OXYGEN DIFFUSION THROUGH HEMOGLOBIN AND Hb^{desFe}

Quenching of the tryptophan and porphyrin emissions

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1. Introduction

Classical models of ligand binding to iron in hemeproteins consider only the diffusion of the ligand through the aqueous phase to the heme pocket. The notion that ligands can diffuse only through a narrow channel to the heme binding site has become almost dogmatic although adequate experimental evidence is lacking. Data on the diffusion path of oxygen in oxygen carrier proteins are clearly important if we are to elucidate the relative contributions of the structural (steric constraints) and dynamics (kinetics of statistical fluctuations) aspects of the protein. Previous studies have characterized the tryptophan emission from hemoglobin [1] and Hb^{desFe} (globin porphyrin) [2] as well as the porphyrin emission properties of Hb^{desFe} [2]. In this work the accessibility of various regions of the hemeprotein to oxygen diffusion was studied by the well-known technique of fluorescence quenching under elevated pressures of oxygen [3]. The quenching of two intrinsic fluorophors, tryptophan and porphyrin, has been studied.

2. Materials and methods

Human adult hemoglobin A was prepared from fresh human blood by well-known procedures [4,5]. Hb^{desFe} was prepared from hemoglobin according to [2]. All fluorescence experiments were performed at $20 \pm 0.5^{\circ}C$ in solutions buffered at pH 7.0 with 0.1 M potassium phosphate and $\sim 5 \times 10^{-6}$ M protein. Oxygen quenching was performed with the pressure cell [3] utilizing a 2 cm square quartz fluorescence cuvet. Under these conditions, and with continuous stirring of the sample, equilibration with each oxygen pressure was complete within 45 min. The intensities of the very weak tryptophan emissions were measured using a high sensitivity photon counting spectrofluorometer [6]. For both tryptophan and porphyrin emissions the intensities were calculated from the total spectral areas corrected for the background consisting of dark counts, Raman and buffer contributions. The porphyrin lifetimes were measured by the phase shift technique using the cross correlation phase/modulation fluorometer [7] with improved electronics from SLM Instruments (Urbana IL).

3. Results

Initial measurements of the porphyrin lifetimes by phase and modulation at 6, 18 and 30 MHz indicated a homogeneous emitting population. In the case of the tryptophan emissions the signal was considered too low for accurate measurement with the conventional analog phase/modulation instrument.

No quenching or modification of tryptophan or porphyrin emission spectra was observed under 1200 lb. in^{-2} of nitrogen. Moreover, the absorption spectra of hemoglobin and Hb^{desFe} remained unchanged under 1200 lb. in^{-2} of nitrogen or oxygen. Since the absorption spectra of these proteins are sensitive to the conformation [8] the absorption studies indicate that elevated gas pressures do not disrupt the protein structure. When oxygen pressure was slowly removed the porphyrin lifetime returned to the unquenched value. The intensity of the tryptophan and porphyrin emission recovered upon pressure release also, though not always completely as some

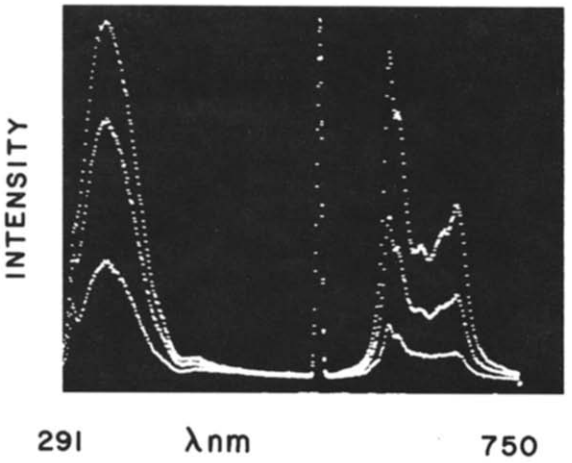

Fig.1. Total fluorescence emission (uncorrected for detector response) of Hb^{desFe} excited at 280 nm and scanned from 291–750 nm. The emissions from tryptophan and porphyrin residues are in the low and high wavelength regions of the scan, respectively. The line near 560 nm is the second order of the exciting light. The 3 traces represent 3, 310 and 900 lb. in^{-2} of O_2 pressure (highest to lowest intensities).

material can be lost by denaturation through bubble formation.

Typical emission spectra for Hb^{desFe} excited in the ultraviolet at varying oxygen pressures are shown in fig.1 demonstrating that both tryptophan and porphyrin emissions are quenched by oxygen. The effect of oxygen pressure upon the tryptophan emission from hemoglobin is shown in fig.2. As evident in fig.2a the emission is weak and the Raman contribution from the water is non-negligible. Fig.2b shows the oxygen-quenched spectra corrected for the Raman contribution. Plots of F_0/F vs $[O_2]$ for the tryptophan emission from hemoglobin and Hb^{desFe} are shown in fig.3. The error bars reflect the divergence in data from several runs; the weak tryptophan emission resulted in a large data-spread as compared to porphyrin. The band shapes and positions of the porphyrin emission remained invariant with increasing oxygen pressure while the tryptophan emission shifted only 2 or 3 nm to the blue at the highest oxygen pressure. This small blue shift for tryptophan has been attributed [9] to the fact that the quenched lifetime approaches the time scale of the proteins residue relaxation around the excited fluorophore. The constancy of the emission distribution allowed us to calculate relative intensities from the uncorrected spectra.

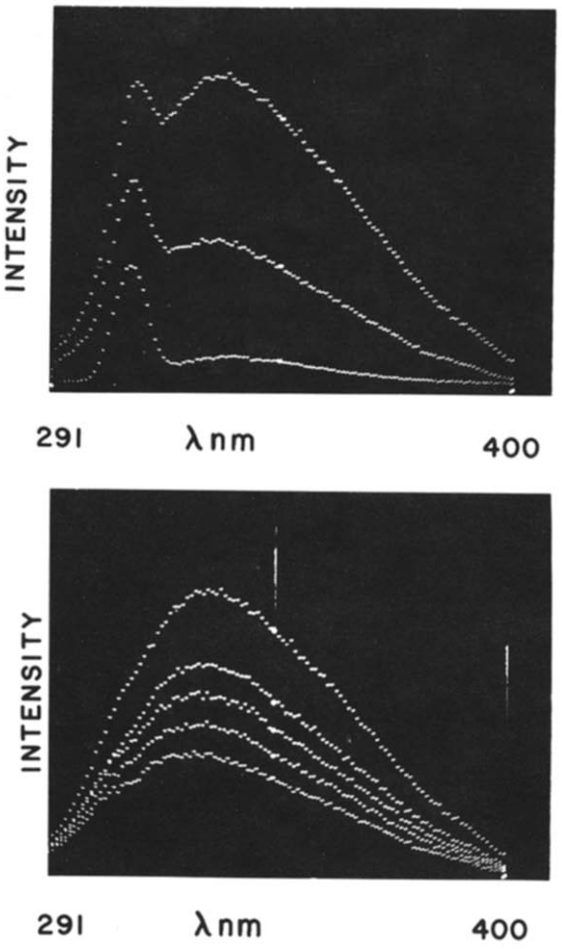

Fig.2. (a) Fluorescence of oxygenated hemoglobin excited at 280 nm and scanned from 291–400 nm. Overlay of buffer background including Raman peak and dark counts (lowest trace) and hemoglobin emission at 3 and 800 lb. in^{-2} of O_2 (highest and intermediate traces, respectively). (b) Fluorescence of hemoglobin excited at 280 nm and scanned from 291–400 nm, corrected for buffer background, at 3, 220, 400, 620 and 880 lb. in^{-2} of O_2 (highest to lowest traces, respectively).

Fig.4 shows the intensity (F_0/F) and lifetime (τ_0/τ) quenching of the porphyrin emission from globin-porphyrin Hb^{desFe} excited at 568 nm. For intensity data, the spectral area from 591–730 nm was measured while the lifetime data was obtained by observation of the emission through a Corning 2-62 cutoff filter. The τ_0 value of 21.3 ns corresponds to the porphyrin lifetime at ambient oxygen pressure.

A Stern-Volmer equation for lifetime quenching by O_2 diffusion may be given as:

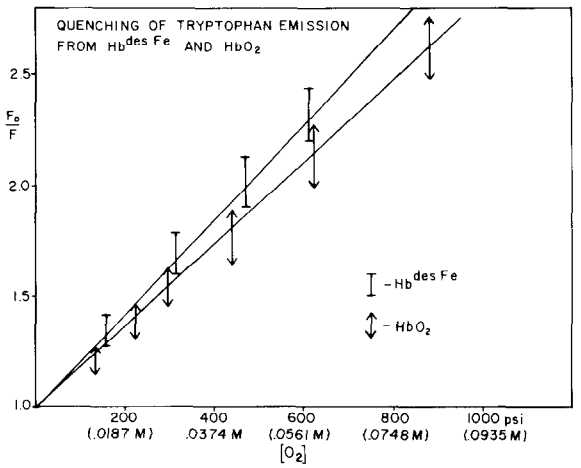

Fig.3. Intensity quenching of tryptophan emission from oxygenated hemoglobin and its globin-porphyrin derivative by oxygen at 20°C. Intensities were calculated from spectral areas of emissions scanned from 291–400 nm, excited at 280 nm and corrected for the buffer background.

$$\tau_0/\tau = 1 + k^*\tau_0[Q]$$

where τ_0 is the lifetime in the absence of quencher, τ the quenched lifetime, k^* the biomolecular rate constant for the quenching process and [Q] the molar concentration of quencher. From the lifetime data on the porphyrin emission (fig.4) we calculate $k^* = 7 \times 10^8\ s^{-1} . M^{-1}$. From data on the lifetime quenching of porphyrin in water ($\tau_0 = 13$ ns) we found a rate constant of $1.3 \times 10^{10}\ s^{-1} . M^{-1}$, indica-

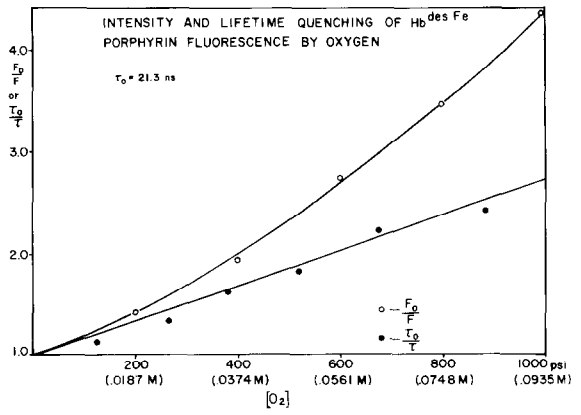

Fig.4. Intensity and lifetime quenching of the porphyrin emission of globin porphyrin (Hb^{desFe}) by oxygen. Intensities were calculated from spectral areas from 591–730 nm, excited at 568 nm. Lifetimes were measured by the phase shift technique at 6 MHz modulation frequency.

tive of a diffusion controlled process. We see then that the dynamic quenching of porphyrin embedded in the heme pocket is factor of <20 from the free diffusion controlled rate.

The intensity data for the porphyrin quenching (fig.4) show a marked positive deviation from the lifetime data as is only possible for heterogeneous quenching mechanisms [10]. In simplest form one requires for description a modified Stern-Volmer expression:

$$F_0/F = (1 + K_1[Q])(1 + K_2[Q])$$

In the case of free fluorophor in solution, K_1 and K_2 represent dynamic and static quenching constants respectively, but in general they need not have this simple interpretation. 'Static' quenching is to be understood as any process that results in quenching with interaction times much shorter than the fluorescence lifetime. Thus, whether the oxygen is associated with porphyrin or its surroundings in the form of a well-defined complex or whether it diffuses from inside the protein in much less than the porphyrin lifetime (~21 ns) the heterogeneous nature of the quenching unequivocally demonstrates the presence of O_2 in the protein structures at typical bulk oxygen levels of $\geqslant$0.02 M. More recent studies indicate that the observed curvature of the intensity quenching and its deviation from the lifetime quenching can be explained qualitatively and nearly quantitatively by straightforward assumptions concerning the partition of oxygen between protein and aqueous phases and a Poisson distribution of oxygen among the protein molecules [11].

The intensity quenching of the tryptophan emission from hemoglobin and Hb^{desFe} (fig.3) indicates that the emitting tryptophan residues are also readily accessible to oxygen. The position of the emission maxima for these proteins (331, 334 nm) as well as the structural studies in [12,13] indicate that the tryptophan residues are not directly exposed to the solvent. The lifetime of the tryptophan emission, however, was too weak to be measured with the same apparatus used for the porphyrin lifetime determinations. In [14,15], the lifetime of these emissions were heterogeneous but with major components at ~1.5 ns. Given these lifetimes and assuming dynamic quenching, the quenching rates calculated from the data of fig.3 are $\sim 7 \times 10^9\ s^{-1} . M^{-1}$, consistent with the rates observed for many proteins in [9].

4. Discussion

In [17] the 4 porphyrin sites of Hb^{desFe} were equally accessible to oxygen [17] although the α and β heme pockets differed in size [18]. Those observations are consistent with these results which indicate that the protein matrix does not provide a decisive restriction to the diffusion of oxygen, i.e., rapid structural fluctuations occur in the protein matrix of hemoglobin and Hb^{desFe}. Considering these findings the hypothesis that the diffusion of ligand is restricted to a well-defined or unique path to the heme pocket [16] appears to be an over-simplification.

Considerations of the accessibility of oxygen to the interior of the protein also have implications for the escape of oxygen and other heme ligands from the protein to the aqueous phase. The results given here are consistent with findings on the cage recombination effect of CO [19] and O_2 [20] studied by flash-laser photolysis. The flash photolysis data indicate that ligand can partition between the aqueous phase and the protein matrix. One expects the kinetics of ligand diffusion through the protein to depend critically on the size and charge characteristics of the ligand and the charge and mobility of the interior protein residues. Further work is necessary to properly evaluate the importance of each of these factors.

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