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THE DETECTION OF HEMOGLOBIN DIMERS BY INTRINSIC FLUORESCENCE

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We have found that the intrinsic fluorescence emission maxima of oxy, met, and cyanmet hemoglobins have a concentration dependent shift to longer wavelengths. For oxy-hemoglobin, this effect is increased in the presence of 3M NaCl. At the protein concentrations studied, these liganded hemoglobins undergo dimerization. In contrast, horse-heart met myoglobin (which is a monomer), and deoxy Hb A and Hb Beth Israel (that have greatly decreased dissociation constants), exhibited a significantly smaller shift in fluorescence maxima. We conclude that hemoglobin dimers exhibit a bathochromic shift with respect to the tetramer. This shift is probably due to the increase in surface exposure of β 37 Trp that occurs during hemoglobin dimerization.

The intrinsic fluorescence of hemoglobins has been shown to be sensitive to tryptophan content (1) and conformational changes at the $\alpha_1\beta_2$ interface upon ligand binding (2,3,4). We suggested that the intrinsic fluorescence of Hb A originated from the β37 Trp residue located at the $\alpha_1\beta_2$ interface. The location of β 37 Trp at the $\alpha_1\beta_2$ interface, known to undergo conformational changes upon ligand binding (5), explained the decrease in fluorescence which we observed upon oxygen binding. Our results of fluorescence changes upon ligand binding and the suggested source of the emission were since confirmed by Itoh et al. (4). Since the surface area of residues involved at the $\alpha_1\beta_2$ contact site become more exposed upon dimerization (6), one can predict that hemoglobin dimers should exhibit differences in fluorescence properties. In this report, we show that the fluorescence of hemoglobin dimers are different from that of the tetramer, most probably due to the increased exposed surface area of β37 Trp.

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MATERIALS AND METHODS

Hemolysates of red blood cells of normal donors (Hb A) were prepared by the method of Drabkin (7) with small modifications. Hb A was separated into purified components by chromatography on columns of DE-52 (Whatman) developed with .05M tris-HCl buffer, pH 8.1, and eluted by addition of 0.5M NaCl and further purified on Sephadex G-200 columns. Hb Beth Israel was isolated by column chromatography on Bio Rex 70 as described in (8). All samples were concentrated, dialyzed, and equilibrated against potassium phosphate buffer (pH 7.35, .05M) and stored in liquid nitrogen. Hemoglobin purity was determined by SDS gels and cellulose-acetate gels. The desired concentrations were obtained by dilution with potassium phosphate buffer, .05M.

Hb A was deoxygenated in the cold by flushing with nitrogen gas and/or using a nitrogen-vacuum flushed atmosphere. Dilutions with deoxy buffer were done anerobically. Experiments were rejected if the met value was greater than 5% since at low dilutions, the presence of met-Hb (known to fluoresce twice as much as oxy Hb A (1)) greatly interferes with the fluorescence spectrum. The unliganded or liganded state was determined by absorption spectrophotometry using a Cary 17. Met Hb A was prepared in a 1.3 molar excess of potassium ferricyanide and then separated on a Sephadex G-25 column, pH 7.35, potassium phospnate, .05M to remove the reagent. Cyanmet hemoglobin was prepared by mixing a 1.3 molar excess of potassium ferricyanide and 2.5 molar excess of potassium cyanide. The reagent was then separated from the liganded hemoglobin on a G-25 Sephadex column.

Horse-heart met-myoglobin solutions (a generous gift of Drs. Jack Peisach and Jonathan Wittenberg) were prepared by Dr. Richard Magliozzo by dissolving the appropriate amount of myoglobin crystals (2x crystallized, Pentex, Inc., Kankakee, Ill.) and rechromatographing on CM 52, according to the methods of Wittenberg and Wittenberg (9). Samples were concentrated and dialyzed against .05 M Tris-HCl, pH 8.2 with 0.2 M KCl or phosphate buffer, pH 7.35, .05 M. Fluorescence emission maxima were similar in both buffers.

It is well known that the tetramer-dimer equilibrium of hemoglobin is shifted to the dimer state by dilution and/or the addition of high salt concentrations (for a review, see (10)). Following Herskovits et al. (11), we used diluted solutions of hemoglobin in 3M NaCl (Baker reagent grade) to shift the equilibrium to dimers. Percent dimerization is calculated by the equation of Herskovits, Cavanagh, San George (11):

$$\alpha = \left[K_D M_4 \ (1 + (16c/K_D M_4))^{1/2} - K_D M_4\right] / 8c$$

where the K_D is the dissociation constant in molarity, M_4 is the molecular weight of the tetramer (64,500), c is the concentration in g/l.

Fluorescence spectra were recorded on a Perkin-Elmer 650-10S recording fluorescence spectrophotometer at ambient temperature. Front-face fluorescence measurements were made using the Perkin-Elmer front-surface accessory specific for the 650-10S spectrophotometer.

RESULTS AND DISCUSSION

We showed in our earlier report (1) that the use of front-face fluorometry results in emission intensity increases with the concentration of hemoglobin up to .16 mM tetramer or .62 mM heme, whereupon the intensity is no longer concentration dependent. This characteristic of front-face fluorometry is

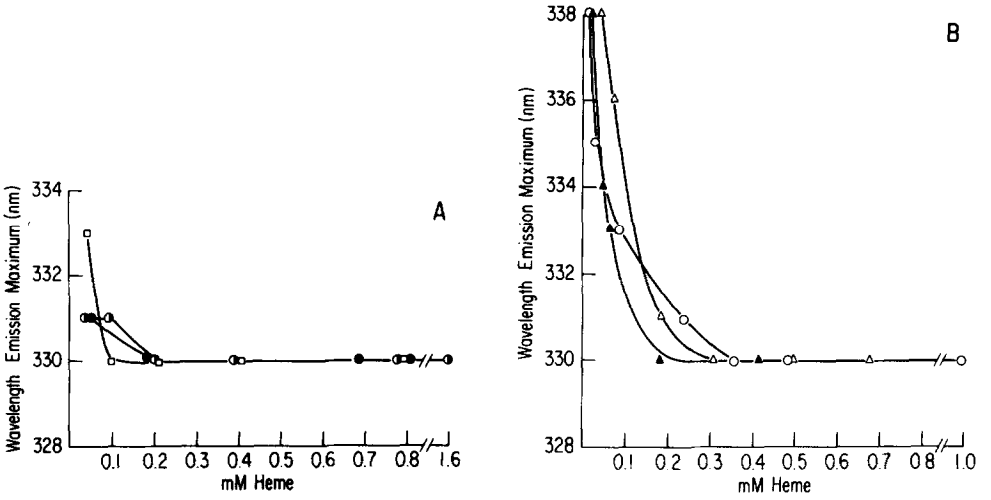

FIG. 1. Concentration dependence of heme-proteins on the emission maximum. Excitation wavelength is 280 nm. Conditions are described in Materials and Methods. Slit widths for excitation and emission sources were open 5 nm. The relative wavelengths are accurate to ± 1 nm. The following symbols denote the respective heme proteins: 1a. ●, deoxy Hb A; ◑, oxy Hb Beth Israel; □, horse-met myoglobin. 1b. ○, oxy Hb A; △, met Hb A; ▲, cyanmet Hb A.

discussed in detail by Eisinger and Flores (12). In the observations presented here (Fig. 1), it is apparent that as the concentration of hemoglobin decreases below .31 mM heme (.5 g%), emission maximum shifts to longer wavelengths, when excited by light at 280 nm. The maximum shift observed amounts to Δ8 nm (± 1 nm) towards longer wavelengths. Excitation of these hemoglobin solutions at concentrations below .31 mM heme (0.5 g%) with light at 296 nm did not result in significant spectra using a Perkin-Elmer 650-10S.

The relative measurements (uncorrected spectra) reported here have emission maxima (wavelength) that are partially a function of properties of the instrument. Thus, in our earlier reports, the uncorrected emission maxima occurred at 325 nm for oxy-Hb A (1 g%) (.62 mM heme) using the Perkin-Elmer MPF-3 rather than 330 nm (.62 mM heme) in the Perkin-Elmer 650-10S used in this present study.

The effects of dimerization on the emission maximum begin to occur below .31 mM heme and resulted in the maximal observed shift at .012 mM heme, within the limitations of our instrument. According to Konev (13) and

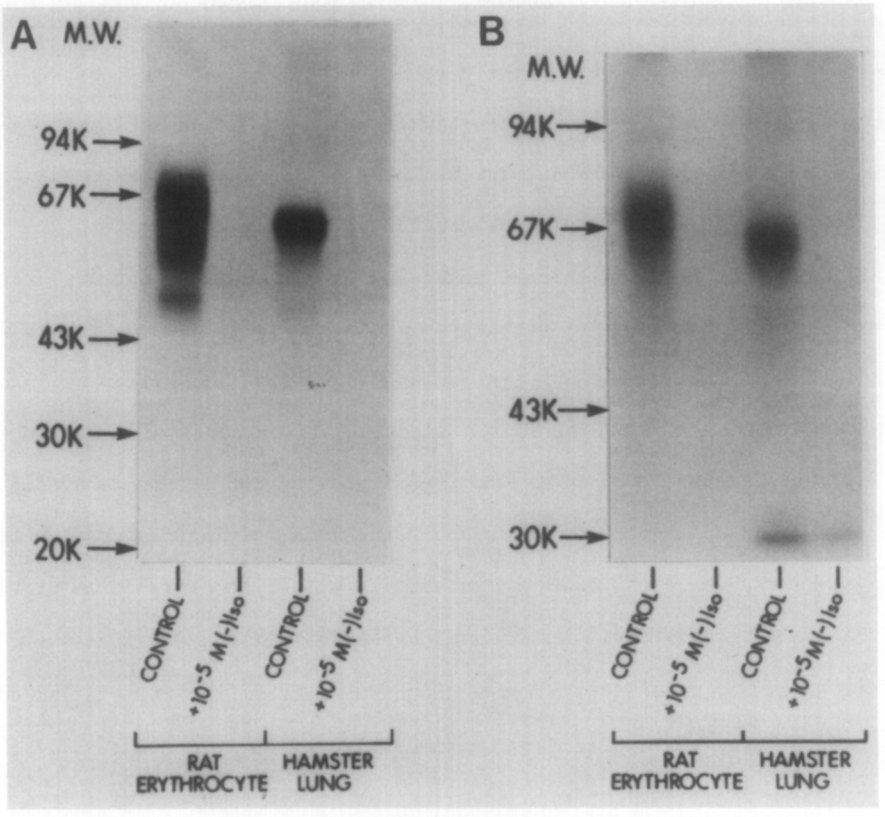

Figure 1 - Photoaffinity labeling of hamster lung and rat erythrocyte β_2-adrenergic receptors with [^{125}I]pABC. Aliquots of each membrane preparation were first labeled in the presence or absence (control) of 10^{-5} M (-)isoproterenol and then solubilized in SDS buffer with (A) or without (B) 5% β-mercaptoethanol as described in Methods. The samples were then electrophoresed on a 8% acrylamide gel. The molecular weights shown (MW) are x 1000 (K) and were determined with iodinated protein standards. (-)Iso, (-)isoproterenol.

presence of 10^{-5} M (-)isoproterenol and electrophoresed under reducing conditions (5% β-mercaptoethanol), the predominant specifically labeled peptides have M_r of 64,000 and 65,000 respectively (see Figure 1A). We have previously demonstrated that the smaller M_r peptides are likely proteolytic products of the major (∿ 60,000) M_r peptide and that their relative proportions can be altered by proteinase inhibitors (particularly EDTA and leupeptin) (2,3). All these peptides have been shown to display all the appropriate pharmacologic specificity and stereospecificity characteristic of a β_2-adrenergic receptor (2,3). When aliquots of these same [^{125}I]pABC labeled membranes are solubilized (5-10% SDS) under non-reducing conditions

TABLE I

DEPENDENCE OF THE FLUORESCENCE EMISSION MAXIMUM IN THE PRESENCE AND ABSENCE OF 3M NaCl

Heme Protein	Conc. (heme)	Fluorescence Emission Maxima (nm) No Salts	3M NaCl	Δnm in presence of salts
Horseheart Mb	.12 mM	330	331	+1
Hb Beth Israel	.04 mM	330	330	0
Deoxy Hb A	.06 mM	330	332	+2
Oxy Hb A	.06 mM	335	339	+4
Met Hb A	.014 mM	337	336	-1
Cyanmet Hb A	.05 mM	335	336	+1

Dependence of the Fluorescence Emission Maximum in the Presence and Absence of 3M NaCl. The excitation wavelength is 280 nm. Slit widths for excitation and emission sources were open 6 nm. Experimental conditions are described in Materials and Methods. The data for oxy, deoxy, met and cyanmet Hb A and myoglobin are each an average of 2 - 5 experiments. Hb Beth Israel was observed as a series of experimental points; however, only 1 preparation was available to us because of the rarity of this mutant.

results show an absence of shift to longer wavelengths upon dilution or in the presence of 3M NaCl (Fig. 1 and Table I).

Horse heart met myoglobin also does not exhibit a comparable shift to longer wavelengths upon dilution (Fig. 1). Furthermore, 3M NaCl has no effect on the emission maximum, as expected for a monomer (Table I). The small shift observed for myoglobin (Fig. 1) may be due to conformational differences at low concentrations or the elimination of intermolecular interaction. This last possibility merits consideration because of the tendency for association between myoglobin molecules as reported by Minton (19).

In conclusion, the observations with deoxy Hb A, Hb Beth Israel, and myoglobin support our conclusion that the wavelength shifts exhibited by oxy, met, and cyanmet hemoglobins are a result of dimerization of these molecules.

These results also clarify some recent observations on Hemoglobin Rothschild. This Hb has been ascertained to be preponderantly a dimer at concentrations where Hb A is a tetramer (20,21). The possibility that the fluorescence differences between Hb A and Hb Rothschild (1) is caused by the difference in their dimer-tetramer equilibrium is ruled out by the present report. We show here that the fluorescence properties of Hb Rothschild (the emission maximum at an excitation of 280 nm is 320 nm) (1) are not associated with hemoglobin dimers (emission

maximum 339-340 nm). This is not surprising as the substitution of β 37 Trp by Arg in Hb Rothschild eliminates the source of fluorescence that is influenced by dimerization. The fluorescence spectra of Hb Rothschild is most likely originating from β35 Tyr resulting in the observed shift to shorter wavelengths characteristic of tyrosine fluorescence.

Our present results may also explain differences between our data and that of others (3) on intrinsic fluorescence changes of hemoglobin upon ligand binding. Fluorescence techniques utilizing very low concentrations of hemoglobin would consist mainly of dimers. Our previous reports (1,2) used hemoglobins at concentrations wherein the hemoglobins were primarily tetramers, taking advantage of front-face fluorometry that allows observations of fluorescence in a concentration independent regime.

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DEDICATION

We want to dedicate this paper to the memory of Eraldo Antonini, whose commitment and contributions to the field of hemoglobin was an inspiration to all of us.

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