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Steady State Fluorescence Energy Transfer Measurements of Human Alpha Apohemoglobin Structure*

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A nonfluorescent reagent, 4-phenylazophenylmaleimide [4-PAPM], was attached to the sole cysteine residue [104(G11)] of alpha apohemoglobin (α^o) and served as an energy acceptor for the single intrinsic tryptophanyl [14(A12)] donor. This novel fluorescence system provided a transmolecular vehicle by which the overall structure of α^o could be monitored in 0.05 M potassium phosphate buffer at 5^0 C. Ratio of the emission intensities at 335 nm for monomeric solutions ($5x10^{-6}$M) of both α^o and α^o [4-PAPM] furnished a measure of the efficiency of energy transfer and average distance of separation (r). An apparent increase in the value of r was observed from pH 6.5 to 8.5, suggesting that the conformation (the structural relationship of the A and G helical segments) of α^o is responsive to its electrostatic environment.

Human hemoglobin, a well characterized oligomeric protein is an ideal system for investigating subunit assembly (1,2). The nascent α and β monomer chains first combine to form dimer intermediates which self-associate to form the final $\alpha_2\beta_2$ tetramer. *In vitro* subunit competition (3,4) experiments have indicated that the rate-limiting step in the assembly of the heme-containing subunits is the monomer combination reaction, and that this association appears to be dependent upon electrostatic interactions (5). Since the exact *in vivo* mechanism is unknown it could be postulated that this combination step may involve heme-free intermediates. The physical properties important to the heme-containing monomer combination reaction may also be influential in heme-free assembly mechanisms. Examination of the structural properties of the apohemoglobin subunits should be useful in understanding their assembly behavior. Here, we employed fluorescence energy transfer to examine the influence of pH on α^o structure.

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EXPERIMENTAL PROCEDURES

Preparation and Labeling of Alpha Apohemoglobin: Human adult hemoglobin and its isolated heme-containing subunits were prepared as previously described (6). Heme-free α chains were obtained by a modification of the acid-acetone method (7). This modification involved an increase in the proportion of 2 N HCl in acetone from 0.2 to 2.0 % (v/v), and was required to ensure maximum removal of the fluorescence quenching heme moiety. The extent of heme removal was confirmed spectrophotometrically and hemin titration indicated complete reconstitution of the α° (7,8). A nonfluorescent reagent, 4-phenylazophenylmaleimide (4-PAPM; Molecular Probes, Inc.) was employed to label the sole cysteine group of α° protein. A solution of 4-PAPM in methanol was prepared immediately prior to use and added to α° subunit (5×10^{-5} M) to yield a 2-fold molar excess of 4-PAPM to protein. Optimal labeling was achieved in 0.05 M potassium phosphate buffer, pH 8.0 for 3 hours at 4 °C. This resultant reaction mixture was immediately chromatographed on Sephadex G-25 (fine) to remove excess 4-PAPM and to adjust the pH of the protein sample for fluorescence studies.

Fluorescent Measurements: All fluorescence measurements were recorded at 5°C on an SLM/Aminco SPF 500 C spectrofluorometer controlled by SLM Software (version 2.4). The emission and excitation bandpasses were routinely set to 2.5 nm and all emission spectra were determined with an excitation wavelength of 290 nm. In order to monitor tryptophanyl emission changes over a wide range (pH 6.5-8.5) free of any dilution artifacts, a series of three overlapping sets of titrations were performed for both the unlabeled and labeled α° samples. The initial sample pH values (6.5, 7.0 and 7.5) were increased by successive additions of 2 M dibasic phosphate. Ratio of the emission intensities for both α° and α° [4-PAPM] samples were recorded and provided a measure of the efficiency of energy transfer. After each spectral study an additional titration was performed and the sample pH following each addition of 2 M dibasic phosphate was measured on a radiometer PHM 83 pH meter. Two sets of titrations were done for each initial pH value.

Analysis of Data: The efficiency of energy transfer (E) between the donor tryptophan residue 14 and the acceptor (4-PAPM) probe at position 104 was calculated from: $E = 1 - F_{DA}/F_D$ where F_{DA} and F_D are the emission intensities at 335 nm for equivalent concentrations of labeled and unlabeled α°, respectively. The efficiency of transfer is related to the apparent distance of separation: $r = R_o(1/E - 1)^{1/6}$ where $R_o = 9786(\kappa^2 \eta^{-4} \phi_D J)^{1/6}$; κ^2 being the geometric orientation factor between donor and acceptor pair, and assumed to be 2/3 for randomly rotating dipoles; η the refractive index of the bulk solvent measured as 1.336; ϕ_D the quantum yield of the unlabeled protein relative to 0.15 for L-tryptophan in water (See Below); and J the spectral integral of overlap, a measurement of the degree of electronic resonance between the donor and acceptor pairs (found to be 2.5×10^{-14} $M^{-1}cm^{-3}$ by rectangular integration method).

RESULTS AND DISCUSSION

The UV-Soret absorbance spectra of α° and α° [4-PAPM] are displayed in Figure 1. The distinct spectral characteristics of the α° [4-PAPM] protein provided convincing evidence for substantial tagging of the protein with our novel probe. Furthermore, titration of α° [4-PAPM]

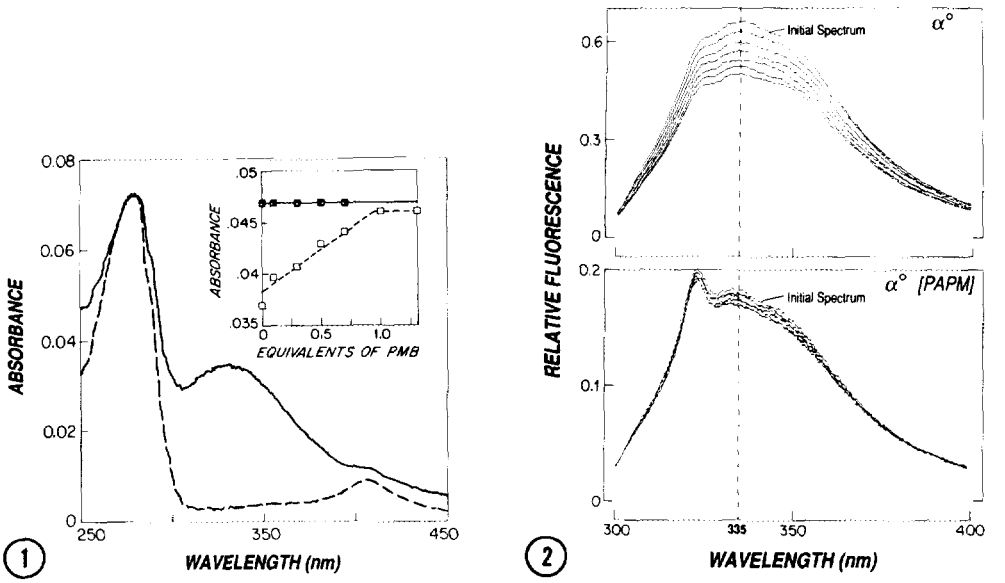

Figure 1. *Absorbance Properties of α° Proteins:* In addition to the protein absorbance observed in the 280 nm region, α°[4-PAPM] (-)possessed a broad absorbance in the 300 to 400 nm region as a result of 4-PAPM labeling. This absorbance completely overlaps the tryptophanyl emission spectrum of α° (See Below) and is a necessary condition for resonance energy transfer. The spectral properties of unmodified α° protein (--) are also included for comparison. The absorbance at 405 nm indicates greater than 99 % heme removal from the α hemoglobin chains (See Experimental Procedure). Insert: *Sulfhydryl Group Titrations:* The α° and α°[4-PAPM] samples were titrated with p-hydroxymercuribenzoate (9) and found to typically exhibit 0.95 and 0.00 available sulfhydryls, respectively, demonstrating essentially complete modification of the 104 cysteine group of the α° molecule. Both samples were at a protein concentration of 5 x 10^{-6} M; a concentration at which α° has been reported to be monomeric (10). Experimental conditions were 0.05 M potassium phosphate buffer, pH 8.0 and 5^0 C.

Figure 2. *Emission Spectra of Alpha Apohemoglobin:* Fluorescence spectral changes of α° and α°[4-PAPM] with successive additions of 2 M dibasic potassium phosphate to a final pH of 8.0. Initial conditions were 0.05 M potassium phosphate buffer, pH 7.5. Overlay of pH titration spectra shown are from an actual experiment and have not been averaged nor corrected for background Raman scatter of buffer.

(Figure 1:Insert) with *p*-hydroxymercuribenzoate (9) revealed no free sulfhydryls confirming the complete modification of the 104 cysteine group. Also note that the absorbance spectrum of α° [4-PAPM] overlaps the tryptophanyl emission spectrum of our donor protein (See Below), thus satisfying a necessary criteria for resonance energy transfer.

The emission spectra of α° and α° [4-PAPM] in 0.05 M potassium phosphate buffer, pH 7.5 and 5^0 C demonstrated a broad peak between 330-350 nm with an emission maximum at 335 (±2)nm (Figure 2). As

expected the presence of the acceptor probe on α° resulted in a dramatic decrease in the emission intensity (Figure 2). Hemoglobin and isolated β hemoglobin chains exhibit similar emission properties in the presence and absence of the heme moiety (10,11); the tryptophanyl emission maximum for α° found here was also similar to that of the heme-containing α chains.

The crystallographic distance for the α chain of oxyhemoglobin (measuring from the base of tryptophan residue 14 to the sulfhydryl group on residue cysteine 104) was found to be approximately 18 Å (12). Furthermore, recent long range electron transfer (13) and two-dimensional NMR (14) studies reported a high correlation between the global structure of α chains in solution and in crystal. Comparison of these distance measurements to the estimated energy transfer distance we report here for α° (See Below) strongly indicates that the global structure (the overall compactness), as monitored by the distance between the A and G helices, is not significantly altered upon heme removal. This finding is in total agreement with other steady state measurements (15,16) aimed at evaluating the structural consequences of heme moiety removal.

Even though the overall compactness is retained, the protein's structure may nevertheless be modulated by environmental factors such as pH. To test this hypothesis, the emission spectra of α° and α° [4-PAPM] were monitored (Figure 2). The average efficiency of transfer, as a function of pH, was determined (Figure 3) and an overall trend was noted in the energy transfer profile. Indeed, an incremental rise in pH of 2 units produced a decrease in the efficiency of energy transfer of 0.1 unit. This corresponded to an increase of 1 Å in the apparent distance of separation between the donor-acceptor pair (Figure 3:Insert) of our monomeric α° system. This may be attributed to fluctuations in the tertiary structure of the protein. (Although a molten globule intermediate (17) has not been demonstrated for α°, it may not be ruled out because of the high homology to apomyoglobin; hence, we may be observing secondary structural changes.)

Thus, we have conducted a systematic evaluation of the effect of pH on α° structure with our novel one donor-one acceptor fluorescence system and have demonstrated a small but significant conformational response of α° to electrostatic manipulation. This approach appears ideal for detecting structural fluctuations in the matrix of the α° protein and should contribute to our understanding of the assembly of α° with a partner β chain, a process presumed to be electrostatically driven at intracellular pH (5).

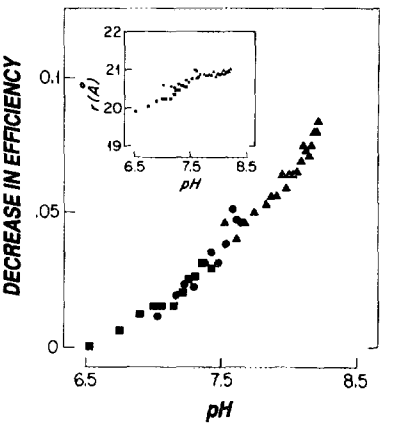

Figure 3. *Effect of pH on Efficiency of Energy Transfer:* A profile of the change in efficiency of energy transfer with pH is shown and was obtained from ratios of emission intensities at 335 nm for α° and α°[4-PAPM] samples. Initial pH values were 6.5 (■-■); 7.0 (●-●), 7.5 (▲-▲). All titrations overlapped irrespective of the starting pH value, implying that neither dilution of the protein (during titration) nor change in ionic strength of buffer contributed significantly to the decrease in efficiency of energy transfer observed. Insert: *Apparent Distance of Separation:* An increase of approximately 1 Å was determined over the pH range from 6.5 to 8.5; two Förster distances were employed [below pH 7.5: R_o = 25.9 Å (Φ = 0.079; above pH 7.5: R_o = 25.6 Å (Φ = 0.066)] to determine r (since the quantum yield for unlabeled α° was found to be sensitive to pH).

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