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Evolutionary Analysis of Functional Properties in Hemoglobins

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The paper presents an analysis of functional innovations in hemoglobins as they evolved from simple monomeric forms to tetramers having two alpha and two non-alpha chains. Our analysis is based on the phylogenetic schema made on the globin sequences of representative species as well as on the information available from oxygenation curves of contemporary hemoglobins, their three-dimensional structure and sequence organization of the globin genes.

We visualise the vertebrate globin ancestor as having been a monomer with high oxygen affinity and possessing weak cooperativity in oxygen binding through association-dissociation phenomenon. We also visualise the myoglobin stem losing self-aggregation properties with time and consequently cooperativity, while the hemoglobin stem retained these properties. Further, it was after the alpha-beta divergence that the hemoglobins acquired the property of pronounced cooperativity. Against this background, we have analysed the ancestor-descendant relationship of sites at heme contact, $\alpha_1\beta_1$ contact and $\alpha_1\beta_2$ contact, over evolutionary time. The role of E7 His in lowering oxygen affinity in the primordial globin has been discussed. This is followed by the suggestion that the central exon-encoded segment of the ancestral vertebrate globin also had the potential for providing most of the heme contact sites and rudiments of $\alpha_1\beta_2$ contact sites (C2, FG3, FG5, G1 and G2). An explanation for the loss of cooperativity in the myoglobin branch, based on changes occurring at functionally important sites during evolution, is given. Finally the emergence of enhanced cooperativity in hemoglobin tetramers as being largely due to changes occurring at sites such as HC3, C5, H9, FG1, C3, C6 and FG4 following alpha-beta divergence, has been discussed.

Key Words: Hemoglobins, Phylogeny of globins, Evolutionary biochemistry, Molecular phylogeny

Introduction

It is generally believed that with the availability of free oxygen in the atmosphere around 2×10^9 yr b.p., oxygen respiration became widespread in prokaryotes (Cloud 1968). Subsequently, evolution advanced beyond the prokaryote level giving rise to the distinct organisation of eukaryotes. It is conceivable that

with the widespread adoption of multicellularity in eukaryotes the need for transport of oxygen within the organism also arose, and one could visualise this need being met by oxygen-carrying proteins. Hemoglobin, the most widespread oxygen-carrier, is a monomer in root nodules of leguminous plants (Keilin & Wang 1945) and in many species of invertebrate phyla in which it is present (Wittenberg et al. 1965, Thompson et al. 1968 and Seamonds et al. 1971). On the other hand, the hemoglobins of vertebrates are tetramers containing two alpha and two non-alpha chains, with the exception of those of cyclostomes which are monomers. The myoglobins found in vertebrate muscle tissue are also monomeric proteins. The hemoglobin-myoglobin family of proteins carry the same heme group, iron (II) protoporphyrin IX (Perutz 1976), and consequently it is the variation in the globin sequence that is responsible for the difference in functional properties.

We analyse here, the evolutionary trends in functional properties of hemoglobins as they evolved from monomeric forms exhibiting high oxygen affinity to the contemporary tetrameric forms which show low oxygen affinity and high cooperativity. Our work is based on the phylogenetic schema made on the sequences of globins of representative species spanning the animal kingdom, as well as on the information available from functional properties of contemporary hemoglobins, their three-dimensional structures and the sequence organization of globin genes.

Pertinent Aspects of Functional Properties of Hemoglobins

Consider the rearranged form of Hills equation,

$$\bar{Y}/1-\bar{Y}=Kp^{n}$$

where $\bar{Y}$ is the fractional saturation and p is the partial pressure of oxygen. The logarithmic form of this equation is given by

$$\log(\bar{Y}/1-\bar{Y}) = \log k + n \log p.$$

Here, a plot of $\log(\bar{Y}/1-\bar{Y})$ against $\log p$ has a slope n (Hills coefficient) which is a convenient index of cooperativity (Roughton et al. 1955 and Roughton 1965). The value of p at $\bar{Y}=0.5$ referred to as p_{50}, gives a measure of oxygen affinity. The lower the p_{50} value the greater is the oxygen affinity and vice-versa. It may be noted that the hemoglobins of invertebrates such as *Chironomus thummi thummi* (Sick & Gersonde 1969) and *Glycera dibranchiata* (Vinogradov et al. 1970) as well as leghemoglobin (Immamura et al. 1972) and vertebrate myoglobins (Rossi-Fanelli & Antonini 1958) show a value of n close to unity and a small p_{50} value. However, lamprey hemoglobin exhibits a value of $n = 1.2$ at pH 6.8, an unusual result in a monomeric protein (Wald & Riggs 1951). The hemoglobins of higher vertebrates on the other hand, exhibit high n values ($n \approx 2.7$) and high p_{50} values (Roughton et al. 1955).

The functional interactions in vertebrate hemoglobins can be explained by the two state model for allosteric proteins proposed by Monod et al. (1965). In this model, the allosteric constant L is defined by $[T]/[R]$ where, T and R represent the two conformational states of hemoglobin with different affinities for ligands. The value of L is a measure of oxygen affinity as is evident from the following equation (Baldwin 1976):

$$\log(p_m/K_r) = 1/4 \log L.$$

Here, p_m is the median ligand activity which is analogous to p_{50} under certain conditions and K_r represents the dissociation constant for subunits in the

R conformation. A plot of log *L* against *n* gives a bell-shaped curve (Rubin & Changeux 1966) which has been elegantly used by Edelstein (1971) to explain the abnormal properties of some mutant hemoglobins. For example, Hb Chesapeake which has a low value of *n* and high oxygen affinity appears on the ascending part of the curve corresponding to a low value of *L*. On the other hand, Hb Kansas which has a low value of *n* and low oxygen affinity appears on the descending part of the curve corresponding to a high value of *L*.

The change in oxygen affinity with *p*H, also known as Bohr effect (Henderson 1920), is another functional property of hemoglobin which is marked in vertebrate hemoglobins. Although not common in monomeric hemoglobins, Bohr effect is exhibited by *Chironomus* (CTT-3) globin (Gersonde et al. 1972 and La Mar et al. 1978) and lamprey globin (Wald & Riggs 1951). The oxygen affinity of hemoglobins can also be altered by organic phosphates such as 2, 3-diphosphoglycerate (2, 3-DPG), inositol hexaphosphate and adenosine triphosphate (Benesch & Benesch 1969, 1974).

From X-ray crystallographic studies, it has become clear that the three-dimensional structure of hemoglobins of root nodules of leguminous plants (Vainshtein et al. 1975), an annelid *Glycera dibranchiata* (Padlan & Love 1968), an insect *Chironomus thummi* (Huber et al. 1971), a lamprey *Petromyzon marinus* (Hendrickson et al. 1973) as well as that of mammalian myoglobins (Kendrew et al. 1958) and hemoglobin chains (Perutz et al. 1960 and Muirhead & Perutz 1963) are remarkably similar. Further studies have led to the identification of amino acid residues involved in the heme contact, $\alpha_1\beta_1$ contact and $\alpha_1\beta_2$ contact (Perutz et al. 1968), the residues responsible for the Bohr effect (Kilmartin & Rossi-Bernardi 1969, Kilmartin et al. 1973 and Perutz et al. 1980) and those necessary for 2, 3-DPG binding (Arnone 1972). Comparison of the structures of liganded and unliganded hemoglobin has also revealed the nature of the structural changes occurring on oxygenation (Perutz 1970, Perutz & TenEyck 1971 and Perutz 1972).

Phylogenetic Schema of Representative Globin Sequences

Evolutionary history of hemoglobins can be envisaged on a phylogenetic tree. The branches of the tree emanate from a single ancestral point (root) and terminate in contemporary protein sequences. Each branching point in the tree represents a time and a globin sequence. The root corresponds to the earliest time and the earliest ancestral sequence. Evolutionary time advances from the root, establishing ancestor-descendant sequences at subsequent branching points. Our aim here, is to utilise contemporary globin sequences to establish ancestral amino acid sequences at the branching points of the phylogenetic tree. Two types of methods are available for constructing phylogenetic trees. The first type uses matrix numbers which are obtained by a count of non-matching amino acids from a set of aligned amino acid sequences of proteins (Camin & Sokal 1965, Fitch & Margoliash 1967, Goodman et al. 1971 and Dayhoff et al. 1972). The second procedure is based on reconstruction of ancestral amino acid sequences which generate descendant sequences through minimum number of mutations (Fitch 1971, Dayhoff et al. 1972, Barnabas et al. 1972 and Moore et al. 1973). It has been shown that the trees derived from the ancestral sequence method give a more accurate reflection of phylogeny than those derived by matrix methods (Goodman

et al. 1974). We have used the parsimony procedures (Moore et al. 1973 and Barnabas et al. 1978, 1980) to derive the phylogenetic tree and to construct the ancestral sequences, from a set of selected globin sequences aligned according to Hunt and Dayhoff (1976). The branching arrangement in our tree (figure 1) are similar to those in a comprehensive tree reported earlier, in which the vertebrate globin ancestor was shown to evolve from a metaphyta-metazoan globin ancestor through an invertebrate-chordate ancestor (Goodman et al. 1974). The new sequences which appear in figure 1 are those of alpha chains of newt, viper and shark. The ancestral amino acid residues at all the positions were worked out.

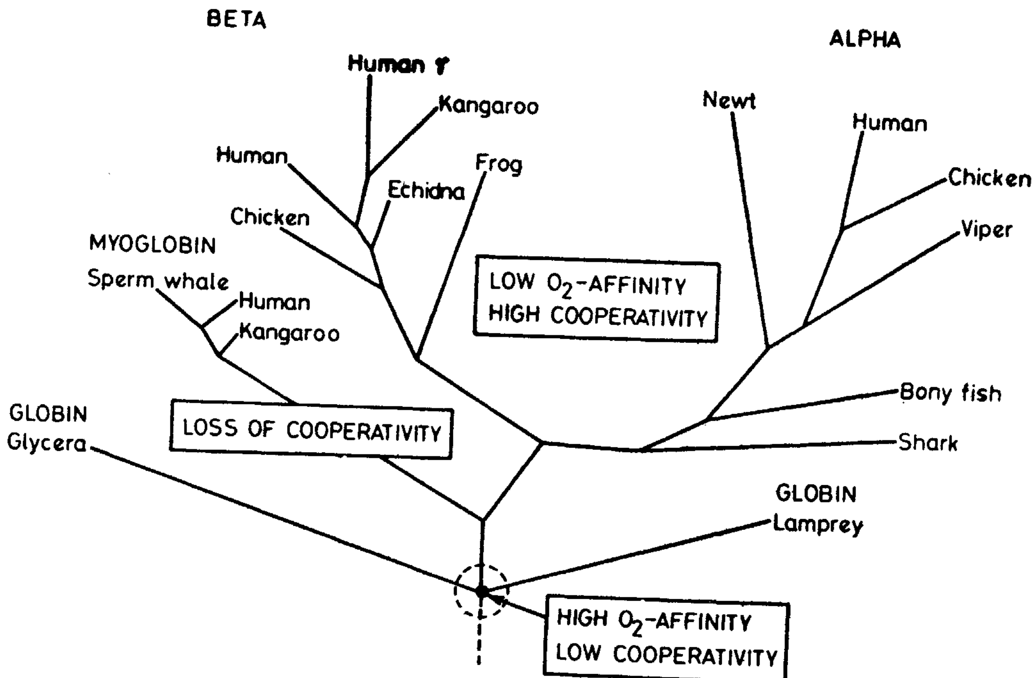

Figure 1. A phylogenetic tree of 17 globin sequences. The branch lengths are proportional to the number of nucleotide replacements between ancestor and descendant sequences. The amino acid sequences are from man (*Homo sapiens*) alpha, chicken (*Gallus gallus*) alpha, viper (*Vipera aspis*) alpha, newt (*Taricha granulosa*) alpha, bony fish (*Catostomus clarkii*) alpha, shark (*Heterodontus portusjacksoni*) alpha, human beta, human gamma, kangaroo (*Macropus giganteus*) beta, echidna (*Tachyglossus aculeatus*) beta, chicken beta, frog (*Rana catesbeiana*) beta, human myoglobin, sperm whale (*Physeter catodon*) myoglobin, kangaroo (*Megeleia rufa*) myoglobin, lamprey (*Petromyzon marinus*) globin and glycera (*Glycera dibranchiata*) globin. The globin sequences have been taken from Hunt & Dayhoff (1976). We have used the maximum parsimony procedure described by Barnabas et al. (1978, 1980) to construct ancestral residues at the branching points as well as to derive the minimum path tree. This method has two calculation cycles referred to as 'zipping' and 'unzipping'. Given the values at exterior points E_i, of a network N, zipping operation identifies amino acids at the interior points I_i, which have the potential of minimizing the total length Σm_r of the network, where m_r represents the length of the rth link (branch). Unzipping establishes a final solution set at I_i', such that Σm_r is minimum possible. Maximum parsimony method of Moore et al. (1973) was also used to check the results at sites which show maximum variation. It has been established earlier that the trees derived by both the methods are equally parsimonious (Barnabas et al. 1978).

However, only the amino acid residues present at sites relevant to our discussion viz. heme contact, and $\alpha_1\beta_2$ contact are given in tables 1 and 2 respectively.

Functional Innovations in Hemoglobin over Evolutionary Time

The most ancestral branching point shown in figure 1 is the vertebrate globin ancestor, which apparently evolved from a metaphyta-metazoan globin ancestor. It is conceivable that the primordial globin carried the heme in a hydrophobic pocket similar to that found in contemporary globins. The heme pocket seems to be a necessary prerequisite for the ferrous iron to react reversibly with oxygen and prevent the irreversible oxidation of ferrous iron to ferric iron which occurs when isolated heme reacts with oxygen (Padlan & Love 1968). It would appear therefore that the amino acid residues which form the heme pocket lower the oxygen affinity of heme and keep it in a physiologically useful range. Table 1 would support this notion in that many of the heme contact sites are evolutionarily stable.

Among the heme contact sites, E7 His, which probably forms a hydrogen bond with oxygen (Pauling 1964), has been suggested to play a significant role in lowering oxygen affinity (Vuk-Pavlovic 1975). In our analysis, histidine appears at position E7 in the vertebrate globin ancestor, hemoglobin-myoglobin ancestor, alpha-beta ancestor, alpha ancestor and beta ancestor. From parsimony considerations, Goodman et al. (1974) have shown the presence of E7 His in the metaphyta-metazoan globin ancestor and invertebrate-chordate globin ancestor. However, monomeric globins of *Glycera* and *Chironomus* do not have histidine at this position (Hunt & Dayhoff 1976). Instead, the spatial position of E7 His is occupied by leucine in *Glycera* globin (Love et al. 1971) and E11 Ile in CTT-3 globin (Huber et al. 1971). These results indicate that while it is highly probable that E7 His played a significant role in the primordial globin in lowering oxygen affinity from the very high affinity of isolated heme to the comparatively lower affinities of monomeric globins and myoglobins, the lowered oxygen affinity was stabilised during the course of evolution by other interactions. Support for this notion indirectly comes from sequence data of opossum alpha chain and Hb-Zurich beta chain. While replacement of E7 His in the latter causes a significant increase in oxygen affinity (Winterhalter et al. 1969), replacement of E7 His in the alpha chain of the former does not significantly alter the functional properties. This would indicate that the role of E7 His may be different in alpha and beta chains. In fact, X-ray crystallographic studies also suggest different roles for E7 His in alpha and beta chains (Fermi 1975 and Perutz 1979).

(i) Low Cooperativity in Vertebrate Globin Ancestor

The ability to form polydisperse aggregates is another property that is common in invertebrate globins (Seamonds et al. 1971 and Okazaki et al. 1965) and lamprey globins (Love et al. 1971). The latter in addition have the ability to form dimers when deoxygenated and dissociate when saturated with oxygen (Briehl 1963). Apparently, this association-dissociation phenomenon is linked to weak cooperativity (Briehl 1963). Li and Riggs (1970) after comparing the amino acid residues forming the $\alpha_1\beta_2$ contact area in horse hemoglobin with residues at analogous positions in lamprey hemoglobin suggested that a contact similar to the $\alpha_1\beta_2$ contact area of mammalian hemoglobins

Table 1 *Ancestor-descendant relationship of amino acid residues at heme contact sites*

Ancestor Descendant	B13	C4	C7	CD1	CD3	CD4	E7	E10	E11	E14	E15	F4	F7	F8	FG3	FG5	G4	G5	G8	H12	H15	H19
Vertebrate	Leu	Thr	Phe	Phe	Lys	Phe	His	Lys	Val	Ala	Leu	Leu	Lys	His	Leu	Val	Tyr	Phe	Leu	Phe	Phe	Leu
Myo-Hemo	Leu	Thr	Tyr	Phe	Lys	Phe	His	Lys	Val	Ala	Leu	Leu	Leu	His	Leu	Val	Tyr	Phe	Leu	Leu	Phe	Leu
Myoglobin	Leu	Thr	Lys	Phe	Lys	Phe	His	Thr	Val	Ala	Leu	Leu	Ser	His	His	Ile	Tyr	Leu	Ile	Leu	Phe	Met
α-β	Leu	Thr	Tyr	Phe	Asn	Phe	His	Lys	Val	Ala	Leu	Leu	Leu	His	Leu	Val	Asn	Phe	Leu	Leu	Val	Leu
α	Met	Thr	Tyr	Phe	His	Phe	His	Lys	Val	Ala	Leu	Leu	Leu	His	Leu	Val	Asn	Phe	Leu	Leu	Val	Leu
β	Leu	Thr	Tyr	Phe	Thr	Phe	His	Lys	Val	Ala	Leu	Leu	Leu	His	Leu	Val	Asn	Phe	Leu	Leu	Val	Leu
Human α	Met	Thr	Tyr	Phe	His	Phe	His	Lys	Val	Ala	Leu	Leu	Leu	His	Leu	Val	Asn	Phe	Leu	Leu	Val	Leu
Human β	Leu	Thr	Phe	Phe	Ser	Phe	His	Lys	Val	Ala	Phe	Leu	Leu	His	Leu	Val	Asn	Phe	Leu	Val	Val	Leu

Table 2 *Ancestor-descendant relationship of amino acid residues at $\alpha_1\beta_2$ contact sites*

Ancestor Descendant	C2	C3	C5	C6	C7	CD2	FG3	FG4	FG5	G1	G2	G3	G4	HC2	HC3
Vertebrate	Pro	Ala	Gln	Glu	Phe	Ala	Leu	Gln	Val	Asp	Pro	Gln	Tyr	Tyr	—
Myo-Hemo	Pro	Ala	Gln	Glu	Tyr	Ala	Leu	Gln	Val	Asp	Pro	Gln	Tyr	Tyr	His
Myoglobin	Pro	Glu	Leu	Glu	Lys	Asp	His	Lys	Ile	Pro	Val	Gln	Tyr	Tyr	Lys
α-β	Pro	Ala	Gln	Arg	Tyr	Ala	Leu	Gln	Val	Asp	Pro	Ala	Asn	Tyr	His
α	Pro	Ala	Lys	Thr	Tyr	Ala	Leu	Lys	Val	Asp	Pro	Ala	Asn	Tyr	Arg
β	Pro	Trp	Gln	Arg	Tyr	Ala	Leu	His	Val	Asp	Pro	Ala	Asn	Tyr	His
Human α	Pro	Thr	Lys	Thr	Tyr	Pro	Leu	Arg	Val	Asp	Pro	Val	Asn	Tyr	Arg
Human β	Pro	Trp	Gln	Arg	Phe	Glu	Leu	His	Val	Asp	Pro	Glu	Asn	Tyr	His

may exist in deoxyhemoglobin of lamprey. In support of this, Love et al. (1971) indicated that the overall configuration of the C helix, CD corner and FG corner of lamprey hemoglobin is similar to that of mammalian hemoglobins. Also, Hendrickson (1973) suggested the involvement of the C helix and the FG corner in the formation of lamprey homodimers. Our analysis shows that vertebrate globin ancestor (table 2) and lamprey globin share common amino acids at all the $\alpha_1\beta_2$ contact sites except at positions CD2 and FG3. These results suggest that the vertebrate globin ancestor had the ability to form both polydisperse aggregates as well as dimers exhibiting weak heme-heme interaction.

Evolutionary history of $\alpha_1\beta_2$ contact sites (table 2) shows that the sites C2, FG3, FG5, G1, G2 and HC2 have been conserved in the hemoglobin stem. Among these sites, G1 and G2 seem to be particularly important in view of the fact that replacement of either G1 Asp or G2 Pro results in increased oxygen affinity and decreased cooperativity. Thus, Hb Yakima G1β (Asp→His), Hb Kempsey G1β (Asp→Asn), Hb Georgia G2α (Pro→Leu) and Hb Denmark Hill G2α (Pro→Ala) all have increased oxygen affinity and decreased cooperativity with n values of 1.1, 1.1, 1.3 and 1.8 respectively (Bellingham 1976). Based on these considerations, it is tempting to speculate that rudiments of the $\alpha_1\beta_2$ contact area were present in the ancestral vertebrate stem.

Recent studies on the structural organization of globin genes suggest that two diverging intervening sequences are present at homologous positions in the alpha and beta globin genes of many vertebrate species (van den Berg et al. 1978 and Abelson 1979). This implies that the origin of the intervening sequences can be traced prior to the alpha-beta divergence. Interestingly, most of the heme contact sites and the $\alpha_1\beta_2$ contact sites are located on the central exon-encoded segment (Eaton 1980). Moreover, this segment has the necessary structural potential for providing specific sites for tight heme binding (Craik et al. 1981). Against this background, we have analysed the ancestor-descendant relationship of globin sequences contained within this segment. Here, we find that from among the amino acid residues at heme contact sites located on the central segment those at sites C4, CD1, CD4, E7, E10, E11, E14, E15, F4, F8, FG3, FG5 and G5 are conserved in vertebrate globin ancestor, myoglobin-hemoglobin ancestor, β-globin ancestor and α-globin ancestor (table 1). Similarly, from among the residues at the $\alpha_1\beta_2$ contact sites belonging to the central segment, those at positions C2, FG3, FG5, G1 and G2 (table 2) are also conserved during descent. These results collectively suggest that the central exon-encoded segment of the ancestral vertebrate globin also had the potential of providing both heme contact sites and rudiments of the $\alpha_1\beta_2$ contact sites.

(ii) Loss of Cooperativity in Myoglobin Branch

Figure 1 shows that as evolutionary time advanced, a gene duplication in the stem line separated the myoglobin branch on the one hand and the hemoglobin branch on the other. Table 2 shows that the hemoglobin-myoglobin ancestor resembles closely the vertebrate globin ancestor at the $\alpha_1\beta_2$ contact site. This would imply that the hemoglobin-myoglobin ancestor also had the ability to form polydisperse aggregates as well as dimers showing weak cooperativity. However, soon after its separation from the vertebrate stem, the myoglobin branch underwent many

substitutions, some of which replaced uncharged amino acid residues at functionally important sites by charged amino acids (table 2). A comparison of amino acid residues of hemoglobin-myoglobin ancestor and mammalian myoglobin ancestor at $\alpha_1\beta_2$ contact sites (table 2) shows five such substitutions, namely C3 (Ala→Glu), C7 (Tyr→Lys), CD2 (Ala→Asp), FG3 (Leu→His) and FG4 (Gln→Lys). Similarly, two such substitutions at $\alpha_1\beta_1$ contact sites are B15 (Thr→Lys) and D2 (Ala→Glu) (not indicated in the tables). In this context, it may be noted that charged amino acid residues in myoglobin attract water molecules and prevent association (Perutz 1976). These observations are suggestive of the fact that after separation from the stem line the myoglobin branch lost the property of self-association and consequently, cooperativity in oxygen-binding.

(iii) Enhanced Cooperativity in Hemoglobin Tetramers

The next major event in the evolutionary history of globins is a gene duplication that separated the alpha and beta globin branches. Subsequently, the evolving monomeric hemoglobins developed the capability to form tetramers of the $\alpha_2\beta_2$ type.

It has been established that the hemoglobin tetramer achieves cooperativity in oxygen binding due to its ability to transit between two conformational states (Baldwin 1976 and Perutz & TenEyck 1971). The low oxygen affinity state, the T state, corresponds to deoxyhemoglobin whereas the high oxygen affinity state, the R state, refers to oxyhemoglobin (Perutz & TenEyck 1971 and Edelstein 1975). The interactions constraining the hemoglobin tetramer in the T structure are salt bridges which are present in deoxyhemoglobin but absent in oxyhemoglobin (Perutz 1970 and Perutz & TenEyck 1971). These salt bridges are formed by the amino acid residues HC3α Arg, HC3β His, NA1α Val, H9α Asp, C5α Lys and FG1β Asp. The relevant point here is that the C-terminal amino acid residues (HC3) of both alpha and beta globins are involved in these salt bridges. More importantly, the removal of these C-terminal residues results in drastic lowering of cooperativity as is evident from the modified hemoglobin Hb des (Arg 141α, His 146β) which has an n value of 1.1 (Kilmartin & Hewitt 1971). Our results show that most of the amino acid residues involved in salt bridges appear only after the alpha-beta divergence. For example, histidine which is present at HC3 in both the hemoglobin-myoglobin ancestor and the alpha-beta ancestor persists in the beta line of descent whereas in the alpha line, it is replaced by arginine. Similarly, a lysine residue at C5α (table 2) evolved only after the divergence of the alpha and beta globin branches. This is also true of FG1β Asp and H9α Asp although these sites are not shown in our tables.

The amino acid residues at the $\alpha_1\beta_2$ contact area of tetrameric hemoglobins are important for cooperativity (Perutz 1968). Many conformational changes occur at this contact surface on oxygenation (Perutz & TenEyck 1971). Comparison of liganded and deoxyhemoglobin structures have indicated that the $\alpha_1\beta_2$ contact region is capable of existing in two alternate stable conformations: one, characteristic of deoxyhemoglobin and the other, characteristic of liganded hemoglobin (Perutz & TenEyck 1971). Table 2, shows that many of the $\alpha_1\beta_2$ contact sites namely C3, C5, C6, C7, CD2, FG4, G3 and HC3 change from those of alpha-beta ancestor following the alpha-beta divergence. Among these

sites, C3α Thr and C6α Thr lie in successive turns of the C helix and occupy the groove of FG5β Val in methemoglobin and deoxyhemoglobin respectively (Perutz 1976). Another $\alpha_1\beta_2$ contact site of interest is FG4. At this site, glutamine is present in the hemoglobin stem. However, following the alpha-beta divergence, arginine appears in the alpha branch and histidine in the beta (table 2). It has been observed that mutations leading to changes at either FG4α Arg or FG4β His back to the ancestral glutamine result in increased oxygen affinity and decreased cooperativity. For example, both Hb J Capetown FG4α (Arg→Gln) and Hb Malmo FG4β (His→Gln) have high oxygen affinity and low cooperativity with *n* values of 2.2 and 1.58 respectively (Bellingham 1976). These results collectively suggest that the $\alpha_1\beta_2$ contact sites that change after the alpha-beta divergence played a significant role in the development of the two alternate positions of the $\alpha_1\beta_2$ contact area and the T→R transition needed for enhanced cooperativity in oxygen-binding.

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