

## Myoglobin/Hemoglobin O<sub>2</sub> Binding and Allosteric Properties of Hemoglobin

- Hemoglobin binds and transports H<sup>+</sup>, O<sub>2</sub> and CO<sub>2</sub> in an allosteric manner
- Allosteric interaction - a regulatory mechanism where a small molecule (effector) binds and alters an enzymes activity

### 'globin Function

O<sub>2</sub> does not easily diffuse in muscle and O<sub>2</sub> is toxic to biological systems, so living systems have developed a way around this.

Physiological roles of:

#### - Myoglobin

- Transports O<sub>2</sub> in rapidly respiring muscle
- Monomer - single unit
- Store of O<sub>2</sub> in muscle high affinity for O<sub>2</sub>
- Diving animals have large concentration of myoglobin to keep O<sub>2</sub> supplied to muscles

#### - Hemoglobin

- Found in red blood cells
- Carries O<sub>2</sub> from lungs to tissues and removes CO<sub>2</sub> and H<sup>+</sup> from blood to lungs
- Lower affinity for O<sub>2</sub> than myoglobin
- Tetramer - two sets of similar units ( $\alpha_2\beta_2$ )

### Myo/Hemo-globin

- Hemoglobin and myoglobin are oxygen- transport and oxygen-storage proteins, respectively
- Myoglobin is monomeric; hemoglobin is tetrameric
  - Mb: 153 aa, 17,200 MW
  - Hb: two  $\alpha$  chains of 141 residues, 2  $\beta$  chains of 146 residues

### X-ray crystallography of myoglobin

- mostly  $\alpha$  helix (proline near end of each helix *WHY?*)
- very small due to the folding
- hydrophobic residues oriented towards the interior of the protein
- only polar aas inside are 2 histidines

### Structure of heme prosthetic group

Protoporphyrin ring w/ iron = heme

Oxygenation changes state of Fe

- Purple to red color of blood, Fe<sup>+3</sup> - brown

Oxidation of Fe<sup>+2</sup> destroys biological activity of myoglobin

Physical barrier of protein is to maintain oxidation state of Fe<sup>+2</sup>

Propionate chain orients heme

### Mb and Hb use heme to bind Fe<sup>2+</sup> / Fe<sup>2+</sup> is coordinated by His F8

- Iron interacts with six ligands in Hb and Mb
- Four of these are the N atoms of the porphyrin
- A fifth ligand is donated by the imidazole side chain of amino acid residue His F8
- When Mb or Hb bind oxygen, the O<sub>2</sub> molecule adds to the heme iron as the sixth ligand
- The O<sub>2</sub> molecule is tilted relative to a perpendicular to the heme plane (*IMPORTANT FOR LATER!*)

### Structure of heme prosthetic group

- heme wedged between hydrophobic pocket of helix E & F
- Iron is out of plane due to his 8 bond
- distal vs. proximal histidines
- 3 states of 6th coordinate site

### free vs. bound heme – role of apoprotein

- Globin binding restricts heme dimers from forming
- Helps keep iron reduced
- Stabilizes transition state (O<sub>2</sub> binding)

CO, NO and H<sub>2</sub>S binding - poison of O<sub>2</sub> binding bind with greater affinity than O<sub>2</sub>

His E 7 decreases affinity of ligands (CO and O<sub>2</sub>) for Fe<sup>+2</sup>

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### Hemoglobin Molecule

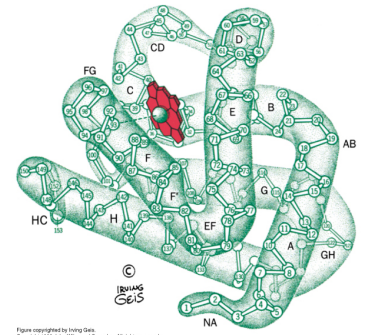
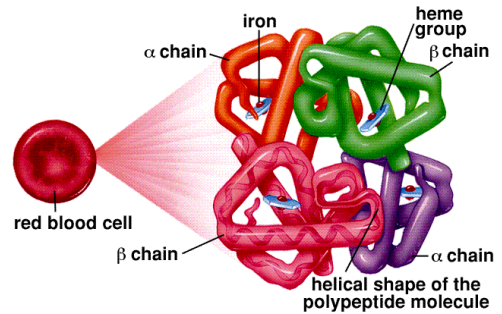
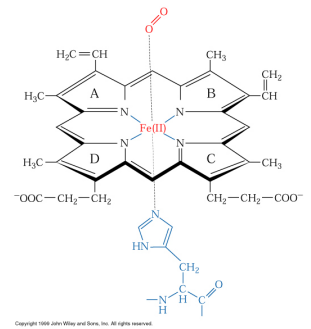


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Form

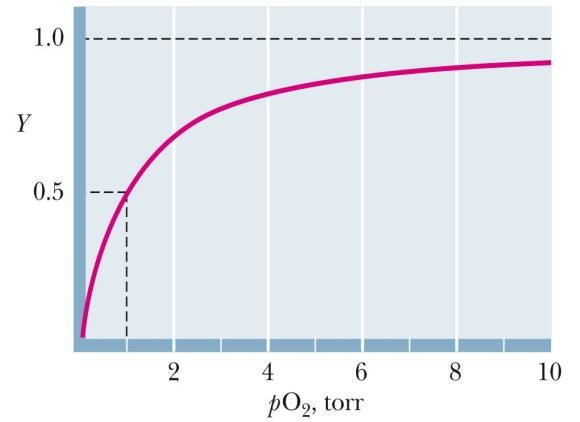
Oxidation

Fe in plane of heme

6<sup>th</sup> Coordinate State

Distal His

### Myoglobin O<sub>2</sub> affinity

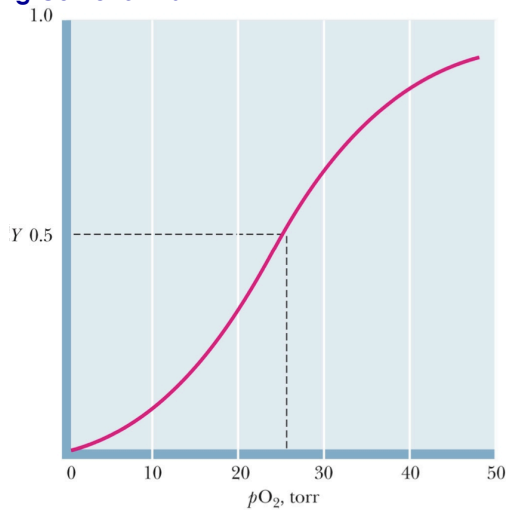


### O<sub>2</sub>-Binding Curve for Hb

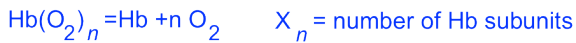
Oxygen saturation curve for Hb in the form of Y versus pO<sub>2</sub> assuming n=4 and P<sub>50</sub> =26 torr.

Y is the fractional saturation of Hb:

$$Y = \frac{[pO_2]^4}{[pO_2]^4 + K}$$



### Hemoglobin -sigmoidal dissociation

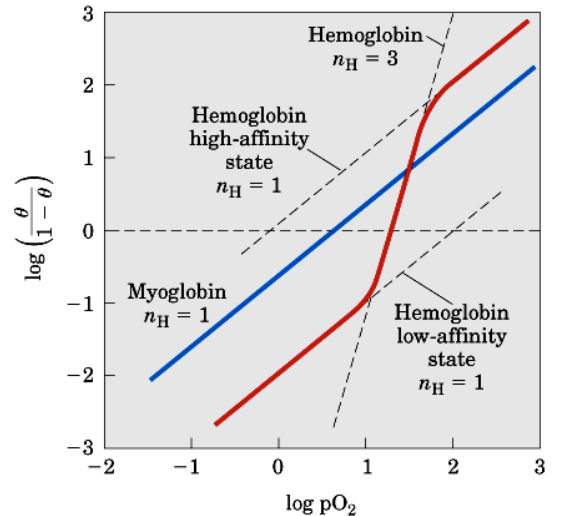


$$\log \frac{Y}{1-Y} = n \log pO_2 - n \log P_{50}$$

Y = fraction of globin bound to O<sub>2</sub>

n = Hill constant - determined graphically by the - hill plot  
 n is the slope at midpoint of binding of log (Y/1-Y) vs log of pO<sub>2</sub>  
 if n = 1 then non cooperativity  
 if n < 1 then negative cooperativity  
 if n > 1 then positive cooperativity

The experimentally determined slope does not reflect the number of binding sites however. It reflects, instead, the degree of cooperativity.



So how does this relate the biological effect of O<sub>2</sub> affinity of Hb?

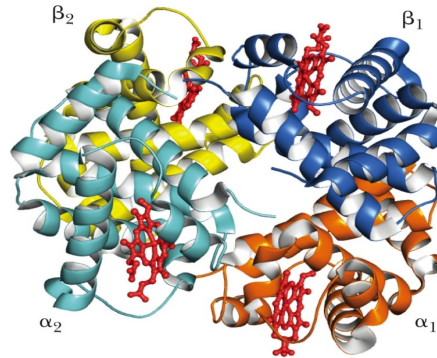
- look at pO<sub>2</sub> of alveoli vs. metabolically active tissue
- What if oxygen dissociation were hyperbolic rather than sigmoidal?

## Myoglobin and Hemoglobin

- Hemoglobin is structurally related to myoglobin
- very different primary sequence about an 18% homology in the primary sequence
- 2 alpha subunits and 2 beta subunits
- in adults there are very small amount of alpha<sub>2</sub>delta<sub>2</sub> hemoglobin



Myoglobin (Mb)



Hemoglobin (Hb)

## Cooperativity

**Cooperativity takes place with multimeric proteins and the term describes impact of one subunit binding one substrate/ligand/effector on other subunit behavior**

- Cooperative binding is a special case of Allosterism
  - Allosteric control- the impact of a protein by a small molecule
  - Binding occurs distal from active site
  - Regulatory binding impacts structure of one subunit and has a neg or pos effect
  - Cooperativity involves multiple subunits EACH with a binding or active site
- Seen as a sigmoidal curve (binding or reaction)
- Positive and Negative cooperativity exist
- Cooperativity results as subunits impact binding of substrate or ligand of other subunits
  - Positive cooperativity happens when substrate/ligand binding increases the affinity for other sites (finding one ligand makes it easier for the protein to bind the second, third... ligand(s))
  - Negative cooperativity – results in a reduced affinity

## Two models explaining Hb Cooperativity – Symmetry and Sequential

*Neither models fully account for allosteric effects*

### Sequential model (D Koshland)

- binding of one O<sub>2</sub> induces T-R conformation change
- 1st change is most difficult due to influence by 3 other subunits
- binding of next three subunits happens sequentially, with higher affinity (easier T-R changes)
- kinetics increase to the fully oxy Hb state as more O<sub>2</sub> is bound

### Concerted Model (J Monod)

- All R or all T no in between as in the Koshland model
- concerted model means as more O<sub>2</sub> binds, the R conformation is favored until all units are in the R conformation regardless of the total units bound to O<sub>2</sub>
- Affinities do NOT change until conformation changes
- 1 O<sub>2</sub> - all T; 2 O<sub>2</sub> - nearly even equilibrium; 3 O<sub>2</sub> mostly R; 4 O<sub>2</sub> - mostly R form
- energy from O<sub>2</sub> binding causes the change in equilibrium
- this model best fits O<sub>2</sub> dissociation curve but with limits.

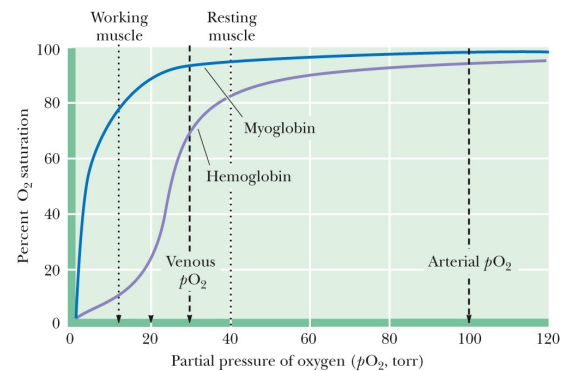
## Cooperative Binding of Oxygen Influences Hemoglobin Function

- Mb, an oxygen-storage protein, has a greater affinity for oxygen at all oxygen pressures
- Hb is different – it must bind oxygen in lungs and release it in capillaries
- Hb becomes saturated with O<sub>2</sub> in the lungs, where the partial pressure of O<sub>2</sub> is about 100 torr
- In capillaries, pO<sub>2</sub> is about 40 torr, and oxygen is released from Hb
- The binding of O<sub>2</sub> to Hb is cooperative – binding of oxygen to the first subunit makes binding to the other subunits more favorable

## Hemoglobin R and T States.

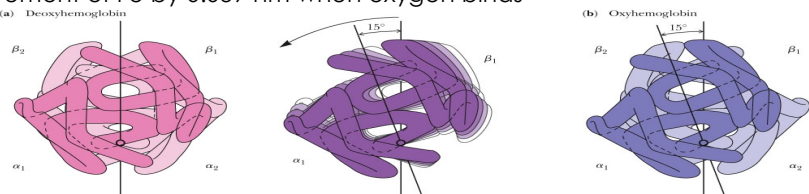
- The interface between alpha and beta subunits of hemoglobin are a network of inter and intra subunit linkages (salt-bridges and other interactions) between amino acid side chains, peptide backbone and the termini of the subunit(s).
- There are two stable states for hemoglobin, each of which are stabilized by these interactions and impacted by the allosteric effectors. Each state differs in their affinity for oxygen as well as the interactions between subunits.
- The tense or taugt (T) state and the relaxed (R) state.
- **The T state is considered a low affinity binding state.**
- **The R state is considered a higher affinity oxygen binding state.**
- Binding of the oxygen ligand destabilizes the T state shifting the equilibria of hemoglobin subunits to the R, oxygen high affinity state. Sometimes the R and T state are called oxy (R) and deoxy (T) state, but this can mislead to thinking of the protein in the respective state when the ligand is present or not. In fact, the R and T state can be stabilized (equilibria shifted one way or the other) independent of oxygen binding.
- Binding of one oxygen enhances the shift of T to R transition of the unbound ligand neighbors creating the positive cooperatively seen with hemoglobin-oxygen binding. Other allosteric effectors stabilize either the R or T form creating the shift in oxygen binding curve observed in your textbook

## Impact of differences of O<sub>2</sub> binding affinity – allosterism



## Oxygen Binding by Hb Induces a Quaternary Structure Change

- When deoxy-Hb crystals are exposed to oxygen, they shatter. Evidence of a large-scale structural change
- One alpha-beta pair moves relative to the other by 15 degrees upon oxygen binding
- This massive change is induced by movement of Fe by 0.039 nm when oxygen binds



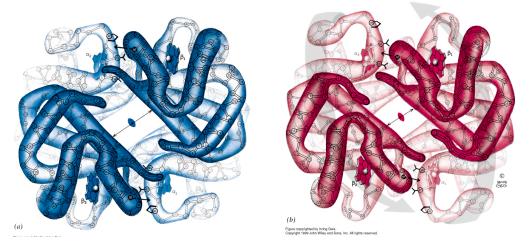
## Fe<sup>2+</sup> Movement by Less Than 0.04 nm Induces the Conformation Change in Hb

In deoxy-Hb, the iron atom lies out of the heme plane by about 0.06 nm. Upon O<sub>2</sub> binding, the Fe<sup>2+</sup> atom moves about 0.039 nm closer to the plane of the heme. As Fe<sup>2+</sup> moves, it drags His F8 and the F helix with it. This change is transmitted to the subunit interfaces, where conformation changes lead to the rupture of salt bridges.

## 3D structure of hemoglobin and myoglobin

- α<sub>1</sub>- β<sub>1</sub> units have 35 interactions
- α<sub>1</sub>- β<sub>2</sub> units have 19 interaction sites
- similar units have few polar contacts
- the two a and two b subunits face each other through aqueous channels

- Binding of oxygen dramatically alters the interactions and brings about a twisting of the two halves (alpha beta pairs)
- Much of the quaternary changes takes place in the salt bonds between the C terminals of all four chains



**Salt bridges that stabilize deoxy-Hb are broken in oxy-Hb**

• There are two general structural states - the deoxy or T form and the oxy or R form.

One type of interactions shift is the polar bonds between the alpha 1 and the beta 2 subunits.

**So... to recap!**

Oxygen binding shifts quaternary structure at long distances

- binding of O<sub>2</sub> ligand at 6th coordinate position pulls Fe into heme
- moves proximal histidine (F8) and the alpha helix it is attached to.
- shift in the helix is transmitted throughout of molecule
- Impacts interactions between Hb subunits.
- Myoglobin? No interactions
- Thus one has allosteric potential while the other doesn't

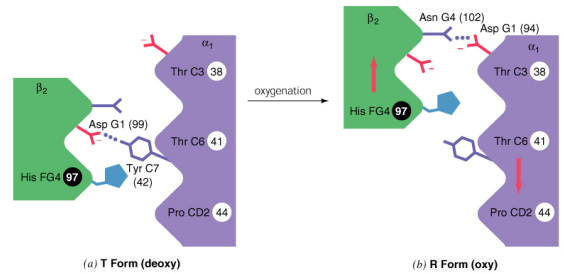


Figure 7-10. Changes at the α<sub>1</sub>-β<sub>2</sub> interface during the T → R transition in hemoglobin. Copyright 1999 John Wiley and Sons, Inc. All rights reserved.

**The Physiological Significance of the Hb:O<sub>2</sub> Interaction**

Hb must be able to bind oxygen in the lungs

Hb must be able to release oxygen in capillaries

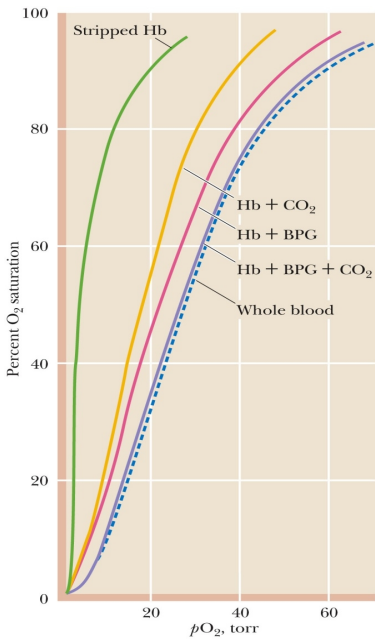
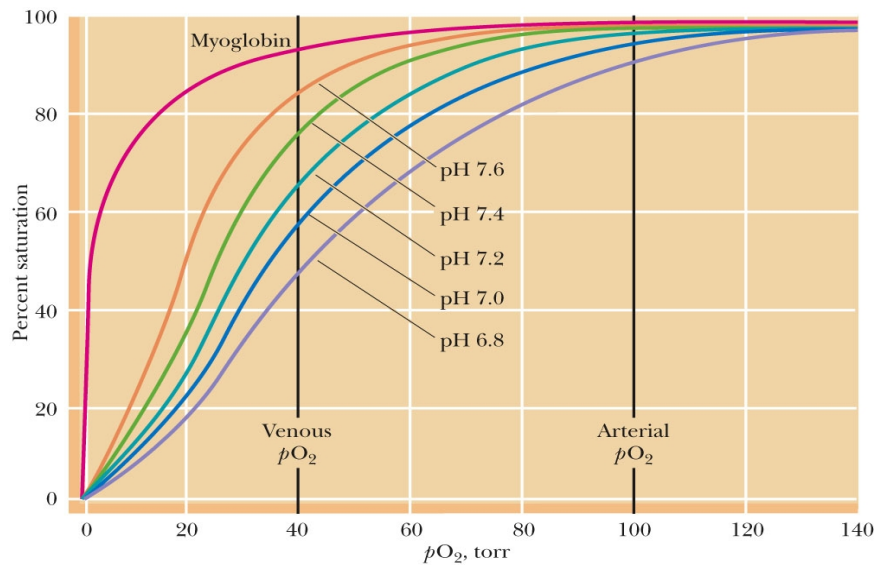
- If Hb behaved like Mb, very little oxygen would be released in capillaries
- The sigmoid, cooperative oxygen-binding curve of Hb makes its physiological actions possible!

**Bohr Effect**

H<sup>+</sup> Promotes Dissociation of Oxygen from Hemoglobin

The effect of H<sup>+</sup> is particularly important  
Deoxy-Hb has a higher affinity for H<sup>+</sup> than oxy-Hb

Thus, as pH decreases, dissociation of O<sub>2</sub> from hemoglobin is enhanced



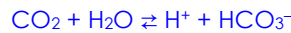
**The Antagonism of O<sub>2</sub> Binding by H<sup>+</sup> is Termed the Bohr Effect**

- The effect of H<sup>+</sup> on O<sub>2</sub> binding was discovered by Christian Bohr (the father of Neils Bohr, the atomic physicist)
- Binding of protons diminishes oxygen binding
- Binding of oxygen diminishes proton binding
- Important physiological significance

**CO<sub>2</sub> Also Promotes the Dissociation of O<sub>2</sub> from Hemoglobin**

*Carbon dioxide diminishes oxygen binding*

Hydration of CO<sub>2</sub> in tissues and extremities leads to proton production:



These protons are taken up by Hb as oxygen dissociates  
The reverse occurs in the lungs

## pH and CO<sub>2</sub> impact

1) In active tissues respiration, (glycolysis) results in lactic acid formation. These tissues need more O<sub>2</sub>. Without the H<sup>+</sup> effect Hb would hold on to more of the O<sub>2</sub>. The increase [H<sup>+</sup>] induces Hb to dump 10% more of it's O<sub>2</sub>.

2) CO<sub>2</sub> reversibly binds to N term (carbamate) to remove remaining CO<sub>2</sub>



The carbamate increases the T formation - deoxy form.

The reverse occurs in the lungs. This results in 1/2 of CO<sub>2</sub> removal from tissues.

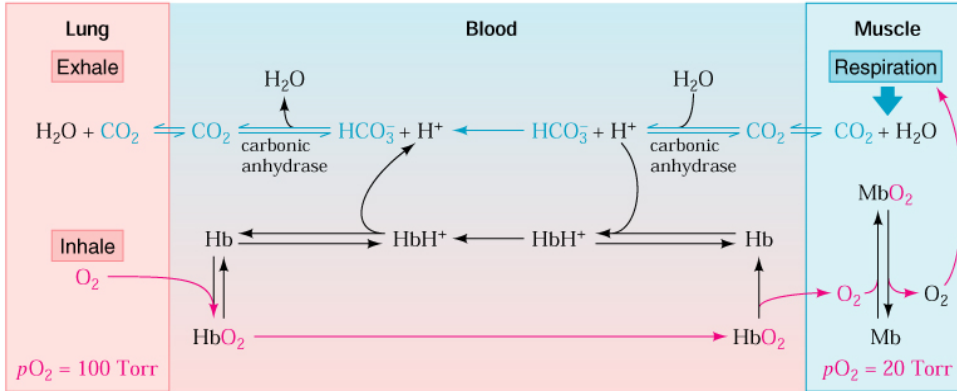
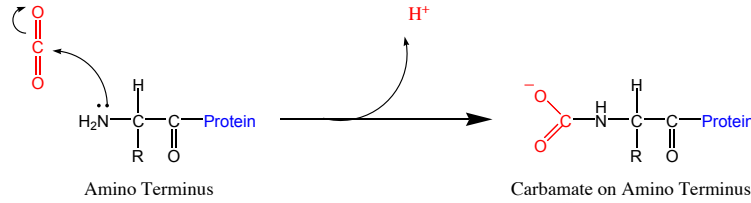
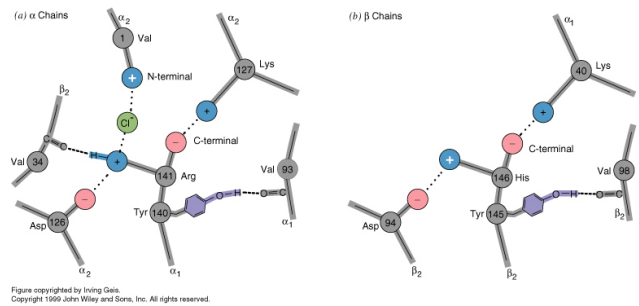


Figure 7-13 Key to Function. The roles of hemoglobin and myoglobin in transporting O<sub>2</sub> from the lungs to respiring tissues and CO<sub>2</sub> (as HCO<sub>3</sub><sup>-</sup>) from the tissues to the lungs.

- The T form finds the terminals in several important H bonds and salt bridges.
- In the T form the C terminus of each subunit are "locked" into position through several hydrogen and ionic bonds.
- Shifts into the R state break these and allow an increased movement throughout the molecule.



## Bohr Effect Continued

The Bohr effect is the reversible shift in Hb affinity for O<sub>2</sub> with changes in pH.

H<sup>+</sup> Transport (effect) - O<sub>2</sub> binding to Hb releases H<sup>+</sup> due to conformational changes in Hb

- deoxyform (T form) brings Asp 94 close to His 146
- the proximity of an acidic amino acid increases the pK of histidine (pKa is now above the pH) and results in H<sup>+</sup> "binding" to deoxyHb
- in other words the His becomes protonated where it normally would be ionized
- increasing pH stimulates Hb to bind to O<sub>2</sub>

- Bottom line - when O<sub>2</sub> binds Hb, H<sup>+</sup> is released from several amino acid's functional groups.

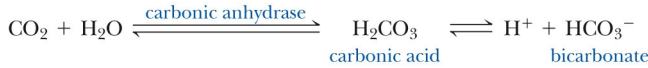
- When O<sub>2</sub> is released, the amino acids become protonized and then "picks" up a H<sup>+</sup>.

So when the H<sup>+</sup> is high (acidic conditions) the H<sup>+</sup> is driven onto the terminal amino acids driving it into the T conformation

## Summary of the Physiological Effects of H<sup>+</sup> and CO<sub>2</sub> on O<sub>2</sub> Binding by Hemoglobin

At the tissue-capillary interface, CO<sub>2</sub> hydration and glycolysis produce extra H<sup>+</sup>, promoting additional dissociation of O<sub>2</sub> where it is needed most

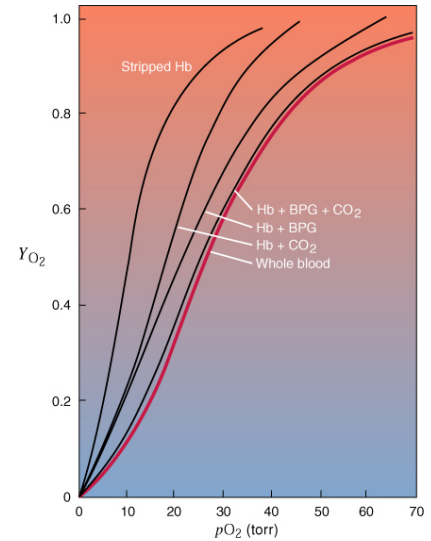
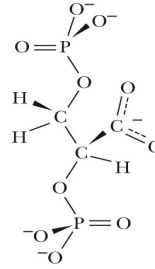
At the lung-artery interface, bicarbonate dehydration (required for CO<sub>2</sub> exhalation) consumes extra H<sup>+</sup>, promoting CO<sub>2</sub> release and O<sub>2</sub> binding



-Purified Hb has a different O<sub>2</sub> affinity than in blood  
 -26 fold decrease change in affinity

### 2,3-Bisphosphoglycerate

- In the absence of 2,3-BPG, oxygen binding to Hb follows a rectangular hyperbola!
- The sigmoid binding curve is only observed in the presence of 2,3-BPG
- Since 2,3-BPG binds at a site distant from the Fe where oxygen binds, it is called an **allosteric effector**



### BPG Binding to Hb Has Important Physiological Significance

Where does 2,3-BPG bind?

- "Inside"
- in the central cavity

What is special about 2,3-BPG?

- Negative charges interact with 8 positive charges in the cavity: 2 Lys, 4 His, 2 N-termini

2,3 bisphosphoglycerate (BPG)

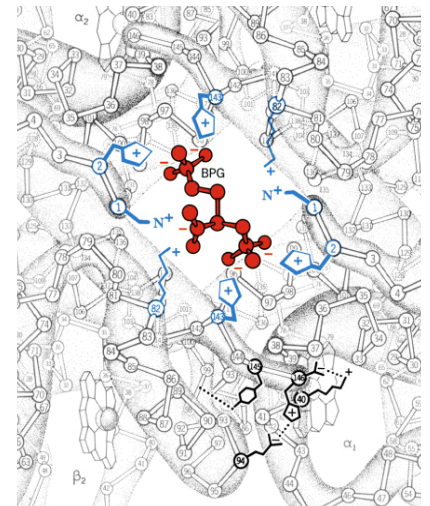
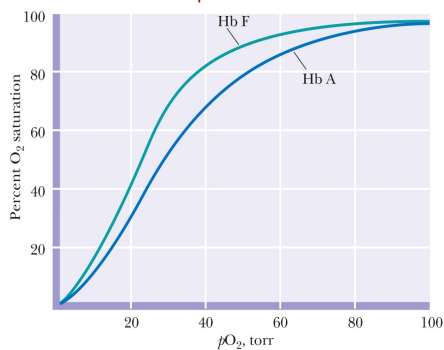
-Purified Hb has a different O<sub>2</sub> affinity than it does in blood

26 fold decrease change in affinity is due to 2,3 diphosphoglycerate BPG

- (BPG replaced by nucleotides IHP and ATP in fish and birds)
- 1 BPG per Hb - binds in central cavity of Hb
- binds preferentially to deoxy Hb
- hydrophobic bonds with Lys and salt bridge with His
- O<sub>2</sub> binding changes conformation and "kicks out" BPG
- change in altitude increases concentration of BPG

Fetal Hb has replaced His 143 with Ser - What might the consequences be?

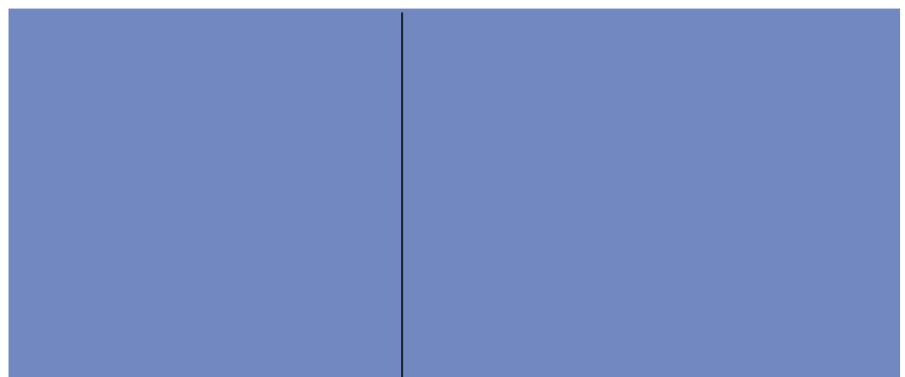
### Fetal and adult forms of hemoglobin



### Expression of fetal Hb

z<sub>2</sub>e<sub>2</sub>, α<sub>2</sub>e<sub>2</sub> and α<sub>2</sub>g<sub>2</sub> chains have a higher affinity for O<sub>2</sub> than α<sub>2</sub>β<sub>2</sub> chains giving fetus ability to get O<sub>2</sub> from mother

Hemoglobin F, the hemoglobin of late fetal life is made of two α and two γ subunits. The beta chain is not fully produced until a few weeks after birth



Postconceptual age (weeks) Birth Postnatal age (weeks)

**Fetal Hemoglobin Has a Higher Affinity for O<sub>2</sub> Because it has a Lower Affinity for BPG**

- The fetus depends on its mother for O<sub>2</sub>, but its circulatory system is entirely independent
- Gas exchange takes place across the placenta
- Fetal Hb differs from adult Hb – with γ-chains in place of β-chains – and thus a α<sub>2</sub>γ<sub>2</sub> structure
- As a result, fetal Hb has a higher affinity for O<sub>2</sub>
- Why does fetal Hb bind O<sub>2</sub> more tightly?
- **Fetal γ-chains have Ser instead of His at aa 143 and thus lack two of the positive charges in the BPG- cavity**
- BPG binds less tightly and Hb F thus looks more like Mb in its O<sub>2</sub> binding behavior

### Sickle-Cell Anemia, a Molecular Disease

One of the first “molecular” diseases found - sickle cell anemia

- sickle cell - blood cell is elongated, mis-shaped (sickle)
  - occurs at low O<sub>2</sub> concentration
  - caused by hemoglobin aggregates
  - inflammation in capillaries and pain
  - red blood cells break down - anemia
- between 10% of American blacks and 25% of African blacks are heterozygous for sickle cell anemia
- homozygous usually do not survive into adult hood
- heterozygous individuals usually have no problem except when in severe oxygen deprivation

### Sickle-Cell Anemia is a Molecular Disease

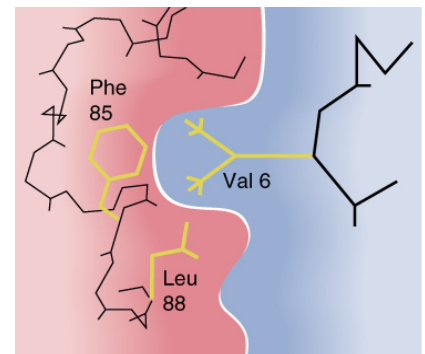
A single amino acid substitution in the β-chains of Hb causes sickle-cell anemia

- Glu at position 6 of the β-chains is replaced by Val

As a result, Hb S molecules aggregate into long, chainlike polymeric structures

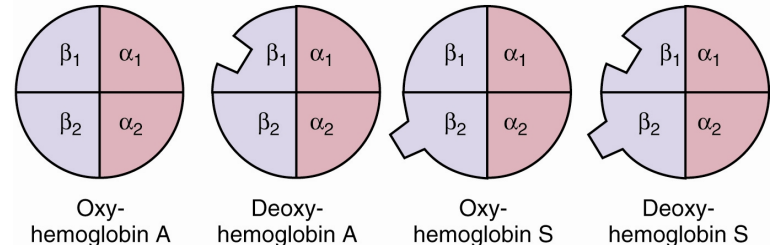
Single amino acid (point mutation) HbS vs. HbA changes structure

- sickle cell β chains have a valine in place of glutamate
- leads to more Hb S (sickle cell) has 2 more + charges than normal hemoglobin
- Glu -Val occurs on exterior of protein - does not change O<sub>2</sub> dissociation/allosteric properties of protein



### Sickle-Cell Anemia is a Molecular Disease

The polymerization of Hb S molecules arises because Val replaces His on the surface of β-chains. The “block” extending from Hb S below represents the Val side chains. These can insert into hydrophobic pockets in neighboring Hb S molecules.



Deoxy HbS precipitates

- oxyHb phenylalanine b85 and leucine b88 interior
- phe and leu shift to exterior
- create a sticky patch with valine (hydrophobic bonding)
- nucleation (cluster of aggregate) occurs logarithmically
- homozygous - 1000 times faster than heterozygous
- that means mixed genes can re-oxygenate faster than polymerization can occur

### How can such a disease occur?

- highest concentration of gene mutation occurs where there is high incidence of malaria
- heterozygous individuals survive this disease better than those without
- malaria causing parasite lives in red blood cells during part of its life cycle
- partial sickling must interrupt life cycle of malaria parasite

Methemoglobinemia - instead of aggregation, mutation leads to changes in O<sub>2</sub> affinity

Hb Boston form: distal his replaced with tyrosine - stabilizes Fe<sub>3</sub><sup>+</sup> state

- heme cannot bind O<sub>2</sub>, T form is favored

Hb Milwaukee Val near distal His site is mutated to a glutamate

- This allows tight association with O<sub>2</sub>. Causes oxidation of iron
- blood is brown (Fe<sup>3+</sup> state)
- only heterozygous individuals survive

Thalassemias - alpha thalassemia, missing alpha chain - usually due to way the DNA mutation in promoter

- heterozygous are usually asymptomatic (show no signs)
- homozygous need blood transfusion to live
- delta chains are very important here



### Hemoglobin and Nitric Oxide

Nitric oxide (NO·) is a simple gaseous molecule that acts as a neurotransmitter and as a second messenger in signal transduction

NO· is a high-affinity ligand for Hb, binding to the heme iron 10,000 times more tightly than O<sub>2</sub>

So why is NO· not bound instantaneously to Hb, preventing its physiological effects?

NO· reacts with the -SH of Cys<sup>93β</sup>, forming an S-nitroso derivative:

The S-nitroso group is in equilibrium with other S-nitroso compounds formed by reaction of nitric oxide with small-molecule thiols such as free Cys or glutathione:

These small-molecule thiols transfer NO· from erythrocytes to endothelial receptors, where it exerts its physiological effects