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HYPOTHESIS

A Proposed Significance of the δ Region and its Implications in the Mechanism of Cooperativity in Hemoglobin

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Abstract. **We propose a functional significance of the delta (δ) band in the UV-visible spectroscopy of hemoglobin. We suggest that the formation of the delta (δ) band correlates with the redox state of both the aromatic and sulphur-containing amino acid residues in the environment of the haem. We suggest also that overlap of electron dense clouds, formed from new molecular orbitals of sterically close atoms, during the transition from the deoxy- to the oxy-conformation also contributes substantially to the phenomenon of cooperativity in Hemoglobin. These mobile and interacting electron clouds occur on ligand binding to the hemoglobin. We propose that oxidation that would dissipate the electron clouds would erase the δ band since the amino acid residues involved in the δ band formation are also redox-active and are thus good sensors to the redox environment of the Soret region.**

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1. Introduction

There has not been a known functional significance of the δ (delta) band unlike other bands in the UV-visible spectrum of hemoglobin (Hb). We suggested that this region might signify cooperativity in the proteins [1]. Our argument then was based on the fact that Mb, a monomer heme protein, lacks this band, which is present in Hb, a tetramer protein. Since Hb is a multimer that exhibits cooperativity based on heme-heme interaction transmitted across the molecule via adjoining amino acid residues (in other words, Hb exhibits the R→T transition), we reasoned that myoglobin (Mb) which is lacking in this band and is monomeric in terms of polypeptide constitution lacked the R→T transition required for this band to develop.

However, some Mb variants while still being monomeric in polypeptide composition still exhibited this regional demarcation even where it occurs as a shoulder. Thus, it seems most plausible that heme–heme interaction might *not* be a sole condition for the formation of this band or even shoulder in the UV-visible spectra of heme proteins in which it occurs. Besides Hb and Mb, this band occurs in some monomer heme proteins under some specified conditions. We, from existing evidence, put forward a hypothesis on the functional significance of the δ band as well as the relationship between the mechanisms of the formation of the δ band and cooperative oxygen binding. We appreciate the shortcomings in this paper based on the fact that majority of our deductions are based on theoretical rather than practical analysis and might in real life not be agreeable in its application both to the mechanism of cooperative oxygen binding in hemoglobins and in the formation of the delta (δ) band in the hemeproteins.

2. Substrate Effects in the Induction of the Delta (δ) Band

Pentacoordinate monomer heme proteins on assuming hexacoordinate heme geometry have developed this band as a result of the interaction of internal field ligands with the heme region of the proteins [2]. As in the case of cytochrome *c* peroxidase (CCP) which is a penta-coordinated high spin ferric protoheme group, the formation of a hexa-coordinated low spin compound accompanied by the appearance of a δ region, could occur by the interaction of CCP with hydrogen peroxides (H_2O_2) or carboxylates (COO^-) [2]. In the case of Mb, this band appears upon interaction of Mb with azides (N_3^-) [3] or cyanides (CN^-) [4], and it also appears in O_2 or CO liganded sperm whale Mb [5, 6].

An interesting observation in the cases of COO^-, N_3^- and CN^- is that the formation and intensification of this blue shifted shoulder (as in CCP) or band (as in Mb) is a function of the concentration of these substances. These substances (COO^-, N_3^- and CN^-) are unsaturated, π-bonding ligands, with available reactive π electron density which they donate to the metal on liganding the Fe of the heme [7].

It has also been reported that all the hemoglobins, which show a low absorption peak in the Soret region in the deoxygenated form, other than having low affinity for oxygen, show no heme-heme interactions [8]. Thus in the Hb, which is multimeric, heme-heme interaction is important for the formation of the δ band. In the Mb, the Fe in the heme could be oxidized from Fe^{2+} to Fe^{3+} as in the metMb formation using N_3^-, or the interaction of high spin ferric protoheme of CCP with H_2O_2 or COO^-, but with accompanying formation of the δ region [2, 3]. In some other instances, the metMb formation is accompanied by the loss of this δ region [6]. Thus, the formation of the δ region is independent of the oxidation status of the Fe in the heme. If this is the case, then the environment of the heme must be responsible for the formation of this region since as argued by Yonetani and Anni [2], the δ region is sensitive to the environment of the heme.

3. Formation of the δ Band in Some

Myoglobin Species

It could be puzzling to note the appearance of this band in the sperm whale Mb when bound to a ligand such as O_2 or CO [5, 6] while not appearing in the presence of similar ligands in horse Mb and or in other Mbs? An attempt to answer this puzzle would require an over view of the amino acid residues in the environment of the heme. There are three tyrosine residues in sperm whale myoglobin (Tyr-103, Tyr-146, Tyr-151), two in horse myoglobin (Tyr-103, Tyr-146), and only one in red kangaroo myoglobin (Tyr-146) [9]. Reactions of these Mbs with H_2O_2 results in covalent dimerization of sperm whale but not horse [10] or red kangaroo [11] Mbs. Replacement of Tyr-151 with a phenylalanine, however, prevents protein cross-linking, in agreement with the absence of cross-linking in the reaction of horse myoglobin with H_2O_2. In contrast, replacement of Tyr-146 with phenylalanine has no effect on the cross-linking ability of the protein. In the case of Tyr-103, the predominant formation of a cross-link between Tyr-103 and Tyr-151 appears to be due to predominant location of the unpaired electron density on Tyr-103, the tyrosine vicinal to the heme group [6]. Computer graphics docking experiments suggest, however, that formation of a dityrosine cross-link between the Tyr-151 residues of two myoglobin chains is sterically allowed [10]. This is in concordance with observations made by Wilks and Ortiz de Montellano [6] that Mb with a single tyrosine at position 151 is readily cross-linked. This confirms that a Tyr-151/Tyr-151 bond is, indeed, feasible and that Tyr-151 is absolutely essential for myoglobin dimerization.

Thus, cross-linking is clearly a relatively specific process determined by the relative distribution and location of the unpaired electron density and by the steric environment of the residues that are involved [6]. In other words, even though Tyr-103 and Tyr-146 are nearer to the heme group, both were not enough to form a *quorum* of unpaired electron density since location wise, Tyr-151 is farthest from the heme group and would thus contribute most probably to the buildup of electron density. This accounts for why the reactive negatively charged groups such as COO^-, N_3^- and CN^- would readily induce the formation of the δ band or even shoulder as the case may be in the presence of a ligand bound to the Fe in the heme. Additionally, the intensity or distinctness of the δ band increases, once it appears, as a function of the concentration of these reactive redox-active, π-bonding negatively charged substances since it would result in a corresponding increase in the electron density required for the interaction of two adjacent heme groups. Importantly too is the issue of enhanced electron density on approach of the environment of two hemes of Mb if the δ band is to form. The redox-active tyrosine amino acids have their own 'halo' of π-electron density each. The process of two hemes dimerizing only brings the separate clusters of π-electron densities together so that their anionic charge radius decreases while there is a build-up of an electron clouds. Thus, the charge density around the environment of the hemes increases as a result of the proximity of their interactions. Arguably, since the Mbs from hemes in the various Mbs, such as sperm whale, horse and kangaroo are all surface exposed, the issue of location to the surface is thus eliminated so that dimerization could occur without much steric constraints. These negatively charged groups increase the electron density around the environment of the heme in such a way that charge transfer between the environments of at least two hemes can occur provided the hemes are *mobile*. Mobility plays an important role here because if dimerization occurs between the environments of the hemes, as in the works of Asakura [12] and then more recently those of Wilks and Ortiz de Montellano [6], the mobility of the electrons required to put the hemes in sterically favourable conformations for their interactions would be halted.

Interestingly, Wilks and Ortiz de Montellano [6] also reported the failure of zinc sperm whale

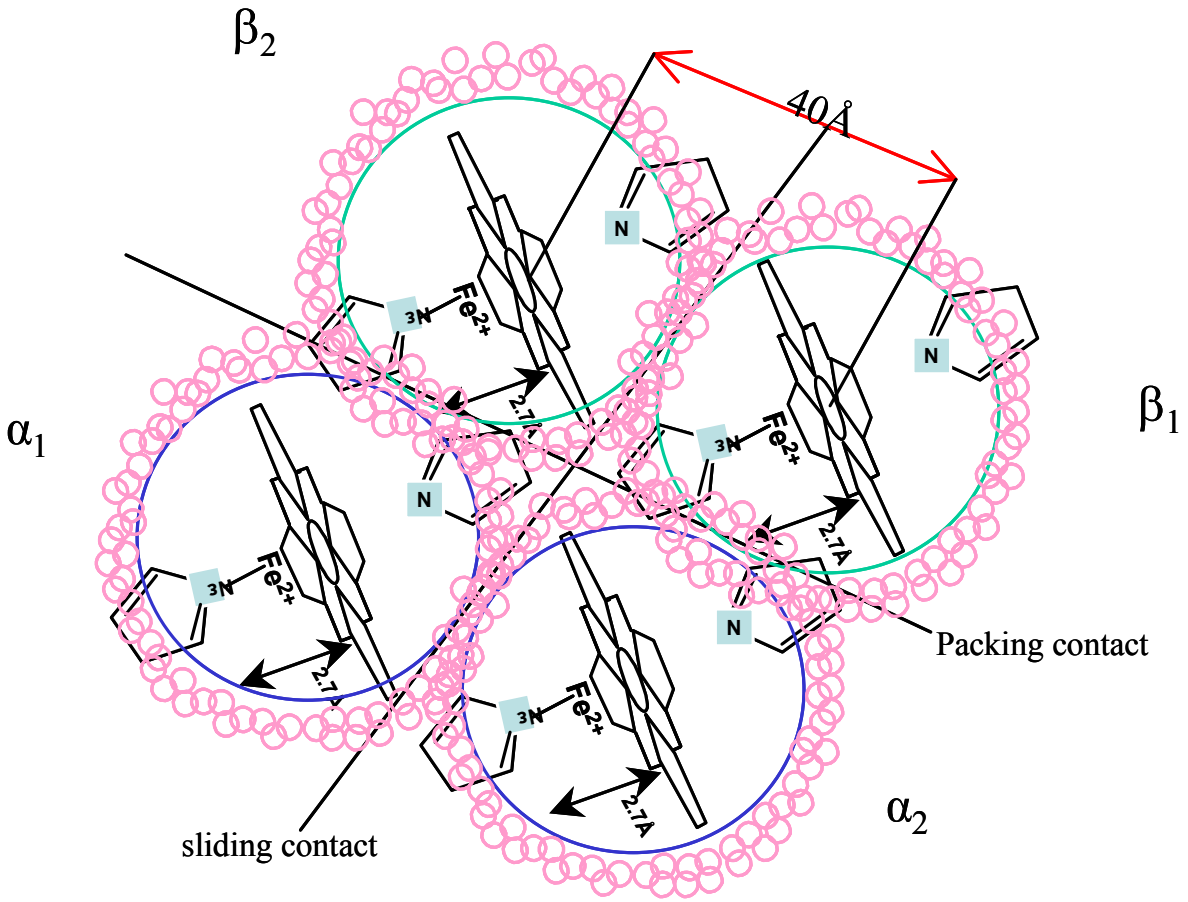

Figure 1A. **A schematic representation of the Hb tetramer in the deoxy or T state showing the Fe^{2+} bound to the proximal histidine (F8) and away from the plane of the heme. The blue and green circles represent the core of the electron clouds around the alpha (α) and beta (β) chains respectively. The small rose circles forming hollows around the blue and green bigger circles represent the diffuse periphery of the electron core. Only the diffuse electrons of the two α or two β chains are in contact and so cannot even mediate oxygen binding since the $\alpha_1\beta_1$ and the $\alpha_2\beta_2$ contacts are required to maintain the stability of the bound dioxygen. In the T state, hydrophobic residues necessary to mediate these mobile electron interactions are buried. In this state, the distance between the Fe^{2+} pair of the two β chains is 40Å. The horizontal contact between the Hb tetramer is referred to as the packing contact while the vertical contacts are termed the sliding contacts.**

myoglobin to dimerize when incubated with ferric horse myoglobin and H_2O_2. This establishes a fact: that the Fe in the heme group is required for dimerization even if it is not directly required in the bond formation as in the case of Tyr-151/Tyr-151 cross-linking where the irons in the hemes were not involved in oxo bond formation. The δ band from the foregoing indicates the interaction, in terms of intermolecular electron density transfer coupled with steric competence, between two or more adjacent heme groups.

4. The Functional Significance of the δ Band and Its Implication in the Cooperative Oxygen Binding to Hemoglobin

In the case of multimer heme groups such as Hb, the δ band would indicate interactions between the different hemes in the molecules when they are bound to ligands since the ligands influence the proximity of the hemes in such a way that the electron clouds of the hemes are

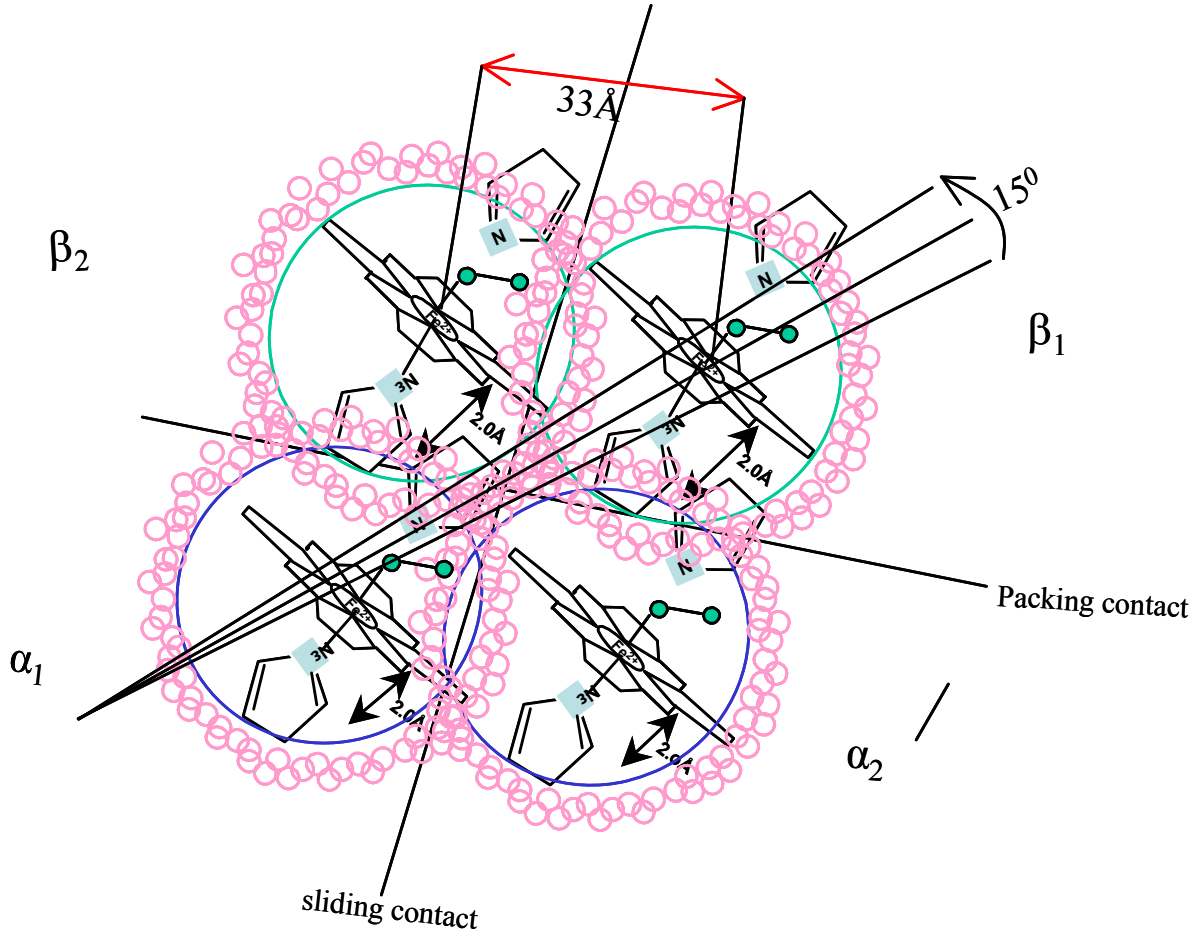

Figure 1B. **A schematic representation of the R state conformation of the Hb tetramer resulting from the T→R transition that occurs when 0_2 binds to the heme $Fe^{(2+)}$ in a non covalent manner. This binding pulls the Fe^{2+} asymmetrically bound to the proximal histidine (F8) into the plane of the heme accompanied by a 15° rotation of the $\alpha_1\beta_1$ pair with respect to the $\alpha_2\beta_2$ pair. The inward movement of the F8 and Fe^{2+} into the plane of the heme results in the increase of the electron density diameter (due to a shortening of the Fe^{2+}-F8 bond from 2.7Å to 2.0Å) with respect to the whole diameter of the heme cavity in the R state conformation to the T state conformation. On going from deoxy- to liganded hemoglobin, the α and β hemes move further into the heme pockets by 0.5 and 1.5Å respectively. This increases the electron cloud diameter and accompanied by the 15^0 movements of the $\alpha_1\beta_1$ pair relative to the $\alpha_2\beta_2$ pair results in the overlap of the electron cloud cores of the $\alpha_1\alpha_2$ pairs as well as those of the $\alpha_2\beta_1$, $\beta_1\beta_2$ and $\beta_2\alpha_1$ pairs. These overlaps mediate cooperative oxygen binding in such a way that one pair overlap results in steric closure of the other chain pairs adjacent to it so that this fastens the rate of 0_2 to that pair and so on until all four sub units are bound. The 0_2 binding results in the merging of the peripheries of the electron clouds of the $\alpha_1\beta_1$ and $\alpha_2\beta_2$ pairs to mediate the stability of the bound dioxygen to each chain. These movements not only result in the rupturing of the salt bridges required to maintain the T state but also shortens the distance between the β pairs from 40 to 33Å. The linked green circles represent the 0_2 molecules.**

interacting and thus acts as a sensor for positive homotropic cooperativity. This is plausible since each chain of the tetrameric Hb when separated can bind O_2 without the induction of this band. O_2 is not bound to Hb as a radical. Then, as individual sub-units bound to O_2, there would be no electron cloud to mediate heme-ligand interaction via the pool of electrons from both the heme and the ligand (as in the case of CCP). When O_2 is bound to tetrameric Hb, this electron cloud forms basically between the different hemes brought into close proximity by the

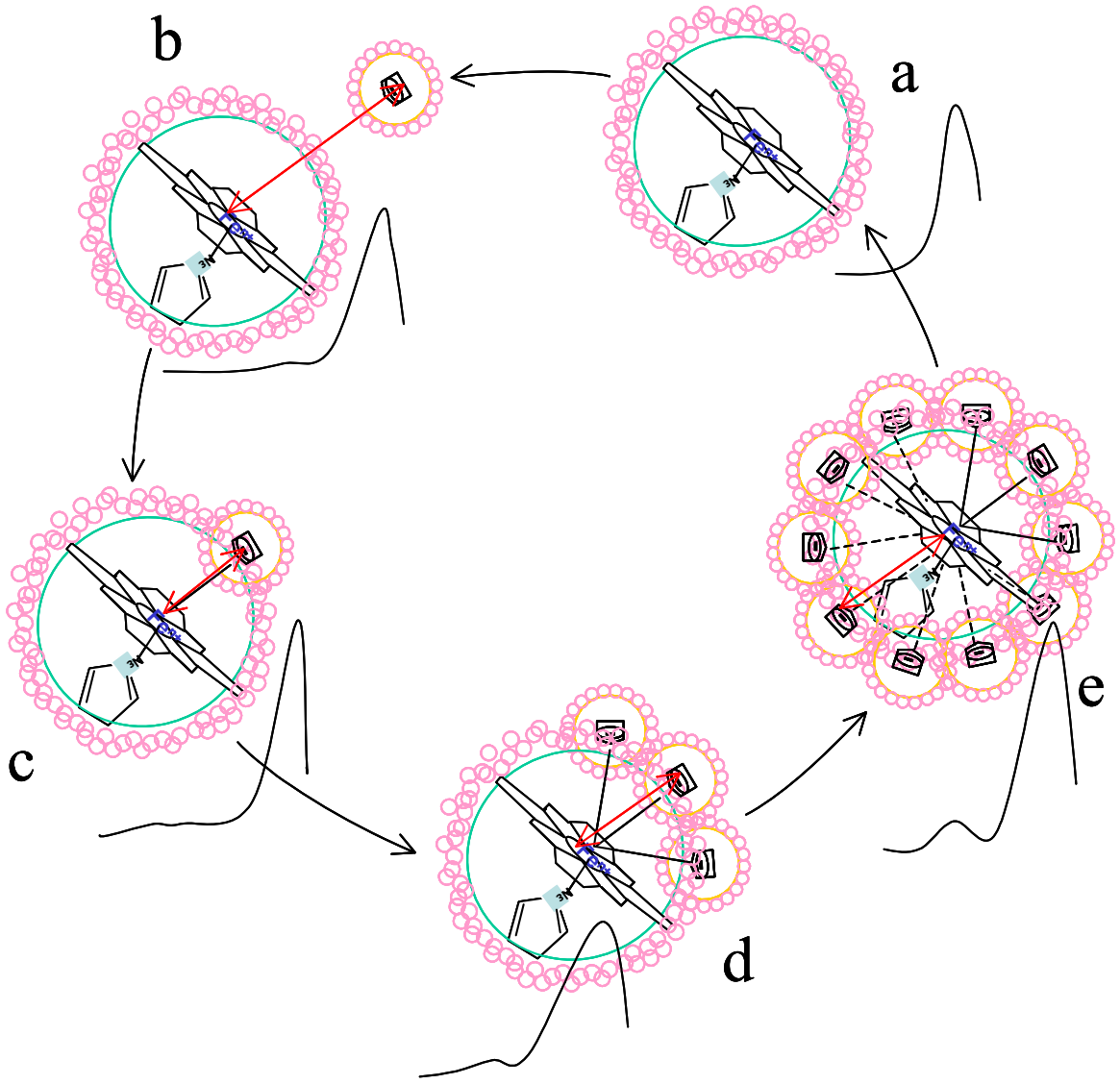

Figure 2. **A schematic depiction of the electron cloud formation as a factor of the negative charge/anion-radius ratio (charge density) and the induction of the δ band in single polypeptide hemoproteins whereby highly negatively charged molecules, which are π-electron donor ligands. a. The hemoprotein with an electron density solely that of the environment around the porphyrin group. There is no ligand at this stage. b. A π-electron donor ligand approaches the environment of the hemoprotein but is outside of the hemoprotein electron density. c. The ligand binds *specifically* to the Fe whose oxidation state is represented by n^+. The electron cloud of the ligand fuses with that surrounding the Fe-porphyrin ring as indicated by the shorter distance of the ligand from the Fe in the porphyrin as compared to b. thus, there is a mergence of π-electron clouds; both from the ligand and the heme. d. The number of ligands bound to the Fe remains one while the other two binds non-specifically to the heme region. The electron density increases as compared to c before it. e. The ligands form a dense cloud of electrons as compared to the event of d before it. Other than the first ligand that binds specifically to the Fe in the heme, the other ligands bind non-specifically to the porphyrin ring as they increase the density of the electron cloud. Even though the ligand binding distance is same as in d before it; the charge density in e is increased over d as a function of the increased number of participating ligands in e over d. As the charge density increases, the prominence of the δ band increases progressively from a to e as shown from the spectral sketches. Keys: the Fe and its oxidation state are written in blue. The bi-directional red arrow represents the distance measurement of the ligand, either approaching or already bound to the heme protein. The solid black line connecting the ligand to the Fe of the heme protein represents specific binding mode of the ligand to the heme courtesy of the Fe of the heme. The broken black line represents non-specific binding of the ligand to the porphyrin ring.**

binding of the ligands.

Ligand binding influences the T→R transition to the R conformational state. The shift from the T to R quaternary structure is primarily triggered by displacement of the Nε-nitrogen of the proximal histidine relative to the mean plane of the porphyrin ring. In deoxyHb, this distance is 2.7Å, whereas, it is 2.0Å in oxyHb. In metHb, the Nε-porphyrin distance is intermediate: 2.3 ± 0.01Å, and is expected to undergo a shift from the R to T quaternary structure more readily than oxyHb [13]. Thus, the ligation state of Hb [14, 15] and or the oxidation state of the iron in the heme group [13] determines which conformation it is in and subsequently the ease of formation of the δ band as a function of the buildup of π-electron clouds. These changes in the iron-bond lengths are intimately linked to larger conformational changes in the polypeptide surrounding the heme group and forming the bridges between the hemes in the Hb tetramer [16]. This results in heme-heme interaction. Thus, heme-heme interaction, consisting of a rise of the oxygen affinity of hemoglobin solutions with rising oxygen saturation, occurs only when the reaction with oxygen is accompanied by a transition between two alternative quaternary structures of the globin [17-19], that is, the R and T states. We postulate thus that mostly aromatic amino acid residues that line the heme cavity contribute the participating electron clouds that interact to mediate cooperativity and thus the formation of the δ band. This, we will come to discuss.

Binding of O_2 only brings the heme groups in close proximity so that an interacting π-electron cloud then builds up (**FIG 1A, 1B**) (interestingly, it is usually suggested that carrier properties, such as O_2 transport by hemoglobin, result from π interactions only [7]). On going from deoxy- to liganded hemoglobin, the α and β hemes move further into the heme pockets by 0.5 and 1.5Å respectively. These movements bring the hemes within each $\alpha_1\beta_1$ dimer closer to each other by 2.0 Å and change their positions relative to the fixed helices of the dimer [20]. We propose thus that cooperativity is mediated by the alignment of the electron clouds at the dimer interfaces as the $\alpha_1\beta_1$ dimer makes an angle of rotation of 15° relative to the $\alpha_2\beta_2$ dimer so that the electron clouds from both dimers can overlap perfectly (**FIG 1B**). From the foregoing, we postulate that it is not only the movements of residues per say that bring about cooperativity; but that the movement of adjacent residues around the environment of the hemes would also help in the proper alignment of the electron density as well as enhancement of the electron cloud density via the decrease in the anion radius as occurs when the deoxy (T) state transits to the oxy (R) state. The major contribution to cooperativity comes from this quaternary change [20] of T↔R transition. This goes to buttress Perutz's proposed stereochemical mechanism for cooperative oxygen binding to hemoglobin [21]. All the same, the contribution of residue-residue interaction cannot be said to be entirely insignificant. Perutz et al. [18] reported that since heme-heme interaction is observed only when reaction with ligands is accompanied by a change of quaternary structure, then by implication, it is coupled to a change of tension at the heme, which is then transmitted, by a change in quaternary structure of the globin. This "change of tension at the heme" results in the decrease in the anion radius and thus an increase in the charge density while the changes in the quaternary structures of the globin would bring about the proper alignment of the electron clouds so as to result in heme-heme interactions.

Thus, in the Hb, there is a greater negative charge/ anion-radius ratio in the oxy state when compared to the deoxy state (**FIG 1A, 1B**). As the Soret $\lambda_{(max)}$ decreases, the interacting electron clouds formed mainly by the cores of the electron density disperses due to the increasing distance of the Fe-histidine bond with respect to the rest of the heme complex. This ultimately results in the loss of the δ band. In the case of single polypeptide hemoproteins that binds highly negatively charged ligands such as N_3^-, CN^- and COO^-, the charge density is a function

of the distance of the ligand from the heme proteins as well as on the charge density borne by the ligands themselves. These ligands in most cases are unsaturated compounds with delocalized π electron clouds. They are electron donors and the merger of the ligand clouds with that of the heme of the hemoprotein enhances the charge density in the heme region and is proportional to the intensity and thus integrity of formation of the δ band. The ligands at saturating concentrations bind non-specifically to the porphyrin of the heme. Prior to this, the ligands bind with specificity to the Fe in the heme (**FIG 2**). From the foregoing therefore, it could be deduced that interacting Hb in the Fe^{3+} with the π electron donating ligands such as N_3^-, CN^- and COO^- would result in the formation of the δ band, as would do O_2 on binding to the deoxy-Hb to form oxy-Hb. However, while the binding of O_2 to the Hb would result in the decrease in the charge radius and increase in the charge density to result in the formation of the δ band, that (i.e., the δ band) formed from the unsaturated, π electron donating ligands would result from the non-specific additive interaction of the π electron clouds with both the Fe^{3+} and porphyrin moieties of the heme and even the amino acid residues around the environment of the heme. On the other hand too, while the δ band formed in the oxyHb would be accompanied by the formation of the Q bands which are indices of the oxygenation status of the protein, that formed from the unsaturated π electron donating ligands would most probably not be accompanied by the formation of the Q band.

We propose thus that the oxidation (loss of electrons) of the Fe^{2+} to Fe^{3+} dissipates the electron clouds required for steric closeness of the tetrameric chain of the Hb. This oxidation, almost always would affect the aromatic amino acids and other redox-sensitive apolar residues lying next door to the heme cavity by bringing about the oxidation of these aromatic amino acids and the other apolar residues; the tyrosines [11, 22-28], tryptophans [24, 26, 29-32], phenylalanines [33], histidines [34, 35] (histidine behaves as an aromatic amino acid residue at low pH [36] which becomes imminent at increased respiratory rate, perhaps due to increased muscular activities), cysteines [24] and methionines [26]. These residues are oxidized to their radicals with the oxidizing equivalents localized on the globin moieties of the hemeproteins to form globin radicals [26, 37]. Moreover, these globin radicals oxidize the Fe^{2+} of the hemeproteins, thus aiding to dissipate even the electron cloud of the porphyrin moiety [26]. Besides, the aromatic amino acids have their own pool of π interactive [38] electrons, which contribute to the pool of π electron clouds in the environment of the heme. Little wonder oxidation of these aromatic amino acids would result in the formation of oxidizing radicals and dissipation of the electron clouds. We propose that these aromatic amino acid residues are the main sources of the electron clouds that mediate heme-heme interactions via heme-protein (Cotton effects) interactions (as we said, the electron clouds that mediate heme-heme interaction is from the aromatic and sulphur-containing amino acids so that heme-protein interactions is first before heme-heme interactions). These amino acids, especially tryptophan, tyrosine and cysteine have been reported to be redox-active [4]. Since the aromatic amino acids are the main source of the electron clouds, oxidation would then result in the dissipation of the clouds and loss in the heme-heme interactions. Oxidation and subsequent dissipation of the electron clouds from the surroundings of the heme cavity is not just a function of the electron-withdrawing effect of the associating ligand (O_2 in the case of oxygen binding to Hb) with the metal prosthetic group; but also a function of the molecular structure of the porphyrin ring itself [39].

5. Probable Evidence for the Involvement of Both Aromatic and Sulphur Containing Residues in the

Formation of the δ Band

The αβ interface which is located just above the heme cavity (actually, the hemes must be surface exposed so as to bind oxygen cooperatively. Moreover, cooperativity on its own is a surface phenomenon) is constituted mainly of sulphur containing amino acid residues such as the cysteines [40] and methionines [41] which are readily oxidized in the face of oxidizing agents that would also generate protein radicals in the core of the Soret region; which of course is bordered by very hydrophobic amino acids in relatively close proximity to the heme group [42-44]. Since the αβ interface is in close proximity to the heme region, oxidizing agents targeted at the heme region as monitored with the Soret region would also affect the αβ interface amino acids and a very good reporter for this event will most probably be sulphur containing amino acids. For example, methionine β(55)D6 in human hemoglobin is located at the $\alpha_1\beta_1$ interfaces. The $\alpha_1\beta_1$ interfaces plays a crucial role in stabilizing the bound dioxygen in HbO_2 [45]. Amiconi et al. [41] reported that when methionine β(55)D6 in human hemoglobin is selectively oxidized to its sulfoxide derivative, the modified protein, although appearing to maintain most of the chemical and structural properties typical of the native protein; undergoes drastic changes in the functional behavior characterized (like that of the isolated chains) by high oxygen affinity, absence of cooperativity (n = l), and lack of Bohr effect.

Also, the cysteines at positions 93 of the β chains lie adjacent to the proximal histidine [16] at the $\alpha_1\beta_2$ ($\alpha_2\beta_1$) subunit interfaces, which mediate cooperative oxygen binding in the human Hb [45]. Thus, the β93 residues play a crucial role in the cooperative oxygen binding of the human Hb. Banerjee et al. [16] reported that modification of the thiol group of the β93 residue with *p*-mercuribenzoate (PMB) results in the oxidation of the thiol group (SH) to a sulphur-PMB linkage with accompanying spectral change to longer wavelengths, associated with diminished oxygen affinity and a probable decline in the prominence of the δ band. Besides, decreased affinity of Hb for oxygen has been correlated with the conducive activation of free-radical processes [46]. Thus, a decrease in oxygen affinity would reflect on the T↔R equilibrium, hamper on the formation of the δ band and thus correlate our proposal that the δ band region would serve as an index of cooperativity in native hemoglobins.

6. Conclusion

We stress the importance of stereochemistry resulting from new molecular orbital formation with accompanying enhancement in electron density and resulting cooperativity in Hb. We stress that these electron clouds are formed by both aromatic and sulphur-containing amino acids. These pools of electron clouds are sensed by redox-active sulphur containing amino acids surrounding the heme groups. A pool of these electron cloud density will not only aid cooperativity, but will also result in the formation of the δ band. We propose thus that the δ band is a sensor for the redox state of the Soret region. We propose also that the overlap of the electron clouds between and within dimmer subunits, with formation of the δ band, would result in cooperative oxygen binding in Hb.

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