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Binding of fluorescent acridine dyes acridine orange and 9-aminoacridine to hemoglobin: Elucidation of their molecular recognition by spectroscopy, calorimetry and molecular modeling techniques

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ABSTRACT

The molecular interaction between hemoglobin (HHb), the major human heme protein, and the acridine dyes acridine orange (AO) and 9-aminoacridine (9AA) was studied by various spectroscopic, calorimetric and molecular modeling techniques. The dyes formed stable ground state complex with HHb as revealed from spectroscopic data. Temperature dependent fluorescence data showed the strength of the dye–protein complexation to be inversely proportional to temperature and the fluorescence quenching was static in nature. The binding-induced conformational change in the protein was investigated using circular dichroism, synchronous fluorescence, 3D fluorescence and FTIR spectroscopy results. Circular dichroism data also quantified the α-helicity change in hemoglobin due to the binding of acridine dyes. Calorimetric studies revealed the binding to be endothermic in nature for both AO and 9AA, though the latter had higher affinity, and this was also observed from spectroscopic data. The binding of both dyes was entropy driven. pH dependent fluorescence studies revealed the existence of electrostatic interaction between the protein and dye molecules. Molecular modeling studies specified the binding site and the non-covalent interactions involved in the association. Overall, the results revealed that a small change in the acridine chromophore leads to remarkable alteration in the structural and thermodynamic aspects of binding to HHb.

1. Introduction

Proteins enact the most dynamic and diverse roles in cells [1]. They perform various functions that include the control of biochemical reactions, providing intracellular and extracellular scaffolding support, transport of molecules, controlling enzymatic action, and building the immune system. Since proteins are the preferred druggable targets for drugs, they represent the best model for the in vitro characterization of the pharmacological action of therapeutic drugs. Prominent among such proteins is hemoglobin, an iron containing respiratory protein, in the red blood cell of vertebrates. Its structure comprises of four polypeptide chains, with two similar α-chains each consisting of 141 amino acid residues and two similar β-chains with 146 amino acid residues. Hemoglobin functions to carry molecular oxygen from the lungs to the tissues and transport CO_2 back. The enzymatic and antioxidant activities of hemoglobin are also well reported in the literature [2,3]. The amino acids in HHb predominantly form α-helices and the helical sections are mostly stabilized by H-bonding interactions which result in the folding of the protein into a particular shape. HHb does not contain any β-strands or disulfide bonds, and is conspicuous by the absence of the amino acid isoleucine [4].

Acridines have a planar polycondensate heteroaromatic nucleus. A number of therapeutic agents based on acridine nucleus are known for example, acriflavine and proflavine are antiseptics, quinacrine is an antimalarial drug while amsacrine and nitracine are anticancer agents. Acridine orange (AO) and 9-aminoacridine (9AA) are two molecules with enormous pharmacological activities. AO is a photochemically active cationic fluorescent dye that binds to nucleic acids (DNA and RNA) in an intercalative mode [5], thought to be the cause of its anticancer activity. Its fluorescence property was used for performing micronucleus test in mice and rats [6], establishing transmembrane pH gradient probe [7,8], staining of bacteria in clinical specimen [9], visualizing the intracellular signal propagation [10], observing change in conformation of RNA in situ [11], investigation of clinical sperm quality [12], rapid diagnosis of malaria [13] etc. It is also used in cell staining of DNA in apoptosis studies, lysosome labeling, flow cytometry, cell-cycle studies etc. On the other hand, 9AA is clinically important as a topical antiseptic. It is an intracellular pH indicator, exhibits mutagenic activities and binds strongly to DNA [14,15]. The better antitumoral activity of 9AA over AO has been ascribed to various activities such as p53 activation and inhibition of NF-κB and Bcl-xL proteins [16]. It has long been known to be a potent frame shift mutagen in viruses and bacteria [17–21]. 9AA inhibits HIV-1 of Tat dependent transcription which activates p53 without inducing apoptosis [22].

Abbreviations: AO, acridine orange; 9AA, 9-amino acridine; HHb, human hemoglobin; UV, ultra violet; CD, circular dichroism; FTIR, Fourier transform infrared spectroscopy; ITC, isothermal titration calorimetry.

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It interacts with guanidinobenzoatase through the active center of the enzyme [23]. Furthermore, while AO has tendency to aggregate/self-associate, this is circumvented in 9AA and many related 9AA derivatives have improved stability and much better anticancer activity than AO [24,25]. Hence AO and 9AA are two structurally closely related molecules with unique and diverse pharmacological functions.

The interaction between proteins and small molecules that bind them is governed by specific molecular recognition, which is the fundamental process governing biological function and activity [26]. Proteins contain binding pockets that can specifically be targeted by small molecules. The binding arises because of the attractive forces between the complimentary loci on the protein (host) and the ligand (guest) molecules and such specific molecular recognition is an indirect measure of the free energy of the system. Hemoglobin is an inert molecule within the erythrocytes, but becomes reactive and toxic upon hemolysis that occurs in some diseased conditions and also due to the usage of some drugs. This may lead to exposure of HHb to drugs and small molecules present in the plasma leading to interaction. A few reports on ligand–hemoglobin interactions have been published recently [27–33]. The molecular aspects of the recognition between acridine dyes and HHb and the related thermodynamics are not yet investigated. Here we have compared the binding of acridine orange and 9-aminoacridine to HHb. We found interesting differences in their interaction profile that are presented here.

2. Materials and Methods

2.1. Materials

Human hemoglobin (HHb, molecular mass = 64.5 KDa), acridine orange (CAS No. 494-38-21) and 9-aminoacridine (CAS No. 52417-22-8) (Fig. 1) were purchased from Sigma-Aldrich Corporation (St. Louis, MO, USA) and re-crystallized twice from ethanol. All other chemicals used were of analytical grade obtained from Sigma-Aldrich.

The protein was purified as described earlier [29,33]. The sample solutions were prepared and the experiments performed in phosphate buffer containing 10 mM [Na^+], pH 7.2.

The buffer was prepared from milli Q water (resistance ~18.25 mΩ) and filtered through membrane filters of pore size 0.22 μm. The concentrations of HHb and the dyes were determined by absorbance measurements using molar absorption coefficient values as follows: $179 \times 10^3\ M^{-1}\ cm^{-1}$ at 405 nm for HHb, $43 \times 10^3\ M^{-1}\ cm^{-1}$ at 470 nm for AO and $9656\ M^{-1}\ cm^{-1}$ at 400 nm for 9AA.

2.2. Spectroscopic Studies

All the spectrophotometric studies were carried out at 20 ± 0.5 °C using a Jasco V-660 spectrometer (Japan Spectroscopic Co. Ltd., Hachioji, Japan) equipped with a thermoelectrically controlled cell holder in 10 mm path length quartz cuvettes (Starna Cells, Inc. CA, USA).

All the fluorescence experiments were carried out at 20 ± 0.5 °C in 10 mm quartz cuvettes using a Quanta Master 400 unit (Horiba PTI, Canada) with a 150 W Xenon lamp controlled by FelixGX spectroscopy software provided with the instrument. The temperature was controlled by the single cuvette Peltier K-155-C of the unit. All fluorescence data were corrected for inner filter effect for the strong absorption of HHb and the dyes in the UV–vis region (vide infra). Synchronous fluorescence spectra were measured at $\Delta\lambda = 15$ nm and $\Delta\lambda = 60$ nm. The wavelength was scanned from 240 to 320 nm.

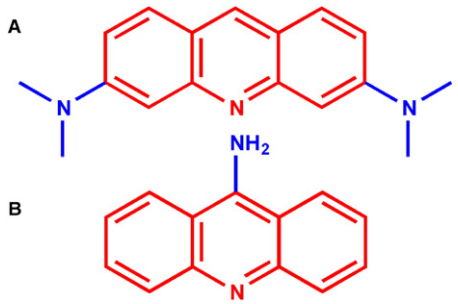

Fig. 1. Chemical structure of (A) acridine orange and (B) 9-aminoacridine.

Three dimensional (3D) fluorescence experiments were recorded on a PerkinElmer LS 55 fluorescence spectrometer (PerkinElmer Inc., Waltham, MA, USA). The fluorescence spectra of HHb were measured in the range of 270–500 nm with an increment of 10 nm; the initial excitation wavelength was set at 200 nm and continued up to 340 nm.

A Jasco J815 CD polarimeter with a peltier cell holder and temperature controller (PFD425 L/15) was used for monitoring the conformational changes in the protein. Rectangular quartz cuvettes of 1 mm and 10 mm path lengths, respectively, were used for CD measurements in the far-UV and Soret band regions. Eight successive scans were performed, averaged and smoothed by the software of the unit to improve the signal-to-noise ratio followed by subtraction of the buffer baseline for each averaged spectrum. For thermal denaturation experiments the ellipticity change at λ_{222} nm was monitored in the temperature range 30–100 °C at a scan rate of 0.5 °C/min. The curves were normalized, assuming a linear temperature dependence of the base lines on native and denatured states. All the CD spectra were presented in terms of molar ellipticity [θ] ($deg\ cm^2\ dmol^{-1}$) except the melting data which are presented in milli degrees.

FT-IR measurements were performed on a Bruker FTIR, TENSOR 27 spectrometer. In a typical experiment, a spectrum of the buffer solution was recorded at first followed by the spectra of HHb and HHb–dye complexes. The buffer spectrum was subtracted from the sample spectra to obtain the corrected IR spectra of HHb and HHb–dye complex.

Melting profiles (absorbance change versus temperature curves) of HHb and HHb–dye complexes were registered on a Shimadzu Pharmaspec 1700 unit (Shimadzu, Kyoto, Japan) equipped with a peltier controlled TMSPC-8 model devise. An eight chambered micro optical quartz cuvette of 10 mm path length was used and the samples were heated at the rate of 0.5 °C/min monitoring the change in absorbance at 295 nm. Analysis of the melting curves for the measurement of T_m was carried out using first derivatives. The accuracy of the reported T_m values is ±1 °C.

2.3. Isothermal Titration Calorimetry

Thermodynamics of the dye–HHb association was studied in a MicroCal VP-ITC unit (MicroCal LLC., Northampton, MA, USA). The titration of the dyes to protein was automated and the data obtained analyzed by Origin 7.0 software supplied with the unit. Programmed injections of 10 μL aliquots of the dye solution (600 μM of AO and 800 μM of 9AA) into HHb solution (50 μM) placed in the calorimeter cell (1.4235 mL) were effected from the rotating injection syringe. For the control dilution experiment the dye solution of the same concentration was injected to the experimental buffer in the same protocol as employed for the samples. The heat absorbed in each injection for the dilution was subtracted from the corresponding heat absorbed for dye–protein association to finally yield the heat change for the dye–protein binding reaction. The final data points were plotted as a function of the molar ratio (dye/HHb), fitted with a model for "one set of binding sites" and analyzed using Origin software to provide thermodynamic parameters along with equilibrium binding constant (K) and the binding stoichiometry (N). The Gibbs energy change (ΔG°) and the entropic contribution for the binding ($T\Delta S^\circ$) were calculated by the following equations:

$$\Delta G^\circ = -RT \ln K \quad (1)$$

and

$$\Delta G^\circ = \Delta H^\circ - T\Delta S^\circ. \quad (2)$$