Course: PG Pathshala - Biophysics

Paper 4: Biomolecules and their interactions

Module 8: Folded conformation of globular proteins and mechanism of action (Hemoglobin, Myoglobin and Oxygen Binding)

Introduction

Proteins, the indomitable work horses of a living cell, are enigmatic biomolecules that bring about functionality. The polypeptide, which is synthesized in a linear form, must fold in an appropriate manner to lend proteins their final native, functional forms. Once folded properly they are amenable to specific chemical reactions and myriads of interactions that define life as we know of today. Understanding how folded conformation of proteins is responsible for their function presents an interesting challenge in biological sciences. This facet of biomolecular interactions is best demonstrated by myoglobin and hemoglobin and their mechanism of oxygen binding so fundamental to life.

The unit constitutes of 4 modules.

Objectives

The objectives of this unit are outlined as follows:

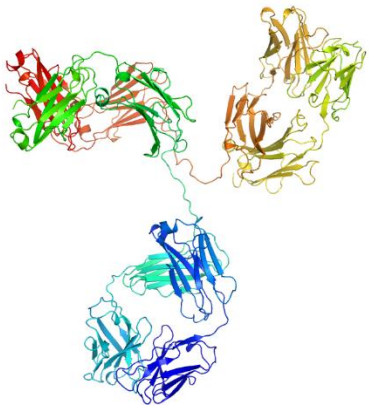
- Structure-function relationship; Importance of folding in globular proteins
- Hemoglobins in general; Myoglobin and Hemoglobin in concert – oxygen transport and storage – CO_2 transport and expulsion
- Structural features of **Myoglobin**
  - Fold; Heme binding; Active Site; Histidines and others
- Mechanism of O_2 binding by Myoglobin
  - Electrostatic interaction; Binding curve - Hyperbola
- Structural features of **Hemoglobin** – subunits
- Conformational changes upon ligand binding
- Mechanism of O_2 binding by Hemoglobin - Cooperativity / Sigmoidal dissociation curves
- Allostery - Effectors (H^+, CO_2, Cl^-, DPG); Root Effect
- Pathology associated – loss of function due to loss of structure / fold

1. Structure-Function relationship in proteins

1.1 **Structure dictates function**: A glance around the macroscopic world will make it evident that an appropriate "shape" is required for any material to be "functional". Thus a ball is spherical since it has to roll, needle is sharp, pointy and long to pierce, a box is cubical for stability and storage and a funnel is conical with long end for pouring liquid with a handle to hold the funnel for easy operation. The microscopic world of proteins is no exception to this general rule and **proteins also attain a proper shape suitable for their function.**

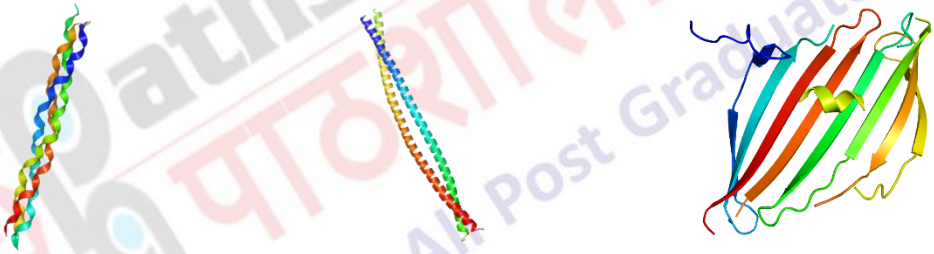
Thus, the well known proteins – antibodies – that are central to our defense against pathogens and other foreign material attain a "Y" shaped structure such that the arms or wings can recognize "antigens" (foreign bodies). The base or stalk of the Y has a shape appropriate for communication with

other components of the immune system. Another well known protein "trypsin", which is a protease (digests peptide bonds) is globular in shape with two well defined domains that produce a cleft for polypeptide binding and cleavage by the active site. To prevent such digesting activity of trypsin nature has also produced "trypsin inhibitor" which is a small molecule with long loops that can insert into the cleft and lock the active site.

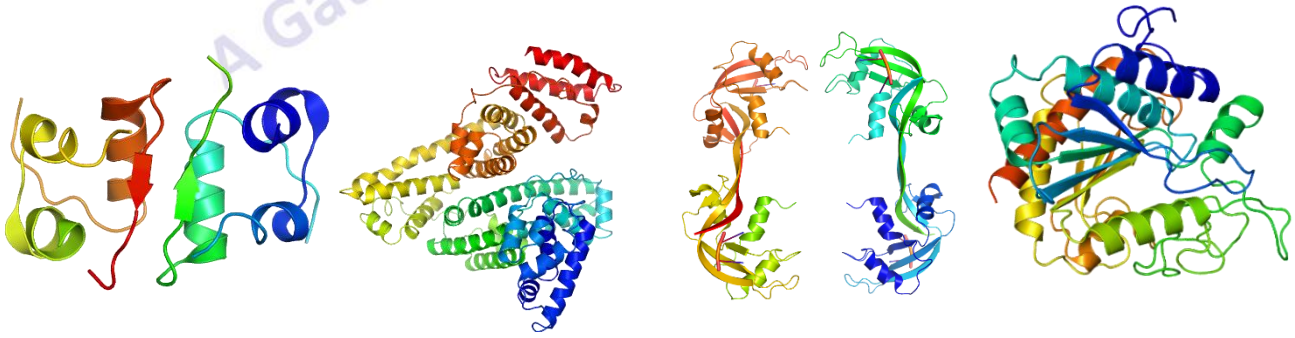


1.2 **Diverse function necessitates diverse structure**: Since proteins perform most of the functions in cells, it is imperative that they will have diverse functions, which in turn necessitates diverse structure, since each structure is associated with a particular function. Thus, proteins can be divided generally into two classes: Fibrous and Golobular proteins. The fibrous proteins contain a single type of secondary structure, are insoluble in water, aggregate together to form long and strong fiber like structures. This shape and structure of fibrous proteins are necessary since they provide mechanical strength, shape and support. Collagen, keratin and silk fibroin are classic examples.

Collagen **α-Keratin** **Silk fibroin**



Globular proteins on the other hand display diverse functions which include regulation, catalysis, defense, transport, signal Transduction, etc. Thus they have diverse structure with a variety of shapes and sizes.



Insulin **Albumin** **Ribonuclease -A** **Carboxypeptidase**

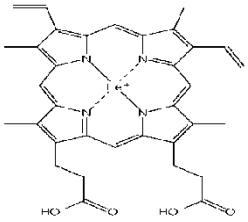
1.3 **Fold dictates structure**: Polypeptide synthesized in the unfolded, linear form (U) undergoes rapid "folding" to attain the native, functional form (N). Thus folding dictates structure of a protein and a functionally active protein must fold accurately. In summary, fold dictates structure, which in turn dictates function. **Folded conformations of globular proteins are thus essential for function/ mechanism of action.**

2. Myoglobin (Mb) and Hemoglobin (Hb) are Paradigms of Globular Proteins

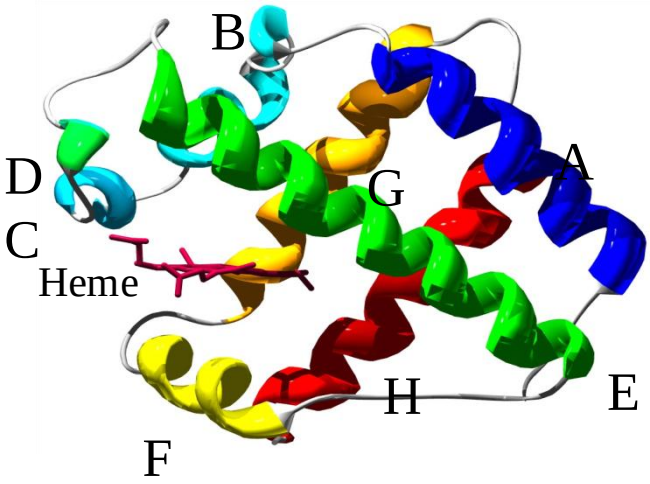
Myoglobin and hemoglobin are the two most popular and investigated proteins. They have been among the first few proteins which were sequenced, molecular weight determined, subjected to ultracentrifugation studies, crystallized and three dimensional structures solved. They have been subjected to a repertoire of biochemical and biophysical investigations as well as extensive site directed mutagenesis. There functions, oxygen transport and storage, are well known and correlated extensively to their molecular structures. Hence these two proteins have served as model proteins for the last several decades and are perfect examples for understanding structure-function relationship and molecular engineering.

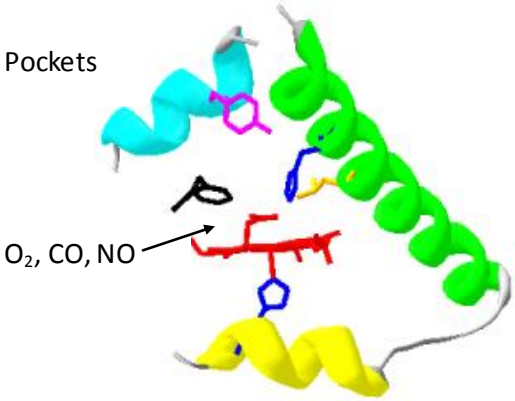
2.1 General features of Hemoglobins (Hbs)

Hemoglobins are members of the globin family formed by the association of a polypeptide chain (called apoglobin) and a heme prosthetic group (heme protoporphyrin IX). The heme binding confers the bright red color to Hbs. The heme contains Fe at its center where oxygen and other ligands can bind.

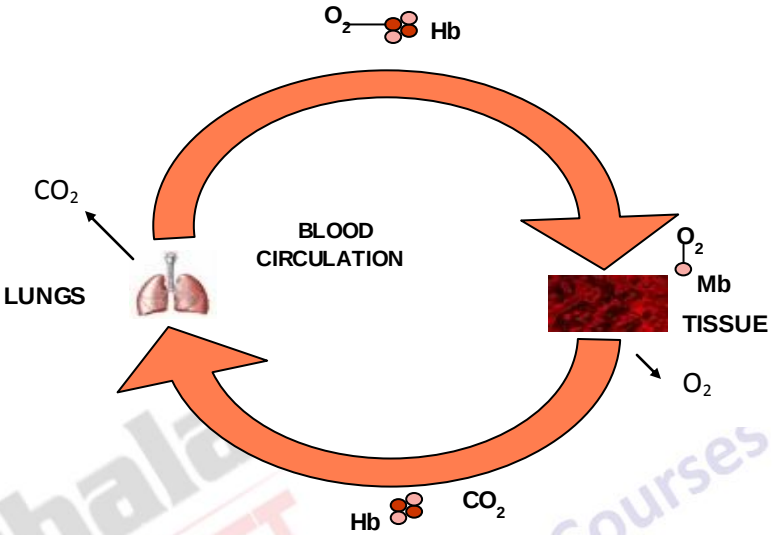
Apoglobin (polypeptide only) +  Heme (Fe center) = Hemoglobin (red)

Hbs are characterized by the presence of eight alpha helices numbered A-H, which forms a typical globin fold with heme sandwiched in between in a non-polar environment. The Fe, which forms the active site, is covalently linked to a His from the F-helix called the proximal His (which is thus in a "proximal pocket"). The other side is open for gaseous molecules like O_2, CO and NO to migrate and bind to iron and this pocket is called the "distal pocket"





Hb and Mb function in synchrony. Hb picks up O2 in the lungs and transports it to Mb in musde tissues. Mb stores the O_2 for later use for oxidative respiration. While Hb unloads O_2, it also carries CO_2 to the lungs, where it dissociates from Hb and is expelled. The structure of Mb and Hb are best suited for the function they perform.



2.2 Kinetics, affinity and transport are linked

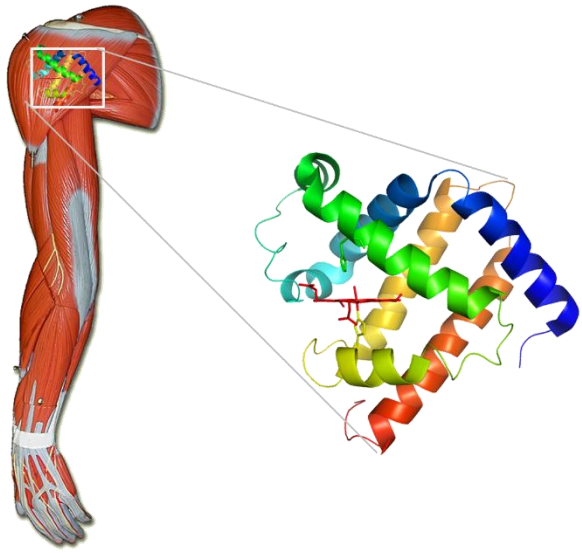
As seen above, Hb transports oxygen in red blood cells and then passes the gaseous ligand to Mb which in turn releases the same to mitochondria for further use. The environment in the lungs, red blood cells, muscle tissues and mitochondria are different and vary in their oxygen tension or oxygen partial pressure (P_{O2}). For the relay process to occur efficiently, Hb and Mb needed to evolve appropriate affinity for O_2, based on the P_{O2} they experience and the kinetics of ligand association and dissociation. Some of these values are listed as follows:

Hb association constants:
α alone = 1500 atm^{-1}, β alone = 2600 atm^{-1}, for 1^{st} O_2 = 5 – 60 atm^{-1}; 4^{th} O_2 = 3000 – 6000 atm^{-1}
For Mb, association constant = 1500 atm^{-1}
P_{50} (K_d) hemoglobin = ~ 27 torr or ~ 27 mm Hg; P_{50} (K_d) myoglobin = 2-3 torr or 2-3 mm Hg (760 torr = 1 atm of pressure).
For reference, P_{O2} in tissues is ~20 torr; in venous blood ~30 torr and in artery is ~100 torr .
In essence, Hb will be saturated with O_2 in the lungs, where the O_2 binding affinity of Hb is maximal. When it moves to tissues, its affinity for O_2 will be lower due to lower P_{O2} and Mb will take up O_2 since it is saturated at the lower P_{O2} of tissues. Mb will store O_2 till it is released in mitochondria for oxidative respiration.

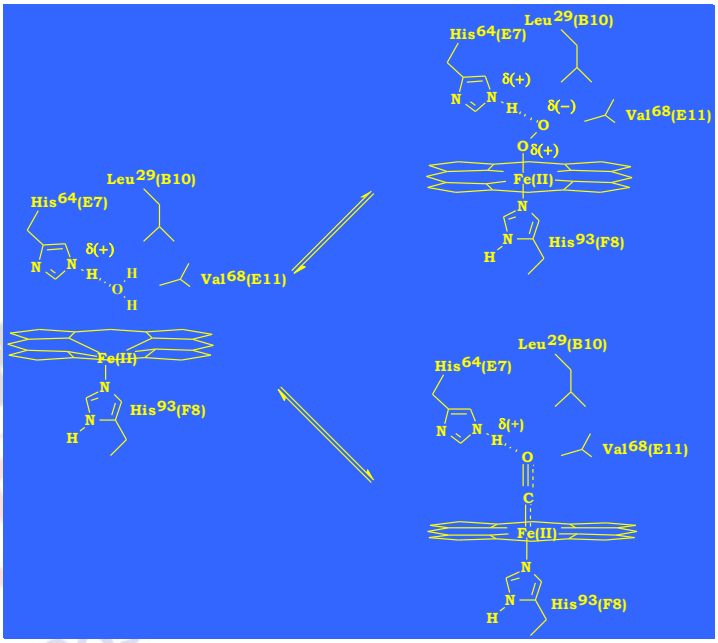
3. Structure-function relationship and mechanism of oxygen binding in Mb

3.1 Structural features of Mb

Mb is a member of the hemoglobin family and located in musde tissues. Its major function is oxygen storage and release to Mitochondria. Mb is the first protein



for which 3D structure was solved (by John Kendrew). Typically, it contains α–helical secondary structure with 8 α-helices, "A" (N-terminus) to "H". Mb also typifies "α-helical globin fold" where 3 helices (A,E,F) are in one plane and 3 helices (B,G,H) in another plane below, with heme (red) sandwiched between the helices. The heme iron within the protoporphyrin is the active site for the globin. Ligands will enter through the open side of the heme pocket, while a heme-histidine covalent linkage holds the heme in its place in the other side of the pocket called "proximal" heme pocket. A second histidine in the open side of the pocket called "distal pocket" is conserved among classical Hbs and acts as a "gate for ligand entry" into the pocket. This distal histidine also stabilizes bound ligand by H-bonding.

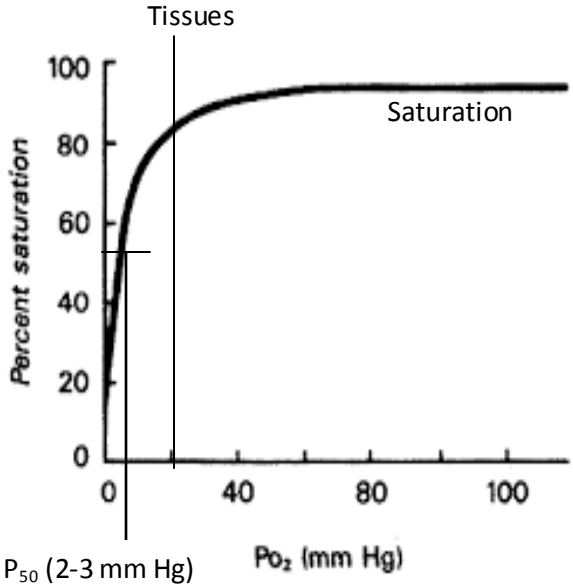


3.2. Oxygen Binding to Myoglobin – Electrostatic Mechanism

In absence of a ligand, a loosely coordinated water molecule occupies the distal pocket of Mb, which is surrounded by His 64 (E7; 7th residue of the E-helix), Leu29 (B10) and Val68 (E11). When ligand comes in (O2 or CO), the distal water is displaced and the ligand binds with the heme iron in the distal pocket. The ligand in turn is stabilized by strong H-bonding with His 64 (E7). Since O_2 is more polar than CO, the former is favorable for such electrostatic interaction. Thus CO binding is discriminated against O_2 binding in Mb (Ref-Springer et al. 1994 Chem Rev 94:699; Hargrove et al 1997 J.Mol. Biol. 266:1032; Olson & Phillips 1997 J. Biol. Inorg. Chem. 2: 544).

3.3 Oxygen Binding to Myoglobin (Mb) – In Tissues

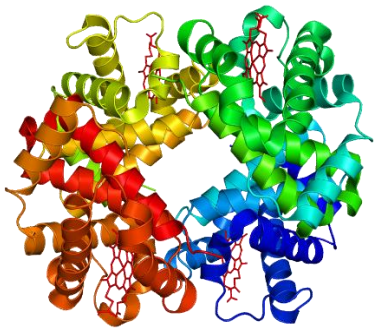
O_2 binding to Mb in tissues can be represented by the dissociation curve shown here. Mb is progressively saturated with O_2 as partial pressure (P_{O2}) increases and the curve is a typical rectangular hyperbola. At P_{O2} of 20 mm Hg, the protein is 90% saturated with the ligand. The partial pressure of O_2 at this point is same as that in tissues, where Mb is localized. Hence Mb is best suited to work in tissue environment. P_{50} of the curve indicates the P_{O2} at which 50% saturation of the globin with O_2 is achieved, which for Mb is ~2-3 mm Hg. Higher P_{50} (curve shifting right) would indicate lower affinity for O_2.



P_{50} (2-3 mm Hg)

4. Structural features of Hb and its ligand binding

Unlike Mb, hemoglobin (Hb) is a tetramer, i.e it contains 4 Mb like subunits, which associate with each other, mainly through non-covalent interactions, to build a quaternary structure. Secondary structure of each of the subunits, their fold, active site and heme pockets are similar to Mb. The three-dimensional (3D) structure of Hb was solved by Max Perutz. Hbs are located in red blood cells. Their functions include oxygen transport from lungs to tissues and CO_2 from tissues to lungs for expulsion.



In adult mammals, Hb contains of 2 alpha subunits (α_1 and α_2) and 2 beta subunits (β_1 and β_2), which differ in their amino acid sequence and length. The protomers form a quaternary structure such that a "central cavity" exists in the Hb molecule.

4.1 Conformational Change Upon O_2 binding

Each subunit of Hb binds one heme prosthetic group (shown in red in the structure). Thus, Hb consists of 4 heme groups and is thus capable of binding 4 O_2 molecules. Heme binds O_2 through its Fe atom in the ferrous state. The ferrous form of iron without O_2 is called the "de-oxy Hb", while the O_2 bound form is often designated as "Oxy-Hb" (the ferric form, which cannot bind O_2 is called the met-Hb form). There is equilibrium between the de-oxy and oxy-Hb brought about by the reversible ligand binding. The quaternary structure of Hb allows it to undergo conformational changes upon ligand binding. **Oxygen binding rotates the α1-β1 dimer with respect to α2- β2 dimer by about 15 degrees thus resulting in a constant motion between the de-oxy Hb and oxy-Hb.** In the distal pocket iron moves into the plane of the heme upon O_2 binding and distal His moves closer to stabilize bound O_2. The size of the central cavity changes as well upon ligand binding.

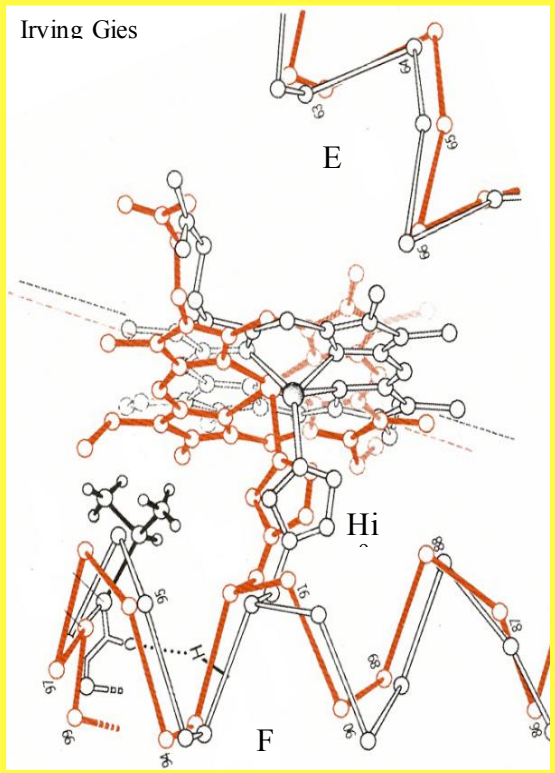
4.2 Conformational Changes in the Proximal Pocket

The proximal pocket witnesses certain conformational changes as outlined below:

De-oxy Hb (white) – F-helix moves away from heme pulling proximal His and thus Fe out of heme plane

Oxy (brown) – F-helix moves towards heme pushing proximal His up and thus Fe moves into heme plane to bind O_2

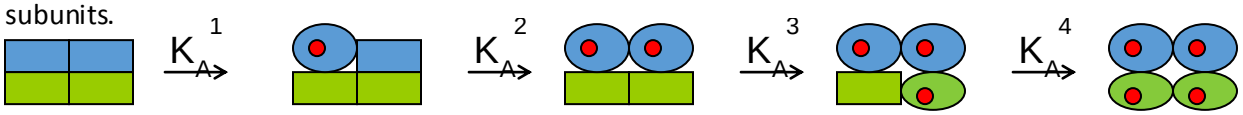
Such motion of F-helix is transmitted from the first subunit to second to third and fourth – helping faster O_2 binding in the subsequent subunits. Such



conformational changes trigger the phenomenon called “Cooperativity”.

5.1. Mechanism of O_2 binding in Hbs - Cooperativity

Binding of one O_2 molecule (●) to one Hb subunit augments the affinity (K_A) of binding to the other subunits due to conformational changes, as shown in the figure below, where the boxes represent de-oxy conformation and the ovals the oxy- conformation. The two colors represent the two different subunits.



$$K_A^4 >>>> K_A^3 >> K_A^2 > K_A^1$$

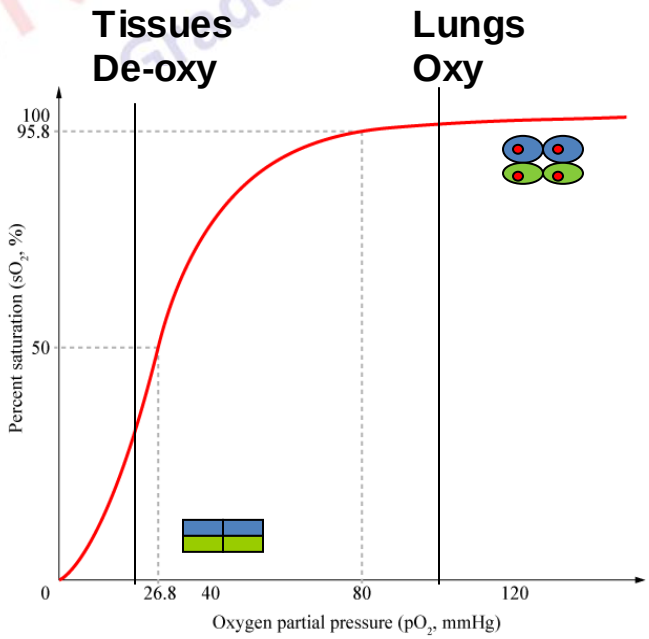
Cooperativity is a means of *regulation of ligand binding* – a threshold concentration of ligand is required to initiate binding. Binding is fast once initiated and finally saturates with ligand, resulting in a “sigmoidal” oxygen binding curve.

5.2. Oxygen Binding to Hemoglobin – The Sigmoidal Curve (Oxygen Dissociation Curve)

- Hb can bind 4 molecules of O_2, one in each subunit.

  $Hb + 4\,O_2 = HbO_2^4$

- Hb is increasingly saturated with O_2 as partial pressure of O_2 increases.
- O_2 binding to Hb follows a Sigmoidal curve (S-shape)
- Initially there is resistance to O_2 binding at lower pO_2 (<20mm Hg; tight state; closed conformation; K_A low)
- Once one O_2 binds, the saturation happens very fast and Hb switches to an open, relaxed state. (K_A high)
- The tight state (de-oxy) exists in musde tissues and open state (oxy) in lungs.



The “All-or-nothing O_2 binding” curve of Hb is thus a typical Sigmoidal curve, with P_{50} = 26.8 mm Hg.

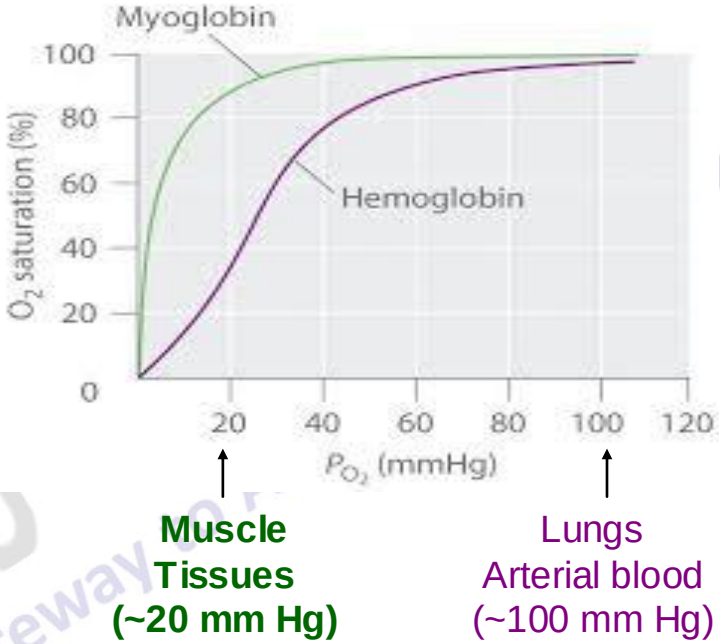
6. Oxygen Binding – Myoglobin vs Hemoglobin

It is thus evident that the Hb O_2 dissociation curve is distinct from Mb, with the former being Sigmoidal while the latter is Hyperbolic. Hb undergoes distinctive conformational changes upon O_2 binding and results in cooperativity unlike Mb.

The P_{50} for Hb is higher than Mb at low pO_2

Mb saturates with O_2 easily at lower pO_2 as exists in muscle tissues; so it can take O_2 from Hb for storage. Mitochondria has still lower pO_2, so Mb releases O_2 for oxidative respiration

Hb dissociates easily in tissue appropriate pO_2 and saturates at higher pO_2, which exists in lungs. Thus it loads in the lungs and transports O_2 to the muscle tissues

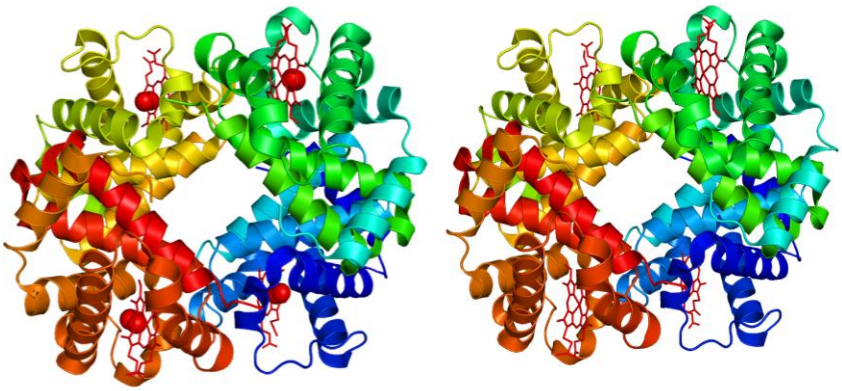


7. T- and R- States Explain Cooperativity in Hemoglobins

It is evident that Hb exists in 2 conformational states.

R-state (relaxed state)
- High affinity conformation :
Oxy Hb form ; Present in lungs where Oxygen tension high – O_2 plentiful; 4 O_2 binds Hb

T-state (tight or taut state)
- Low affinity conformation :
De-oxy Hb ; Present in tissues where oxygen tension lower; O_2 released by Hb



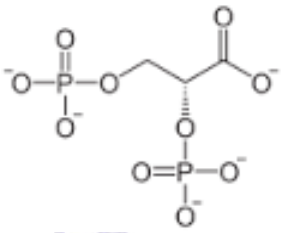
R-state T-state

Hb moves fast between these 2 conformations to bring about cooperativity – all-or-nothing O_2 binding; any conformation in between is transient. Thus oxygen dissociation curve is S-shaped for Hb.

8. Allosterism in Hemoglobins - Effectors

The S-shape of HbO_2 dissociation curve allows it to be manipulated by other effector molecules or ambient conditions such that conditions that lower O_2 affinity shift curve right - O_2 dissociates easily and vice versa. This results in the phenomenon of "allostery".

Allostery ("different shape") – change in conformation by molecules ("effectors") not similar to ligand binding away from active site. Hb is model for allosteric proteins.

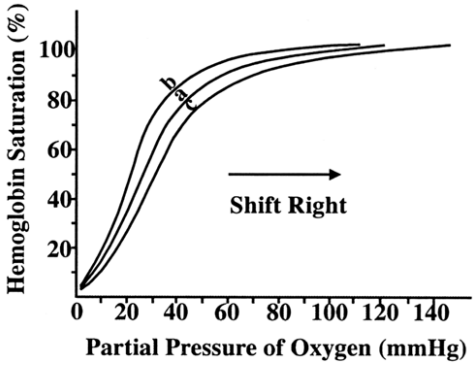


The subunit cooperativity / conformations of Hb is influenced by the presence of some physiologically significant effector molecules: **H^+, CO_2 , Cl^-, 2,3-Bisphosphoglycerate or 2,3-BPG; also called 2,3-diphosphoglycerate or 2,3-DPG**

These molecules encourage oxy-Hb to drop its oxygen in tissues, thus providing an effective means of regulating O_2 dissociation.

Each of these molecules functions by binding more strongly to deoxy-Hb (T-state) than to oxy-Hb (R-), shifting the equilibrium towards deoxy-Hb and release of O_2. The S-Curves shift right.

b < a > c

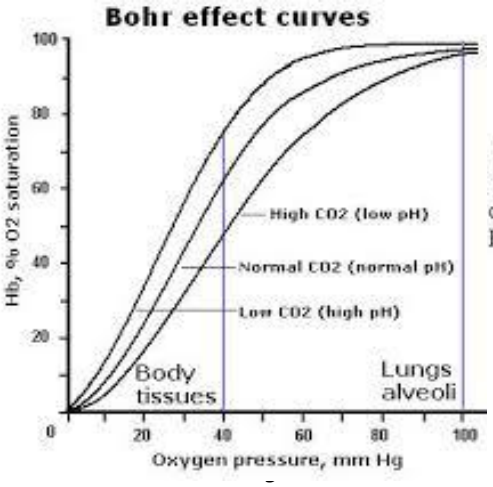


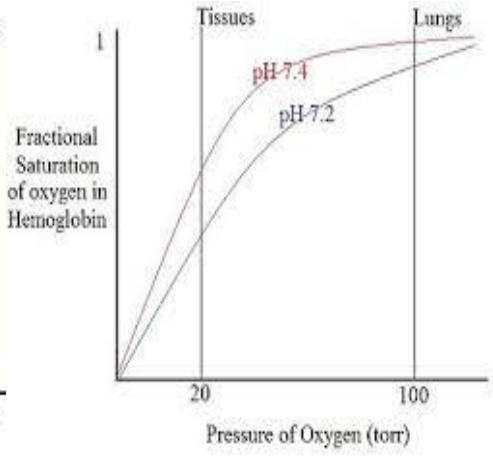
8.1 Bohr Effect – H^+ (proton as an effector)

In 1904, Christian Bohr discovered that the O_2 binding affinity of Hb is related to acidity (pH) of the blood.

If blood pH is high; O_2 binding affinity of Hb is high and it loads more O_2 at lower PO_2.
At low blood pH; Hb unloads more O_2 at lower PO_2. The equilibrium shifts towards de-oxy Hb

($HbO_2 + H^+ = HbH^+ + O_2$)



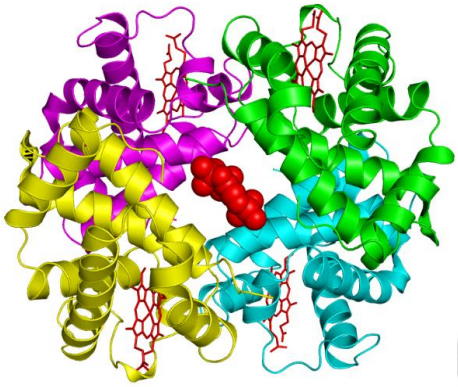


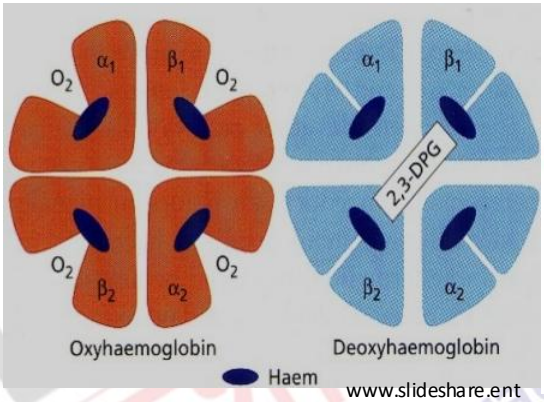
Blood pH changes with dissolved CO_2 concentration; High CO_2, Low pH.

Physiological Relevance : Bohr effect facilitates O_2 transport – Metabolic tissue cells produce higher CO_2, lowering pH thus Hb dissociates O_2. During exercise, CO_2 & lactic acid produced – blood pH lowers to 7.2; ~10% more oxygen unloaded by Hb.

8.2 Effect of DPG / BPG – Allosteric Feedback

BPG or DPG is the predominant effector molecule in blood that regulates O_2 binding to Hb, resulting in allosteric feedback. Red blood cells contain almost as many DPG molecules as Hb.





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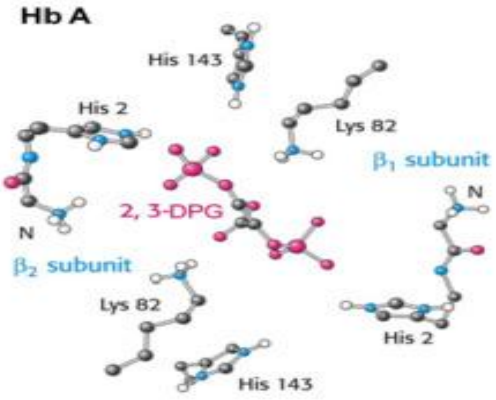
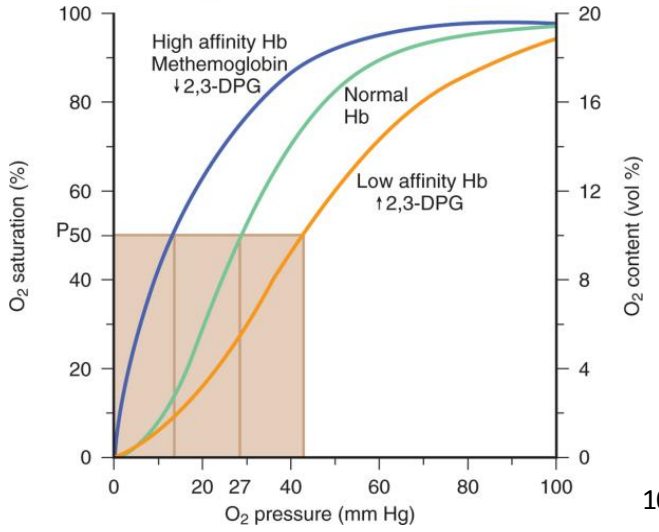


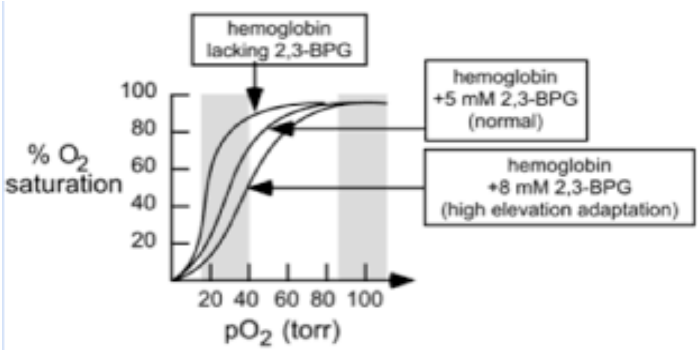
R-state **T-state**

The central cavity in the quaternary structure of Hb plays a key role as DPG (red-space filling model) binds here (left panel figure). Multiple His and Lys residues interact with BPG (right panel figure).

DPG binds only to the T-state, de-oxy Hb, between β_1 and β_2 subunits with several charged and polar residues surrounding it. This closes the Hb molecule and "squeezes" O_2 out of it; O_2 is released into the surrounding. This is known to occur in tissues under low pO_2. Under high pO_2, O_2 binding induces conformational change and R-state cannot bind DPG.

DPG is thus a significant allosteric feedback molecule and it Influences the oxygen dissociation curve.



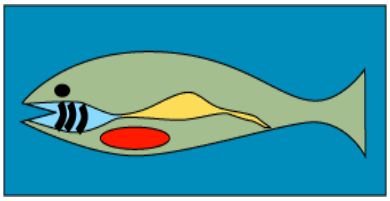


High altitude adaptation : Humans at high altitude have higher concentration of DPG which forces Hb to release more O_2 for higher metabolic use.

(Also the volume of red blood cells is higher for greater O_2 binding.)

9. Root Effect – Fish Hemoglobin

Fish changes the amount of gaseous O_2 in its swim bladder (red) behind the gills to produce appropriate buoyancy at various depths of water. It takes help from Hb for the same.



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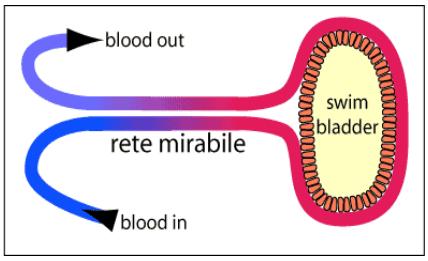
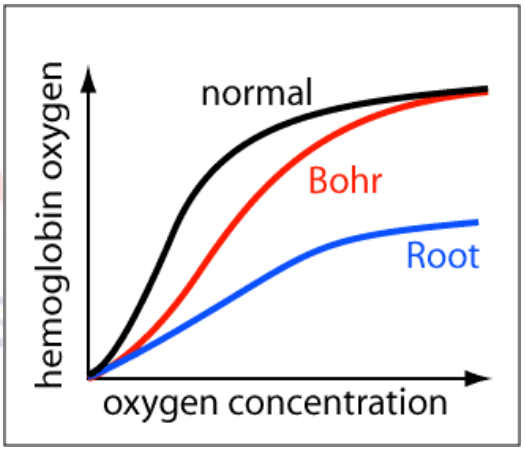


(www.scienceisart.com)

Fish releases enough lactic acid into blood stream to lower the pH significantly (red area around swim bladder).

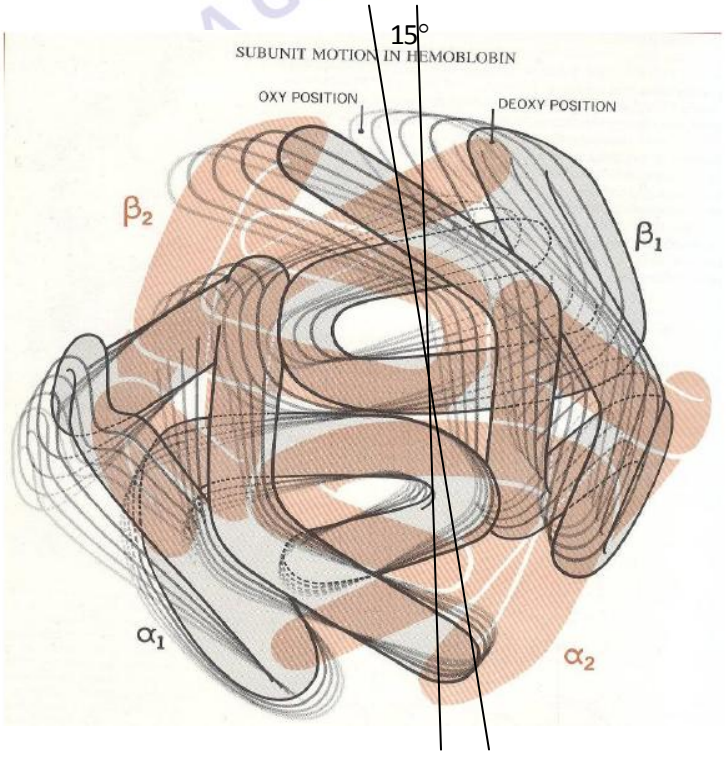


As a result Hb unloads O_2 and the dissociation curve shifts significantly to the right.

This effect is named Root effect to honor the discoverer of this phenomenon - R.W. Root

10. Structure – Function Relationship in Mb and Hb: A Summary

It is evident that the function of Mb or Hb is intricately linked to their structural features, dictated by a proper fold. The various structural features that help function are summarized here:



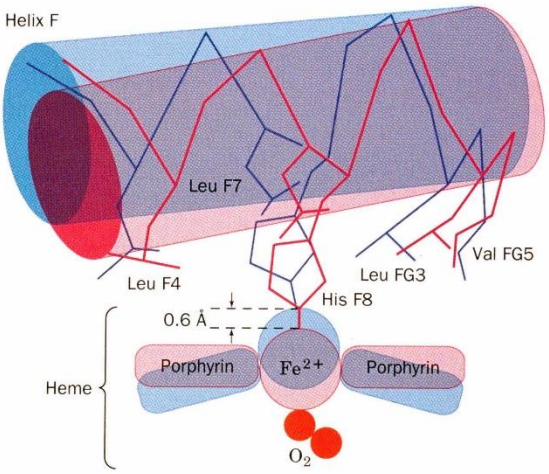
It is obvious that the key difference in the structure of Mb and Hb that lends these molecules their respective properties lie in their quaternary structures. Hb, with four subunits can easily undergo conformational changes to regulate O_2 binding. Oxygenation rotates the α1-β1 dimer with respect to α2- β2 dimer about 15°. The Hb molecule thus switches between two conformations like a stroboscope. Without the multiple subunits Mb cannot do so.

Interlocking contact of side chains are extensive between unlike subunits and little

between like ones : $\alpha 1\beta 1$ & $\alpha 2\beta 2$ (packing contacts) = 32 residues; $\alpha 1\beta 2$ & $\alpha 2\beta 1$ (sliding contacts) =27 residues in interface (deoxy). These contacts, which are made by different but equivalent sets of non-covalent interactions, act as a binary switch between the T and the R states and promote rotation by 15°.

The T/R states, in turn, dictates O_2 binding in Hb. Mb is a single subunit with lack of cooperativity and tight control on O_2 binding; thus its is a storage molecule. Hb, with its structural properties, is suitable for transport.

Within subunits, in the deoxy state, heme Fe is ~ 0.6 Å out of the heme plane. Upon oxygen binding, Fe is pulled into the heme plane. Proximal His F8 being attached to the Fe, the entire F helix is pulled down.



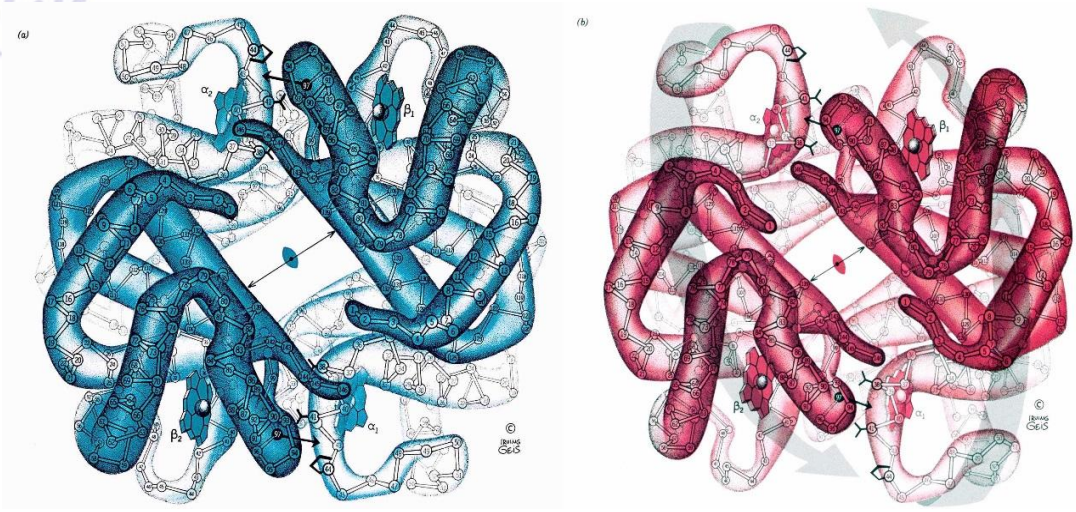
Such F-helix motion is possible due to the in concert quaternary shift that moves the $\alpha 1$ C-$\beta 2$ FG contact one turn along the $\alpha 1$ C helix.

The first subunit to bind the 1st O_2 is α-subunit

Binding of 1st O_2 to one α-heme is difficult but the binding results a shift in the $\alpha 1$-$\beta 2$ contacts. Thus, the distal HisE7 and ValE11, which block Fe in β-subunit, moves out of the path of O_2. This process increases the affinity of O2 binding triggering cooperativity.

Bohr effect - In deoxy-Hb, C-termini of the α and β chains engage in a network of H-bonds and salt bridges, which are broken in oxy-Hb and Bohr effect emerge. The N-terminal amino groups also cause Bohr effect (slide 24). His146$\beta 2$ is salt bridged with Asp 94$\beta 2$ in T-state and this interaction is lost in the R state, causing Bohr effect.

The central cavity within the quaternary structure also plays a central role. DPG binds here in the T-state expelling O_2 from the subunits. The cavity is much smaller in size in the R-state, since ends of H-helices move 7Å closer. Hence DPG is released from R-state and O_2 binds.



T-state **R-state**

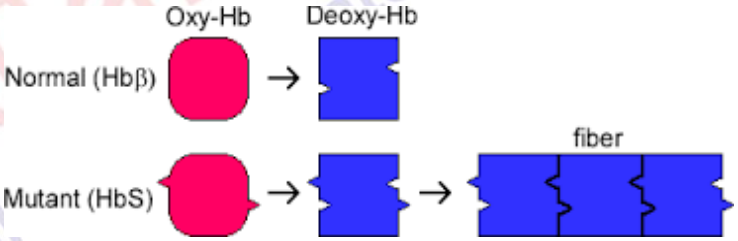
11. Pathology – Sickle Cell Anemia (Hemoglobin S)

If function in Hb is so intricately linked to structure, even single amino acid changes can result in loss of Hb function and properties. Most well known example is that of HbS where Gluβ6 to Val (helix A; 3rd) point mutation results in red blood cells being sickled and assume long crescent shapes – a disease named sickle cell anemia.

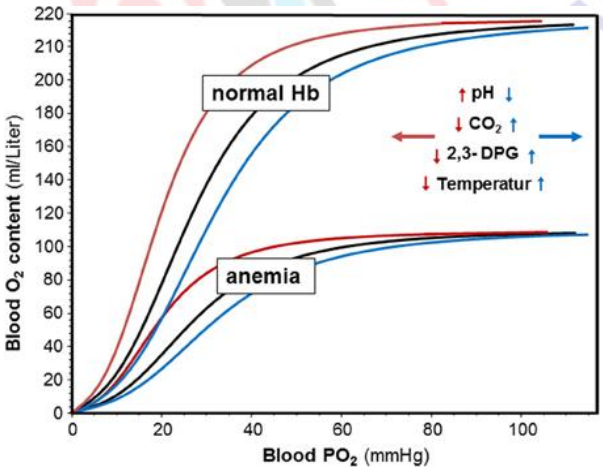
Sickled cells are deformed and rigid and trapped in capillaries causing excruciating pain and inflammation. These abnormal cells damage erythrocyte membranes. The defective cells are fragile and have shorter lifetime, making patient anemic. This causes load on the bone marrow to produce more blood cells.

(www.sicklecellga.org)

The mutation (charged to apolar aa) in HbS exposes hydrophobic patch at a corner of the tetramer in deoxy form. The high concentration of Hb in red blood cells means frequent contact and proximity; so these patches lead to aggregation and thus polymerization of deoxy HbS (fibers) resulting in sickling. When oxygenated, aggregation is much less since the patch gets hidden.



(www.chem.wisc.edu)



(www.journal.frontiersin.org)

The oxygen dissociation curves of anemic Hb are thus different from normal.

12. Summary

The folded conformation of Mb and Hb evolved to support their function through distinct mechanism of oxygen binding.

With single subunit, Mb displays a hyperbolic ligand binding and is saturated at lower partial pressure of O_2 normally present in muscle tissues.

Hb uses 4 subunits to undergo conformational changes that help it to bind O_2 in a relaxed state shifting from a more tensed or strained state which cannot bind O_2. Such a mechanism supports cooperative ligand binding and displays sigmoidal O_2 dissociation curves, with Hb being loaded in lungs.

Effector molecules like H^+, Cl^-, CO_2, 2,3-DPG bind deoxy Hb in the tensed state and help regulate O_2 binding by promoting Hb to shed O_2 appropriately.

Change in any of the properties that perturb the requisite conformation can result in abnormal Hb leading to pathological conditions.