Sickle Cell Anemia

From Basic Science to Clinical Practice

Fernando Ferreira Costa
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Editors

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To all those with sickle cell disease, their families and their caregivers.

Preface

Although sickle cell anemia was the first molecular disease to be identified, its complex and fascinating pathophysiology is still not fully understood. A single mutation in the beta-globin gene incurs numerous molecular and cellular mechanisms that contribute to the plethora of manifestations and complications associated with the disease. Knowledge regarding sickle cell disease mechanisms, while still not complete, has broadened considerably over the last decades.

Sickle Cell Anemia: From Basic Science to Clinical Practice aims to provide an update on some aspects of our current understanding of the disease's pathophysiology and use this information as a basis to discuss its manifestations in childhood and adulthood, as well as therapeutic approaches to the disease. An introductory chapter (Chap. 1) describes the structure and function of hemoglobin, giving us a clue as to why a single gene mutation can wreak such havoc in the red blood cell. Chapter 2 describes the current theories regarding the emergence of the sickle mutation and the subsequent epidemiology of sickle cell anemia. Chapter 3 presents an overview of sickle cell disease pathophysiology, describing how the polymerization of the abnormal sickle hemoglobin injures the red cell, causing its membrane injury and ultimate failure, and producing a population of heterogeneous red blood cells, hemolysis, and reduced nitric oxide bioavailability. In a process that is driven by vascular inflammation and oxidative stress, interactions of the sickle red cells and leukocytes with the endothelium prolong the transit of the red cells through hypoxic vascular beds, resulting in red cell sickling and ultimately in the vaso-occlusive processes that are the hallmark of the disease. Some of these aspects are described in greater detail in Chaps. 4 (red cells), 5 (leukocytes), and 8 (inflammation), while Chap. 6 relates the evidence for a hypercoagulable state in sickle cell anemia and its role in disease pathophysiology and Chap. 7 looks at the endothelium and how the anemia that results from red cell destruction affects the cardiovascular system.

Recurrent vaso-occlusive processes, together with hemolytic anemia, result in end-organ damage and the diversity of complications of this disease, which can include stroke, pulmonary hypertension, osteonecrosis, leg ulcer, nephropathy, retinopathy, and priapism among numerous others. The complications of childhood sickle cell anemia, and their treatment, are considered in Chaps. 9 and 10, while

priapism and the manifestations and current treatment and therapy of adult sickle cell anemia are presented in Chaps. 11 and 12, respectively. Some of the other more common sickle cell diseases (caused by the inheritance of the HbS gene along with another abnormal Hb variant) are explored in Chap. 13, while Chap. 14 looks at the management of sickle cell disease in Africa, the region with the highest burden of sickle cell disease, and in the Arabian Peninsula, which displays a great variety in terms of sickle cell disease genotype and phenotype; the challenges faced by clinicians in these regions are discussed. The clinical severity of sickle cell disease is extremely heterogeneous, and co-inheritance of numerous other genetic factors can significantly alter the course of the disease, for better or for worse; some of these genetic modifiers are discussed in Chap. 15. Finally, prospects for the development of new approaches for the management of the disease, many of which have been developed based on our progressive understanding of the pathophysiology of the disease, are explored in Chap. 16, in addition to discussion regarding the expansion of the use of hematopoietic stem cell transplantation as a curative option for sickle cell anemia and perspectives for the development of gene therapy/gene editing approaches for the disease.

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Chapter 1
Hemoglobin: Structure, Synthesis and Oxygen Transport

Susan E. Jorge, Daniela M. Ribeiro, Magnun N.N. Santos, and Maria de Fátima Sonati

Abstract Human hemoglobin (Hb) is the erythrocyte hemeprotein resulting from the combination of one pair of α-like (α or ζ) chains and another pair of β-like (β, δ, γ or ε) chains. Each of these chains is associated with a *heme* prosthetic group, a tetrapyrrole ring (protoporphyrin IX) containing a central ferrous atom (Fe^{2+}), which can reversibly bind to a molecule of O_2, being, therefore, responsible for its transport from the lungs to the tissues. This introductory chapter summarizes these important aspects, including findings of protein structure, synthesis and function, as well as its gene organization and regulation. We also describe the developmental switches in globin chain production (from the embryonic period until hematological adult life), *heme* synthesis and globin gene expression/regulation, besides functional aspects of the hemoglobin molecule. The chapter also includes models that predict the mechanisms of Hb-O_2 ligation, mediated by the presence of allosteric effectors, such as H^+/CO_2, Cl^- and organic phosphates, such as 2,3-biphosphoglycerate (2,3-BPG, from erythrocyte metabolism).

Keywords Hemeproteins • Human hemoglobin • Globin chains • Globin genes • Oxygen transport

1.1 Human Hemoglobins

Hemoglobins (Hb) are hemeproteins that transport oxygen (O_2). These proteins, or the genes expressing them, seem to be present in all living organisms. Like other vertebrate organisms, human hemoglobins are found in high concentrations in erythrocytes (around 640 million molecules/cell), and are their main component

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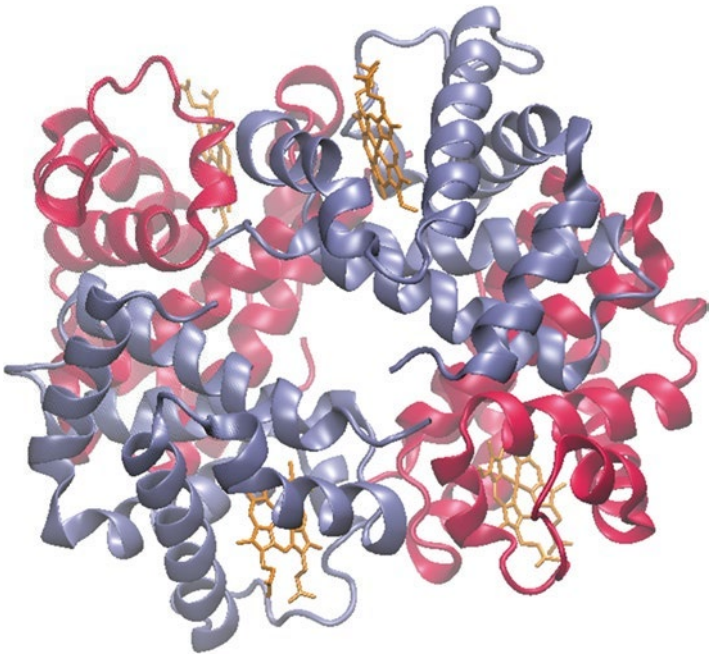

Fig. 1.1 Human Hb A, represented by two α-chains (in ice *blue*), two β-chains (in *red*) and four *heme* groups (in *orange*). Obtained from *Protein Data Bank* 1ZGX coordinates, using Visual Molecular Dynamics (VMD) software

(Shikama 2006). Despite hemoglobin diversity, the molecular structures of hemoglobins are very similar, showing a high degree of conservation during evolution. Hemoglobins are globular tetramers (molecular weight, 64,450 Daltons), comprised of two pairs of polypeptide chains (globins); one pair of α-like (α or ζ) chains and another pair of β-like (β, δ, γ or ε) chains. Each chain is associated with a *heme* prosthetic group, a tetrapyrrole ring (protoporphyrin IX) containing a central ferrous atom (Fe^{2+}), which can reversibly bind to a molecule of O_2 to transport oxygen from lungs to tissues (Fig. 1.1) (Antonini and Brunori 1971; Hoffbrand and Moss 2011).

The α and ζ globin chains have 141 residues, while the β, γ, δ and ε globins have 146 residues. Different combinations of these chains result in the formation of different types of hemoglobins, adapted for distinct periods of human development. During the embryonic period, hemoglobin synthesis starts at the end of the third week of pregnancy in primitive erythroblasts derived from the hematopoietic stem cells in the vitelline sac, with the production of the embryonic hemoglobins Gower 1 ($\zeta_2\varepsilon_2$), Hb Gower 2 ($\alpha_2\varepsilon_2$), Hb Portland I ($\zeta_2\gamma_2$) and Portland II ($\zeta_2\beta_2$). After the tenth week of pregnancy, hemopoiesis occurs in the liver and spleen (visceral phase) and the embryonic hemoglobins are replaced with fetal Hb, or Hb F ($\alpha_2\gamma_2$), which is predominant during the entire fetal period. During adult life the main site of erythropoiesis is the bone marrow and Hb F is replaced by hemoglobins A ($\alpha_2\beta_2$) and A_2 ($\alpha_2\delta_2$). Hb A predominates, comprising more than 95 % of the total hemoglobin,

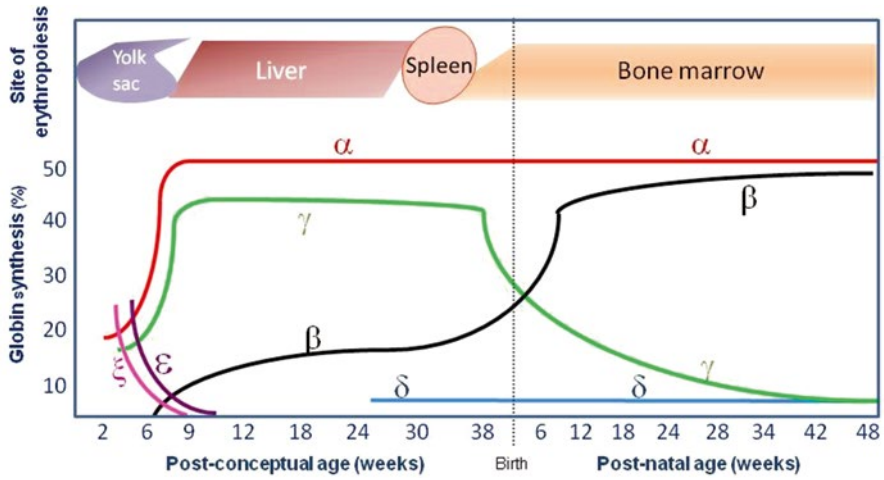

Fig. 1.2 Normal developmental switches in globin chain production. Adapted from Hoffbrand and Moss (2011)

Table 1.1 Normal hemoglobin profile in healthy adults

	Hb A	Hb F	Hb A_2
Globin chains	$\alpha_2\beta_2$	$\alpha_2\gamma_2$	$\alpha_2\delta_2$
Normal (%)	95–98	up to 2.0	2.0–3.0

while Hb A_2 corresponds to 2–3 % and Hb F to a maximum of 2 % of total hemoglobin. The 'adult' hemoglobin profile is generally established by the sixth post-birth month (Steinberg et al. 2001; Hoffbrand and Moss 2011) (Fig. 1.2) (Table 1.1).

1.1.1 Heme Synthesis

As previously mentioned, the tetrapyrrole ring (protoporphyrin IX) containing a bivalent iron atom (*heme*) constitutes the hemoglobin core, where the reversible binding of O_2 occurs. During the process of *heme* synthesis, the beginning and the end of the protoporphyrin production and the incorporation of iron take place in the mitochondria, which are arranged around the nuclei of erythroid precursors. The intermediate steps of protoporphyrin synthesis occur outside these organelles, in the soluble portion of the cytoplasm. Initially, the process involves condensation of succinyl-coenzyme A, formed in the mitochondria after the Krebs cycle (aerobic respiration), with amino acid glycine, to produce δ-Aminolevulinic acid (δ-ALA), a reaction stimulated by erythropoietin and catalyzed by ALA-synthetase, employing vitamin B6 as a coenzyme. Consequently, two molecules of δ-ALA produce porphobilinogen, a pyrrole, where four molecules of porphobilinogen, arranged in a ring structure (tetrapyrrole ring), yield uroporphyrinogen, which, after successive decarboxylation of side-chains, originates coproporphyrinogen, followed by

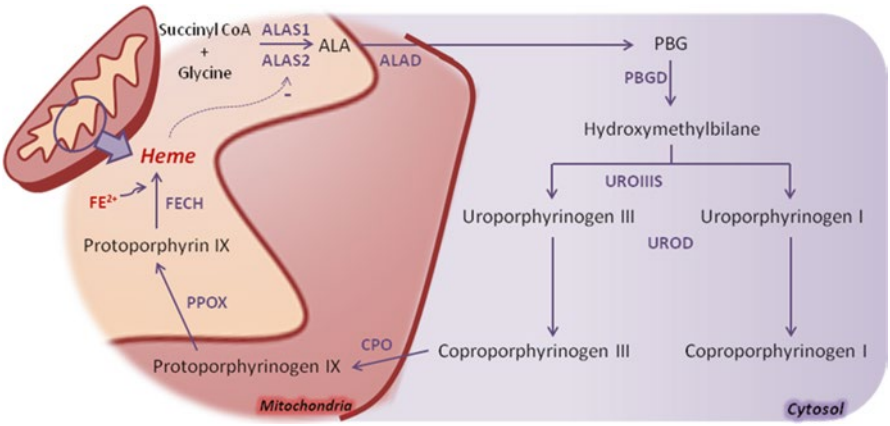

Fig. 1.3 *Heme* biosynthetic pathway. *ALA* 5-aminolaevulinic acid, *PBG* porphobilinogen, *ALAS1* ALA synthase 1, *ALAS2* ALA synthase 2, *ALAD* ALA dehydratase, *PBGD* porphobilinogen deaminase, *UROIIIS* uroporphyrinogen-III synthase, *UROD* uroporphyrinogen decarboxylase, *CPO* coproporphyrinogen oxidase, *PPOX* protoporphyrinogen oxidase, *FECH* ferrochelatase. Adapted from Puy et al. (2010)

protoporphyrin (protoporphyrin IX). In the final phase, protoporphyrin binds to an atom of iron in the ferrous state to produce *heme*. All these reactions are mediated by enzymes, including alanine synthetase, alanine dehydratase, porphobilinogen deaminase, uroporphyrinogen synthetase, uroporphyrinogen decarboxylase, coproporphyrinogen oxidase, ferrochelatase and *heme* synthetase (Fig. 1.3). In the human organism, biosynthesis of *heme* occurs predominantly in erythroid cells, where it is incorporated into recently synthesized hemoglobin, although *heme* can also be produced in hepatic cells, where it is used as part of cytochrome p-450 (Puy et al. 2010; Hoffbrand and Moss 2011).

Each gram of hemoglobin contains 3.4 mg iron which, before being incorporated into the molecule, is stored in macrophages located in the liver, spleen and bone marrow, and in cells of the liver parenchyma. Iron is transported in plasma by the transferrin protein and delivered to transferrin receptors present on the surface of the red blood cell membrane, before its incorporation into hemoglobin. Iron can be stored for prompt use in ferritin, a water-soluble protein, comprised of 22 subunits (apoferritin), arranged as a shell around a central storage cavity, where variable amounts of iron are found as ferric hydroxyphosphate microcrystals. Iron moves freely inside and outside this cavity, through channels in the protein shell and, therefore, is readily available for metabolic use. Iron can also be stored in the form of hemosiderin, an insoluble iron-protein complex, comprised of aggregates of partially denatured ferritin molecules. Iron release from hemosiderin is slower than from ferritin. In both ferritin and hemosiderin, iron is in the ferric state and, to be mobilized, it must be reduced to the ferrous state in a reaction that involves vitamin C (Puy et al. 2010; Hoffbrand and Moss 2011).

1.1.2 Globin Synthesis

Globin synthesis occurs in the polyribosomes, in the cytoplasm of erythroblasts and reticulocytes, where specific messenger RNAs (mRNAs) are translated into different globin chains. The globin mRNA is relatively stable and, therefore, reticulocytes are able to synthesize hemoglobin for at least 2 days after loss of the nucleus. A balanced globin synthesis is critical to the development and function of erythroid cells; every α chain should have a non-α chain, so that neither is in excess or deficient. Although the quantity of mRNA encoding α globins in normal reticulocytes exceed the quantity of mRNA encoding β globins, the efficiency of the translation of β globin mRNA seems to be higher to compensate and maintain the important balance between both chains (Stamatoyannopoulos et al. 2001; Weatherall et al. 2001). Moreover, during the synthesis process, free alpha globin chains are stabilized by the alpha-hemoglobin stabilizing protein (ASHP), an erythroid molecular chaperone that binds to α-hemoglobin (α-Hb) or α-globin until its association with beta globin to form the Hb tetramer (Khandros et al. 2012; Domingues-Hamdi et al. 2014).

The polypeptide chains (primary structure), released by ribosomes, assume their tridimensional configuration spontaneously due to the interactions of their residues (Fig. 1.4). The protein folding of the alpha helix structure of hemoglobin (secondary structure) involves a very stable ligation to the *heme* group, in the globin chain core, favored by the creation of a hydrophobic environment that protects the *heme* group from oxidation (tertiary structure) (Figs. 1.4 and 1.5). Subsequently, a tetramer of globin chains is created by the association of the tertiary conformations (quaternary structure) (Figs. 1.4 and 1.5). Each chain is constituted by seven or eight α-helical segments (named A through H, from the amino-terminal) followed by non-helical segments (Fig. 1.5a). The flexibility of the chain permits oxygen to access the *heme* pocket (Antonini and Brunori 1971; Mairbäurl and Weber 2012) (Fig .1.5b).

1.1.3 Globin Chain Genes

Globin genes are arranged in groups, or gene clusters, that organize the balanced production of globins during the different pre- and post-birth stages. Cluster α, located in a 30-kb DNA segment on the short arm of chromosome 16 (16p13.3), contains embryonic gene ζ, pseudogenes ψζ and ψα_1, double α globin genes (α_2 and α_1) and genes θ and α^D, of undetermined functions. The arrangement in the chromosome is the same as that expressed during human development, i.e., 5′ - ζ - ψζ - α^D - ψα_1 - α_2 - α_1 - θ - 3′ (Weatherall et al. 2001; Harteveld and Higgs 2010) (Fig. 1.6). Cluster β, located in an approximately 50-kb DNA segment on the short arm of chromosome 11 (11p15.5), includes genes ε, $^G\gamma$, $^A\gamma$, δ, β and pseudogene ψβ, in the following order: 5′ - ε - $^G\gamma$ - $^A\gamma$ - ψβ - δ - β - 3′ (Fig. 1.7).

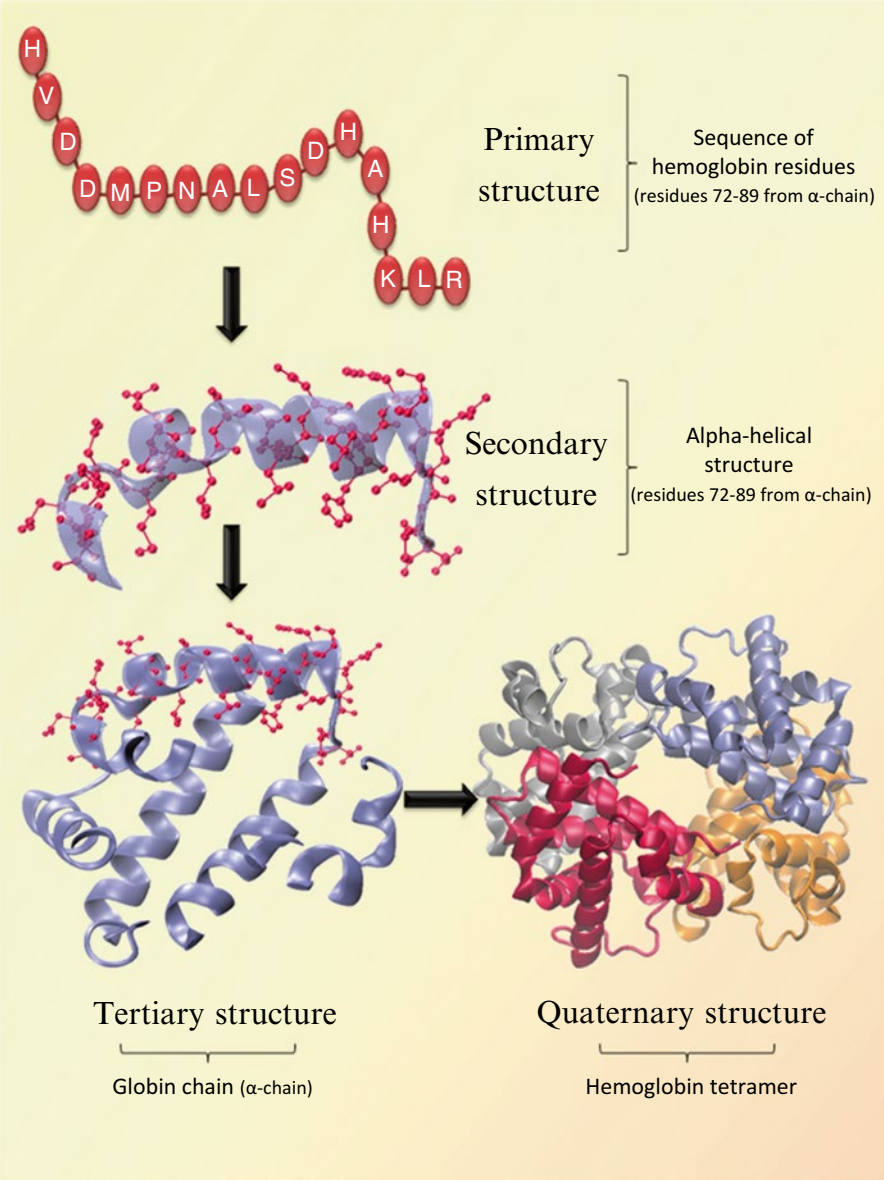

Fig. 1.4 Hemoglobin formation. A fragment of the alpha globin chain (residues from the position 72 to 89, in *red*), was used to represent primary, secondary and tertiary conformation. Structure obtained from *Protein Data Bank* 1GZX coordinates, using Visual Molecular Dynamics (VMD) software

Pseudogenes (ψ) are not functional genes, i.e. they are unable to encode proteins, although it has been demonstrated that they may have an important role in the regulation of transcription and translation in human cells. The globin pseudogenes have

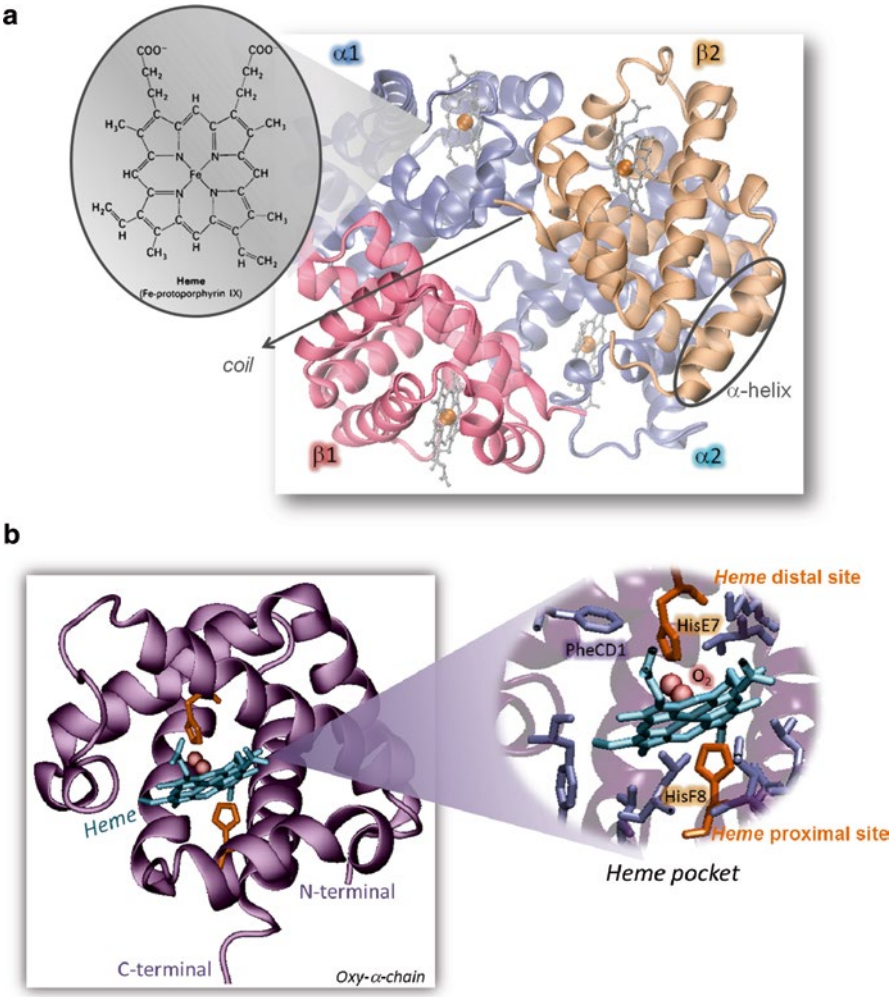

Fig. 1.5 Hemoglobin structure. (**a**) Tetramer of Hb A with *heme* featured and globin chains highlighted; coil and α-helical regions are indicated. Structure obtained from *Protein Data Bank* 1GZX coordinates, using Visual Molecular Dynamics (VMD) software. (**b**) Structure of the Oxy-alpha chain in globin (*purple*); C- and N-terminals are indicated. *Heme* in *cyan blue*, distal and proximal histidines in *orange*, oxygen in *red* and other residues from the *heme* pocket (active site) in *purple*. Structure obtained from *Protein Data Bank* 1GZX coordinates, using Visual Molecular Dynamics (VMD) software

developed from protogenes α and β through replicating events and have been disabled by mutations (substitution of bases, deletions and/or insertions) that have determined their loss of expression. Genes θ and α^D do not have a specific function; they are expressed at very low levels in vivo and their protein products have not been identified (Johnsson et al. 2013).

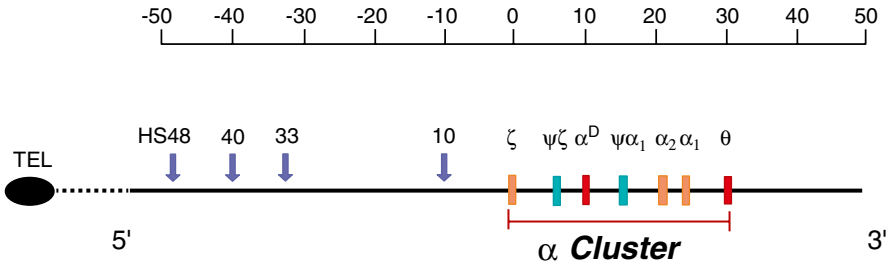

Fig. 1.6 Structure of the α cluster on chromosome 16. The telomere is shown as an oval, the *orange boxes* represent functional genes, while *blue boxes* are pseudogenes and the *red boxes* represent genes of undetermined function. *Arrows* represent the α-globin regulatory region. The scale is in kilobases as indicated above, counting from the ζ-globin gene. Adapted from Harteveld and Higgs (2010)

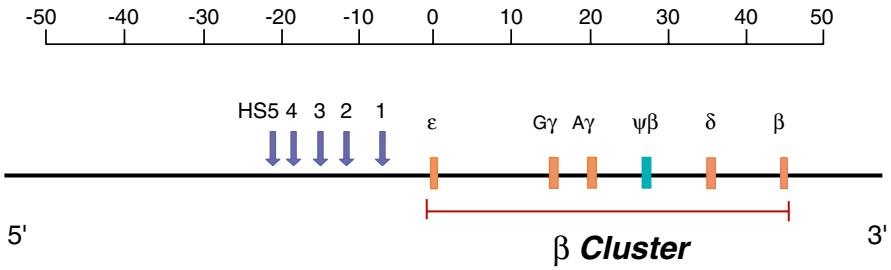

Fig. 1.7 Structure of the β cluster on chromosome 11. The *orange boxes* represent the functional genes and the *blue box* depicts a pseudogene. The *arrows* represent the β-globin regulatory region. Distances are in kilobases, counting from the ε-globin gene, as indicated above. Adapted from Patrinos et al. (2004)

Globin genes are compact, measuring 1–2 kb in length, and they have three exons and two introns. Amino acids involved in binding to the *heme* group (*heme* pocket), essential for the ability of hemoglobin to bind O_2 and total stability of the molecule, and involved in $\alpha_1\beta_2$ contacts, are mainly coded by exon 2, while those that intermediate $\alpha_1\beta_1$ contacts (increasing molecule stability and involving numerous amino acids in the chain interaction) are mostly coded by exon 3; the residues related to the binding affinity of hemoglobin for O_2 (Bohr effect and binding to 2,3- BPG) are randomly distributed among exons (Steinberg et al. 2001; Hoffbrand and Moss 2011). Genes γ and α are duplicated in chromosomes 11 and 16, respectively. Gene γ encodes different polypeptide chains ($^{G}\gamma$ and $^{A}\gamma$), which contain alanine or glycine, respectively, at position 136 of the chain. Genes α_2 and α_1 encode identical proteins and are very similar, presenting only 17 % structural divergence, limited to intron 2 (IVS-II) and exon 3, in the 3′ non-coding region (Weatherall et al. 2001; Higgs et al. 2012; Richard et al. 2012). Although it produces identical α chains, the expression level of gene α_2 is around 2.5 times greater than the expression level of gene α_1, as evaluated by the proportion of synthesized messenger RNA (mRNA) and experiments

with mutants (Liebhaber and Kan 1982). Another particularity of the α genes is that they are inserted, as every group, in a GC-rich DNA segment, characteristic of genes expressed in all tissues (housekeeping genes); however, although it has been demonstrated that endothelial cells can also produce alpha-hemoglobin, the expression of these genes is highly erythroid specific (Straub et al. 2012).

A balanced globin synthesis is critical for the development and function of erythroid cells. As mentioned above, the quantity of mRNA encoding α globin in normal reticulocytes exceeds the quantity of mRNA encoding β globin, but the efficiency of the translation of this second mRNA seems to be higher to compensate and maintain the balance between both chains. Although a balanced expression of globin chains occurs during development, no feedback mechanism has been identified through which the expression of a globin may affect the expression of another globin; both seem to function coordinately, but with independent regulation (Liebhaber and Kan 1982; Weatherall et al. 2001; Higgs et al. 2012; Richard et al. 2012).

1.1.4 Regulation of Globin Gene Expression

Globin gene expression in both clusters is controlled according to the human developmental stage and in a tissue-specific manner. This complex control is dependent on *cis-acting* regulatory sequences (such as promoters and 3′ non-coding regions) that are close to and far from the globin genes (enhancers and negative regulatory elements), and the interaction of trans-acting factors with proteins, i.e. transcription factors. The interaction of transcription factors and regulatory elements close to the globin genes is similar to the regulatory mechanisms used by other human genes; however, the remote *cis-acting* control involves some particularities (Weatherall et al. 2001).

The expression of genes in the β cluster is controlled by regulatory sequences located upstream of the ε gene, which are five erythroid-specific DNase I hypersensitive sites (HS 1-5) (Fig. 1.7). The DNA segment that contains these sites is called the β Locus Control Region (β-LCR). The β-LCR, besides acting as an enhancer that activates the expression of β genes, has the additional function of changing the chromatin structure where these genes are inserted, allowing their transcription (Patrinos et al. 2004).

The β cluster is located in an AT-rich genome region of highly condensed chromatin. A few cell types have active genes in these regions. In non-erythroid cells, the chromatin domain in which this group is located is not sensitive to DNase I, demonstrating delayed replication (in the second half of the S phase of the cell cycle) and transcriptional inaction. In contrast, in erythroid cells, this domain is sensitive to DNase I, replicates at the beginning of the S phase and is transcriptionally active. Tissue-specific changes in chromatin structure are attributed to the β-LCR, which interacts via a loop with promoters of the active genes, creating a single chromatic structure, known as the active chromatin hub (ACH), causing chromatin decondensation and promoting the interaction of regulatory proteins with

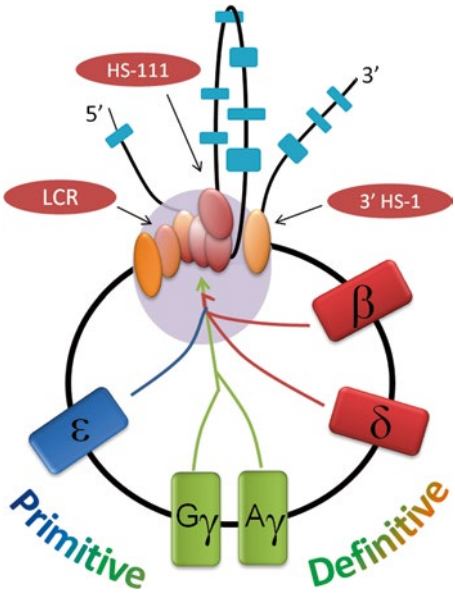

Fig. 1.8 Representation of the interactions between the β-Locus Control Region (LCR and the β cluster genes in erythroid cells. The Active Chromatin Hub (ACH), indicated as a lilac sphere, is formed by LCR and Hypersensitive Sites (HSs). The genes are depicted in different colors, indicative of their interaction with the β-LCR within the ACH during development. The embryonic ε- and fetal γ-globin genes are expressed during primitive hematopoiesis and the switch to the fetal γ- and adult δ- and β-globin genes occurs during early definitive hematopoiesis. Adapted from Patrinos et al. (2004)

DNA and the activation of the expression of genes from the respective group. This erythroid-specific chromatic structure is also regulated according to different stages of human development as, during each developmental stage, only one gene interacts with the β-LCR to yield a single ACH (Patrinos et al. 2004) (Fig. 1.8).

Due to the common ancestry of the α and β clusters (they diverged around 500 million years ago) and their similar arrangements, it was assumed for some time that the expression of these genes was controlled similarly. However, in vitro and in vivo studies employing transgenic mice suggest that these clusters are controlled in different manners. The regulation of the expression of the α cluster in humans is dependent upon a regulatory element located 40 kb upstream of the ζ gene, close to the telomere, denominated HS-40, which is an erythroid-specific DNase I hypersensitive site (Fig. 1.6). Its existence was perceived at first, due to deletions in this region that resulted in an α-thalassemia phenotype, although the α genes in these individuals presented a normal structure (Weatherall et al. 2001; Higgs et al. 2012).

HS-40 (today referred to as α-Major Regulatory Element, or α-MRE) does not change the chromatic structure in which the α cluster is inserted; its function is to act as an enhancer, activating the expression of genes in this cluster, located in a GC-rich genome region, of decondensed chromatin in both erythroid and

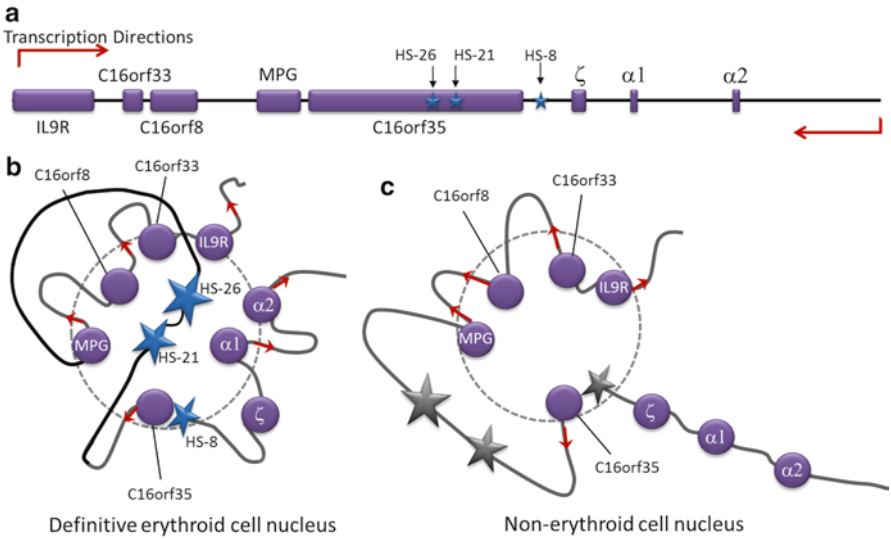

Fig. 1.9 The linear structure of the mouse α cluster (**a**) and putative chromatin structure in murine definitive erythroid cells (**b**) and murine nonerythroid cells (**c**). In panel **a**, the *blue stars* indicate hypersensitive sites (HSs) and the *purple boxes* indicate genes. In panels **b** and **c**, the *blue* and *gray stars* show the positions of HSs, the *purple spheres* represent protein complexes on gene promoters, the *gray* and *black strings* represent chromatin and the *red arrows* indicate transcription direction. Adapted from Zhou et al. (2006)

non-erythroid cells. Thus, the main question is to understand how HS-40 controls the expression of the α genes without interfering in adjacent gene expression. In fact, a permanent chromatin structure is created and maintained (Vernimmen et al. 2009). In murine erythroid cells, the recruitment of promoters of active genes from the α cluster and the regulatory element for this ACH results in the gene expression of globins. Furthermore, the exclusion of α globin genes and their regulatory element from this chromatin structure in non-erythroid cells disables gene expression (Zhou et al. 2006) (Fig. 1.9).

1.2 Oxygen Transport

Hb A results from the combination of two α and two β chains. These segments consist, respectively, of seven and eight α-helical structures, named from A to H, which are intermediated by non-helical sequences (AB, BC, and so on). The terminal portions of each globin, NA and HC, are short non-helical extensions from helix A to the C-terminal. Thus, all residues are numbered according to the coordinates presented in the structure, from the N-terminal and/or according to its position on the segment (Antonini and Brunori 1971; Mairbäurl and Weber 2012) (helix, coil, etc.—Fig. 1.5a).

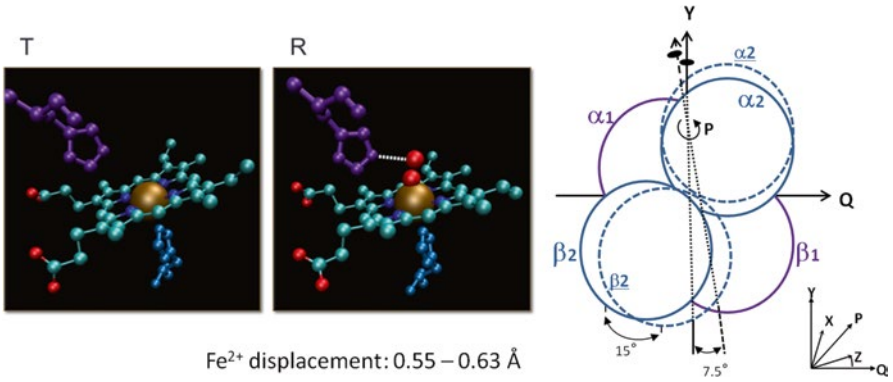

Fig. 1.10 Proximal (*blue*) and distal histidines (*purple*) in the *heme* pocket, in the T (deoxy) and R (oxy) hemoglobin states. Oxygen (*red*) is bound to Fe^{2+} (*brown*) in the R conformation of hemoglobin—figure produced from *Protein Data Bank* 1GZX and 2DN1 coordinates, using Visual Molecular Dynamics (VMD) software. The chain movements are represented in the *right panel*

The *heme* group is located under the E, F and G helixes and CD contacts. The stability of the binding of O_2 to *heme* is, therefore, affected by a number of residue interactions located at these contact regions. Of these, the most important are formed by proximal histidines [α87 (F8) or β92 (F8)], which bind directly to the iron of protoporphyrin IX, and distal histidines [α58 (E7) or β63 (E7)], which then interact with iron-bound O_2 (Antonini and Brunori 1971; Shikama 2006; Mairbäurl and Weber 2012) (Fig. 1.5b). During oxygen binding, the iron atom moves 0.55–0.63 Å, which results in conformational changes on the residues surrounding the active *heme* pocket site. This event also causes significant changes in the remaining globin chains, in both the tertiary and quaternary structures (Antonini and Brunori 1971; Mairbäurl and Weber 2012). These structural alterations confer two stable states to the hemoglobin molecule; the tense form (T or "*deoxy*-Hb", with a lower affinity for O_2) and the relaxed form (R or "*oxy*-Hb", with increased affinity for O_2), as described by Perutz, in 1970, using crystallography (Perutz 1970) (Fig. 1.10).

The protein stability of the tetramer is controlled by the interface contacts $\alpha_1\beta_1$ and $\alpha_2\beta_2$, also called packing contacts, where the α- and β- chains are connected by 34 residues located in the C, G and H helices and the BC corner. The $\alpha_1\beta_2/\alpha_2\beta_1$ interdimer interface is less extensive and contains 19 residues in total. This characteristic ensures the stability of the T-R transition during oxygen binding. Besides being shorter, the $\alpha_1\beta_2/\alpha_2\beta_1$ interface is located close to the *heme* group, promoting conformational changes in the active *heme* site. Simultaneously, structural changes in the *heme* pocket also mediate allosteric interactions in the $\alpha_1\beta_2$ area. Therefore, any substitution of residues at the $\alpha_1\beta_2/\alpha_2\beta_1$ interface may result in reduced *heme-heme* cooperativity and changes in the affinity of hemoglobin for O_2, demonstrating the extreme importance that $\alpha_1\beta_1$ and $\alpha_2\beta_2$ dimer interactions have in balancing T-R conformational changes when binding to O_2 (Antonini and Brunori 1971; Eaton et al. 2007).

1.2.1 Functional Aspects of Hemoglobin

The main function of hemoglobin is to transport O_2 from the lungs to peripheral tissues, and to take carbon dioxide from tissues back to the lungs. Usually, 95 % of O_2 molecules transported from the lungs to tissues are maintained in chemical combination with hemoglobin in the red blood cells, while the remaining 5 % are transported dissolved in plasma and cell water (Hoffbrand and Moss 2011).

The mechanism of the Hb-O_2 ligation is mediated by homotropic (*heme-heme* cooperativity) and heterotropic events, such as differences in O_2 pressures. Under high pressures of O_2 (PO_2), as in pulmonary capillaries, O_2 binds to hemoglobin; when PO_2 is decreased, as in tissue capillaries, O_2 is released from the hemoglobin, in an event that is modulated by allosteric effectors, such as H^+/CO_2, Cl^- and organic phosphates, such as 2,3-biphosphoglycerate (2,3-BPG). O_2 binding can be mathematically represented by the oxygen-dissociation curve (ODC), which is obtained from the PO_2 *versus* saturated *heme* site under standard conditions of temperature, pH, atmospheric pressure, PCO_2 and concentration of organic phosphates, such as 2,3-BPG. Due to *heme-heme* cooperativity, the ODC results in a sigmoidal shape, and any change in the concentrations of gases, ions or 2,3-BPG concentration, among other factors, may affect the affinity of hemoglobin for O_2 (Antonini and Brunori 1971; Shikama 2006; Mairbäurl and Weber 2012) (Fig. 1.11).

The affinity of hemoglobin for O_2 is expressed by the P_{50}: the partial pressure of O_2 required for 50 % hemoglobin saturation (Fig. 1.11). The standard P_{50} value for

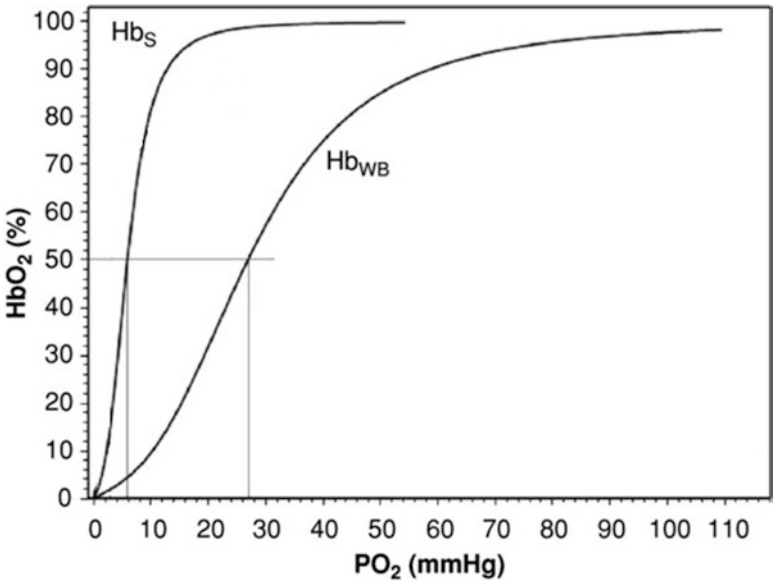

Fig. 1.11 Oxygen dissociation curves (ODCs) for stripped Hb in buffered solution (Hb_S), and human RBCs in whole blood (Hb_{WB}). Adapted from Mairbäurl and Weber (2012)

Hb A is 26.6 mmHg; thus, higher P_{50} values signify a decreased affinity for O_2, and vice versa. Several genetic and environmental factors may affect O_2 affinity and the great variation observed in hemoglobin P_{50} values occurs, partially, due to structural differences among the hemoglobins and, partially, due to differences in the intracellular environment. Native human hemoglobin affinity may vary by as much as 100-fold, as a result of changes in pH and/or PCO_2 (Bohr effect), salt ion concentrations (such as Cl^-) and, in particular, due to the concentration of organic phosphates (e.g. 2,3-BPG), which can shift the dissociation curve to the right (favoring the T conformation and a decreased oxygen affinity) (Antonini and Brunori 1971; Shikama 2006; Mairbäurl and Weber 2012) (Fig. 1.11).

During the last two decades, a number of different quaternary states of hemoglobin, such as R_2, RR_2, RR_3, T_{high}, have been reported. These states represent different conformations and comprise from canonical T-(deoxy) to R-(ligated) quaternary structures, depending on the experimental conditions, although the T and R states are the most stable. It is assumed that hemoglobin exists in allosteric equilibrium between two functional states, the **T** (lower-affinity) and **R** (higher-affinity) states, and that successive O_2-binding shifts the allosteric equilibrium towards the higher-affinity R states, therefore, explaining the sigmoidal Hb-O_2 binding curve (Fig. 1.11). The allosterism of the T-R transition is modulated by homotropic and heterotropic mechanisms, which comprehend the *heme-heme* cooperativity and the allosteric modifications promoted by non-specific ligands that interfere in the affinity for O_2 at the active *heme* site, respectively (Bruno et al. 2001; Eaton et al. 2007; Yonetani and Laberge 2008).

1.2.2 Homotropic Interactions: Heme-Heme Cooperativity

Heme-heme cooperativity can numerically be represented by the Hill coefficient ('*n*') as a global event, or calculated by the Adair equation, which considers four O_2-binding *hemes*, with, therefore, four equilibrium constants ($K_1 < K_2 < K_3 < K_4$). Several models predict the T-R transition due to *heme-heme* cooperativity and heterotropic interactions (Antonini and Brunori 1971; Eaton et al. 2007).

Allosteric Regulation: Symmetry Model—MWC

The first T-R transition model was proposed by Monod, Wyman and Changeux (MWC, symmetry model) in 1965. The model describes two stable states for hemoglobin: the T and R states, which are independent of the presence of O_2, where hemoglobin has a higher affinity for O_2 in its R state, and a lower affinity for O_2 in the T conformation. This model predicts the T-R transition as a global allosteric event, not based on chain-by-chain conformational changes; as such, in this model, hemoglobin has only two constants of O_2 ligation-dissociation K_{high} or K_R for the R state and K_{low} or K_T for the T state. The Hb-O_2 ligation occurs, therefore, as the