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A biophysical investigation on the binding of proflavine with human hemoglobin: Insights from spectroscopy, thermodynamics and AFM studies

Anirban Basu, Gopinatha Suresh Kumar *

Biophysical Chemistry Laboratory, Organic & Medicinal Chemistry Division, CSIR-Indian Institute of Chemical Biology, Kolkata 700 032, India

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ABSTRACT

Interaction of proflavine with hemoglobin (Hgb) was studied employing spectroscopy, calorimetry, and atomic force microscopy. The equilibrium constant was found to be of the order $10^4\ M^{-1}$. The quenching of Hgb fluorescence by proflavine was due to the complex formation. Calculation of the molecular distance (r) between the donor (β-Trp37 of Hgb) and acceptor (proflavine) suggested that energy can be efficiently transferred from the β-Trp37 residue at the α1β2 interface of the protein to the dye. Proflavine induced significant secondary structural changes in Hgb. Synchronous fluorescence studies showed that proflavine altered the microenvironment around the tryptophan residues to a greater extent than the tyrosine residues. Circular dichroism spectral studies showed that proflavine caused significant reduction in the α-helical content of Hgb. The esterase activity assay further complemented the circular dichroism data. The Soret band intensity of Hgb decreased upon complexation. Differential scanning calorimetry and circular dichroism melting results revealed that proflavine induced destabilization of Hgb. The binding was driven by both positive entropy and negative enthalpy. Atomic force microscopy studies revealed that the essential morphological features of hemoglobin were retained in the presence of proflavine. Overall, insights on the photophysical aspects and energetics of the binding of proflavine with Hgb are presented.

1. Introduction

Proflavine is a novel acridine analog consisting of a planar polycyclic monocationic aromatic moiety with two amino groups exhibiting wide range of pharmacological activities [1–3]. Proflavine was used as an antiseptic as well as a disinfectant bacteriostatic [4,5]. It can cause frameshift mutations in viruses, bacteria and bacteriophages [6], and inhibit replication in cancer cells by intercalating between the DNA base pairs [7–13]. So, it has the potential to be developed as a chemotherapeutic agent [14–16].

Proteins perform a wide range of functions in living organisms including transportation of endogenous and exogenous molecules form one site to another and hence they are the prime choice for probing the pharmacological action of drugs [17]. Understanding the dynamics and biochemical consequences of drug–protein interactions is essential in pharmacology and drug development. Although the interaction of acridines with nucleic acids is well documented their interaction with proteins have not yet been properly characterized [18–27]. Hgb is an important protein whose structure and function are well established [28–31]. Hgb is present inside the red blood cells but it can become reactive and toxic on hemolysis, under diseased conditions or owing to the action of some drugs. This leads to its presence in the plasma getting exposed to various small molecules and drugs. Since Hgb can interact with a variety of small molecules the metabolism, distribution and efficacy of many drugs and biologically important compounds *in vivo* may be influenced by their affinity to Hgb [32–34]. The therapeutic effect of a drug is directly linked with its concentration in blood. The unbound drug can easily diffuse from blood to tissue/organ where the pharmacological activity occurs. Strong association between protein and drug molecules reduces the free drug concentration in blood and also decreases its pharmocodynamic effect. However, weak binding between protein and drug molecules results in shorter lifetime [35]. Thus, complexation between protein and drug molecules can influence the release of drugs from blood to receptors and also inhibit their rapid metabolism [36]. For this reason, it is pertinent to investigate the interaction of potential drugs like proflavine with Hgb to understand their metabolism and distribution *in vivo*. In this report, we investigated the effect of proflavine on Hgb studying the excited-state photophysics of proflavine within the microheterogeneous proteinous environment. Furthermore, we have evaluated the energetics of the interaction using sensitive calorimetric techniques to correlate the structural aspects with the energetics.

* Corresponding author at: CSIR-Indian Institute of Chemical Biology, 4, Raja S. C. Mullick Road, Jadavpur, Kolkata 700 032, India.
E-mail address: gskumar@iicb.res.in (G.S. Kumar).

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2. Experimental

2.1. Materials

Hgb and proflavine hydrochloride (PFV hereafter, Fig. 1) were obtained from Sigma–Aldrich Corporation (St. Louis, MO, USA). All the sample solutions were prepared in 10 mM citrate-phosphate buffer, pH 7.0 at (298.15 ± 0.01) K unless otherwise specified. The concentrations of PFV and Hgb were determined using molar absorption coefficient (ε) values of 42,000 M^{-1} cm^{-1} at 444 nm and 1,79,000 M^{-1} cm^{-1} at 405 nm, respectively [37–42].

2.2. Spectroscopic Studies

Absorbance spectral studies were performed on a Jasco V660 double beam double monochromator spectrophotometer (Japan International Co., Hachioji, Japan). Steady state and time resolved fluorescence experiments were performed on either a Shimadzu RF-5301 PC (Shimadzu Corporation, Kyoto, Japan) or Quanta Master 400 unit (Horiba PTI, Canada) controlled with FelixGX spectroscopy software. Fourier transform infrared (FTIR) measurements were performed on a Bruker FTIR, TENSOR 27 spectrometer (Bruker Corp, USA) while circular dichroism (CD) studies were performed on a Jasco J815 spectropolarimeter (Japan International Co.).

2.3. Microcalorimetric Studies

Differential scanning calorimetry (DSC) and isothermal titration calorimetry (ITC) experiments were performed on MicroCal VP-DSC and MicroCal VP-ITC units, respectively, (MicroCal, Inc., Northampton, MA, USA, now Malvern Instruments, UK) following the procedures reported earlier [24,25,41]. In ITC, the heats of PFV-Hgb binding reaction, after appropriate correction for dilution, were plotted as a function of the corresponding molar ratio. The data were then analyzed to obtain the thermodynamic parameters and the equilibrium constant (K_a) [24,25].

2.4. Dynamic Light Scattering Experiments

Dynamic light scattering (DLS) measurements were performed on a HORIBA (SZ-100 OZ) dynamic light scattering particle size analyser [43]. The temperature was maintained at 298.15 K during the measurement.

2.5. Esterase Activity Assay

Esterase activity of Hgb was assayed by using *p*-nitro phenyl acetate (*p*-NPA) as the substrate according to the procedure reported previously [44]. The reactions were initiated by adding 1.5 mM alcoholic *p*-NPA to a reaction mixture containing 5.0 μM of Hgb and absorbance change at 400 nm due to the addition of *p*-NPA was recorded. The absorbance data were further corrected for *p*-NPA hydrolysis by the buffer. Thereafter, relative esterase activity was plotted as a function of the changing PFV concentration.

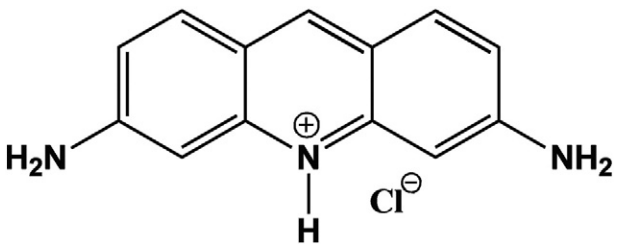

Fig. 1. Molecular structure of PFV.

2.6. Atomic Force Microscopy Studies

Atomic force microscopy (AFM) studies were performed for Hgb and Hgb-PFV complexes. Hgb solution was diluted to a final concentration of 200 nM. Thereafter, it was centrifuged and filtered through 0.22 μm filter paper. 5 μL aliquot of Hgb was adsorbed onto a muscovite Ruby mica sheet (ASTM V1 grade Ruby Mica from MICAFAB, Chennai) and dried for 30 min in vacuum drier under inert atmosphere. For the Hgb-PFV complex, an equimolar mixture of Hgb and PFV was prepared, incubated for 10 min and adsorbed onto the mica. AAC mode AFM was done with a Pico plus 5500 ILM AFM (Agilent Technologies, USA) equipped with a piezo scanner. The images were processed using Picoview version 1.1 software (Agilent Technologies) while it was manipulated with the help of Pico Image Advanced version software.

3. Results and Discussion

3.1. Electronic Absorption Spectroscopy

Hgb exhibits two major peaks in the 190–500 nm region. The first peak arises owing to π–π* transition of the carbonyl groups of the amino acid residues while the other one (~405 nm) is from the π–π* transition of porphyrin-Soret band [39,40]. The Soret band arises from the heme group embedded within the hydrophobic pocket formed through appropriate folding of Hgb's backbone [45–47]. Both these bands showed a decrease in absorbance on addition of PFV. The absorbance spectral changes in the Hgb spectrum upon addition of PFV are shown in Fig. 2A and the changes associated with the Soret band are highlighted in Fig. 2B. The Soret band's position remained virtually unaltered. The hypochromic change in the Soret band suggested the complexation between PFV and Hgb occured at the ground state. The changes in the absorbance of the Soret band were analyzed using the Benesi–Hildebrand equation [48].

$$\frac{1}{\Delta A} = \frac{1}{\Delta A_{max}} + \frac{1}{K_{BH}(\Delta A_{max})} \times \frac{1}{[PFV]} \quad (1)$$

where ΔA = absorbance change at the Soret band, ΔA = maximum absorbance or absorbance of Hgb at the Soret band in absence of PFV, K_{BH} = Benesi–Hildebrand binding constant and [PFV] = Concentration of PFV. The Benesi–Hildebrand association constant for PFV-Hgb complexation (K_{BH}) was deduced to be 2.73×10^4 M^{-1} (Fig. S1A).

3.2. Effect of Proflavine on the Intrinsic Fluorescence of Hemoglobin

Intrinsic fluorescence of Hgb is due to the tryptophan (Trp) and tyrosine (Tyr) moieties in the polypeptide chain. The intrinsic fluorescence of Hgb is essentially due to the β-Trp37 residue at the α1β2 interface [49], and indicates transition from relaxed (*R*) to taut (*T*) form. Ligand bound (oxy) form is the *R* form while the deoxy form is the *T* form [50], and the relative fluorescence intensities of these two forms have significant differences. The fluorescence emission spectrum of Hgb was monitored by exciting at 295 nm which preferentially excited the Trp residues [51]. Hgb exhibited emission maximum at 329 nm revealing that the β-Trp37 residue is buried or is in a hydrophobic region [52]. The fluorescence intensity of Hgb steadily decreased in the presence of increasing PFV concentration. This suggested that PFV interacted with Hgb and the quenching was owing to specific complex formation.

3.3. Effect of Hemoglobin on the Fluorescence of Proflavine

Proflavine is a highly fluorescent with emission maximum at 509 nm when excited at 444 nm. Since this peak is far away from the Hgb fluorescence maximum (329 nm) it provided a convenient handle to probe the effect of Hgb on the PFV fluorescence. Fluorescence intensity of PFV gradually decreased upon addition of increasing concentration of Hgb