studies (6–8) which indicate that the intrinsic tryptophan emission from Hb can be detected. Recent time-resolved studies on Hb (9–11) indicate the decay times are 100 picosecond or less, which are consistent with quenching by energy transfer to the heme. In all these studies the authors also detected longer lived components on the nanosecond timescale. The origin of these components is difficult to determine, and, in at least one case (Aplysia myoglobin), has been interpreted as the result of difficult to remove impurities (12). It should be stressed that picosecond timescale decay times are consistent with the values expected for the tryptophan residues, after consideration of the 100-fold quenching produced by the heme. In this report we emphasize interpretation of the picosecond decay times, which we believe are due to the intrinsic tryptophan emission.

In general it is difficult to obtain picosecond resolution of fluorescence decay times. If one uses time-correlated single photon counting, then the pulsed source must have a comparably short pulse width and the detector must have picosecond resolution. The more exotic probe-pump technique circumvents the time response of the detector by using the optical pulse as a gate. However, the sensitivity of this method is low, and the method has only occasionally been applied to protein fluorescence. In this report we used the frequency-domain method (13). The resolution of this technique was recently extended to picosecond processes by extending the frequency range to 2 GHz (14). This was possible by using the intrinsic high frequency harmonics of a picosecond laser source and a microchannel plate detector. Additionally, the sensitivity is high because the excitation source is not attenuated by a number of optical elements, which is necessary for intensity modulation of continuous light sources (13). The GHz instrument was shown to provide reliable measurements of 25 picosecond time delays (14) and has been used to measure correlation times as short as 8 picoseconds (15).

Using this instrument we have found that the emission of tryptophan in extensively purified samples of hemoglobin was largely dominated by lifetimes in the picosecond region which were sensitive to ligation. Longer lived components in the nanosecond range were also present, which accounted for less than 1% of the emitting tryptophans and were not sensitive to ligand binding. We suggest that only the lifetimes in the picosecond range belong to hemoglobin. The correction times detectable in the system indicate rapid mobility and extensive rotational freedom of the tryptophans.

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**The abbreviations used are: Hb, hemoglobin; apohemoglobin, (apoHb) heme-free hemoglobin; χ², reduced χ-square.

MATERIALS AND METHODS

Human hemoglobin was prepared from washed red cells obtained from fresh blood samples donated by the local blood bank. They were hemolyzed in 0.005 M phosphate buffer at pH 7.0, and the stroma were eliminated by filtration through a 0.45 μm pellicon cassette. The protein was concentrated by ultrafiltration through a M, 10,000 pellicon cassette, dialyzed against water, recycled through a mixed-bed resin cartridge for removing polyphosphates and other ions, and stored at -90 °C.
The extent of purification is critical to these experiments. Manipulation of hemoglobin solutions was avoided as much as possible. Pipetting the solutions from one container to another exposes the protein to surface denaturation, which even if minimal may influence the long-lived emission of the samples. For this reason we choose chromatographic procedures which automatically eliminated denatured products, producing samples at the proper concentration (a few milligrams per milliliter) for single dilutions with the necessary buffers. The solutions were stored in the cold and used within a few days.

Oxyhemoglobin was purified by high pressure liquid chromatography through a DEAE-PW5 preparative column (Waters) using a gradient formed by 0.015 M Tris acetate at pH 8 and 0.015 M Tris acetate at pH 7.7 in 0.2 M sodium acetate. The procedure lasted less than 1 h at room temperature. Fig. 1 shows the chromatographic profile of an elution followed at 280 nm. We used the major peak near 35 min, which on rechromatography showed only one single peak in the elution profile.

Deoxygenation of hemoglobin was achieved by diluting the protein with nitrogen-bubbled interface. Na* dithionite was added with a syringe through a serum stopper, to a final concentration of 0.01 mg/ml. Spectrophotometric analyses indicated the complete deoxygenation of the samples.

Carbonmonoxyhemoglobin was obtained by flushing the air phase of a closed fluorimetric cuvette with CO and then gently inverting a few times. The complete saturation of the samples with carbon monoxide was monitored by spectrophotometry.

All measurements were conducted at a protein concentration near 0.2 mg/ml in 0.05 M phosphate buffer at pH 7.0 at 4 °C. Protein concentration was measured spectrophotometrically using $E = 0.868 \text{cm}^{-1} \text{mg}^{-1}$ for the carbonmonoxy derivative at 540 nm. Optical densities were measured using a Cary 14 spectrophotometer.

Steady state fluorescence measurements were performed with a SLM photon-counting spectrofluorometer. Lifetimes and correlation times were measured using a fluorometer operating between 4 and 2000 MHz (14). The modulated excitation was provided by the harmonic content of a laser pulse train with a repetition rate of 3.76 MHz and a pulse width of 5 picosecond, from a synchronously pumped harmonic content of a laser pulse train with a repetition rate of 3.76 MHz and a pulse width of 5 picosecond, from a synchronously pumped KDP crystal. The emitted signals were observed with a microchannel photomultiplier, and cavity-dumped rhodamine 6G dye laser. The dye laser was nearly completely vertically polarized. It was necessary to eliminate this scattered light prior to the detector. Otherwise, the 0 picosecond time delay due to scattered light would corrupt the data and prevent reliable determination of the tryptophan decay times. Also shown in Fig. 2 are the emission spectra recorded through two emission filters, Corning 0–52 and 7–51. The 0–52 eliminates light below 340 nm but transmits longer wavelengths. Since some emission was seen near 450 nm (−−−), we also used the 7–51, which transmits a broad band centered at 360 nm. In this way the Raman scattering was eliminated, along with residual emission above 400 nm. All subsequent measurements were performed using the two filters in the optical path and observing the emission at the resultant maximum of 365 nm.

One possible source of non-Hb fluorescence is heme-free hemoglobin. Small percentages of apohemoglobin could make a major contribution to the total emission. Suppose a solution contains 1% apoHb and that the tryptophan emission is quenched 100-fold in Hb. Then, 50% of the total emission would be from the 1% contaminant. We attempted to detect the presence of apoHb by titrating the solutions with hemin chloride, while monitoring the emission at 365 nm. If the emission was due to apoHb, then titration with hemin should

The frequency-domain anisotropy data were fit to

$$r(t) = \sum \alpha_i e^{-\lambda_i t},$$

where $\alpha_i$ are the pre-exponential factors, $\lambda_i$ the decay times, and $\Sigma \alpha_i = 1.0$. The frequency-domain anisotropy data were fit to

$$r(t) = \sum \alpha_i e^{-\lambda_i t}.$$
result in ligation and quenching. Fig. 3 shows that the decreasing emission followed a simple exponential function, produced by the increasing optical density of the solutions. This argues against the presence of detectable amounts of apohemoglobin, at least in a form capable of binding hemin.

To further demonstrate that the emission is due to tryptophan, we examined the wavelength dependence of the intensity and the steady state anisotropy (Fig. 4). The weak signals prevented a full investigation of the excitation and polarization spectra of our samples. However, the emission intensity decreased as the excitation wavelength was increased from 290 and 300 nm, and the anisotropy increased (Fig. 4). These features are consistent with the spectral properties of tryptophan.

We also examined the emission in the presence of increasing concentration of acrylamide, which is an efficient quencher of tryptophan fluorescence (18,19). It is known that collisional quenching is proportional to the fluorescence lifetime. Hence, acrylamide should selectively quench the long-lived emission. We reasoned that the measured intensity would then contain an increase proportion of the presumed intrinsic Hb fluorescence. If this emission was dominated by scattered light (with a decay time of 0) then the anisotropy in the quenched samples may rise above 0.4 (20). However, the anisotropy rose gradually, and the values remained reasonable (Fig. 5). Additionally, acrylamide was not able to quench more than 50% of the emission, which is consistent

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**Fig. 3.** Effect of hemin chloride on the emission intensity of oxyhemoglobin solutions. Excitation at 295 nm and emission at 365 nm. Both the 0-52 and 7-51 filters were used in the emission path. The emission intensity is shown in arbitrary units, on a logarithmic scale. The total optical density at 295 nm is indicated on the right-hand axis. Other conditions are as in Fig. 2.

**Fig. 4.** Dependence on excitation wavelength of the 365 nm emission intensity and anisotropy of oxyhemoglobin samples. Conditions are as in Fig. 2.

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**Fig. 5.** Static anisotropy of the 365-nm emission of oxyhemoglobin in the presence of increasing quantities of acrylamide. Quenching of fluorescence (○); steady state anisotropy (●). Excitation at 295 nm and emission at 365 nm. The values of F0/F were corrected for inner filtering due to light absorption by acrylamide at 295 nm. Other conditions are as in Fig. 2.

**Fig. 6.** Frequency-resolved intensity decay of oxy- (△), deoxy- (●), and carbonmonoxyhemoglobin (○) at 4 °C. Excitation at 300 nm and emission at 365 nm. For carbonmonoxyhemoglobin the excitation was at 295 nm. The continuous lines were computed using the parameters listed in Table I. The lower panels show the distribution of the residuals. The dotted lines without symbols are the simulated data for an intensity decay with a 1-nanosecond lifetime. Other conditions are as in Fig. 2.
The modulation was not sensitive to these short variations in the short decay time, affecting the frequency responses near 20 picosecond, 0.8 and 8 nanosecond. The picosecond components dominate the decays ($\alpha_t \approx 0.99$) but contribute only 40–55% to the integrated intensities. As expected, quenching with acrylamide did not affect the picosecond lifetime of oxyhemoglobin, but did decrease the decay times and intensities of the longer components.

It is of interest to estimate the uncertainties in the picosecond decay times. If the uncertainties are less than the differences between the various liganded states, then one can interpret the picosecond decay times with respect to the molecular properties of the ligation states. Estimation of uncertainties for the decay times is complex. The decay times are correlated with the other parameters describing the decay, while the usual assumption of nonlinear least squares fitting is the lack of correlation. To circumvent this problem, we examined the $\chi^2$ surface for each of the picosecond decay times (Fig. 7). These curves were constructed by fixing the picosecond decay times at the values on the $x$ axis, while the other parameters were adjusted to minimize $\chi^2$. We believe this method accounts for all possible correlations between the parameters and hence provides an estimate of the maximum uncertainty of the decay times. A very conservative estimate of the uncertainties is given by the standard deviations around the minima of the curves, which are indicated by the horizontal lines in Fig. 7. By this criterion the COHb decay time is distinct from the other two decay times, and it is likely that the oxy and deoxy decay times are also distinct. The uncertainty in these components appears to cover a range of about 3 picosecond.

And finally, we examined simulated data to determine how variations in the short decay time affect the frequency response. The simulated curves were generated using the parameters ($\alpha_t$ and $r_t$) found for oxy Hb (Table I), except for the picosecond correlation time which was varied from 9 to 27 picosecond (Fig. 8). These values bracket the recovered value of 16 picosecond. The modulation was not sensitive to these short decay times (9–27 picosecond), at least to our current measurement limit of 2 GHz. However, the high frequency phase

| Sample | $\tau_1$ | S.D. | $a_1$ | $f_1$ | $x^2$ |
|--------|---------|------|-------|-------|-------|
| Oxy Hb | 0.016   | 0.001| 0.992 | 0.596 | na    |
|        | 0.820   | 0.025| 0.007 | 0.190 |       |
|        | 8.306   | 0.240| 0.01  | 0.274 | 2.8   |
|        | 0.349   |       |       |       | (117.0)' |
| Deoxy Hb | 0.009 | 0.001 | 0.991 | 0.349 |       |
|         | 0.901   | 0.025| 0.008 | 0.226 |       |
|         | 7.479   | 0.270| 0.001 | 0.385 | 3.5   |
|         | 3.5     |       |       |       | (165.8)' |
| CO Hb   | 0.027   | 0.001| 0.985 | 0.394 |       |
|         | 1.118   | 0.037| 0.011 | 0.177 |       |
|         | 7.508   | 0.160| 0.004 | 0.541 | 2.4   |
|         | 2.4     |       |       |       | (90.4)' |
| Oxy Hb 0.5 M acrylamide | 0.016 | 0.001 | 0.995 | 0.578 |       |
|         | 0.660   | 0.100| 0.003 | 0.076 |       |
|         | 3.948   | 0.100| 0.002 | 0.346 | 2.6   |
|         | 2.6     |       |       |       | (14.7)' |

*Excitation at 300 nm and emission 365 nm by Corning 7-51 and 0–52 filters, 0.05 M phosphate buffer at pH 7.0, 4 °C.

†As calculated from the diagonal elements of the covariance matrix (34).

The values in parenthesis are the values of $\chi^2$ found for analyses of the data using two decay times only.

‡Excitation at 295 nm. CO Hb, carbonmonoxyhemoglobin.

Absence of overlap of the values of the various parameters. Analyses based on only two lifetime components gave larger values of $\chi^2$ and an unsatisfactory match between the data and the simulated curves.

In each state the Hb decays show components with decay times near 20 picosecond, 0.8 and 8 nanosecond. The picosecond components dominate the decays ($\alpha_t \approx 0.99$) but contribute only 40–55% to the integrated intensities. As expected, quenching with acrylamide did not affect the picosecond lifetime of oxyhemoglobin, but it did decrease the decay times and intensities of the longer components.

It is of interest to estimate the uncertainties in the picosecond decay times. If the uncertainties are less than the differences between the various liganded states, then one can interpret the picosecond decay times with respect to the molecular properties of the ligation states. Estimation of uncertainties for the decay times is complex. The decay times are correlated with the other parameters describing the decay, while the usual assumption of nonlinear least squares fitting is the lack of correlation. To circumvent this problem, we examined the $\chi^2$ surface for each of the picosecond decay times (Fig. 7). These curves were constructed by fixing the picosecond decay times at the values on the $x$ axis, while the other parameters were adjusted to minimize $\chi^2$. We believe this method accounts for all possible correlations between the parameters and hence provides an estimate of the maximum uncertainty of the decay times. A very conservative estimate of the uncertainties is given by the standard deviations around the minima of the curves, which are indicated by the horizontal lines in Fig. 7. By this criterion the COHb decay time is distinct from the other two decay times, and it is likely that the oxy and deoxy decay times are also distinct. The uncertainty in these components appears to cover a range of about 3 picosecond.

And finally, we examined simulated data to determine how variations in the short decay time affect the frequency response. The simulated curves were generated using the parameters ($\alpha_t$ and $r_t$) found for oxy Hb, except for the picosecond correlation time which was varied from 9 to 27 picosecond (Fig. 8). These values bracket the recovered value of 16 picosecond. The modulation was not sensitive to these short decay times (9–27 picosecond), at least to our current measurement limit of 2 GHz. However, the high frequency phase

**FIG. 7.** Dependence of $\chi^2$ on the picosecond decay times on the picosecond for oxy-, deoxy-, and carbonmonoxyhemoglobin. The dashed line shows the dependence for oxyhemoglobin in the presence of 0.5 M acrylamide. The horizontal line shows the range of values encompassed by the standard deviation of the minima of the curves.

**FIG. 8.** Comparison of the measured frequency response of oxy Hb with simulated data. The lines represent simulated data using the parameters observed for oxy Hb (Table I), except for the shortest lifetime which was varied from 9 to 27 picosecond. The solid dots (⊗) represent the experimental data.

**FIG. 9.** Frequency-resolved anisotropy decay of oxy- (◇), deoxy- (⊗) and carbonmonoxyhemoglobin (○). The continuous lines were obtained using the parameters listed in Table II. Other conditions are as in Fig. 6.
question whether the emission was due to the hemoglobin molecules or to impurities present in the samples.

The anisotropy analyses in Table II assume that a single anisotropy decay. However, an impurity would be a distinct emitting species, and its intensity decay would be associated with its own anisotropy decay. The formalism for analyzing the sum of the anisotropy amplitudes is

\[ \chi^2 = \frac{1}{n} \sum_{i=1}^{n} \left( \frac{\chi_{i,\text{obs}} - \chi_{i,\text{calc}}}{\chi_{i,\text{err}}} \right)^2 \]

which illustrates our ability to recover these values with reasonable precision.

We also examined the frequency-domain anisotropy decays (Fig. 9). The lines show the best fits to the data obtained using two correlation times, and the parameters are summarized in Table II. Fig. 10 is self-explanatory. The tryptophan residues appear to be remarkably mobile. Most of the anisotropy decayed by a 50-60-picosecond correlation time. This emitting species, and its intensity decay would be associated with the 4-fold symmetry axis passing through the center of the iron atom perpendicular to the plane of the tetrapyrole ring. The former are \( z \)-polarized in the direction of the 4-fold symmetry axis passing through the center of the iron atom perpendicular to the plane of the heme. The latter transitions are \( x,y \)-polarized on the plane of the heme and show equal absorption for all directions of the electric vector parallel to the heme plane. For such transitions the heme behaves like a planar, circular absorber.

In deoxy- and carbonmonoxyhemoglobin the transition moments associated with the absorption in the 300-450 nm region are \( x,y \)-polarized, therefore, a tilting of the plane of the heme can justify a different efficiency of energy transfer from tryptophans. Crystallographic studies have given clear indications that a tilting of the heme occurs upon ligand binding transfer from the tryptophan residues to the heme. Using the Forster equation (5), with the assumption that the transition moments of the donor and the acceptor are randomly oriented, one can calculate that transfer occurs with an efficiency of 50% at a distance of 70 Å. Hemoglobin contains three different types of tryptophan residues, one in the \( \alpha \) and two in the \( \beta \) subunits. The distances between heme and the tryptophans vary from 13 and 17 Å. The intersubunits distances are near 35 Å. In view of the 6th power relationship between distance and transfer efficiency, the quenching of tryptophan is expected to be between 100- and 10,000-fold. The lifetime of unquenched tryptophan is near 2 nanosecond (25). Hence, the 10-20-picosecond components we detected imply a modest 100-200-fold quenching. Probably, these quantities represent an average including even shorter lifetimes.

The picosecond decay times were affected by changing the ligation state of the protein. The lifetimes varied from 9 picosecond in deoxyhemoglobin to 16 picosecond in oxyhemoglobin and to 26 picosecond in carbonmonoxyhemoglobin. The sharp curvature functions shown in Fig. 7 indicate very little, if at all, overlap between these values. It should be stressed that these values are very consistent with the values reported by Hochstrasser and Negus (11) for ferric and CO myoglobin, whose intensity decays displayed components of 14 and 26 picosecond, respectively (11). These values imply a different quenching of the tryptophans produced by the oxy-, carbonmonoxy-, or deoxyheme.

The origin of the differences can be either a modification of the overlap integral in the 300-450 nm region, a change of the distance, or a modification of the angular relationships between tryptophan and heme. With regard to the overlap integral, it should be noted that in the 300-400 nm region the absorption spectra of oxy- and carbonmonoxyhemoglobin are practically superimposable. The absorption of deoxyhemoglobin is slightly shifted so that below 350 nm is lower than that of the liganded derivatives, while above 350 nm is higher, therefore compensating the areas in the overlap integral. In the 400-450 nm region, carbonmonoxyhemoglobin has the highest values of extinction coefficient, while its picosecond lifetime is three times longer than that of deoxyhemoglobin. Thus, the origin of the different efficiency of transfer must be either in the distance or in the angular relationships between tryptophans and heme or both.

The distance is probably a minor factor. At high levels of quenching, the transfer efficiency is not sensitive to modifications of 1 Å or less (26) and would not explain the 3-fold change of the picosecond lifetime in carbonmonoxy- and deoxyhemoglobin. A major role is most likely played by the angular relationships. As described by Eaton and Hofrichter (27), for reasons of symmetry, the transition moments associated with the absorption of the light in the heme are polarized either perpendicular or parallel to the plane of the tetrapyrole ring. The former are \( z \)-polarized in the direction of the 4-fold symmetry axis passing through the center of the iron atom perpendicular to the plane of the heme. The latter transitions are \( x,y \)-polarized on the plane of the heme and show equal absorption for all directions of the electric vector parallel to the heme plane. For such transitions the heme behaves like a planar, circular absorber.

In deoxy- and carbonmonoxyhemoglobin the transition moments associated with the absorption in the 300-400 nm region are \( x,y \)-polarized, therefore, a tilting of the plane of the heme can justify a different efficiency of energy transfer from tryptophans. Crystallographic studies have given clear indications that a tilting of the heme occurs upon ligand binding
the pico- and nanosecond lifetimes. These values are uncertain because the amplitudes are small (Table II) and because they were determined primarily by the nanosecond timescale emission. This emission was probably contaminated with the emission from non-Hb impurities. Nonetheless, these values are comparable to those obtained in this laboratory for extrinsically labeled Hb and its isolated subunits (32, 33).

In summary, our data provide reliable measurements of the intrinsic tryptophan decay times in Hb. These values are sensitive to the ligation state and may provide a sensitive probe for the structure and dynamics of Hb.

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