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Absorption Spectra of Normal Adults and Patients with Sickle Cell Anaemia Treated with Hydrogen Peroxide at Two pH Values

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Abstract

Background: The aim of the present study was application of haemoglobin absorption spectroscopy as a distinguishing tool for identification of two haemoglobin types-HbA and HbS.

Material and Methods: 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).

Results: The absorption spectra of HbA and HbS exhibited the characteristic Soret band with maximum absorptivities at wavelenght (λmax) = 415 nm. Maximum absorptivities of HbA and HbS treated with H_2O_2 showed hypochromic red shift of the Soret band from (λmax) = 415 to 420 nm regardless of changes in pH values. In addition, increasing concentrations of H_2O_2 at the two pH conditions caused differential distortion and obliteration of the Soret band. Whereas 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, maxima absorptivities of HbS were 1.8 and 2.3 mmol-1•cm-1 respectively.

Conclusion: The spectra patterns of HbA and HbS treated with H_2O_2 were non-identical under the two experimental pH conditions. Therefore, these spectra absorptivity patterns can serve for the identification of HbA and HbS and to distinguish between the two haemoglobin types.

Keywords: Haemoglobin, hydrogen peroxide, Soret band, absorption spectra, sickle cell anemia.

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. Haemoglobin subunits are transformed directly to haemichrome using oxidation with H_2O_2 without the generation of methaemoglobin for specific absorption spectrometric studies[4] and UV-Visible absorption spectroscopy shows that haemoglobin exhibits intense absorption in wavelengths above 320 nm[5]. Strong absorption occur at near 400 nm and this peak region is known

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as the Soret band[6;7]. The Soret band is characteristic of hematoporphyrin proteins[8;9]. Specifically, deoxy-, oxy-, met- and ferri- haemoglobins have their corresponding absorbance maximum in the Soret region at 430, 415, 405 and 418 nm respectively[10]. Thus, haemoglobin maximum absorbance 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) can be readily distinguished by their different mobility in electrophoresis, the aim of the present study was application of haemoglobin absorption spectroscopy as a distinguishing tool 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 ten (n = 10) human volunteers between the age t of 21 and 34 years 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, Nigeria. This study was in accordance with the ethical principles that have their origins in the Declaration of Helsinki.

Preparation of haemolysate haemoglobin: The two variant haemoglobins were subjected to the same purification and analytical procedures and investigations were carried out to ascertain the absorption spectra of the two harmoglobins. The absorption spectra of HbA were considered as the control/reference, whereas HbS represented the test analysis.

The erythrocytes were washed by centrifugation method as described by Tsakiris et al., [11] with minor modification according to Pennings et al., [12]. 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. To remove platelets and leucocytes, the sediment was re-suspended in 3 ml of phosphate-buffered saline (PBS) solution, pH 7.4, and passed through a column (3.5 cm in a 30 ml syringe) of cellulose-microcrystalline cellulose (ratio w/w 1:1) as described by Kalra et al., [13]. The eluted fraction was passed twice through a new column of cellulose-microcrystalline cellulose (ratio 1:1 w/w)

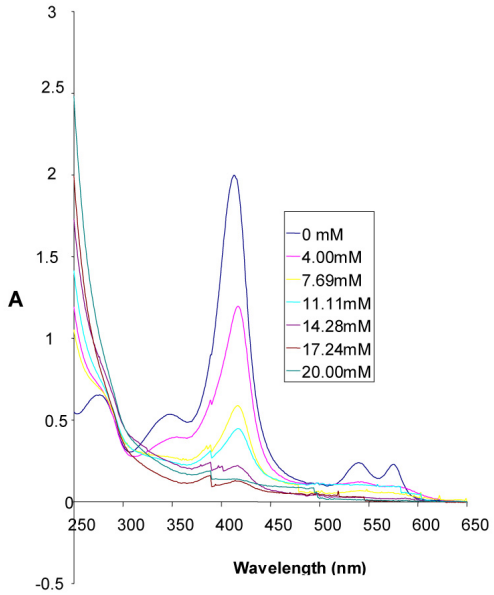


Figure 1: Absorption spectra of HbA in the presence of H_2O_2 (4.00 mM – 20.00 mM) at experimental pH = 7.2. The absorptivity is expressed in $A \bullet mmol^{-1} \bullet cm^{-1}$.

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to obtain erythrocyte suspension sufficiently devoid of leucocytes and platelets. A 2.0 ml of 0.05 M Tris-HCl buffer (pH = 8.5) was re-introduced to eluted fraction and kept at refrigerated temperature of 4° C for 20 min. The erythrocytes were 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, [14] and Kamber et al., [15].

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)[7]. 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 pellet 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 separate crude haemoglobins were further purified using the DEAE-cellulose ion-exchange (TOYOPEARL MD-G SP-650S; TOSOH BIOSCIENCE LLC, PA, USA) chromatography as described by Sun and Palmer [16], with modifications. A 5.0 ml of the dialyzed haemoglobin suspension was introduced into the packed and equilibrated gel in a column (10 mm (i.d) x 6.8 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 different 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[17], based on cyanomethaemoglobin reaction was used for 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 was read at λmax = 540 nm (UV-Visable Spectrophotometer, Jenway 6405) against

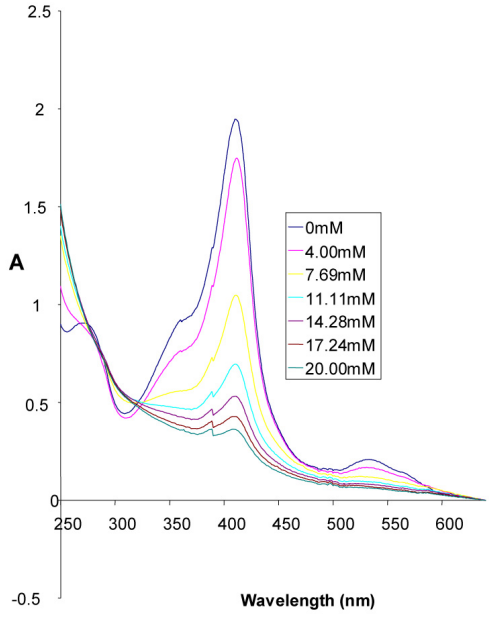


Figure 2: Absorption spectra of HbA in the presence of H_2O_2 (4.00 mM – 20.00 mM) at experimental pH = 5.0. The absorptivity is expressed in A•mmol^{-1}•cm^{-1}.

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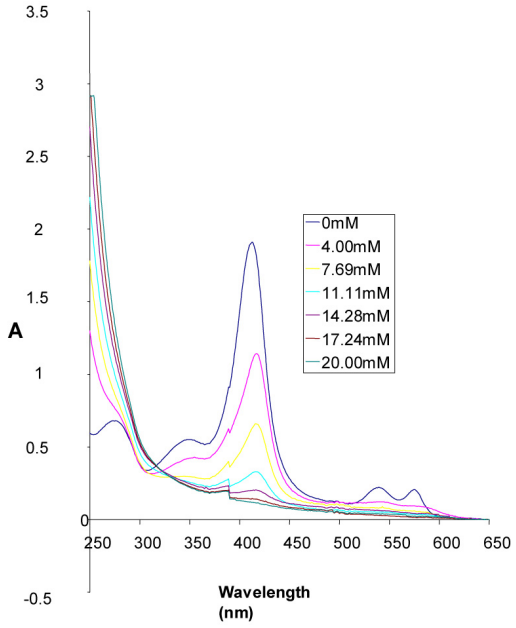


Figure 3: Absorption spectra of HbS in the presence of H_2O_2 (4.00 mM – 20.00 mM) at experimental pH = 7.2. The absorptivity is expressed in A•mmol^{-1}•cm^{-1}.

a blank. The absorbance was used to evaluate for haemoglobin concentration by comparing the values with the standards[18].

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 control sample (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), considered as the test analyses. The absorbtivity expressed in A•mmol^{-1}•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, as shown in figures 1-4 the absorption spectra of HbA and HbS exhibited a charateristic Soret band. Figure 1 shows that within the spectral range of 250-650 nm, maximum absorptivities of HbA and HbS occured at wavelenght (λmax) = 415 nm. At the two experimental pH conditions of 7.2 and 5.0, the maximum absorptivity of HbA was 2.0 mmol^{-1}•cm^{-1}. HbA and HbS exhibited lower peak absorptivities in the presence of hydrogen peroxide (H_2O_2) in a concentration dependent manner. Maximum absorptivity 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 shows that increasing concentrations of H_2O_2 at the two experimental pH values cause a differential distortion and obliteration of the Soret band. For instance, whereas 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 show that at the two experimental pH values of 5.0 and 7.2, maxima absorptivities of HbS were 1.8 and 2.3 mmol^{-1}•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

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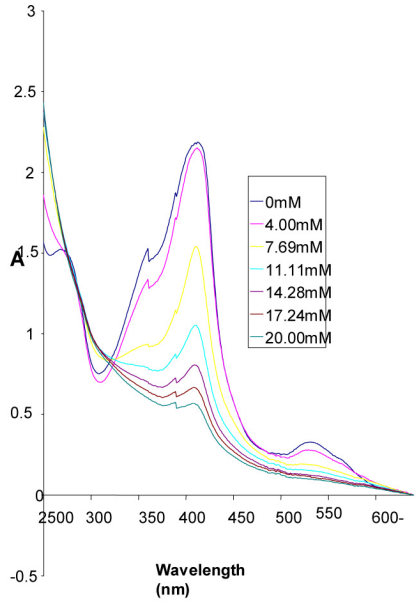


Figure 4: Absorption spectra of HbS in the presence of H_2O_2 (4.00 mM – 20.00 mM) at experimental pH = 5.0. The absorptivity is expressed in $A \bullet mmol^{-1} \bullet cm^{-1}$.

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 $\lambda < 320$ 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.

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[5; 6; 7]. 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. [7], 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.

Suzuki,[19] 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[19]. The absorption spectra at $\lambda < 320$ nm region is the result of aromatic amino acids and peptide bonds with distinct peaks at 280 nm 205 nm respectivly[1;20]. Furthermore, the presence of carboxylic acid moieties are reponsible for absorbance peaks around 274 nm[21].

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 are confined within the visible/ ultraviolet portion of the spectrum; although in certain cases observations extended into the near infra-red region indicating the presence of an interesting oxyhemoglobin band [5,22, 23].

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Conclusion

The spectra patterns of HbA and HbS treated with H_2O_2 were non-identical under the two experimental pH conditions. Therefore, these spectra absorptivity patterns can serve for the identification of HbA and HbS and to distinguish between the two haemoglobin types.

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