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STEADY-STATE FLUORESCENCE EMISSION FROM THE FLUORESCENT PROBE, 5-IODOACETAMIDOFLUORESCEIN, BOUND TO HEMOGLOBIN

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In the past, fluorescence emission from an extrinsic fluorophore bound to heme-proteins would only be studied with the removal of the heme since fluorescence from the flurophore could not be detected using right-angle optics. Using front-face fluorometry, a significant steady state emission signal originating from the probe bound to hemoglobin is detected. This is the first report of the detection of extrinsic fluorescence of a probe bound to a heme-protein. We also demonstrate that the extrinsic probe, 5-iodoacetamidofluorescein, is covalently bound to hemoglobin, specifically at β93 Cysteine. Ligand binding results in a change in the fluorophore fluorescence intensity as predicted by hemoglobin crystallographic studies. Efficiency of energy transfer measurements are made.

Extrinsic fluorescent probes are bound to proteins in order to monitor conformational changes, ligand interactions, and to estimate intermolecular and intramolecular distances (1,2). With heme-proteins, the high extinction coefficient of absorption coupled with the use of right-angle optics, typically used in a standard fluorometer, prohibits the detection of significant emission by the bound fluorophore. Thus, in heme-proteins bound with an extrinsic fluorophore, quenching by the hemes of the extrinsic fluorescence emission signal is the usual means by which the interaction is studied (3). Alternatively, the heme is removed and the extrinsic fluorophore emission is studied in the apoprotein (4,5). However with the use of front-face optics, we demonstrate that a significant steady-state fluorescence signal originating from the extrinsic probe, 5-iodoacetamidofluorescein (5-IAF), bound to hemoglobin can be detected. Furthermore, we show that (a) fluorescein is specifically bound to β93 Cys, (b) a conformational change occurs in the molecule upon binding to fluorescein; (c) a local conformational change occurs upon ligand binding as

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predicted by hemoglobin crystallographic studies (6); and (d) measurements of efficiency of energy transfer can be made.

MATERIALS & METHODS

Preparation of Human Hemoglobin solutions. Hemolysates of red blood cells of human donors were prepared according to the method of Drabkin (7) with small modifications. HbA or HbS was separated into purified components by chromatography on columns of DE-52 (Whatman) developed with 0.05M Tris-HCl buffer, pH 8.1,eluted by addition of 0.5M NaCl and further puified and equilibrated on Sephadex G-200 columns, pH 7.35, .05M phosphate. All samples were concentrated, dialyzed, and equilibrated against potassium phosphate buffer and stored in liquid nitrogen. Hemoglobin purity was determined by starch-gel and cellulose-acetate electrophoresis. Saturated helium was used to deoxygenate the hemoglobin solutions which was judged complete by absorption spectroscopy (8).

Binding of 5-IAF to Hemoglobin. The dye, 5-IAF (Molecular Probes, Eugene, Oregon), was experimentally attached to purified Hb A or S by described procedures (9) in 50 mM potassium phosphate buffer, pH 7.35, in excess of 5:1 5-IAF:heme for 3 hours in the cold. Separation of the free dye from the bound dye was done by column chromatography on Sephadex G-25, equilibrated with the aforementioned buffer. The sample was concentrated in an Amicon concentrator. In order to ensure separation of the sample from the free dye, membrane dialysis was also performed with the hemoglobin bound dye preparation. Complete and covalent modification was shown by starch-gel electrophoresis (10). (See Results & Discussion).

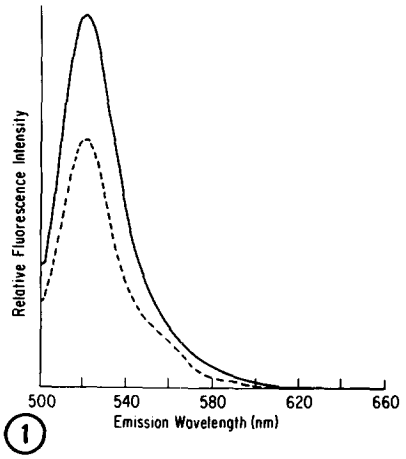
Fluorescence Measurements. Front-face fluorescence measurements are made on a Perkin-Elmer 650-10S spectrofluorometer with the Perkin-Elmer front-face accessory as described in (11,12).

Oxygen equilibrium measurements are made using an Imai cell in conjunction with a Cary 17 spectrophotometer as described in (13).

RESULTS & DISCUSSION

We have detected a significant fluorescence spectrum of fluorescein bound to hemoglobin (Hb-AF) using front-face fluorometry (Fig. 1). In the past, it was assumed that the fluorescence of any extrinsic probe bound to hemoglobin would be quenched by the heme moieties. Right-angle optics, typically used in standard fluorescence measurments, cannot detect significant fluorescence of an extrinsic fluorescent probe bound to hemoglobin. Our observation of significant fluorescence with front-face optics (14) indicates that total quenching by the hemes does not occur and that the lack of fluorescence with right-angle optics is a result of inner-filter effects(11,12,15-17).

Starch-gel electrophoresis (Fig. 2) and extensive dialysis (3 days) of an aliquot of the hemoglobin-AF solution both demonstrate that the probe is covalently bound to hemoglobin. The migration of hemoglobin on the



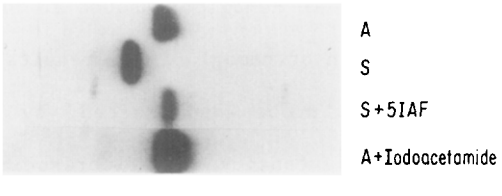


Fig.1. FLUORESCENCE EMISSION SPECTRA OF LIGANDED AND UNLIGANDED HbA-AF. The fluorescence of HbA-AF (1g%) in 0.05M potassium phosphate buffer, pH7.35 at 25 C. Emission is detected by front-face optics as described in Materials & Methods. Excitation light is 480 nm. Both excitation and emission slit widths are 6 nm. Solid line (——), deoxygenated HbA-AF; broken line (----), oxygenated HbA-AF.

Fig.2. STARCH GEL ELECTROPHORESIS. Gel is stained with O-tolidine. Note that HbS-AF results in one band at a position different from unmodified Hb S. HbA-AF (not in this gel) also results in one band at a position different from unmodified HbA.

starch-gel exhibits a single band at a site different from that of the unmodified hemoglobin. After 3 days of dialysis, the surrounding buffer does not show any significant fluorescence. The starch-gel also demonstrates that hemoglobin-AF and hemoglobin covalently modified with iodoacetamide migrate exactly the same. This strongly suggests that 5-IAF and iodoacetamide have affected the hemoglobin similarly. Iodoacetamide has previously been shown to modify hemoglobin specifically at β93 Cys(18). A titration of these modified hemoglobins with PCMB results in no binding by PCMB which leads us to conclude that β93 Cys is the specific site of modification by 5-IAF.

Our oxygen-equilibrium studies show that when hemoglobin is modified with 5-IAF, the oxygen affinity is significantly increased: the p50 is 0.9 mm Hg as compared to 11.2 mm Hg for unmodified hemoglobin. This implies that a conformational change in the hemoglobin occurred upon binding with 5-IAF.

In HbA-AF, the emission maximum of the intrinsic hemoglobin emission (exc. 280 or 296 nm) remains the same as that of the unbound hemoglobin (15,16), suggesting that the conformational change is localized to the site

of binding, β93. A general conformational change would be expected to result in changes in the tryptophan and/or tyrosine fluorescence emission maximum (11,12,17).

A comparison of the fluorescence spectrum of the extrinsic probe in the presence and absence of oxygen (Fig. 1) also suggests a local conformational change in hemoglobin bound to fluorescein occurs upon ligand binding. Deoxygenation of hemoglobin-AF results in a 50% increase in the emission of the extrinsic probe when excited by light at 480 nm. An increase in the fluorescence intensity of fluorescein (emission maximum, 520 nm) implies that the probe is in a more hydrophobic environment (19); hence, we conclude that β93 Cys is now less exposed upon deoxygenation. This is in agreement with known structural changes of β93 Cys upon deoxygenation (6).

In a previous report, we have shown that the intrinsic fluorescence of hemoglobins can be detected with front-face fluorometry (11,12,15-17) and that the signal is primarily emanating from β37 Trp (15,20). Energy transfer from tryptophan to fluorescein has been documented in other proteins (9,21). Resonance energy transfer in hemoglobin between tryptophan and fluorescein is indicated in this study by (a)partial quenching of the intrinsic fluorescence of hemoglobin with bound probe; and (b)excitation (at 280 or 296 nm) of tryptophan in HbA-AF or HbS-AF results in an emission peak at 520 nm, characteristic of bound 5-IAF. Fluorescence emission at 520 nm (exc. 280 or 296 nm) is not observed with unbound hemoglobin. The efficiency of energy transfer from the tryptophan to the fluorescein is calculated by comparing the relative intensity of the intrinsic fluorescence of tryptophan (exc.296 nm), with and without 5-IAF bound to the hemoglobin according to the following relationship (1,22):

$$E = 1 - (Fo/F) \qquad \text{Eq. 1}$$

where Fo is the intensity of the fluorescence emission with bound probe and F is the intensity of emission without bound probe. Using Eq. 1, the efficiency of energy transfer is calculated to be 0.32.

In Förster's theory of energy resonance transfer (22), the transfer efficiency is related to the distance between the donor and acceptor, r, by (1,22):

$$E = \frac{R_o^6}{R_o^6 + r^6} \qquad \text{Eq. 2}$$

where, $R_o = 9.79 \times 10^3 \, (K^2 n^{-4} \phi_d \, J)^{1/6}$ (in Å) Eq. 3

To calculate the distance between the fluorescent tryptophan and the bound fluorophore, we have used the Förster relationships (Eqs. 1-3), where K^2 is the orientation factor for dipole-dipole transfer; ϕ_d is the quantum yield of the donor in absence of transfer; n is the refractive index of the medium; and J, the spectral overlap integral which can be measured for tryptophan and fluorescein (21).

As a first approximation, we have used the quantum yield of hemoglobin calculated by Alpert and colleagues (23,24) at an excitation wavelength of 280 nm (which excites both tyrosines and tryptophans): 1.34. Although these authors noted that the quantum yield for tryptophan alone, obtained by excitation at 296 nm (25), could not be measured because of the low yield at that wavelength, we find that this value gives us an adequate approximation since the efficiency of transfer determined at 280 or 296 nm results in a similar estimation of r. Assuming the usual value for the other Forster terms (26), we obtain a calulated distance of 2Å (approximated to the nearest whole Ångstrom), as a lower limit, between the bound fluorophore and the emitting tryptophan. The model of hemoglobin, derived from x-ray crystallography (27), shows a distance on the order of 7Å between β37 Trp and β93 Cys. Using 1.4Å as the carbon-carbon bond angle distance, the size of fluorescein-acetamide may be estimated as a minimum of 9x10Å. Considering the size of the bound probe, the distance between β37 Trp and β93 Cys, the likelihood of a small contribution of fluorescence emission from α14 and β15 Trp (28), and the numerous assumptions involved in the Förster equation, along with our oxygen-binding studies showing that the structure of hemoglobin has been altered, probably at the site of binding (β93), then the

calculated distance of 2Å is not only within reason as a relative approximation of the lower limit, but may also indicate how the molecule has been altered by this covalent modification. Future studies are planned with fluorescent probes bound to tryptophan mutants of hemoglobin in order to determine the significance of this distance calculation.

The ability to detect the fluorescence of probes bound to intact hemoglobin has applicability in the study of polymerization of sickle-cell hemoglobin and other aggregating hemoglobins. Such studies can parallel investigations of polymerizing microtubules with fluorescent probes (29) which should provide information about nucleation size during polmerization. Minimum gelling concentrations, determined by the method of (30), demonstrate that HbS polymerization is not significantly altered with bound probe: 26.1 g% for deoxy HbS-AF vs. 23.0 g% for deoxy HbS.

In conclusion, we have demonstrated that (a) the steady- state fluorescence emission of a probe bound to hemoglobin can be detected using front-face optics; (b) 5-IAF is covalently bound to β93 Cys, resulting in an altered hemoglobin structure, (c) efficiency of energy transfer measurements may be made; and (d) binding of probes to hemoglobins may be useful in the study of polymerizing or aggregating hemoglobins.

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