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INTRINSIC FLUORESCENCE EMISSION OF INTACT OXY HEMOGLOBINS

Rhoda Elison Hirsch, R. Suzanne Zukin and Ronald L. Nagel
Department of Medicine and Department of Biochemistry
Albert Einstein College of Medicine
Bronx, N.Y. 10461

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SUMMARY - Fluorescence has not been previously detected in intact hemoproteins. We have been able to measure significant fluorescence emission in purified oxy HbA using front-face fluorometry. The excitation maximum (293 nm), the emission maximum (325 nm) and the fluorescence spectra of Hb Rothschild (β 37 Trp → Arg) allows us to conclude that β 37 Trp is primarily responsible for the fluorescence signal of HbA. We propose that this intrinsic fluorescence of hemoglobin may be used as a probe to study conformational changes in hemoglobin and possibly other heme-containing proteins.

It has been generally accepted that heme-proteins exhibit no detectable tryptophan fluorescence. Characterizations of heme proteins by tryptophan fluorescence analysis have not been attempted because total quenching by heme groups was assumed (1,2). Such quenching occurs when conventional right-angle optics are used for fluorescence measurements. With the application of front-face fluorometry, however, we have been able to detect a significant fluorescence emission signal from intact, chromatographically pure hemoglobins. With the use of various tryptophan mutants of hemoglobin, we can make tentative assignments as to the specific amino acid residues from which the signal is emanating. The fluorescence of proteins containing tryptophan and tyrosine can be used as an important probe in understanding conformational characteristics of proteins, protein-protein interactions, and arrangement of proteins within membranes (3).

METHODS

Hemolysates of red blood cells of normal donors (HbA), abnormal donors (Hb RC; HbH Disease), and fetal cord blood (HbF) were prepared by the method of Drabkin (4) with small modifications. HbA was separated into purified components by chromatography on columns of DE-52 developed with 0.05M tris-HCl buffer, pH 8.1 and eluted by addition of 0.5M NaCl. Hb RC was a generous

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gift of Drs. G. Gacon and D. Labie (Institut de Pathologie Moleculaire, Paris, France) and purified as described by Gacon et al. (5). HbH was isolated by column chromatography on CM23, pH 6.6, 0.01M phosphate buffer. HbF was separated on Bio-Rex 70, equilibrated with pH 6.9, 0.05N sodium phosphate buffer. All samples were concentrated, dialyzed, and equilibrated against potassium phosphate buffer (pH 7.35, .05M) and stored in liquid nitrogen. The desired concentrations were obtained by dilution with the potassium phosphate buffer. The hemoglobins were studied in the oxy-liganded state.

Fluorescence measurements were made on the Perkin-Elmer MPF-3 recording fluorescence spectrophotometer with thermostatically regulated cell holders (25°C). Slit widths for excitation and emission sources were open 6 nm. Front-face fluorescence measurements were performed using either the Perkin-Elmer triangular cell (model #010-2112) with a cell holder modified for front-face optics by Dr. Zaharia Hillel (Albert Einstein College of Medicine, Bronx, N. Y.); or, using the front-face cell and adaptor block designed by Eisinger and Flores (6) and manufactured by Aviv Associates (Lakewood, N.J.).

RESULTS

Figs. 1 and 2 show the excitation and emission spectra of the intrinsic protein fluorescence of various hemoglobins. Front-face optics were used and exciting light was at either 280 nm or 296 nm (Figs. 1 and 2). The hemoglobins are observed to fluoresce in increasing magnitude in the order: HbH >> HbF > HbA > HbRC. At a concentration of .07mM hemoglobin, the emission of HbH is approximately 9 times greater than that of HbA (excitation wavelength = 280 or 296 nm). In the case of HbF, the fluorescence emission is approximately 1.5 - 2 times larger than that of HbA (depending upon the exciting wavelength). HbRC, on the other hand, fluoresces less than HbA when excited at 280 nm. When excited by light at 296 nm, the emission of HbRC is observed as a shoulder superimposed on apparently non-specifically emitted fluorescence without an emission maximum. In addition, HbRC exhibits an emission maximum at 310 nm (uncorrected) when excited by light at 280 nm.

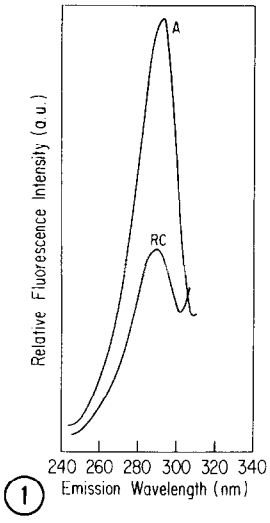
TABLE I

RELATIVE INTENSITIES OF FLUORESCENCE FROM INTACT HEMOGLOBINS

Hb Variant	Chain Composition	Tryptophans (*)	Total # of Tryptophans	Excitation λ, 280 nm: Emission max. (nm)	Excitation λ, 280 nm: Ratio of Intensities Hb variants: Hb A	Excitation λ, 296 nm: Emission max. (nm)	Excitation λ, 296 nm: Ratio of Intensities Hb variant: Hb A
Hb A	$\alpha_2\beta_2$	α14(2) β15(2) β37(2)	6	325	1.0	325	1.0
Hb H	β_4	β15(4) β37(4)	8	325	8.8	325	9.1
Hb F	$\alpha_2\gamma_2$	α14(2) γ15(2) γ37(2) γ130(2)	8	325	1.5	325	2.0
Hb RC	$\alpha_2\beta_2^{RC}$	α14(2) β15(2)	4	310	0.7	no max.	-

Concentration of the hemoglobins are .07 mM hemoglobin. Temperature is maintained at 25°C. Slit widths for both excitation and emission light are 6nm. The hemoglobin solutions consist of .05M potassium phosphate buffer at pH 7.35. All solutions are oxygenated. The values presented here are averages of several independent measurements.

*Figure between parenthesis corresponds to the number of each Trp residue per tetramer.



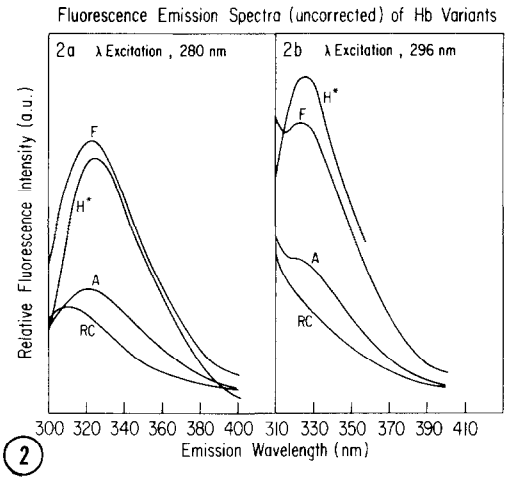


Fig. 1. Excitation Spectra (uncorrected) of HbA and HbRC. The emission wavelength is constant at 325 nm. The concentration of HbA is .16 mM while the concentration of HbR is .06 mM. All other conditions are as described in Table 1. The excitation spectra of HbH and HbF are the same as that of HbA except for the relative intensity.

Fig. 2. Emission spectra (uncorrected) of hemoglobin variants. Conditions are as described in Table 1. HbH (Curve 1); HbF (Curve 2); HbA (Curve 3); HbRC (Curve 4).
*Note that the sensitivity of the recorder for HbH is 1/3 less than that recorded for the other hemoglobins.
2a) The excitation wavelength is 280 nm.
2b) The excitation wavelength is 296 nm.

This corresponds to a blue shift of about 15 nm when compared to the emission maxima of 325 nm of HbA, HbF and HbH (Fig. 2; Table 1).

Addition of 1M KI to the hemoglobin solutions does not result in any detectable fluorescence quenching.

Fig. 3 illustrates the effect of hemoglobin concentration on fluorescence emission. The lack of dependence of hemoglobin fluorescence upon protein concentration (above approximately .16mM hemoglobin) is expected when using front-face optics with samples of high absorbance (6).

A linear increase in fluorescence emission intensity is observed with an increasing proportion of Met-Hb. The fluorescence intensity of oxy HbA increases by 52% when converted to 100% met-HbA.

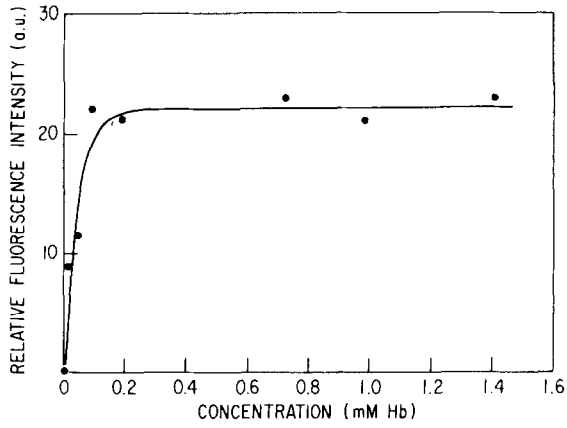


Fig. 3. Concentration dependence of HbA on fluorescence emission. Exciting wavelength is 280 nm. Conditions are described in Table 1.

DISCUSSION

The present study shows that oxy HbA exhibits a detectable fluorescence emission spectrum when front-face fluorescence optics are used. Several findings indicate that the HbA fluorescence is that of protein tryptophans. First, the excitation and emission peaks obtained are typical of tryptophan residues in proteins (Figs. 1 and 2) (7). Secondly, the use of a longer excitation wavelength (296 nm) results in the predicted increase of fluorescence intensity for proteins containing tryptophan. Thirdly, when HbA is excited with 296 nm light (a wavelength at which tryptophans are the only amino acids which are excited (7)), a significant peak (λ max = 325 nm) is observed (Fig. 2). The intensity of this peak is different for HbA, HbF, HbH and HbRC, proteins which differ with respect to their tryptophan content, a finding which allows us to assign the emission spectra to particular tryptophan residues in the amino acid sequence of these hemoglobins.

At an excitation wavelength of 296 nm, HbRC clearly exhibits a lower intensity and no distinct maximum in comparison to HbA. Unless there is an unlikely concommitant conformational change in HbRC involving α14 Trp and β15 Trp, this data suggest that the β37 Trp residue is primarily responsible for the fluorescence signal observed in HbA.

At the same excitation wavelength (296 nm), the intensity of fluorescence of HbF is almost double that of HbA. As HbF contains an additional tryptophan at position δ130, it is reasonable to assume that the increase in fluorescence is derived from this residue, particularly in light of the demonstration by Frier and Perutz (8) that HbA and HbF are nearly identical in conformation.

Finally, the emission intensity of HbH (λ excitation = 296 nm) is significantly higher than would be predicted on the basis of the additional two β37 Trp residues/tetramer in this variant. The explanation of this discrepancy must lie in the different conformational states that β-chains adopt in a homotetramer as compared to their conformational state in a heterotetramer.

Let us now turn to the data obtained with an excitation frequency of 280 nm. It has been previously shown that tyrosine is responsible for fluorescence maxima observed at 303 or 313 nm in proteins (3). When HbRC is excited by light at 280 nm (known to excite both tyrosine and tryptophan residues (7)), the emission maximum is 310 nm (uncorrected) and consequently, blue-shifted by 15 nm from that of HbA. This anomaly probably results from the environmental change of β35 Tyr in HbRC. The mutation in HbRC results in an amino acid sequence change (5)

	35 36 37	
from	-Tyr-Pro-Trp-	β^{A}
to	-Tyr-Pro-Arg-	β^{RC}

If we assume that in HbA, β35 Tyr is not fluorescent as a consequence of quenching (3) by the β37 Trp, the mutation in HbRC will release this constraint.

The 4 nm difference seen in the excitation maximum of HbRC (289 nm, uncorrected) with respect to that of HbA (Fig. 1) further suggests that tyrosine is contributing to the fluorescence of this mutant. The amino acid tyrosine typically exhibits a long wavelength excitation maximum (276 nm) which is 4 nm shorter than the excitation maximum of tryptophan (280 nm) (9). From the aforementioned, it is suggested that α14 Trp and β15 Trp contribute minimally to the fluorescence observed in oxy HbA, HbF and HbRC.

Crystallographic evidence is compatible with our contention that β37 Trp is mainly responsible for the fluorescence emission difference observed between HbA

and HbRC. This residue is hydrogen bonded to β94 Asp in deoxy HbA, but this bond is probably broken in the liganded state (10). These conformational events involving β37 Trp opens the possibility of utilizing its fluorescence spectra as a probe of the R → T transition. We are now actively exploring this possibility.

The finding of fluorescence emission maxima of 325 nm for HbH, HbF and HbA may suggest that the fluorescent tryptophans are located internally, in a low-polar hydrophobic environment, according to the interpretation of Burstein (7). According to that study nevertheless, the maximum of fluorescence is at 330 nm for tryptophans buried amongst hydrophobic residues. The 5 nm difference which we observe may be a result of increased scatter occurring with front-face optics and/or inner-filter effects. The observation that the fluorescence emission of hemoglobins cannot be quenched by 1M KI further suggests that the fluorescent amino acid residues are located in an internal, hydrophobic environment (11). The assigned residue, β 37 Trp, complies with these characteristics (10).

In summary, this study demonstrates that the quenching of the fluorescence of the aromatic amino acids by the hemes of a series of hemoglobin variants is incomplete as intrinsic fluorescence in hemoglobin is detectable. The intrinsic fluorescence of hemoglobin should provide a valuable probe to study conformational changes in hemoglobin and other heme-containing proteins.

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