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Band Assignment in Hemoglobin Porphyrin Ring Spectrum: Using Four-Orbital Model of Gouterman

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Abstract: Band assignment for oxy, deoxy and methemoglobin using orbital promotion is crucial to understanding inter-relation of electronic transitions. Spectral changes may be correlated with conformational alterations. Conformational changes of hemoglobin were interpreted using four-orbital model of Gouterman. Our results indicated that Goutherman model can predict the predominant conformations of hemoglobin.

Keywords: Porphyrin, Hemoglobin spectrum, Gouterman model, Band assignment

INTRODUCTION

The porphyrin molecule is a 24-member ring with 11 conjugated and delocalized double bonds. The electronic absorption spectrum of porphyrin ring shows several transitions: Q-band in visible region (490-650 nm), Soret or B band in near-UV (400-436 nm), N, L and M bands in deep UV regions (200-350 nm). In four-orbital model of Martin-Gouterman, Q-band and Soret bands are interpreted as $\pi \rightarrow \pi^*$ electron transitions [1-10]. In Gouterman model these bands are considered as electronic transitions from two HOMO orbitals (Highest Occupied Molecular Orbital), a_1u (π) and a_2u (π), to two LUMO orbitals (lowest Unoccupied Molecular Orbital), e.g. (π^*) orbitals [3-5, 11]. A weak transition from ground state S_0 to the first excited state S_1 results in four Q-bands with weak intensity. The strong transition from ground state or S_0 to the second excited state S_2, yields Soret band, with strong intensity [8-9]. Further studies of molecular fluorescence of porphyrins showed that there is another transition at about 630 nm, which is considered as $n \rightarrow \pi^*$ transition [8, 10-14]. Metal ions like Fe^{+2}, Zn^{+2}, Cu^{+2} and Co^{+2} can be inserted in the center of porphyrin ring via accepting lone pairs electrons of nitrogen atoms, forming metalloporphyrin. Metalloporphyrin formation blocks $n \rightarrow \pi^*$ transition and omits the 630 nm band. Four Q-bands of free or porphyrins changed to two bands, α and β bands, in case of metallation [11, 15]. The Q-bands of porphyrins is sensitive to the changes exerted by surroundings, probably through internal nitrogens of porphyrin heterocycle, so Q-band splitting pattern can be considered as an indicator for the interactions between the surroundings and the porphyrin rings [16-24]. The spectrum of normal oxy hemoglobin mimics that's of metalloporphyrins. The first transition in hemoglobin produces Q-bands, α band at about 560 nm and β band at about 528 nm. The second transition produces B-band or Soret band at about 422 nm. The molecular interpretation of spectral changes in hemoglobin spectra in different physiological and pathological conditions e.g. deoxygenation and oxidation taking in account the conformational changes in globin structure and d orbital hybridization through bands assigning, is not well understood yet. In the present work, using absorption spectra of oxy, deoxy and met-hemoglobin and present a precise model by which the hemoglobin spectrum could be interpreted in deferent conditions. The gradual alteration in hemoglobin structure also could be followed by this model by a simple absorption spectrum.

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METHODS AND MATERIALS

Sodium n-dodecyl sulfate (especially pure grade) was obtained from Sigma. CM-Sephadex and Sephadex G-25 were obtained from Pharmacia fine chemicals. Other reagents were of analytical grade. All solutions were prepared with double distilled water.

Hemoglobin Preparation

Human adult hemoglobin (HbA) was prepared from human red blood cells of healthy donors according to the method of Williams *et al.* [25] and stripped of anions according to the procedure reported by Riggs [26]. The heparinized blood was washed twice with ten volumes of isotonic saline. The washed, packed cells were lysed with an equal volume of deionized water. The stroma was removed after 1 hour at 0˚C by centrifugation at 10,000 rpm for 25 min. The solution was applied to a Sephadex G-25 column (1.5×45 cm) equilibrated with 0.1 M phosphate buffer pH 7.0. Elution was carried out with 0.1 M phosphate buffer pH 7.0 at a flow rate of 25 ml per hours [27]. CM-Sephadex cation exchanger at pH 6.5 was used for further purification of human adult hemoglobin.

Preparation of Hemoglobin Variants:

Fresh human hemoglobin samples equilibrated with air were used as oxy hemoglobin [25]. Deoxy hemoglobin was prepared by addition of enough dithionite to the fresh hemo-

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globin samples [25]. Cyanomet hemoglobin was prepared by addition of Drabkin's solution to oxy hemoglobin samples [25].

Spectroscopy Experiments

The method of Benesch, *et al.* [28] was used with minor modifications of equations at pH 7.0. The concentrations of oxy-, deoxy- and met- forms of hemoglobin were determined using a Shimadzu model UV-3100 (Japan) spectrophotometer and a thermostatically controlled cell compartment with Haak D8 water bath.

RESULTS AND DISCUSSION:

This paper aimed at understanding the species of hemoglobin through studying the hemoglobin spectra at different conditions, trying to interpret the electronic transitions according to tertiary structure of hemoglobin, and assigning the absorption bands to these transitions in oxy, deoxy and met conformations of hemoglobin. The visible spectra of hemoglobin originated from electronic transitions of porphyrin ring were studied. It is found that the ligand binding to hemoglobin and the surrounding environment affect the spectrum pattern.

Visible Spectrum of Porphyrin Ring

Table **1a** and Table **1b** summarize Soret band, Q-bands (bands I to IV) and n $\rightarrow\pi^*$ band wavelengths and mean values for different porphyrins used by Dinello and Chang [19]. Clearly almost all porphyrins show a Soret band in 400-436 nm range with a mean wavelength of 413 nm, four Q-bands include band IV in 497-526 nm range with mean of 504 nm, band III in 532-563 nm range with a mean wavelength of 540 nm, band II in 566-595 nm range with a mean wavelength of 574 nm and band I in 593-650 nm range with a mean wavelength of 596 nm and finally n $\rightarrow\pi^*$ transition in 620-630 nm range with a mean wavelength of 623 nm. Furthermore, Table **1c** shows the collection of epsilon (molar extinction coefficient) values for four bands of porphyrin studied by Dinello and Chang [19]. Band I among the Q-bands of different porphyrins, has the lowest epsilon value that could be the reason behind its absence from some porphyrins spectrum (see Table **1c**).

Ferrous Ion Binding to Porphyrin Ring

As shown in Table **1d**, the binding of Fe^{+2} to porphyrin ring exerted vast alterations in absorption spectrum of porphyrin. First of all, as depicted in Scheme **1**, Fe^{+2} with filled $d\pi$ orbitals contributed a significant metal $d\pi$ to porphyrin π^* orbital interaction (metal to ligand back bonding), increasing the energy gap between π and π^* orbital and causing blue shift in porphyrin $\pi \rightarrow \pi^*$ transitions both in Soret and Q-bands. In the reverse experiment showed in Fig. (**1**), using Drabkin's solution to oxidizing ferrous ions caused red shift in Soret band, alike the conversion of metalloporphyrin to free porphyrin and may infer to the release of ferric ion from heme. Second, Table **1d** shows that metal binding was followed by combination of the four split Q-bands to emerge into two bands, namely α and β bands, which means the blockage nitrogen protonation in following the porphyrin metallation. Fig. (**1**) curve c depicts the appearance of three Q-bands (except band I) in the presence of Drabkin's solution. Third, Table **1d** indicates that the n $\rightarrow \pi^*$ transition at about 630 nm was absent in metalloporphyrins in contrast to metalloporphyrins at Table **1a**, because of a participation of lone pair electrons of nitrogen atoms in covalent bonding with ferrous ion and as shown in Fig. (**1**). This band is gradually appeared after ferrous oxidation by Drabkin's solution. The presence or absence of n $\rightarrow \pi^*$ band at about 630 nm may be used as a criterion for the conversion of the spectroscopic properties of metalloporphyrin to free porphyrin.

Effect of Ferricyanide on Hemoglobin Spectrum

In this study, Drabkin's reagent was used for hemoglobin oxidation. As shown in Fig. (**1**), the intensity of α and β bands decreased with increasing the Drabkin's solution con-

Table 1a. Q-Bands I to IV, Wavelengths for Different Porphyrins Studied by Dinello and Chang

Porphyrin Substrates	Band IV (nm)	Band III(nm)	Band II(nm)	Band I (nm)
A.protoporphyrin	506	541	576	605
B.Deuteroporphyrin	497	539	566	593
C.Hematoporphyrin	499.5	532	569.2	596
D. Mesoporphyrin	498	532	567	595
E. Diacetyldeuteroporphyrin	517	552	587	No Band
F. Diformyldeutroporphyrin	526	562	595	No Band
H. porphyrin c	504	538	570	No Band
J. Uroporphyrin I and III	502	536	572	No Band
K. Coproporphyrin I and III	498	532	566	594
L.Pemptoporphyrin	502	536	572	No Band

Table 1b. Soret Band, Q-bands (bands I to IV) and n→π* Band's Mean Values for Different Free Porphyrins Studied by Dinello and Chang

Band type	Absorption wavelength (nm)	Mean wavelength (nm)
Soret Band	400-436	413.2
Band IV	497-526	504.9
Band III	532-563.5	540
Band II	566-595	574
Band I	593-650.5	596.6
n → π*	620-631	623.2

Table 1c. Collection (Molar Extinction Coefficient) Values for four Q Bands of Different Free Porphyrins Studied by Dinello and Chang

Porphyrin substrates	$\varepsilon \times 10^{-3} M^{-1} cm^{-1}$			
	Band IV	Band III	Band II	Band I
A.protoporphyrin	14.89	11.87	7.48	2
B.Deuteroporphyrin	14.5	7.84	6.32	1.32
C.Hematoporphyrin	14.7	9.04	6.57	1.26
D. Mesoporphyrin	14.37	10	6.92	1.68
E. Diacetyldeuteroporphyrin	13.3	7.3	6.1	No Band
F. Diformyldeutroporphyrin	12.6	7.7	6.48	No Band
H. porphyrin c	12.1	8.4	6.2	No Band
J. Uroporphyrin I and III	15.8	9.35	6.85	No Band
K. Coproporphyrin I and III	14.34	9.92	7.13	1.48
L.Pemptoporphyrin	13.65	10.4	6.45	No Band

Table 1d. Soret, Alpha and Beta Band's Mean Values for Different Metalloporphyrins Studied by Dinello and Chang

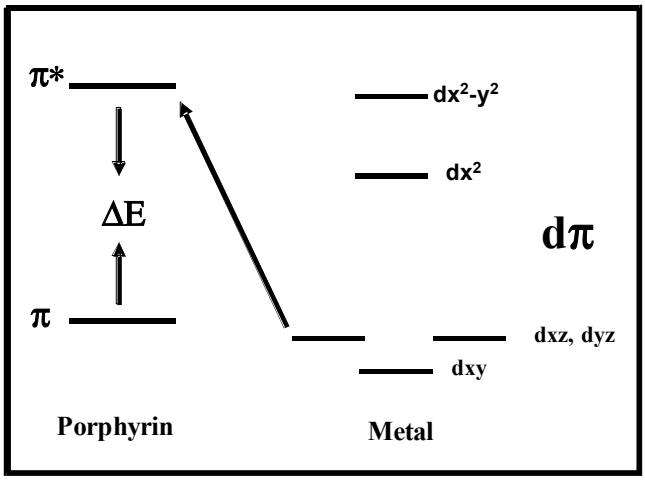
Metalloporphyrin(Fe^{+2})	Absorption wavelength (nm)	Mean wavelength (nm)
Soret Band	405.5-450	422.3
Beta Band	514-549.5	528.1
Alpha Band	544-586.5	560.1

centration and gradually changed to four Q-bands, I to IV at about 596.6 nm, 574 nm, 504.9 nm and 450 nm respectively and n→π* band at about 623.2 nm. However, at higher concentration, because of close proximity of band I (597 nm) and n→π* band (630 nm), the latter band with strong intensity overlapped band I with lower intensity, hence its disappearance in free heme rings.

Effect of Acidic pH on Hemoglobin Spectrum

Increasing proton (H^+) concentration via affecting hemoglobin conformation, facilitated the release of oxygen from hemoglobin and converted oxy to deoxy conformation. Thus, typical spectrum of deoxy hemoglobin may be obtained in acidic condition. Moreover, at more prolonged acidic conditions the internal nitrogen atoms of heme ring become protonated. The protonation of heme ring led to release of Fe^{+2} ion, hence the formation of typical free porphyrin spectrum, with a Soret band, four Q-band and a band at 630 nm, as shown in Fig. (**2**). To magnifying the small bands the absorbance difference is drawn instead of simple absorbance against wavelength in Fig. (**2**). There are two transitions at pH 7 and pH 6 in Q-band region in accordance with normal

oxy hemoglobin. At pH 5 the two transitions changed to four bands which shows the release of Fe^{+2} from heme ring.



Scheme 1.

Effect of Sodium Dedecyl Sulfate on Hemoglobin Spectrum

Sodium dodecyl sulfate (SDS) is an anionic detergent which can denature hemoglobin at > 2 mM concentrations. As already reported [29] 1 mM concentration of SDS, at pH 7, folds the hemoglobin to more compacted deoxy conformation. This effect of SDS is because of repulsive forces between the negatively charged hemoglobin at pH 7 and the negatively charge of SDS. Fig. (**3**) shows the effect of 1-4 mM concentrations of SDS on UV-Vis spectrum of hemoglobin. Using the Eq (1) [30] to calculate the oxy, deoxy and met hemoglobin concentrations, we indicated that the deoxy conformation of hemoglobin was the prevalent species at 1 mM concentration of SDS.

$$[Oxy] = (1.0154A_{576} - 0.2772A_{630} - 0.742A_{560})\times10^{-4}$$

$$[Deoxy] = (1.357A_{560} - 0.7376A_{576} - 0.6254A_{630})\times10^{-4}$$

$$[Met] = (2.6829A_{630} + 0.174A_{576} - 0.3614A_{560})\times10^{-4}$$

Equation (1)

A small band at 630 nm in HbA sample, inset of Fig. (**3**), curve I, shows that some populations of oxidized hemoglobin are present. The folding effect of SDS in 1 mM concentration pushed Fe^{+2} ions from out of plane to ring plane as reported previously [29]. This condition increases the reduction potential of Fe^{+3} ions reducing it to Fe^{+2} ions via water oxidation. The $n\rightarrow\pi^*$ transition in 1mM concentration of SDS was not observed, inset of Fig. (**3**), curve II. More increase in SDS concentration caused denaturation of hemoglobin protein resulting Fe^{+2} settled out of plane (see Fig. **3** curves a to c). This claim is supported by blue shifts in Q and Soret bands (data not shown) and by changing α and β bands to four bands and the gradual appearance of $n\rightarrow\pi^*$ transition in high concentration of SDS.

Effect of Dithionite on Hemoglobin Spectrum

It was shown that in Hypsoporphyrins - open-shell metalloporphyrins with d^m orbitals (m=6-9 electrons like Fe^{+2} with 6 electrons)- $d\pi \rightarrow \pi^*$ backbonding increases the energy gap between π and π* orbitals energy causing blue shift in absorption spectrum (see Scheme **1**).

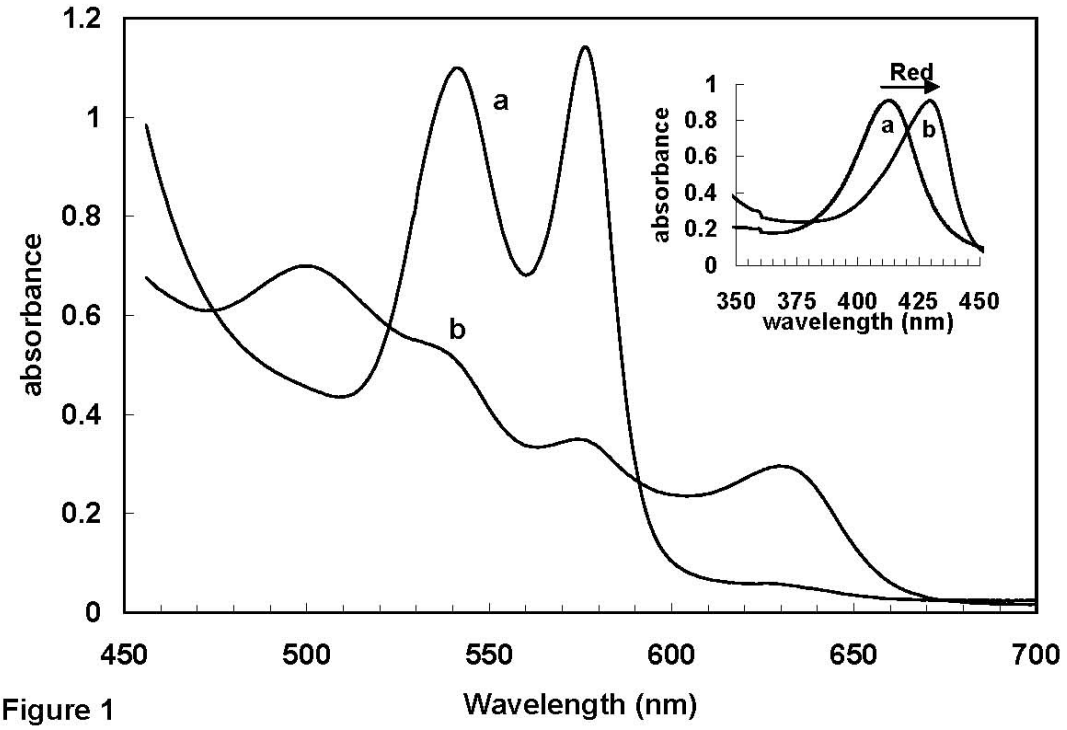


Figure 1. The absorption spectrum of oxy hemoglobin in the presence of different concentrations of Drabkin's solution. Inset shows the red shift in Soret band in the presence of Drabkin's solution. a, 0 mM; b, 0.5 mM and c, 1.5 mM concentration of Drabkin's solution.

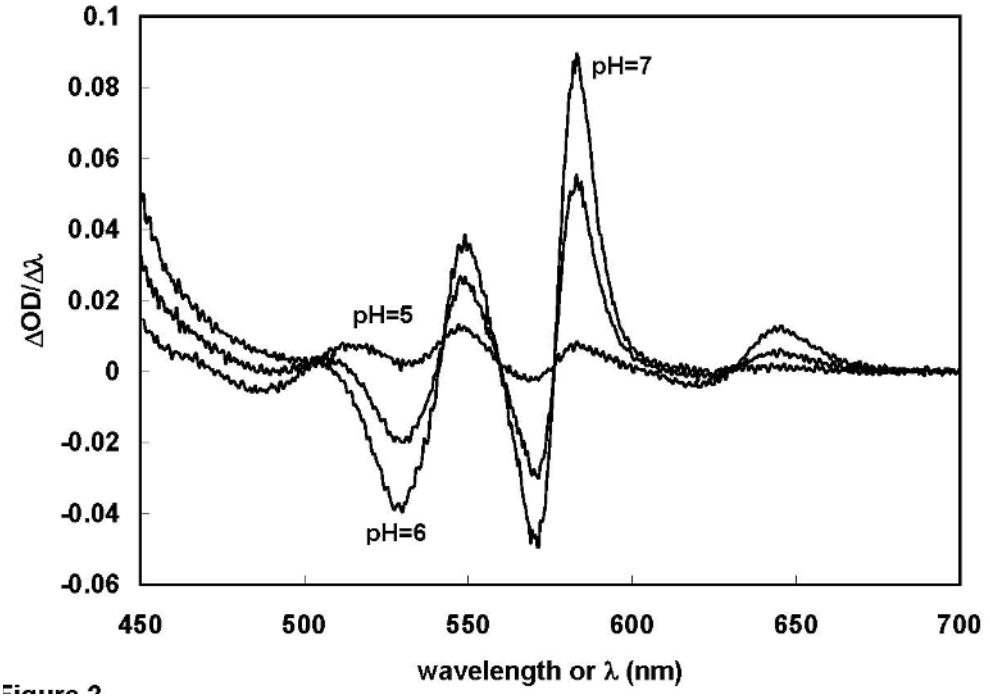


Figure 2. The difference absorption spectrum (ΔOD/Δλ against λ wavelength) of oxy hemoglobin in100 mM phosphate buffer and 10 min incubation time at pH 5, 6 and 7.

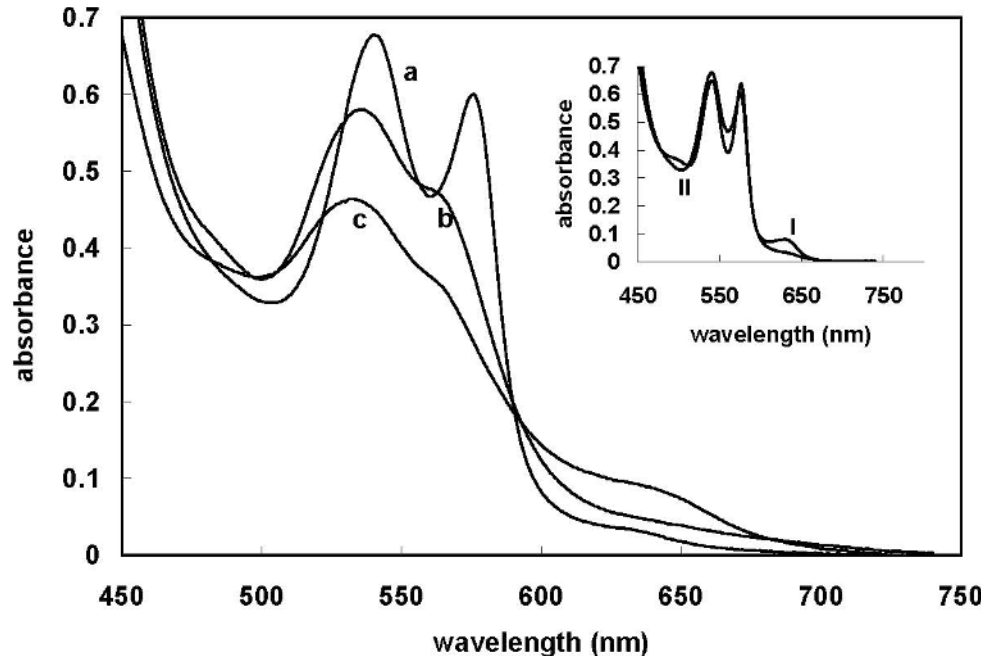


Figure 3. Absorption spectrum of oxy hemoglobin in the presence of different concentrations of SDS. a,0 mM; b,1 mM; c, 2 mM; d, 4 mM concentration of SDS. Inset is absorption spectrum of partially oxidized hemoglobin in the absence of SDS (spectrum I) and in the presence of 1 mM concentration of SDS (spectrum II)

Dithionite has been known to reduce the oxygen to water and used for long time to convert oxy hemoglobin to deoxy hemoglobin. In this study, dithionite was used to obtain deoxy hemoglobin spectrum. As presented in Fig. (**4**), in deoxy hemoglobin spectrum, Soret and Q bands were red shifted (Fig. **4a-c**). Two Q-bands at 541 nm and 576 nm (Fig. **4a**, inset) became broad and converted to a very broad band at about 554 nm (Fig. **4b-c**, inset). In deoxy hemoglobin, Fe^{+2} is a five coordinated complex with square pyramidal configuration. As depicted in Scheme **2**, in this configuration the energy of dxz and dyz orbitals become lower than that of six octahedral coordinated complex of oxy hemoglobin, hence $d\pi \rightarrow \pi^*$ backbonding becomes less favorable, so a red shifts in absorption spectrum takes place. During changing iron

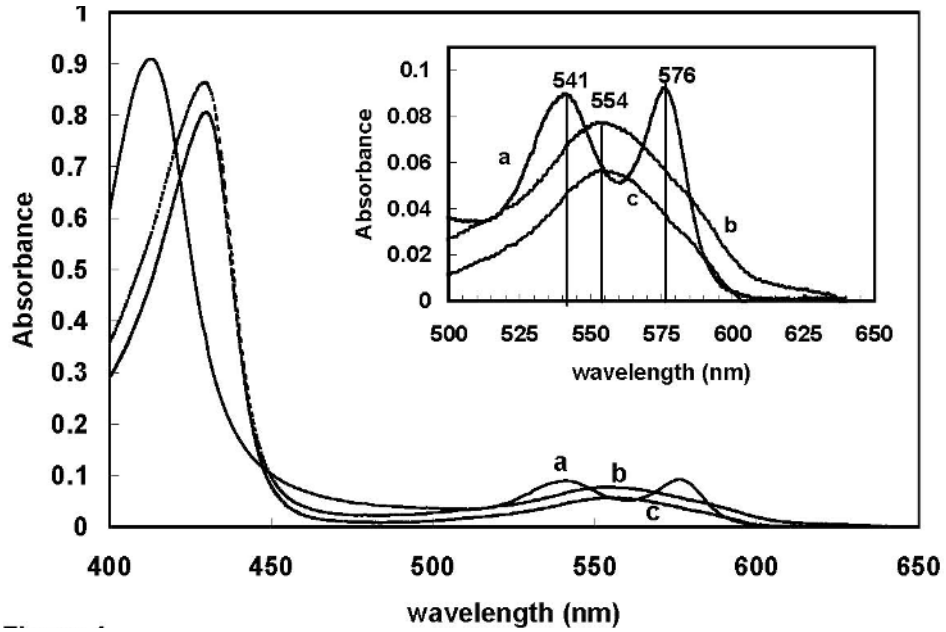
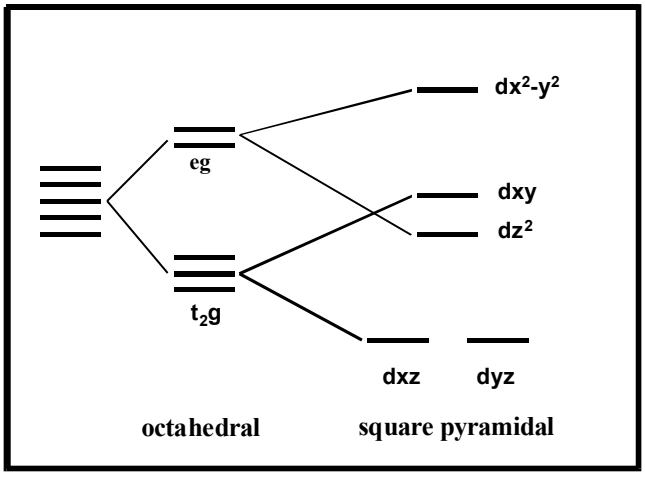


Figure 4. Absorption spectrum of oxy hemoglobin in the presence of different concentrations of dithionite. a=0 mM, b=0.5 mM concentration and c=3 mM concentration of dithionite.

configuration, from octahedral to square pyramidal, a gradual moving of Fe^{+2} ions from inside to outside of ring, because of the absence of the sixth ligand, makes different conformational populations with differently positioned Fe^{+2} ions. This phenomenon produces different electronic transitions with slightly different energy causing broadened and overlapped α and β bands. This broadening effect is because of oxidation and out going of Fe^{+2} in the presence of oxidant like Drabkin's solution (Fig. **1**).



Scheme 2.

CONCLUSION

In biochemical studies, band assignment and interpretation of band changes in physiologically functioning hemoglobin is important for assessment and validation of the accepted models. In this work, oxygenation, deoxygenation and oxidation of hemoglobin were thoroughly studied via absorption spectrum analysis. The collected data was found to be in conformity with others works, indicating that the hemoglobin absorption spectrum in the range of 350-700 nm could be simply interpreted using the four-orbital model of Gouterman. Our data shows that there are three bands in fully oxygenated hemoglobin, Soret band at about 422 nm, beta band at 540 nm and alpha band at 576 nm. We suggest that deoxygenation of hemoglobin may change the conformation of Fe^{+2} d-orbitals from octahedral to square pyramidal leading which is weakening of $d\pi \rightarrow \pi^*$ backbonding. As shown in Scheme **2**, this changes caused a decrease in energy gap between dxy and π* orbitals and induced a red shift in absorption spectrum. Moreover, the two bands α and β changed to a broad band with a maximum at 554 nm. It is well known that there is a slight movement of ferrous ions inside and outside the heme ring upon oxygenation and deoxygenation respectively. Gradual outside going of Fe^{+2} on deoxygenation produces different populations of hemoglobin with different position of ferrous ions and makes different electronic transitions with slight deference in energy. This may be the cause of broad bands. In met or cyanomethemoglobin, as shown in Fig. (**1**) at excess concentrations of ferricyanide (1.5mM), Fe^{+2} went completely out of heme plane and the spectrum resembled that of free porphyrin ring with a strong and red shifted Soret band at about 413 nm, four Q-bands in 495-650 nm range and $n \rightarrow \pi^*$ band at 630 nm which overlapped band I of Q-bands. Finally, it can be concluded that conformational changes in hemoglobin during its physiological role are concomitant with band shift and changes in absorption spectrum. Moreover, our finding shows that band alterations upon hemoglobin conformational changes are exerted via axial bands of Fe^{+2} ions and changes the Fe^{+2} position, but not via heme ring members.

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