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Research Paper

Absorption Spectra of Normal Adult and Sickle Cell Haemoglobins Treated With Hydrogen Peroxide at Two pH Values

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ABSTRACT
Millimolar absorptivities of normal adult haemoglobin (HbA) and sickle cell haemoglobin (HbS) were monitored at pH values of 7.2 and 5.0, within the ultra violet and visible spectral range (250-650 nm) in the presence of varying concentrations of hydrogen peroxide (4.00-20.00 mM). The absorption spectra of HbA and HbS exhibited the characteristic Soret band with maximum absorptivities at wavelenght (λ_{max}) = 415 nm. HbA and HbS exhibited lower peak absorptivities in the presence of hydrogen peroxide (H_2O_2) in a concentration dependent manner. Maximum absorptivities of HbA and HbS treated with H_2O_2 showed hypochromic red shift of the Soret band from (λ_{max}) = 415 to 420 nm regardless of changes in experimental pH values. In addition, increasing concentrations of H_2O_2 at the two experimental pH conditions engendered differential distortion and obliteration of the Soret band. Whereas at pH = 7.2, [H_2O_2] > 14.28 mM caused obliteration of the Soret band of HbA, 20.00 mM H_2O_2 at lower pH = 5.0, obliterated the Soret band. In contrast to the absorption spectra of HbA, at experimental pH values of 5.0 and 7.2, maximum absorptivities of HbS were 1.8 and 2.3 $mmol^{-1}\cdot cm^{-1}$ respectively. However, HbA and HbS showed decreasing peak absorptivities with increasing concentrations of H_2O_2 regardless of changes in pH values. The spectra signatures of HbA and HbS treated with H_2O_2 were non-identical under the two experimental pH conditions which are diagnostic of the two haemoglobin types.
KEYWORDS: *Haemoglobin, absorptivity, hydrogen peroxide, Soret band, absorption spectra.*

INTRODUCTION

Haemoglobins are tetrameric conjugate proteins comprised of pairs of two different polypeptide subunits and prosthetic haem group which contains iron [1]. Greek letters are used to designate each subunit type. The subunit composition of the principal haemoglobins are $\alpha_2\beta_2$ (HbA; normal adult haemoglobin), $\alpha_2\gamma_2$ (HbF; foetal haemoglobin), α_2S_2 (HbS; sickle cell haemoglobin), and $\alpha_2\delta_2$ (HbA_2; a minor adult haemoglobin). The primary structures of the β, γ, and δ chains of human haemoglobin are highly conserved. The ferrous iron is coordinated to four pyrrole nitrogen of protoporphyrin IX and to imidazole nitrogen of a histidine residue of porphyrins [2]. The sixth coordinate position is available for binding to small molecules such as O_2, CO or CO_2 [3].

Spectroscopic techniques have been widely applied in various biochemical analyses. These techniques take advantage of the interactions of molecules with spectrum regions of electromagnetic radiation. UV-Visible absorption spectroscopy shows that haemoglobin exhibits intense absorption wavelengths above 320 nm [4]. Strong absorptions occur near 400 nm and this peak region is known as the Soret band [5,6]. The Soret band is characteristic of hematoporphyrin proteins [7, 1].

Specifically, deoxy-, oxy-, met- and ferri- haemoglobins have their corresponding absorbance maximum in the Soret region at 430, 415, 405 and 418 nm respectively [8]. Thus, haemoglobin absorbance maximum in the Soret region can be used in differentiating these states.

Although it is well known that the various vertebrate haemoglobins as well as the individual variants of adult human haemoglobin (HbA) may be readily distinguished by their different mobility in electrophoresis, the present study applied haemoglobin absorption spectroscopy as a distinguishing feature for identification of two haemoglobin types-HbA and HbS.

MATERIALS AND METHODS

Collection of blood samples: Four milliliters (4.0 ml) of human venous blood samples of electrophoretic confirmed HbAA and HbSS genotypes were collected by venipuncture from volunteers between the age bracket of 21 and 34 yr and stored in Na_2EDTA anticoagulant tubes. Blood samples of HbSS genotype were from individuals attending clinics at University of Nigeria Teaching Hospital (UNTH), Nsukka, Nigeia. This study was in accordance with the ethical principles that have their origins in the Declaration of Helsinki.

Preparation of haemolysate haemoglobin: The erythrocytes were washed by centrifugation method as described by Tsakiris *et al.*,[9] with minor modification according to Pennings *et al.*, [10]. Blood volume of 4.0 ml was introduced into centrifuge test tubes containing 4.0 ml of buffer solution pH = 7.4: 250 mM tris (hydroxyl methyl) amino ethane–HCl (Tris-HCl)/ 140 mM NaCl/ 1.0 mM $MgCl_2$/ 10 mM glucose) and centrifuged at 4000 rpm for 10 min at 4 ˚C. The supernatant was carefully removed with a Pasteur pipette. This process was repeated until the supernatant became clear. A 2.0 ml of 0.05 M Tris-HCl buffer (pH = 8.5) was re-introduced to the sediment and kept at refrigerated temperature of 4 ˚C for 20 min. The sedimented erythrocytes was made up to 5.0 ml with 0.05 M Tris-HCl buffer (pH = 8.5) and lysed by freezing/ thawing as described by Galbraith and Watts, [11] and Kamber *et al.*, [12].

Salting out/Dialysis: While the lysed erythrocytes were centrifuged at 4000 rpm for 10 min at 4 ˚C to recover crude haemoglobin, the sedimented erythrocyte membrane "ghosts" were discarded. The crude haemoglobin was finally suspended in 5% NaCl (w/v) [6]. The suspension was allowed to stand at 4 ˚C for 10 min, after which it was centrifuged at 4000 rpm for 20 min at 4 ˚C. The sediment of precipitated anions and erythrocyte proteins were discarded. The supernatant was subjected to dialysis at 4 ˚C in a beaker containing 800 ml of 0.05 M Tris-HCl buffer (pH = 8.5) for 12 h. The buffer was replaced at the 7th hour. The dialysate was finally stored in a freezer at regulated temperature of -32 ˚C.

DEAE-cellulose ion-exchange chromatography: The crude haemoglobin was further purified using the DEAE-cellulose ion-exchange chromatography. A 5.0 ml of the dialyzed haemoglobin suspension was introduced into the packed and equilibrated gel in a column (2.5 cm (i.d) x 13.0 cm). One hundred (100) ml of 0.001 M Tris-HCl (pH = 8.5) was gradually introduced into the column to wash off any unbound protein molecules. A pH gradient between 250 ml of 0.05 M Tris-HCl buffer (pH = 8.5) and 250 ml of 0.05 M Tris-HCl buffer (pH = 6.5) was generated by a gradient mixer (Pharmacia Gradient Mixer, GMI, Pharmacia, IL, USA). The eluate was collected in 3.0 ml fractions, their absorbance taken at 541 nm and stored at -32 ˚C. To remove traces of 2, 3-bisphosphoglycerate, the various eluates of HbS and HbA with high absorbances were pooled and dialyzed at 4 ˚C for 12 h in 0.05 M Tris-HCl buffer (pH = 7.2) and 0.05 M acetate buffer (pH = 5.0) respectively.

Determination of haemoglobin concentration: A modified method [13], based on cyanomethaemoglobin reaction was used for the determination of haemoglobin concentration in mmol/L. A 0.05 ml portion of the eluate (haemoglobin suspension) was added to 4.95 ml of Drabkin reagent (100 mg NaCN and 300 mg $K_4Fe(CN)_6$ per liter). The mixture was left to stand for 10 min at room temperature and absorbance read at λ_{max} = 540 nm (UV-Visable Spectrophotometer, Jenway 6405) against a blank. The absorbance was used to evaluate for haemoglobin concentration by comparing the values with the standards [14].

Spectroscopic analysis of HbA and HbS: The separate dialysate containing HbS and HbA was mixed with corresponding buffer solution (ratio 1:3 v/v) for spectroscopic analysis at pH 7.2 and 5.0 respectively. The content (3 ml) was transferred into a curvette and scanned at wavelenghts within the range of 250-650 nm (UV-Visable Spectrophotometer, Jenway 6405). Also, spectroscopic analyses were carried out using HbA and HbS treated with 0.1 ml of hydrogen peroxide concentrations (4.00-20.00 mM). The absorbtivity expressed in $A\cdot mmol^{-1}\cdot cm^{-1}$ was calculated by dividing the absorbances at corresponding wavelenght by haemoglobin concentration (mmol/L) and length of light path (l = 1.0 cm).

RESULTS

Generally, the illustrations of Figure 1-4 demonstrated that absorption spectra of HbA and HbS exhibited a charateristic Soret band. Figure 1 showed that within the spectral range of 250-650 nm, maximum absorptivities of HbA and HbS occured at wavelenght (λ_{max}) = 415 nm. At the two

experimental pH conditions of 7.2 and 5.0, the maximum absorptivity of HbA was 2.0 $mmol^{-1}\cdot cm^{-1}$. HbA and HbS exhibited lower peak absorptivities in the presence of hydrogen peroxide (H_2O_2) in a concentration dependent manner. Maximum absorptivities of the two haemoglobin molecules showed hypochromic red shift of the Soret band from 415 to 420 nm with increasing concentrations of H_2O_2 regardless of changes in experimental pH values (Figures 1-4).
Furthermore, Figures 1-4 showed that increasing concentrations of H_2O_2 at the two experimental pH values engendered a differential distortion and obliteration of the Soret band. For instance, Whereas at pH = 7.2, $[H_2O_2]$ > 14.28 mM (Figure 1) caused obliteration of the Soret band of HbA, 20.00 mM H_2O_2 at lower pH = 5.0 (Figure 2), obliterated the Soret band.
In contrast to absorption spectra of HbA, Figures 3 and 4 showed that at the two experimental pH values of 7.2 and 5.0, maximum absorptivities of HbS were 1.8 and 2.3 $mmol^{-1}\cdot cm^{-1}$ respectively. However, HbA and HbS showed decreasing peak absorptivities with increasing concentrations of H_2O_2 regardless of changes in pH values. Comparatively, millimolar absorptivities within the absorption spectra of HbA and HbS were relatively higher at experimental pH = 5.0 than pH = 7.2.
At pH values of 5.0 and 7.2 the absorption spectra within the wavelenght range (λ > 450 nm approx.), of both free and H_2O_2 treated HbA and HbS, displayed low light absorption and did not show appreciable difference in millimolar absorptivity. Furthermore, all absorptivity curves converged at the isosbestic point which was slightly above 625 nm. The absorption spectra showed upward displacement at λ < 320 nm approx. which gave a crossover point on the right of the Soret band (Figures 1-4). Also, the two haemoglobins exhibited absorption peaks within wavelenght region of 250-280nm.

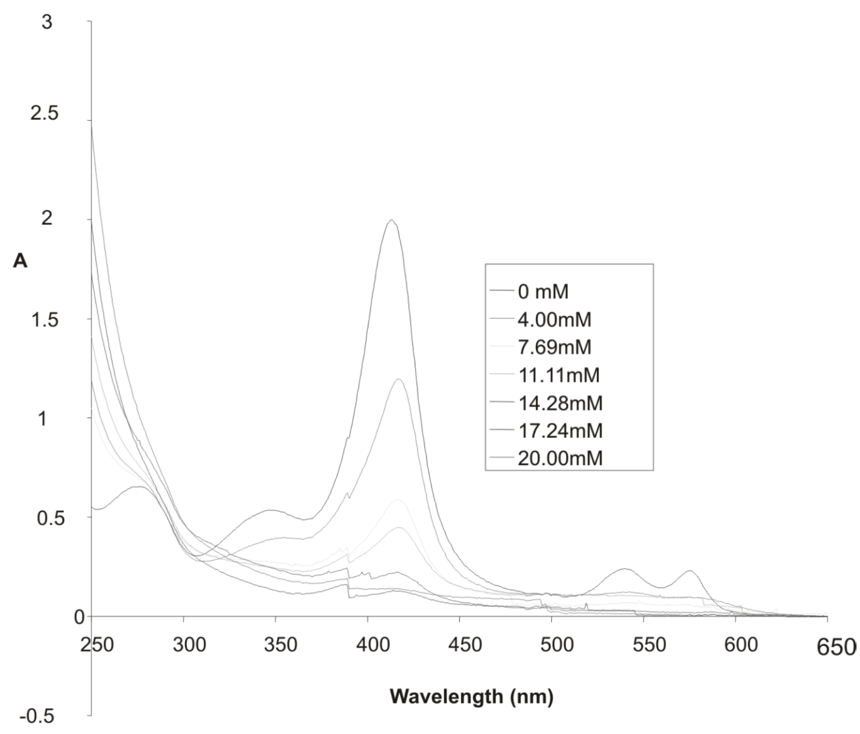

Figure 1: Absorption spectra of HbA in the presence of H_2O_2 (4.00 mM – 20.00 mM) at experimental pH = 7.2. The absorptivity is expressed in $A\cdot mmol^{-1}\cdot cm^{-1}$.

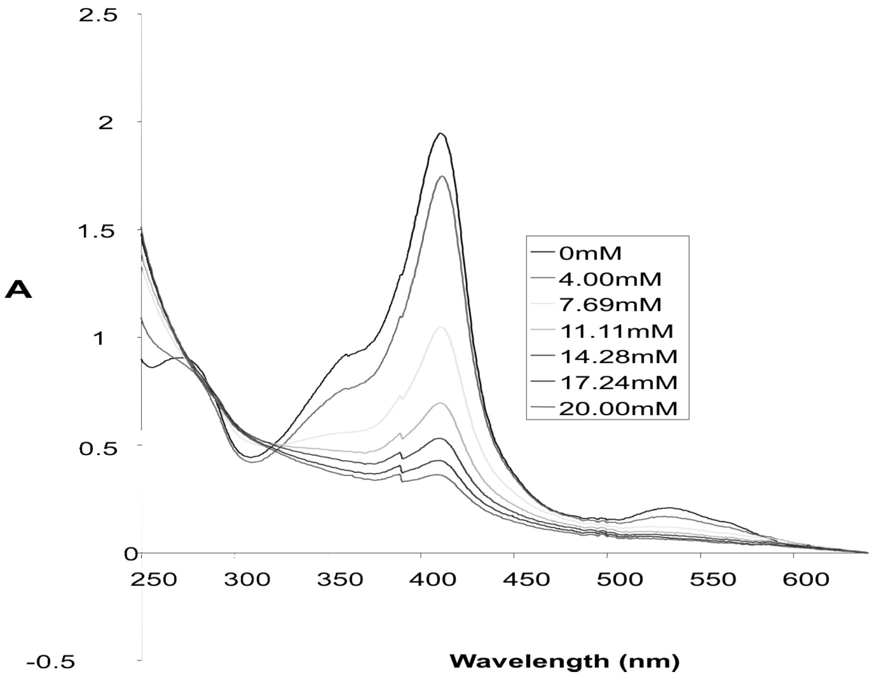

Figure 2: Absorption spectra of HbA in the presence of H_2O_2 (4.00 mM – 20.00 mM) at experimental pH = 5.0. The absorptivity is expressed in $A \cdot mmol^{-1} \cdot cm^{-1}$.

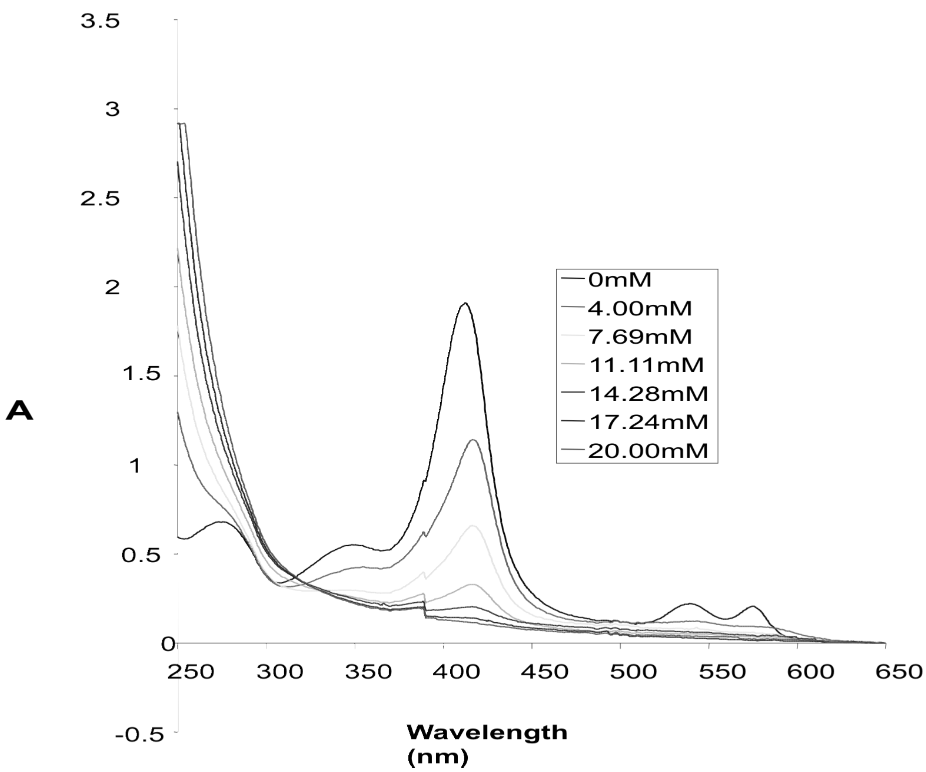

Figure 3: Absorption spectra of HbS in the presence of H_2O_2 (4.00 mM – 20.00 mM) at experimental pH = 7.2. The absorptivity is expressed in $A \cdot mmol^{-1} \cdot cm^{-1}$.

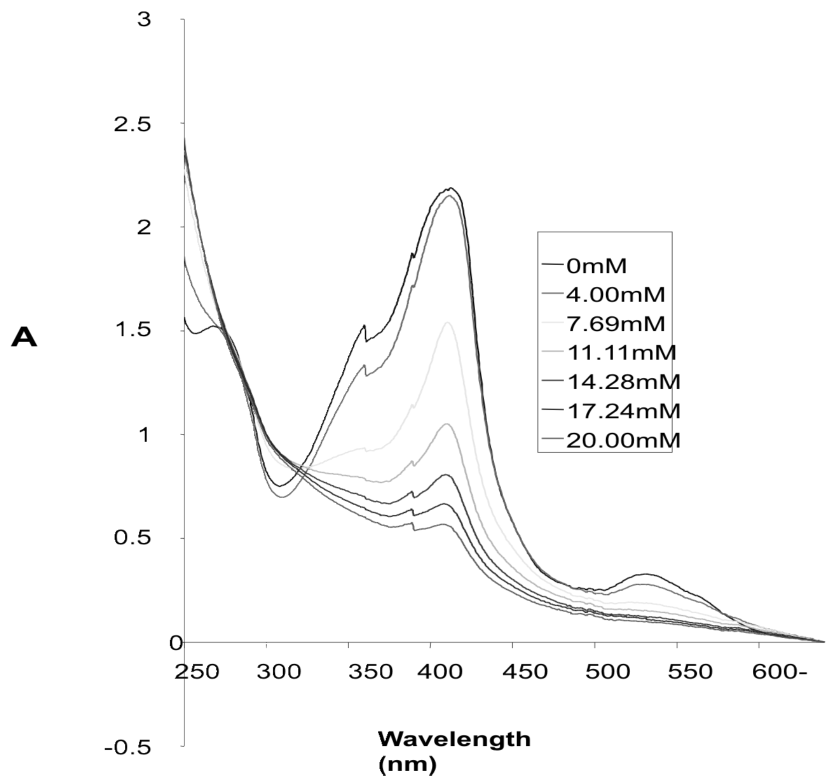

Figure 4: Absorption spectra of HbS in the presence of H_2O_2 (4.00 mM – 20.00 mM) at experimental pH = 5.0. The absorptivity is expressed in $A{\cdot}mmol^{-1}{\cdot}cm^{-1}$.

DISCUSSION

The absorption spectra of HbA and HbS within the range of 250-650 nm displayed the characteristic Soret band of hematoporphyrins. The spectral features are in conformity with previous reports elsewhere [4,5,6]. In addition, the maximum wavelenght of peak absorptivity and corresponding spectra shift of the two haemoglobins were not affected by changes in experimental pH conditions. In a related investigation, Denninghoff *et al.*, [6], averred that blue-green spectral shift with changing oxyhemoglobin saturation was preserved in blood samples and was relatively unaffected by physiological changes in blood pH values of 6.6, 7.1, and 7.4 units.

The reports of Suzuki, [15] stated that liganded derivative (oxyhaemoglobin) exhibited two absorptivity maxima in the same region i.e. two sharp peaks at about 540 nm and 576 nm, with a large bathochromic shift upon the addition of water and soluble organic solvents. The present study showed that treatment of HbA and HbS with H_2O_2 distorted their characteristic spectral properties but with a hypochromic red shift of the Soret band from 415 to 420 nm, which is thought to be related to the dielectric constraint of the solvent [15]. The absorption spectra at $\lambda < 320$ nm region is as a result of aromatic amino acids and peptide bonds with distinct peaks at 280 nm 205 nm respectivly [1, 16]. Furthermore, the presence of carboxylic acid moieties are reponsible for absorbance peaks around 274 nm [17].

In the present study, much difficulty was encountered in preparing pure hemoglobin and in establishing adequate criteria for its purity. Therefore, discrepancies might arise with respect to absolute values of millimolar absorbtivity, depending on the purity of haemoglobin preparation, analytical methods used and dispersing power of the spectrophotometers employed by other investigators. Also, the present investigations were confined within the visible/ultraviolet portion of the spectrum; although in certain cases observations extended into the near infra-red region indicated the presence of an interesting oxyhemoglobin band [18, 4,19]. Nevertheless, our investigations have established that spectra signatures of HbA and HbS treated with H_2O_2 were non-identical under the two experimental pH conditions which are diagnostic of the two haemoglobin types.

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