Biochemistry - HLS

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Hemoglobin : An overview and more

Prof. Mamoun Ahram
Hematopoietic-lymphatic system

this lecture will discuss the following :

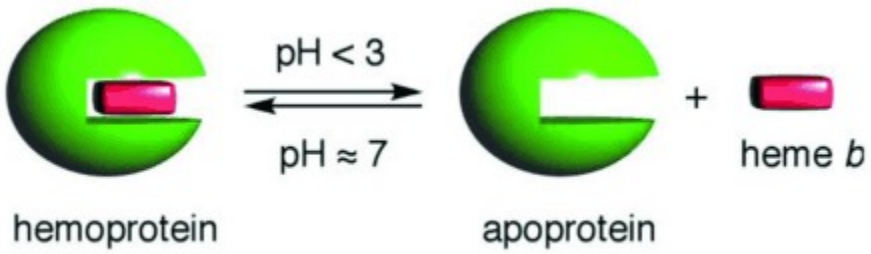
1. The structure of hemoglobin and important amino acids.
2. Hemoglobin glycosylation and its importance in diagnosing diabetes.
3. Genetics of hemoglobin molecules.

Resources

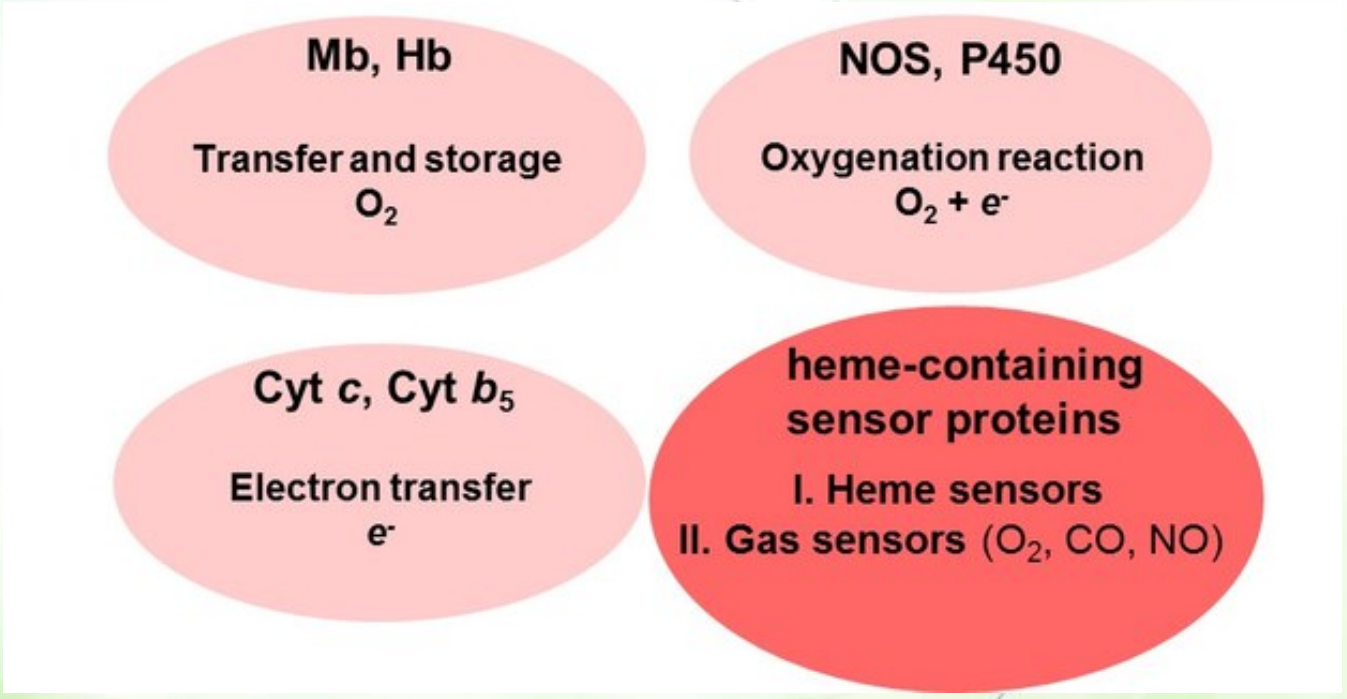
- This lecture
- Myoglobin/Hemoglobin O2 Binding and Allosteric Properties of Hemoglobin (http://home.sandiego.edu/~josephprovost/Chem331%20Lect%207_8%20Myo%20Hemoglobin.pdf)
- Lecture 3: Cooperative behaviour of hemoglobin (https://www.chem.uwec.edu/chem452_f12/pages/lecture_materials/unit_III/lecture-3/overheads/Chem452-lecture_3-part_1-overheads.pdf)

Hemoproteins

- Many proteins have heme as a prosthetic group called hemoproteins.



A prosthetic group *is a tightly bound, specific non-polypeptide unit required for the biological function of some proteins. The prosthetic group may be organic (such as a vitamin, sugar, or lipid) or inorganic (such as a metal ion), but is not composed of amino acids.*



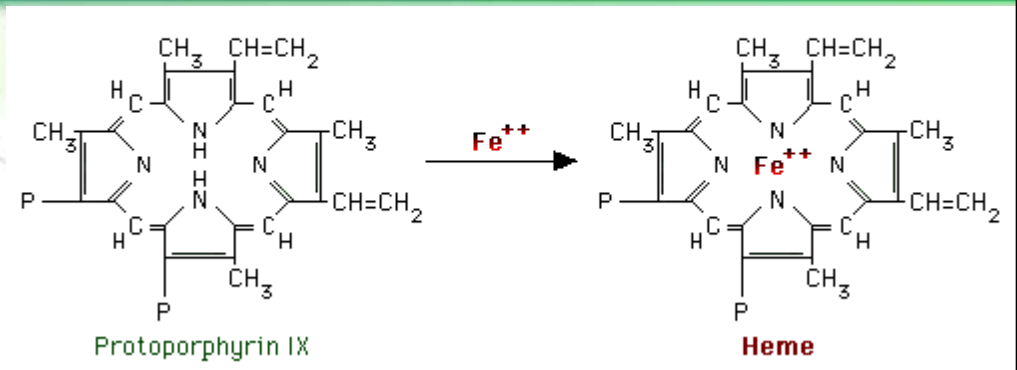
Hemoproteins

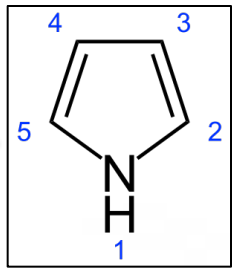
1. Holoproteins are proteins that have non-protein groups attached to them. Removing this group turns them into apoproteins.
2. According to this definition, hemoproteins are hollow proteins with a specific non-protein group (heme) attached to them.
3. Hemoproteins belong to a large class of proteins with different functions.
4. The most important/famous ones are the Myoglobin and Hemoglobin, why is that ? because they are involved in binding oxygen for the sake of storing or transferring.
5. Cytochrome P450 proteins are found in the liver, and they are important in detoxification of xeno molecules.
6. Heme groups involved in the electron transport chain and other protein that function as sensors (heme being part of them playing a major role) can also be found.

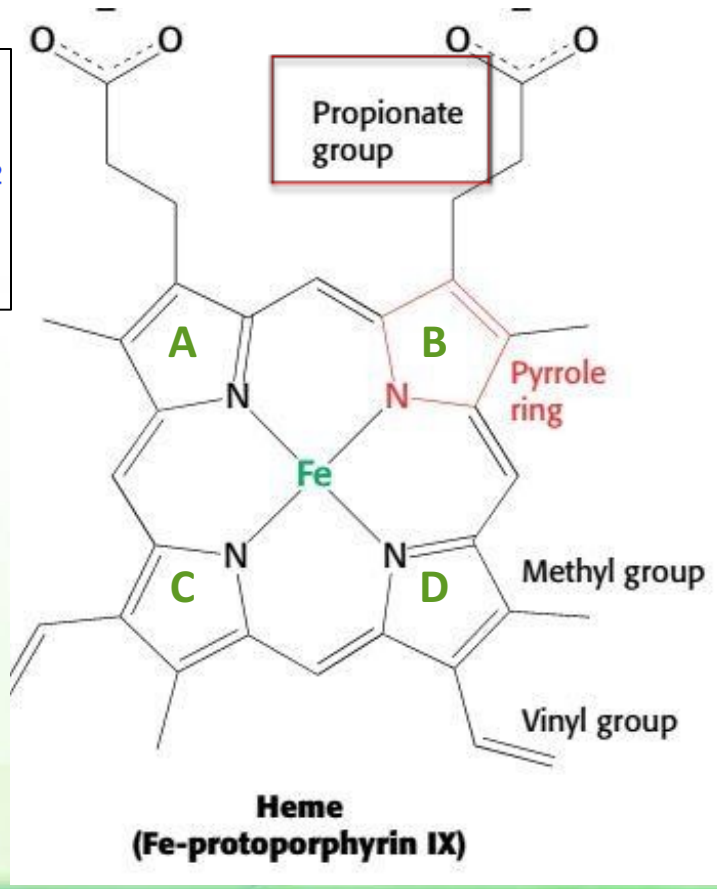
Heme structure

- Heme is an organic cyclic molecule (a ring molecule to be specific).
- It is a complex of protoporphyrin IX + Iron (Fe^{2+}).
- The active form of heme has an iron attached to it in the center, if we remove the iron, we get a molecule known as protoporphyrin IX, which is a precursor of heme.
- The porphyrin is planar and consists of four rings (designated A-D) called pyrrole rings.
- Each pyrrole can bind two substituents.
- It has branches that extend out of the heme molecule (primarily we have methyl & vinyl groups)
- Two rings have a charged propionate group each.

- *Note: the molecule is hydrophobic*. (in an overall view, it is even though we have a propionate group)
- Fe has six coordinates of binding, it can form 6 covalent bonds (4 are with the heme molecule itself)



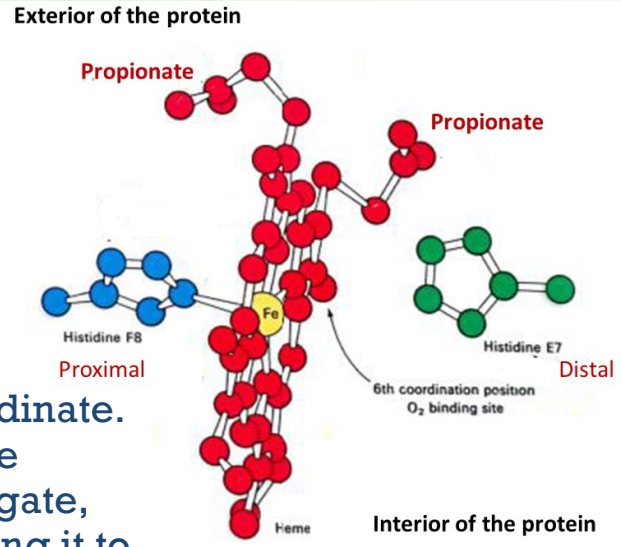


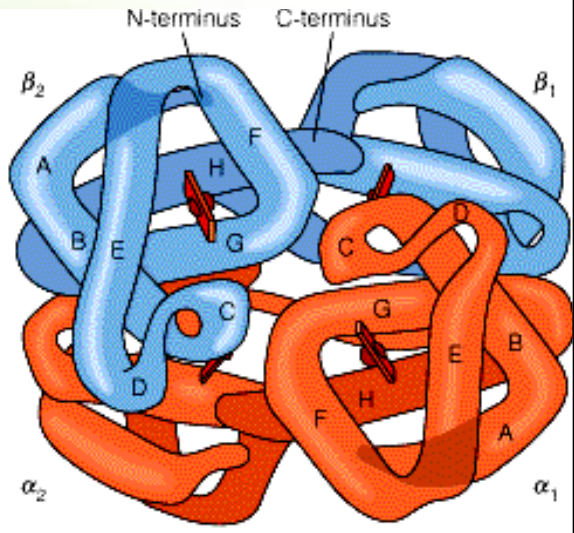


Structure of hemoglobin

- Hb is a globular protein.
  - Amino acid distribution (Globular protein: hydrophilic amino acids located on the outside of the protein while hydrophobic ones are located on the inside except for 2 His residues)
    - Positions of two histidine residues (in relation to the heme)
      - Proximal (within helix F8) and distal (within Helix E7)

1. Proximal histidine binds covalently with iron, it is the 5th coordinate.
2. The other histidine residue found on the other side of the heme molecule doesn't interact covalently, rather it functions like a gate, controlling what goes inside the heme molecule like O_2 allowing it to bind to the iron atom forming the 6th coordinate.





- It is an allosteric protein (has more than one structure) → T (taut) , R (relaxed))
  - Multiple subunits (2α + 2β)
    - α polypeptide = 141 amino acids (Arg141)
    - β polypeptide = 146 amino acids (His146)
  - Altered structure depending on bound molecules
  - Positive cooperativity towards oxygen
  - Regulated by allosteric effectors

This is a primary feature; allosteric proteins must have a quaternary structure.

The number of mentioned amino acids is of functional importance.

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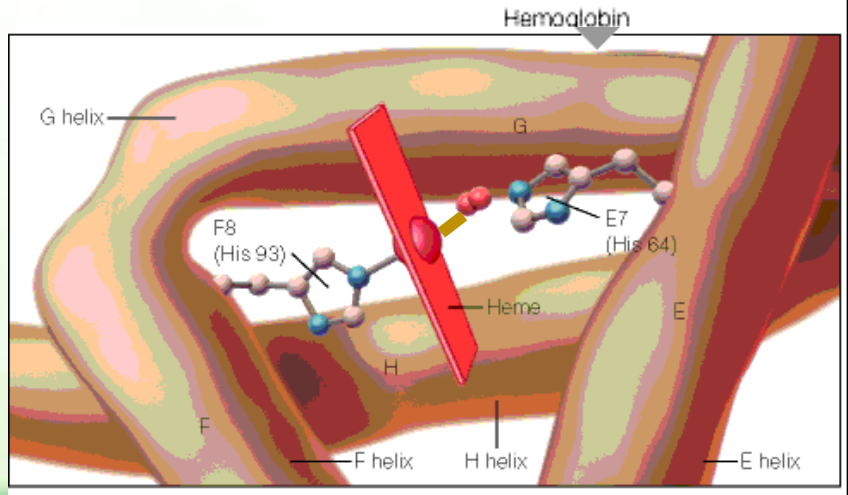
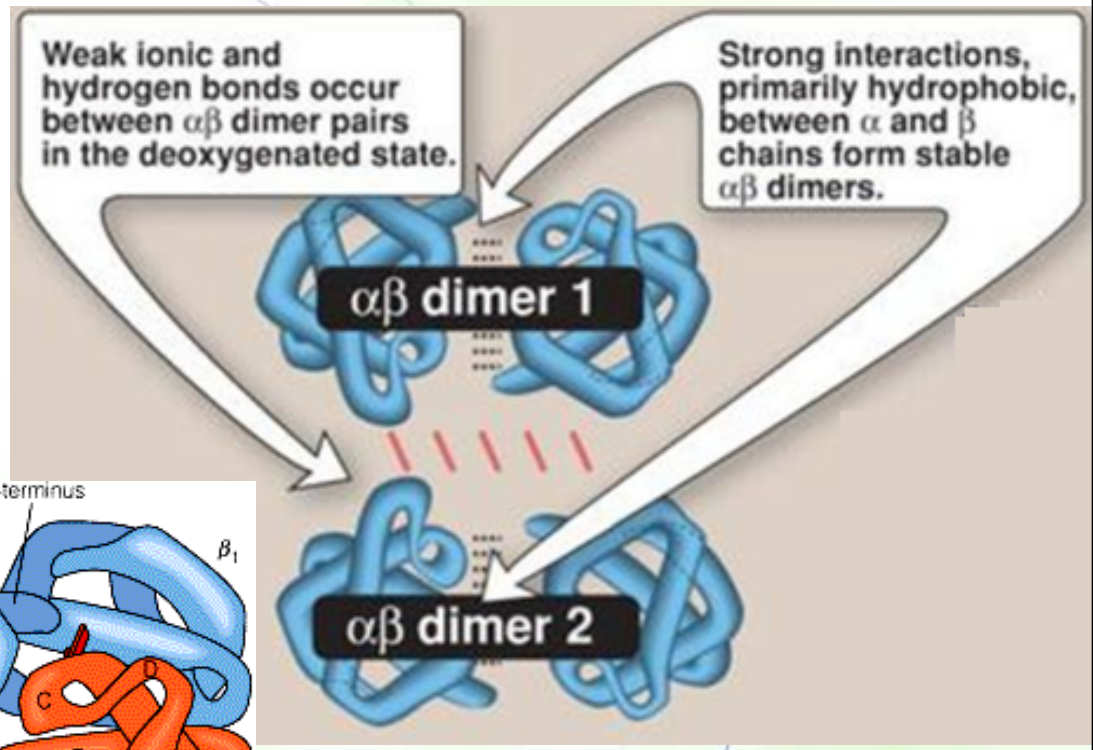


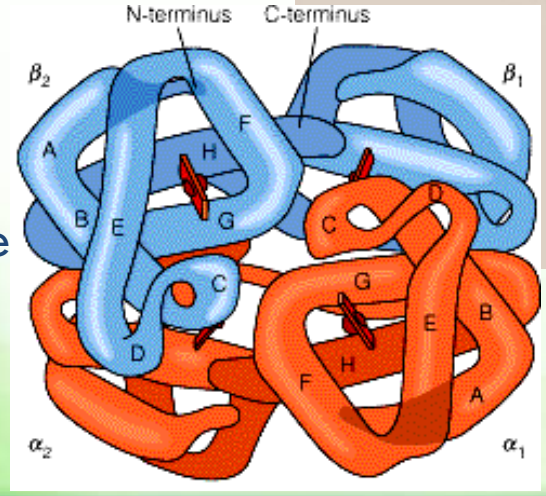
How are the subunits bound?

1. Cooperativity : Oxygen binds to one of the heme molecules (each polypeptide contains a heme molecule) and that binding facilitates the binding of the 2nd O2 to the 2nd heme, that makes it even easier for the 3rd O2 to bind the 3rd heme and so on.
   There is positive cooperativity where the binding of one ligand makes it easier for the second one to bind and so on.
2. The switching between the 2 states (T & R) is controlled by binding to O2 as well as other allosteric effectors (will be mentioned later).

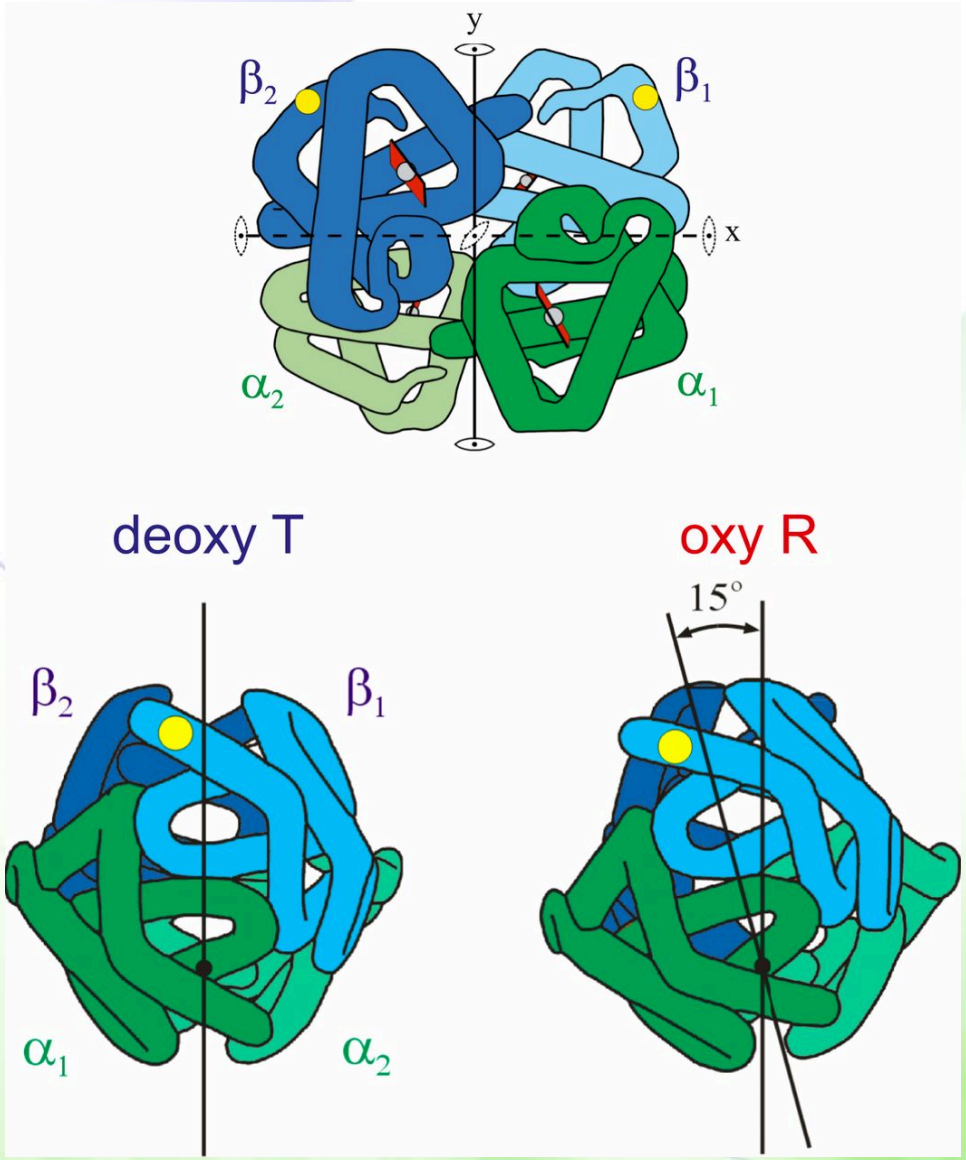
- A dimer of dimers (I made up this term)
  - (α-β)2
  - Note how they interact with each other.

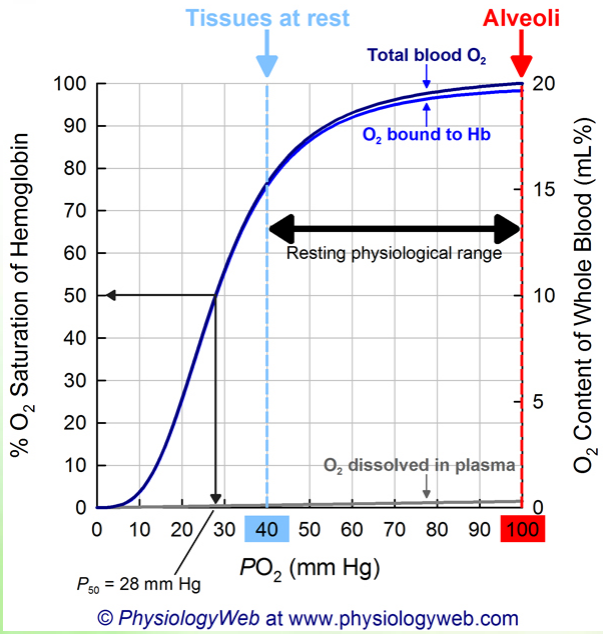


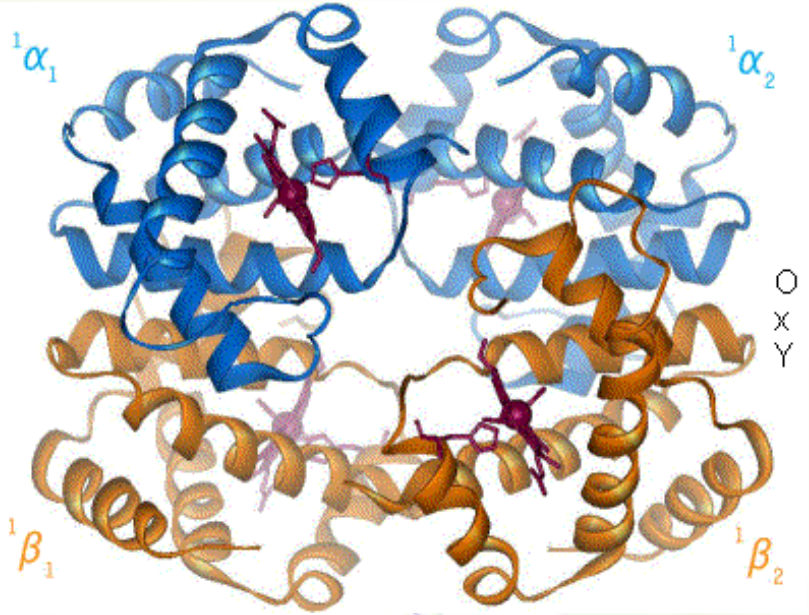
1. The 4 peptides interact with each other in different manners.
2. The interactions between alpha and beta in dimer 1 are hydrophobic interactions.
3. The interactions between dimer 1 & dimer 2 are electrostatic interactions with some hydrogen bonds.



Structural change of hemoglobin







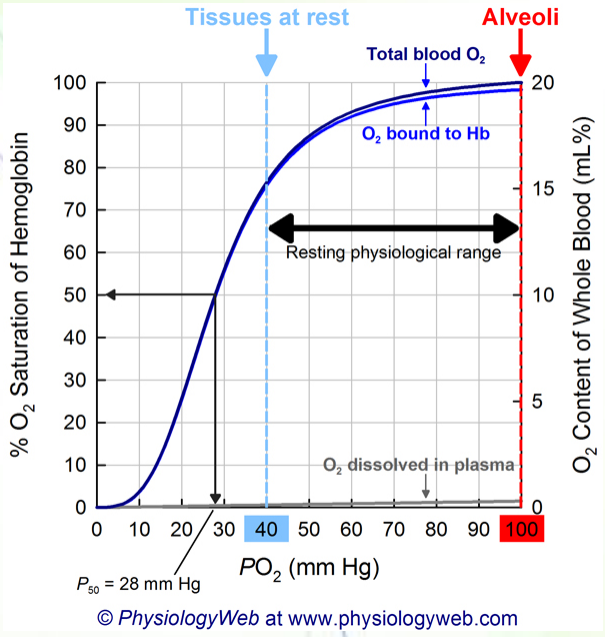
Structural change of hemoglobin

Note that sigmoidal means we have 2 conformations (active and inactive)

- Hemoglobin has 2 structures (T & R).
- These structures differ in the following :
  1. The orientation.
  2. The 3D position of amino acids (due to the movement of polypeptides as a result of oxygen binding).
  3. Oxygen affinity (different binding affinity to O2 → T = low affinity / R = high affinity).

- This curve is known as the oxygen saturation curve
  1. X axis : different pressures/amounts of oxygen.
  2. Y axis : saturation of oxygen in hemoglobin.

- As the O2 level increases, more oxygen is bound to the hemoglobin.
- This curve looks sigmoidal (a characteristic of allosteric proteins).
- The significance of having a sigmoidal curve is to indicate the existence of 2 shapes of the protein (T structure having low affinity towards oxygen while the R structure is having a high affinity towards oxygen)



Structural amplification change

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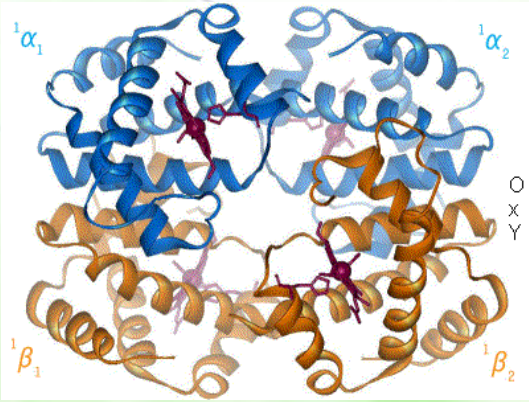
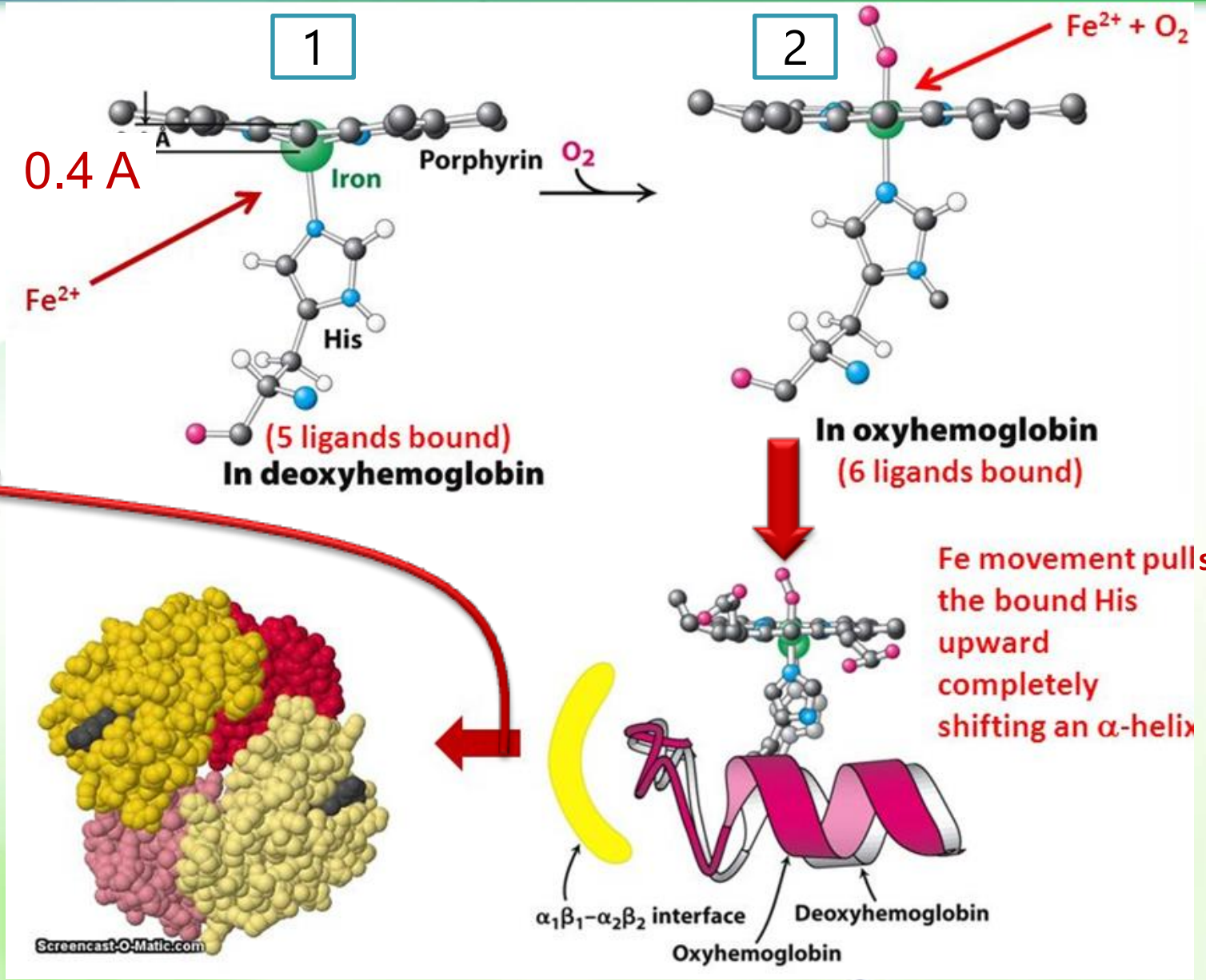
- Changes in tertiary structure of individual hemoglobin subunits
- Breakage of the electrostatic bonds at the other oxygen-free hemoglobin chains.





Structural amplification change

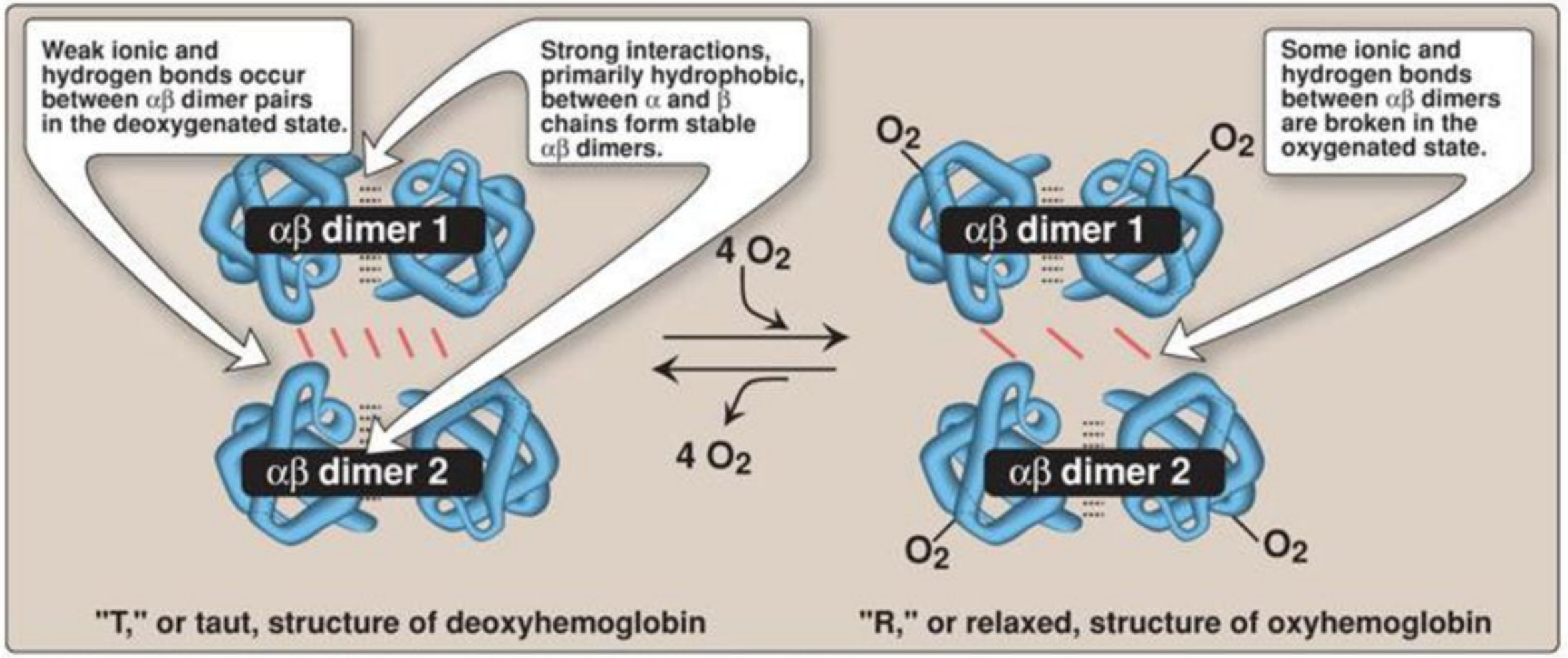
- Hemoglobin has 2 structures due to gradual changes in its quaternary structure.
  - Remember proteins have four levels of structures. Quaternary is the overall structure of proteins that are made of more than one polypeptide/multiple subunits. Tertiary is the 3D structure of a single polypeptide. Secondary is the structure of secondary structures like alpha helices (enriched in the hemoglobin molecule). Primary is the sequence of amino acids that make up the polypeptide, or the order of amino acids.

1. These changes start with a little change in the heme molecule itself.
2. (In deoxyHb (figure 1)) The heme molecule in the hemoglobin is bent because there is repulsion between the proximal histidine and the hydrophobic heme molecule, so the heme molecule is bent, and it has a dome-like structure, and iron is outside the plan of the heme molecule.
3. When O2 comes in and binds to the iron, the repulsion between the proximal histidine and heme is gone or it is reduced. So, heme takes this flat/planner shape which is supposed to be its normal shape. (In oxyHb (figure 2))
4. When the structure changes into a flat/planner shape, it pulls the proximal histidine towards the heme molecule. (this movement is little = 0.4 A)
5. BUT, this little movement results in a shift in the structure of the helix that contains the proximal histidine, so the structure and position of that alpha helix also changes.
6. This change affects the tertiary structure of the polypeptide that is now bound to the O2 which causes breakage of the electrostatic interactions between the dimers.

All these changes and processes are what changes a T hemoglobin into an R hemoglobin (low affinity → high affinity)… and that's what makes hemoglobin functional

Electrostatic interactions are broken

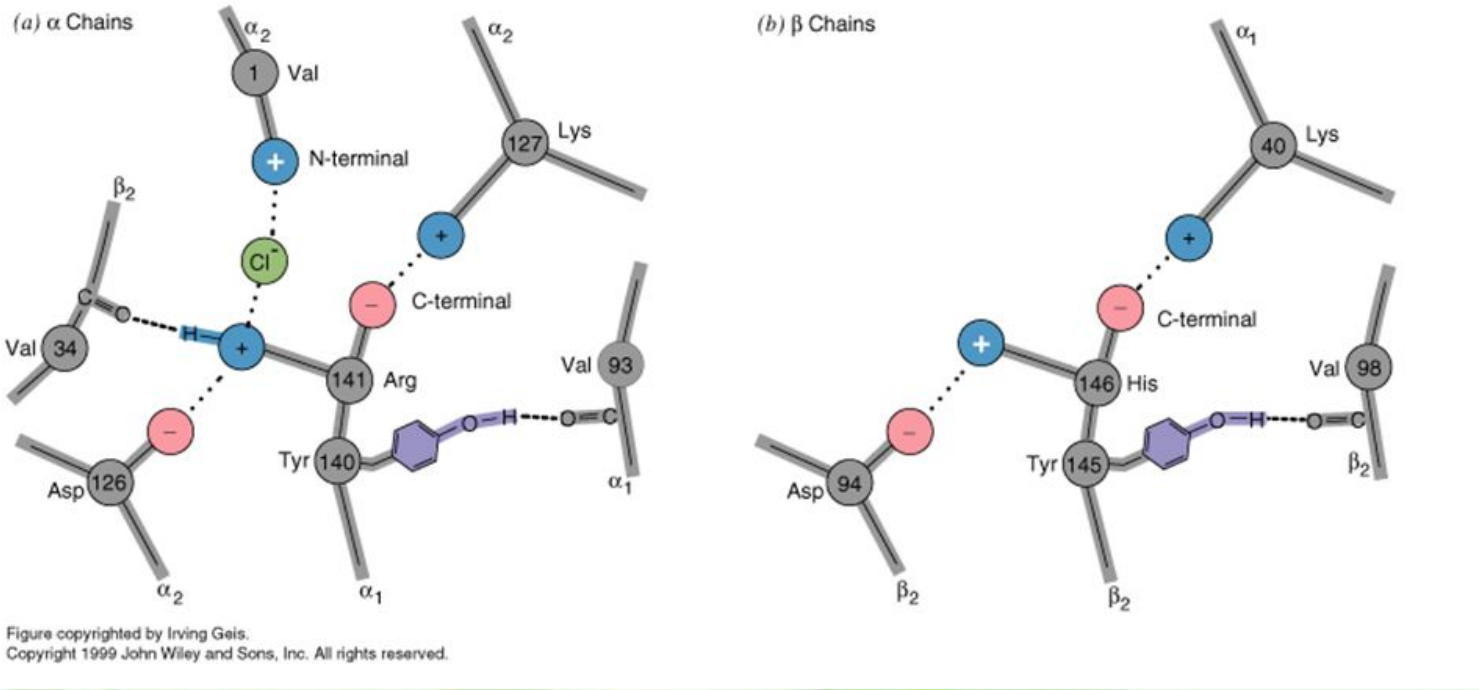
When oxygen binds, the number of electrostatic interactions between the 2 dimers is reduced and that results in the relaxation of the hemoglobin molecule (R state)

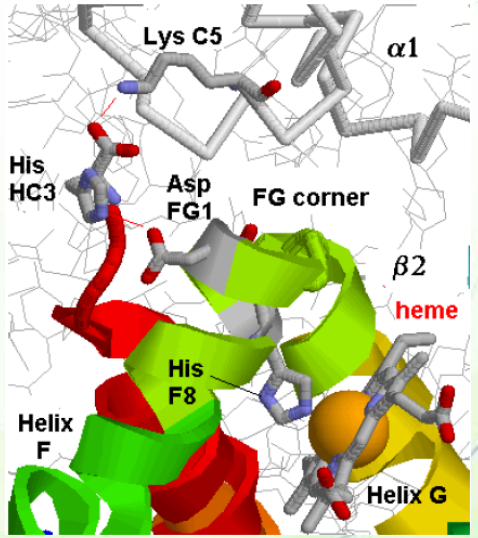


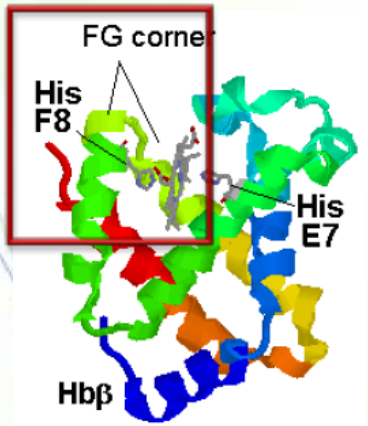
Note that the dimers are bound to each other in a Criss Cross pattern (Alpha 1 with Beta 2) (Alpha 2 with Beta 1)

The broken electrostatic interactions

- Electrostatic interactions and hydrogen bonds (at the C-termini of the alpha and beta chains) that stabilize the T-form of hemoglobin are broken upon movement of the alpha-helix.
  - Note the groups, the protonation status, and the allosteric effectors (protons and chloride ions)

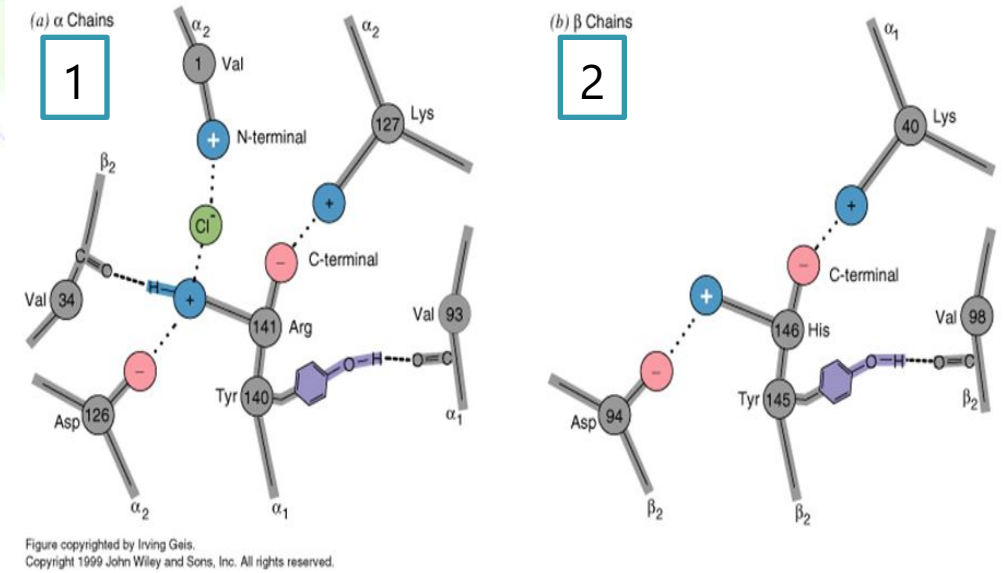






The broken electrostatic interactions

- We are going to zoom into the structure of the hemoglobin molecule and see exactly what happens at the molecular level.
- There are electrostatic interactions taking place, primarily, there is the interaction between the very last histidine (146) of the beta 2 subunit with a lysine on the alpha 1 subunit (figure 2)
  (this interaction is between beta 2 & alpha 1 but the same interaction is taking place between beta 1 and alpha 2 so it's reciprocal).
- 146 histidine can form 2 electrostatic interactions. (figure 2)
  1. The carboxy terminus itself (the carboxyl group of the histidine residue), it forms electrostatic interactions with a lysine on alpha 1.
  2. The R group of histidine can form electrostatic interactions with aspartate 94 which is located on the same chain so it's an intra-molecular interaction.
- This takes place on the surface of the hemoglobin molecule, so while 146 histidine is forming 2 electrostatic interactions with lysine and aspartate.
- At the same time, there are electrostatic interactions taking place at the N-terminus of the alpha subunit with the C-terminus of the other alpha subunit. (figure 1)
  1. The very last amino acid of the alpha polypeptide is an arginine (a possibly charged amino acid), and it has connections with a carboxyl and a possibly charged R groups that is forming electrostatic interactions with different subunits on the alpha and beta subunits.
  2. The involvement of the chloride ions mediate electrostatic interactions between the very last arginine of alpha with the N-terminus of the alpha 2 subunit. (chloride ions have a role in stabilizing and regulating the R → T switch and vice versa).



- There are different allosteric effectors here, we are talking about H+ (protons and Cl- (chloride ions)
- The point of having protons, is that we have these groups (like histidine 146) being protonated. If it is protonated, it would carry a positive charge that can form electrostatic interaction. If it was not protonated, then these interactions won't be formed.

And reformation of hydrogen bonds

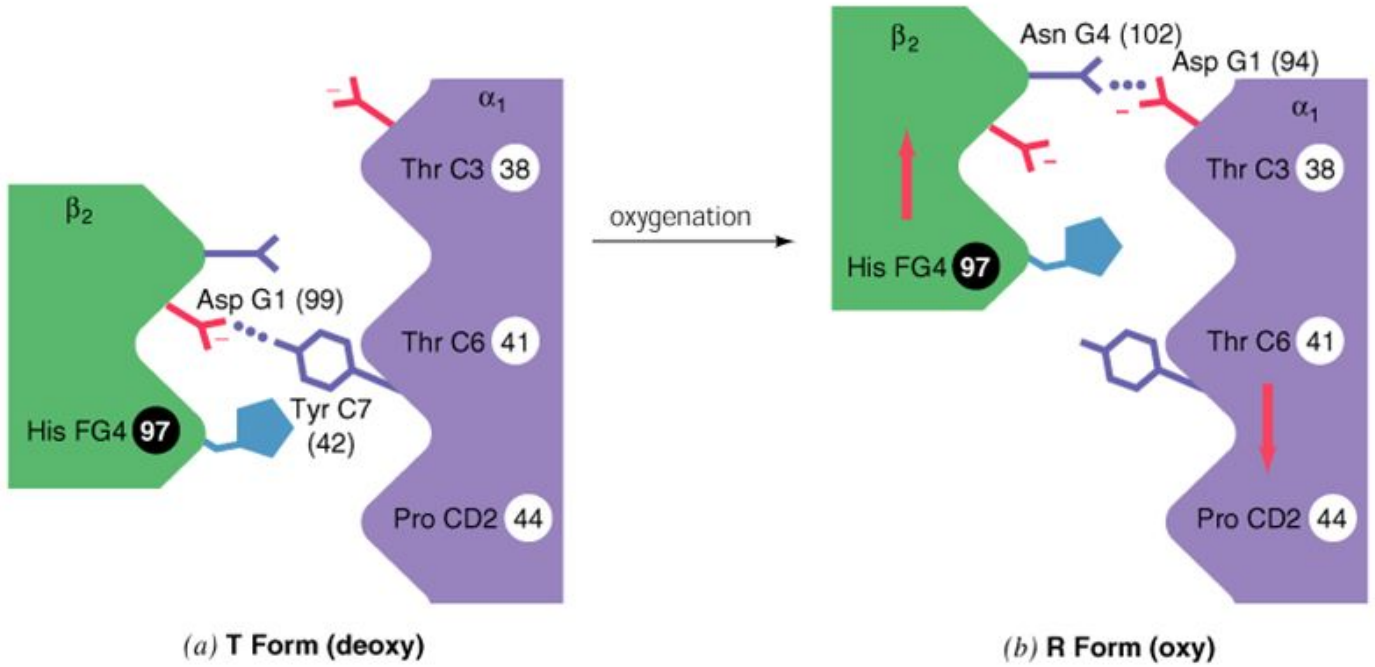
Note that we have breakage of electrostatic interactions in more than one site, 20 and even more interactions are broken and modified but we're focusing on specific amino acids and certain electrostatic interactions.

- T-state hemoglobin (deoxyhemoglobin) is stabilized by a hydrogen bond between Asp G1 of β 2 with Thr C6 of α1. (during breakage of electrostatic interactions , there's a formation of hydrogen bonds)
- When O_2 binds, the α1 surface slides and a hydrogen bond is formed between AspG1 of α chain with AsnG4 of β chain stabilizing the R form of hemoglobin.

Remember what happens when O2 binds to the heme (it changes from a dome-like structure to a flat planner structure with a rotation of the polypeptides along the axis).

When the polypeptides change position, the same change happens to different alpha helices in different parts of the protein.

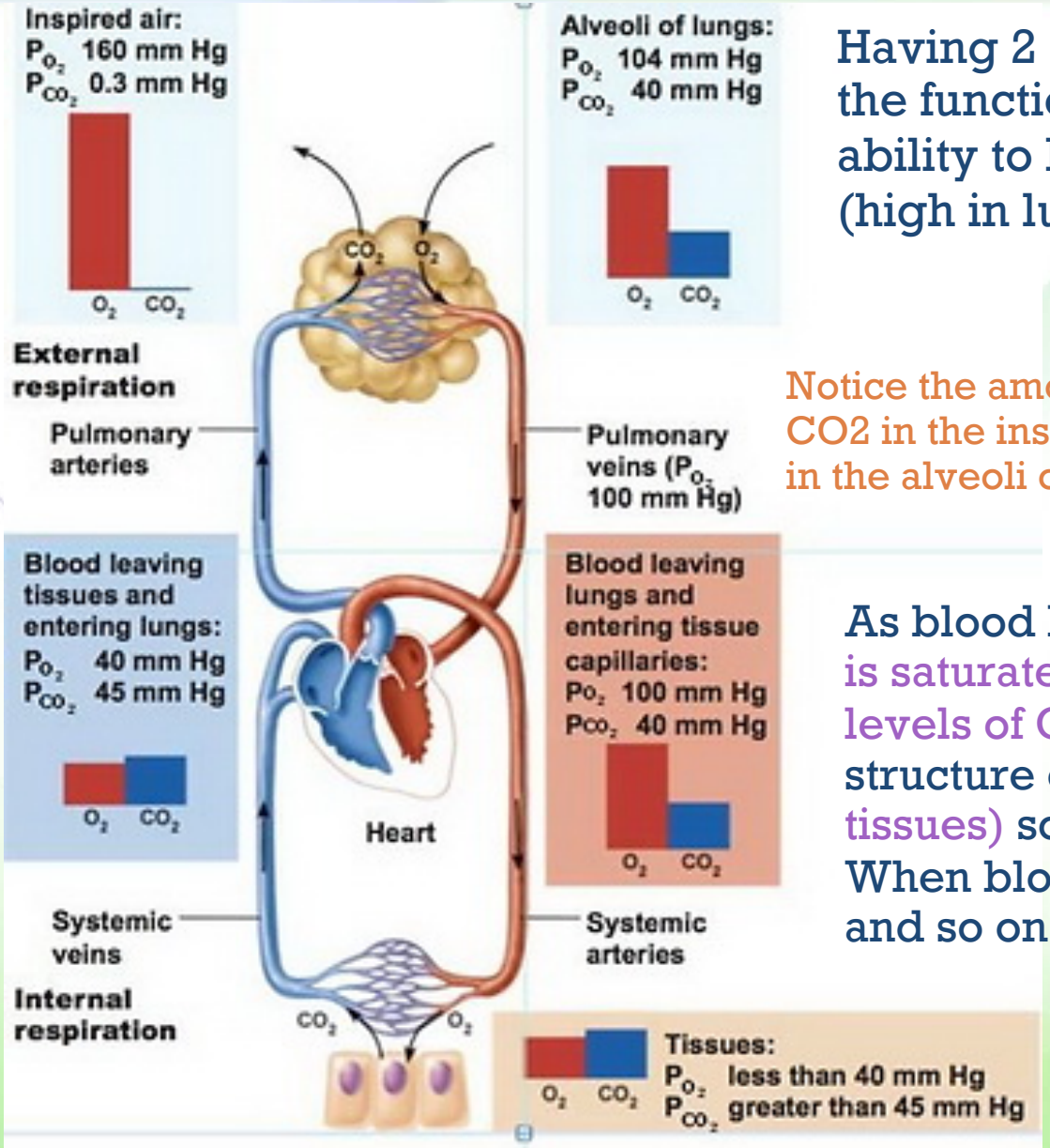
The green part (in Beta 2 subunit) *slides* away from where hydrogen bonds are taking place, so instead of forming hydrogen bonds between Asp 99 & Thr 41, the bonds forms between Asn 102 & Asp 94. so it's a process of sliding and reforming of hydrogen bonds.



Note that in this figure, the hydrogen bond is formed between Asp 99 and Tyr 42 but the doctor said we should stick to what the textbook mentioned (Thr 41 NOT Tyr 42)

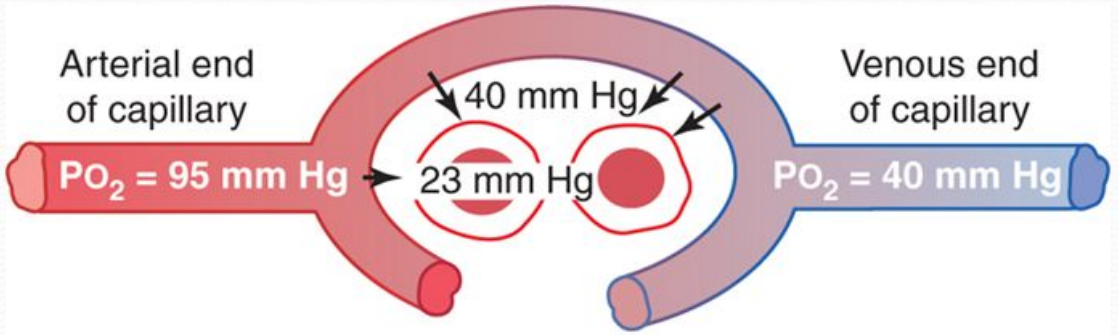
Hydrogen bonds between Asp 99 & Thr 41 stabilize the T state. Similarly, Hydrogen bonds formed between Asn 102 and Asp 94 stabilize the R state of the hemoglobin molecule.

Oxygen distribution in blood versus tissues



Having 2 structures for hemoglobin makes a huge significance in the function of hemoglobin itself, because it needs to have the ability to bind to O2 with high and low affinity in different places (high in lungs and low in tissues).

Notice the amount of O2: CO2 in the inspired air and in the alveoli of lungs.



As blood leaves the lungs, the level of oxygen is 100 torr (hemoglobin is saturated with O2). Once blood reaches tissues (where we have low levels of O2 with high levels of CO2 due to metabolism), a shift in the structure of hemoglobin is caused (R state in the lungs → T state in the tissues) so O2 can be released due to low affinity with hemoglobin. When blood leaves the tissues (O2 level is 40 torr, CO2 level is 45 torr) and so on.

Oxygen saturation curve

- The saturation curve of hemoglobin binding to O_2 has a sigmoidal shape.
  - It is allosteric.
- At 100 mm Hg, hemoglobin is 95-98% saturated (oxyhemoglobin).
- As the oxygen pressure falls, oxygen is released to the cells.
- *Note: at high altitude (~5000 m), alveolar pO2 = 75 mmHg.*

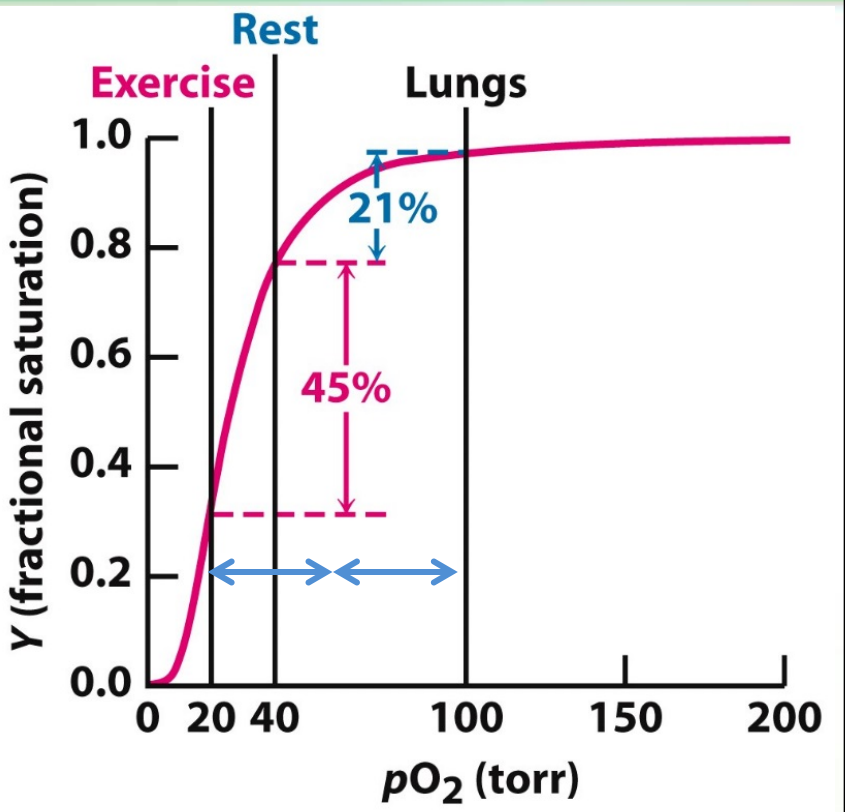
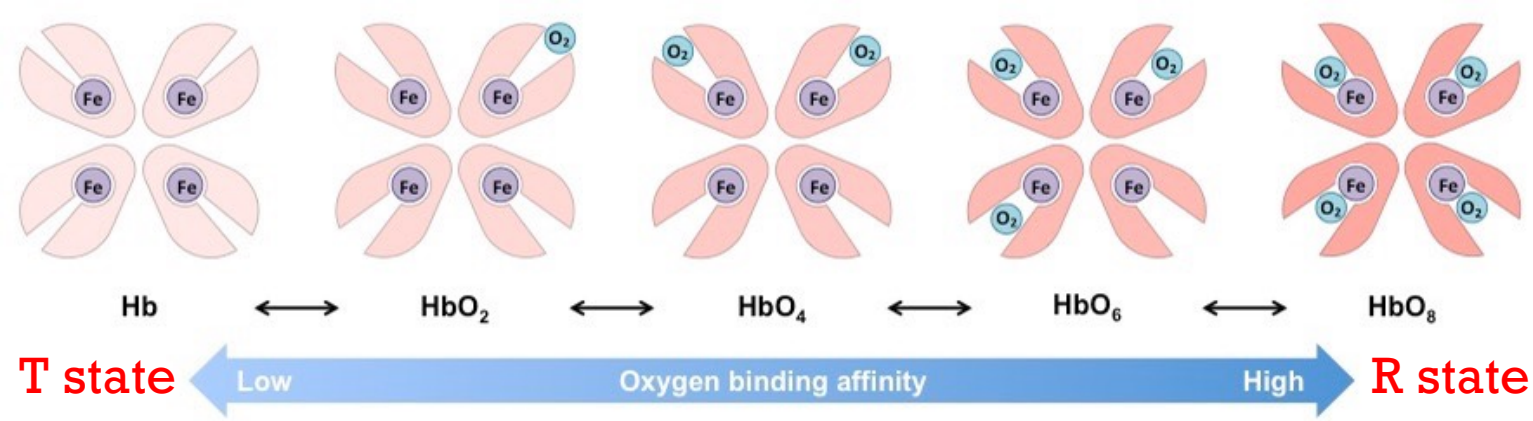


Figure 7.10
Biochemistry, Seventh Edition

You can see in the figure that in low affinity state, the interaction between hemoglobin and O2 is low (not much hemoglobin is saturated with O2). During exercise, about 40% of hemoglobin is saturated whereas in lungs, when O2 pressure is 100 torr, most hemoglobin molecules are saturated with oxygen(almost 100%)

Positive cooperativity



A hemoglobin molecule in the T state with no oxygen bound has low affinity towards oxygen when one oxygen binds to the first heme, it makes it easier for the second oxygen to bind to the second heme and so on. so the more oxygen atoms bind to, hemoglobin the higher the affinity to oxygen

Increasing ligand concentration drives the equilibrium between R and T toward the R state (**positive cooperativity)** → **sigmoidal curve**

- The effect of ligand concentration on the conformational equilibrium is a **homotropic effect (oxygen)**. (it influences the binding of other oxygen molecules)
- Other effector molecules that bind at sites distinct from the ligand binding site and thereby affect the R and T equilibrium in either direction are called **heterotropic effectors (e.g. CO_2)**.

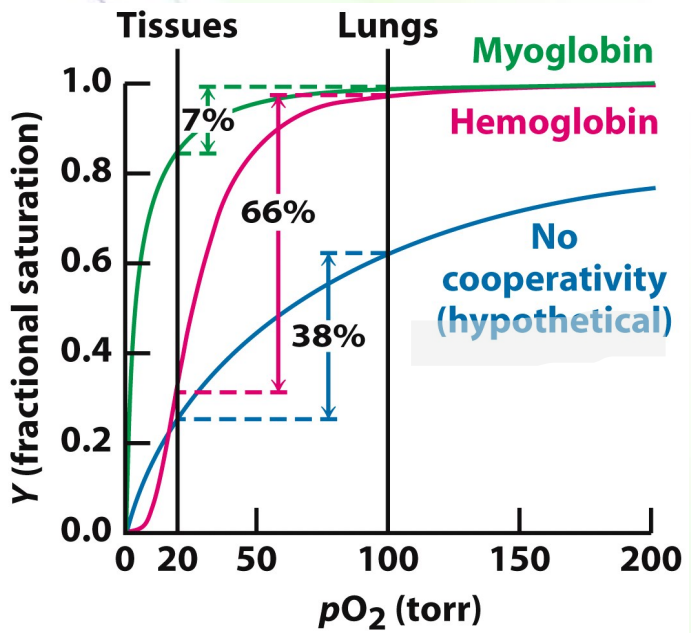


Figure 7.9
Biochemistry, Seventh Edition

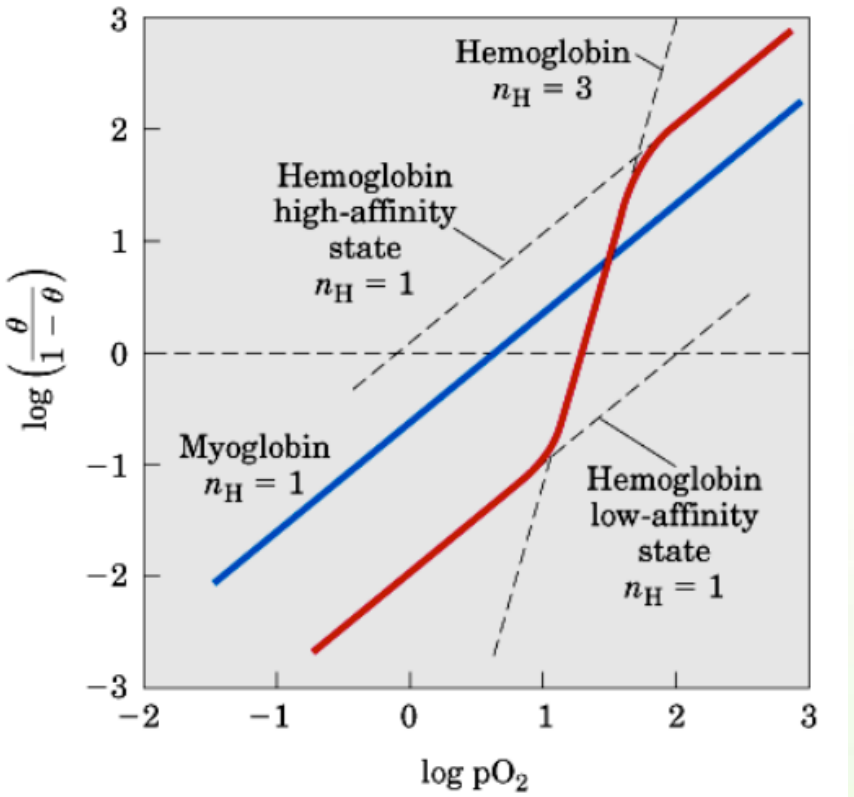
In a hypothetical situation, where we have no cooperativity, we'd need to have high pressure of O2 to saturate the heme prosthetic groups.
At atmospheric and sea level oxygen pressure (normal situation), if O2 pressure goes down from 100 → 20, the amount of released O2 (in this hypothetical protein) would be less than the allosteric protein, the protein that shows cooperativity. So, it's an advantage having hemoglobin as an allosteric protein that shows cooperativity.

The Hill constant (coefficient)

- The Hill plot is drawn based on an equation (you do not have to know it).
- 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 pO2
  - if n = 1 then non cooperativity such as myoglobin, that is not allosteric nor shows cooperativity
  - if n < 1 then negative cooperativity
  - if n >1 then positive cooperativity
- *The slope reflects the degree of cooperativity, not the number of binding sites.*

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

Y or θ is the fraction of oxygen-bound Hb



Y axis: logarithm of the fraction of hemoglobin molecule that is saturated
X axis: logarithm of the pressure of O2

Based on Y and X axis, we have a straight line (slope) that gives an indication of cooperativity (absent or not, degree of cooperativity, positive or negative)

Considering hemoglobin which has 2 states, there's 2 different slopes:

1. Low affinity (T) state , slope = 1
2. High affinity (R) state , slope > 1 (2.6-2.8)

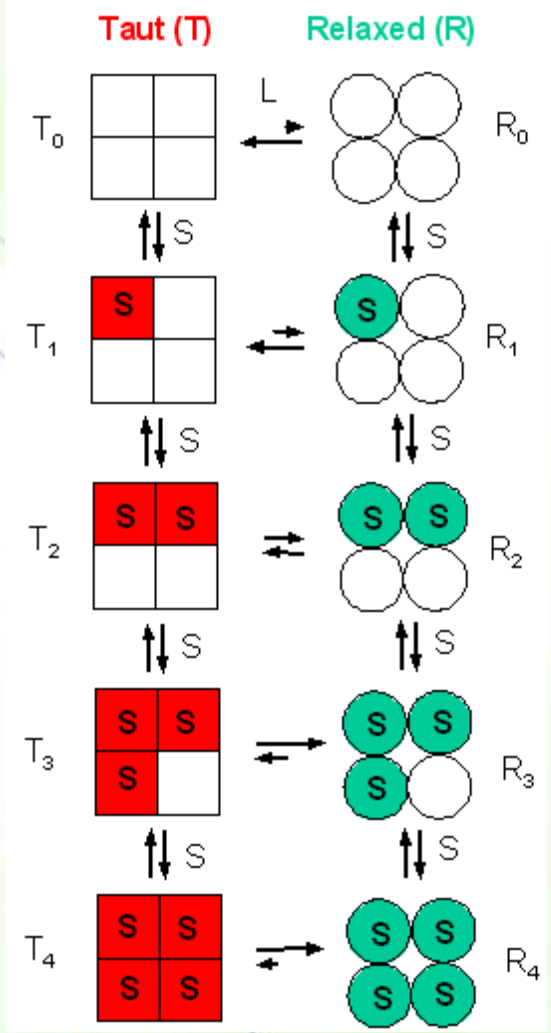
If n=3 that doesn't mean that if 3 sites are saturated or bound to O2 then the protein changes from T to R state, rather it gives a measure of the extent of cooperativity.

The concerted model (MWC model)

Most accurate for Hb

Each letter In MWC refers to a name of scientist that participated in coming up with this model

- The protein exists in two states in equilibrium: T (taut, tense) state with low affinity and R (relaxed) state with high affinity.
- Increasing occupancy increases the probability that a hemoglobin molecule will switch from T to R state.
- This allows unoccupied subunits to adopt the high affinity R-state.
- If the hemoglobin molecule is free of O2, it's mainly in the T state (some of it can form a high affinity R state but most probably it'll exist in the T state)
- The binding of O2 to the first heme results in a change in the equilibrium (it's still in the T state but more hemoglobin molecules are in the R state)
- Equilibrium keeps changing whenever a new heme binds to O2, meaning that more hemoglobin molecules are changing from T → R
- Until all 4 heme molecules are bound to O2, only then can we say that most of the hemoglobin molecules are in the R state

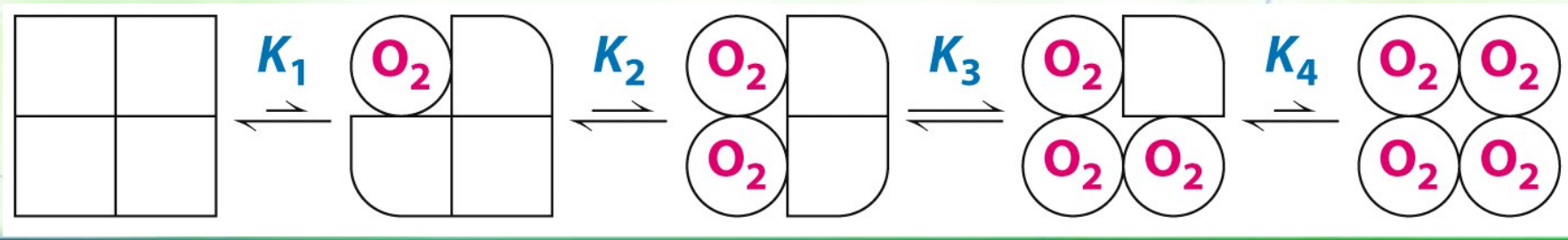


Note direction of arrows

The sequential, induced fit, or KNF model

Each letter In KNF refers to a name of scientist that participated in coming up with this model

- Induced fit happens when a ligand/substrate binds to the active site and changes the shape of the active site in order to fit the substrate.
- Sequential means that we have intermediates instead of only 2 states (T & R).
- In the figure below, hemoglobin exists in the T state. when oxygen comes in and binds to one of these subunits, the shape of this subunit changes to the R state and it affects the shape of the neighbouring subunits. When the 2nd O2 binds the 2nd subunit changes to the R structure also influencing neighbouring subunits and so on. When the 4th binds, the whole molecule now exists in the R state (extra explanation of sequential)
- The subunits go through conformational changes independently of each other, but they make the other subunits more likely to change, by reducing the energy needed for subsequent subunits to undergo the same conformational change.
- Which one is better? Both can explain the sigmoidal binding curve, but, in general,
  - The MWC model explains positive cooperativity & the change of structure of hemoglobin better than KNF
  - The KNF model works well for negative cooperativity (hemoglobin).

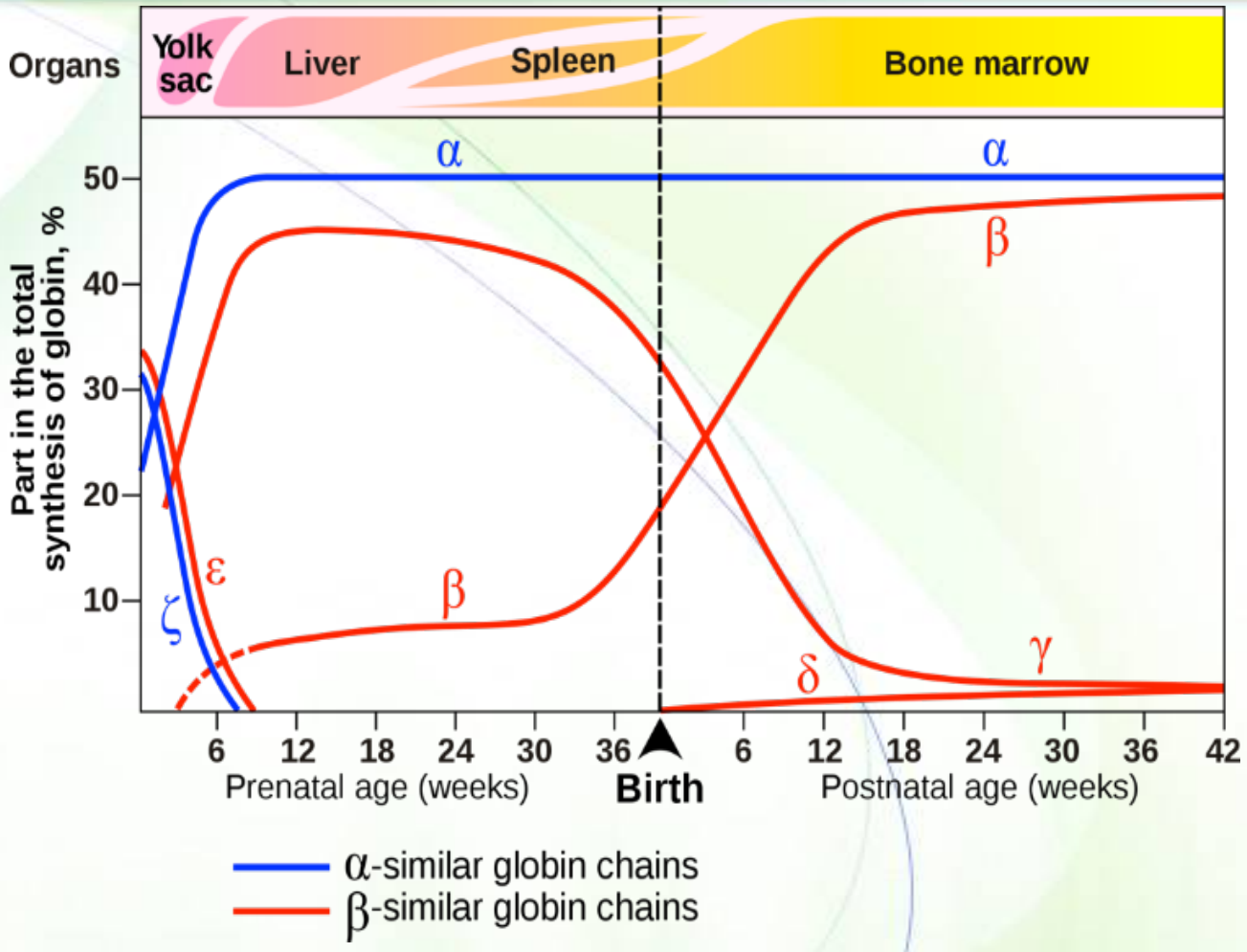


It is not only one hemoglobin

There are more forms of hemoglobin molecules that appear during development

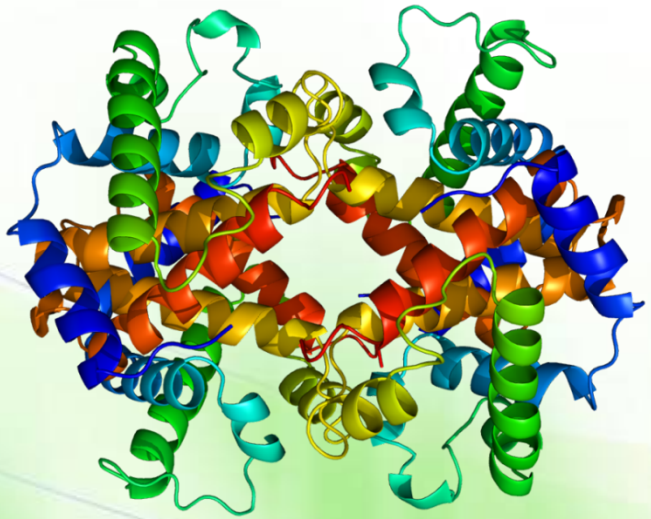
Developmental transition of hemoglobins

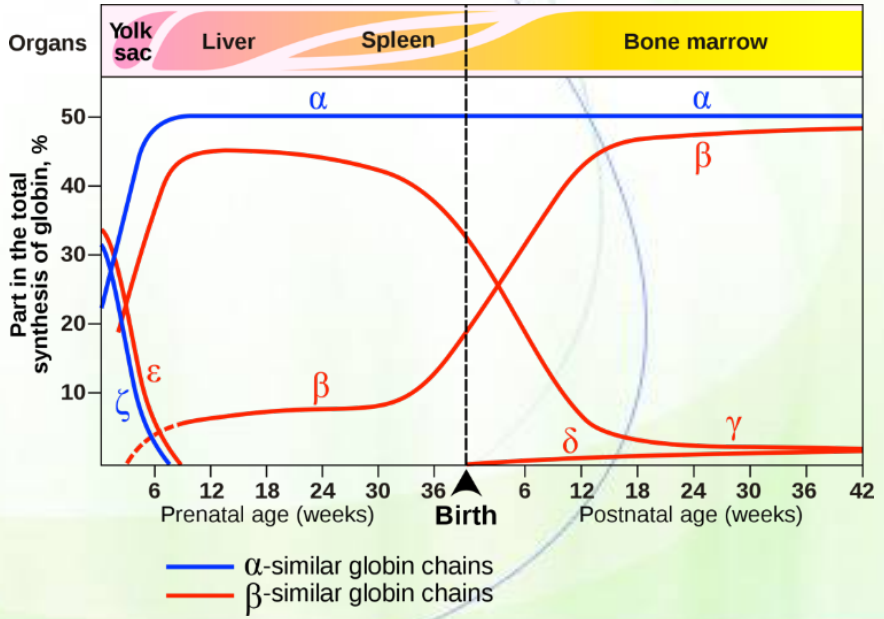
- During development, hemoglobin molecules change polypeptides.
- In your adult stage, your hemoglobin molecules are predominantly made of 2 alpha & 2 beta chains.
- In the embryonic stage, we have a hemoglobin made of 2 different polypeptides compared to the adult or fetal stage.
- In the fetal stage, we have a hemoglobin made of 2 alpha and 2 gamma chains.
- In the adult stage, we have an increase in the expression of the beta polypeptide and a decrease in the expression of the gamma polypeptide.
- We have a delta polypeptide which is not expressed highly in adults.
- The expression of these polypeptides is different during developmental stages



The embryonic stage

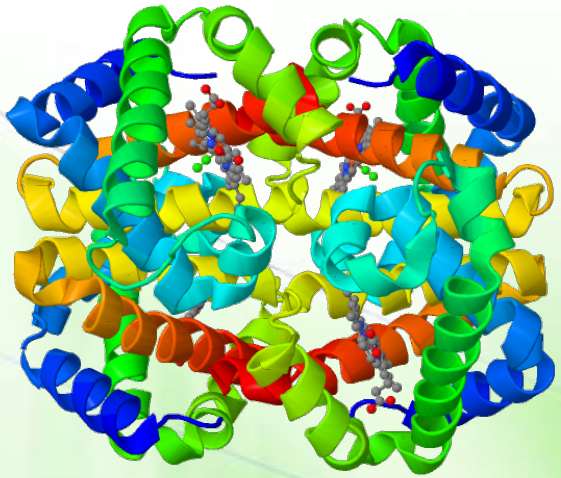
- Hemoglobin synthesis begins in the first few weeks of embryonic development within the yolk sac.
- The major hemoglobin (HbE Gower 1) is a tetramer composed of 2 zeta (ξ) chains and 2 epsilon (ε) chains. (E in HbE, stands for embryonic)
- Other forms exist due to expression of the alpha, and a little expression of gamma with some expression of beta : HbE Gower 2 (α2ε2), HbE Portland 1 (ζ2γ2), HbE Portland 2 (ζ2β2). HbE Gower predominates in the embryonic stage

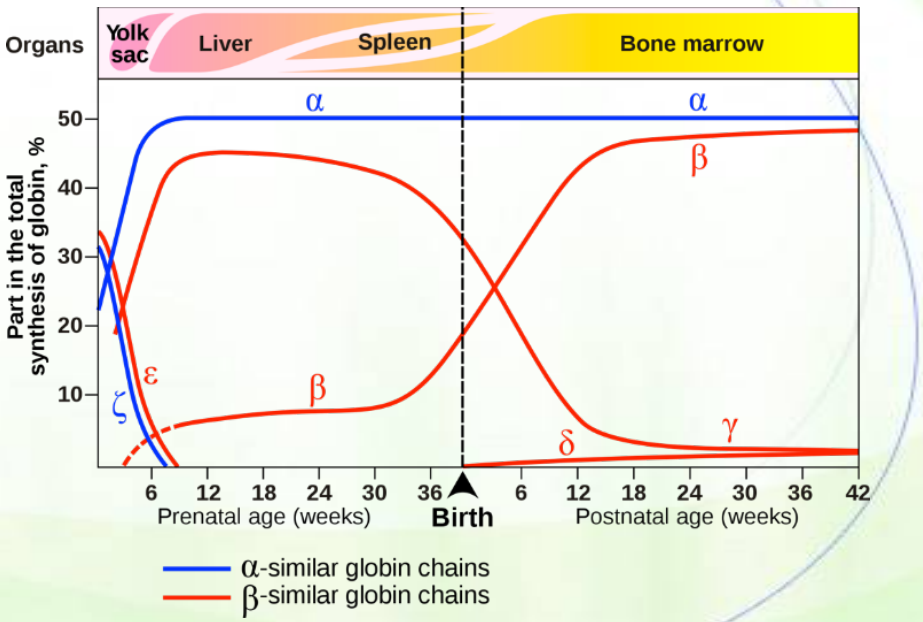




Beginning of fetal stage

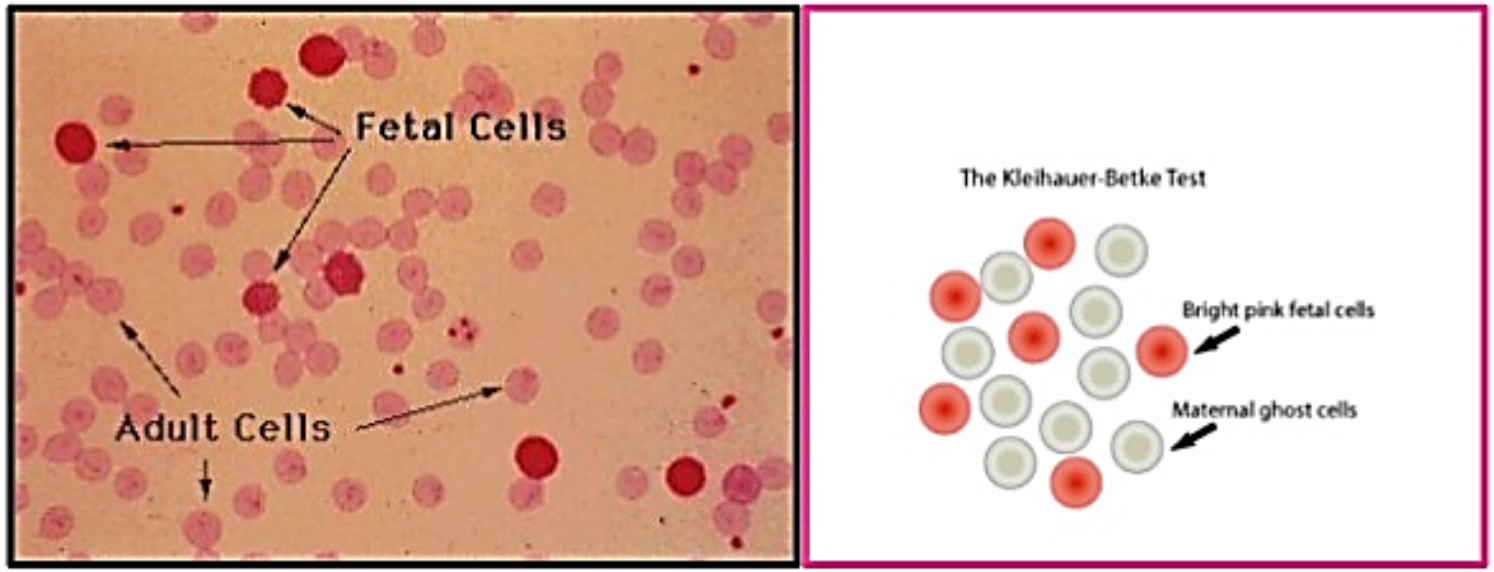
- By 6-8 weeks of gestation, the expression of embryonic hemoglobin declines dramatically and fetal hemoglobin synthesis starts from the liver.
- Fetal hemoglobin consists of two α polypeptides and two gamma (γ) polypeptides HbF (α2γ2) There is a decrease in the expression of zeta and epsilon and an increase in the expression of alpha and gamma
- The α polypeptides remain on throughout life.

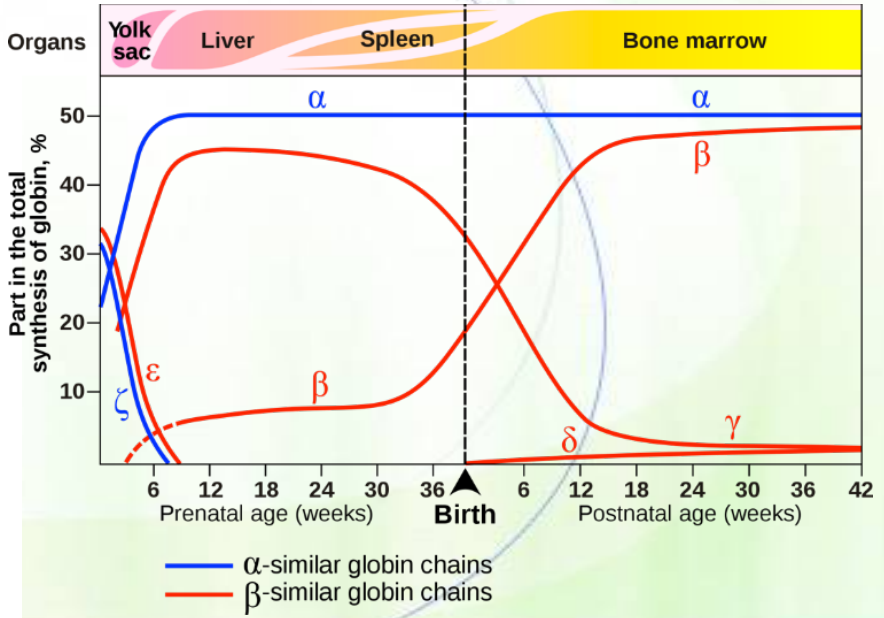




Beginning of adult stage

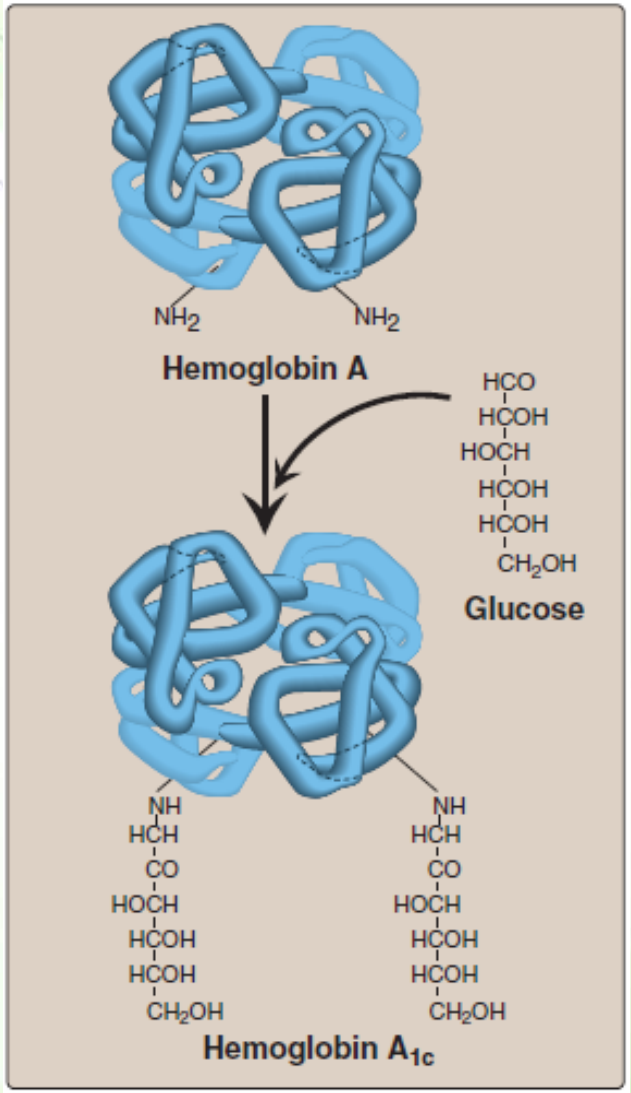
- Shortly before birth, there is a gradual switch to adult β-globin. (Increase in expression of beta polypeptide and decrease in expression of gamma poly peptide)
- Still, HbF (fetal hemoglobin) makes up 60% of the hemoglobin at birth (while HbA makes up 40%), but 1% of adults.
- At birth, synthesis of both γ and β chains occurs in the bone marrow.





Adult hemoglobins

- The major hemoglobin is HbA1 (a tetramer of 2 α and 2 β chains).
  - A minor adult hemoglobin, HbA2, is a tetramer of 2 α chains and 2 delta (δ) chains. (The promoter region of the delta gene is not very active)
- HbA (HbA1 specifically) can be glycosylated with a hexose (specifically at a Val) and is designated as HbAc.
  - The major form (HbA1c) has glucose molecules attached to valines of β chains.
  - HbA1c is present at higher levels in patients with diabetes mellitus.
  - Glycosylation is important because it can be used as a marker for diabetes mellitus (diabetic patients would have a higher proportion of glycosylated hemoglobin compared to a normal individual)



Advantages of HbA1c testing

- There are advantages for measuring the amount of glycosylated hemoglobin in the blood especially for diabetes.
- We can measure the level of glucose in two ways:

1. Blood fasting glucose level is the concentration of glucose in your blood at a single point in time, i.e. the very moment of the test. You fast overnight, you don't eat for 8-12 hours and then you do the test (numbers should be between 90-120), note that these numbers keep changing. This test tells us how much at the very moment, how much glucose there is in the blood.

2. **HbA1c** provides a longer-term trend, similar to an average, of how high blood sugar levels have been (controlled) over a period of time (2-3 months). It tells us if a patient was committed to taking his medication, if he was overeating sugar or if the medications are working for him or not.

- HbA1c can be expressed as a percentage (DCCT unit, used in the US/Jordan) or as a value in mmol(of HbA1c)/mol (total Hb in blood)(IFCC unit). ***IFCC is new and used in Europe***.
- Limitations of HbA1c test:
  - It does not capture short-term variations in blood glucose, exposure to hypoglycemia and hyperglycemia, or the impact of blood glucose variations on individuals' quality of life.

Table

- Normal individuals would have levels of glucose between 80-120
- Looking at glycosylated hemoglobin, 5-6 DCCT unit is normal. 5-6 means that 5%-6% of the hemoglobin is glycosylated.
- IFFC units rely more in millimole of hemoglobin per mole of total hemoglobin.
- IFFC is more accurate than percentages (you have no accurate measure for 5.5 for example) so using IFFC is more accurate because you can have more variation and indication of the actual amount of glycosylated hemoglobin.
- Fasting glucose levels for severe diabetes are really high, same thing goes to their level of glycosylated hemoglobin

BLOOD GLUCOSE		STATUS	HbA1c	
mmol/L	mg/dL		%	mmol/mol
5.4	97	Normal	5	31
7.0	126		6	42
8.6	155	Pre-Diabetes	7	53
10.2	184	Diabetes	8	64
11.8	212	Diabetes	9	75
13.4	241		10	86
14.9	268	Diabetes	11	97
16.5	297	Severe	12	108

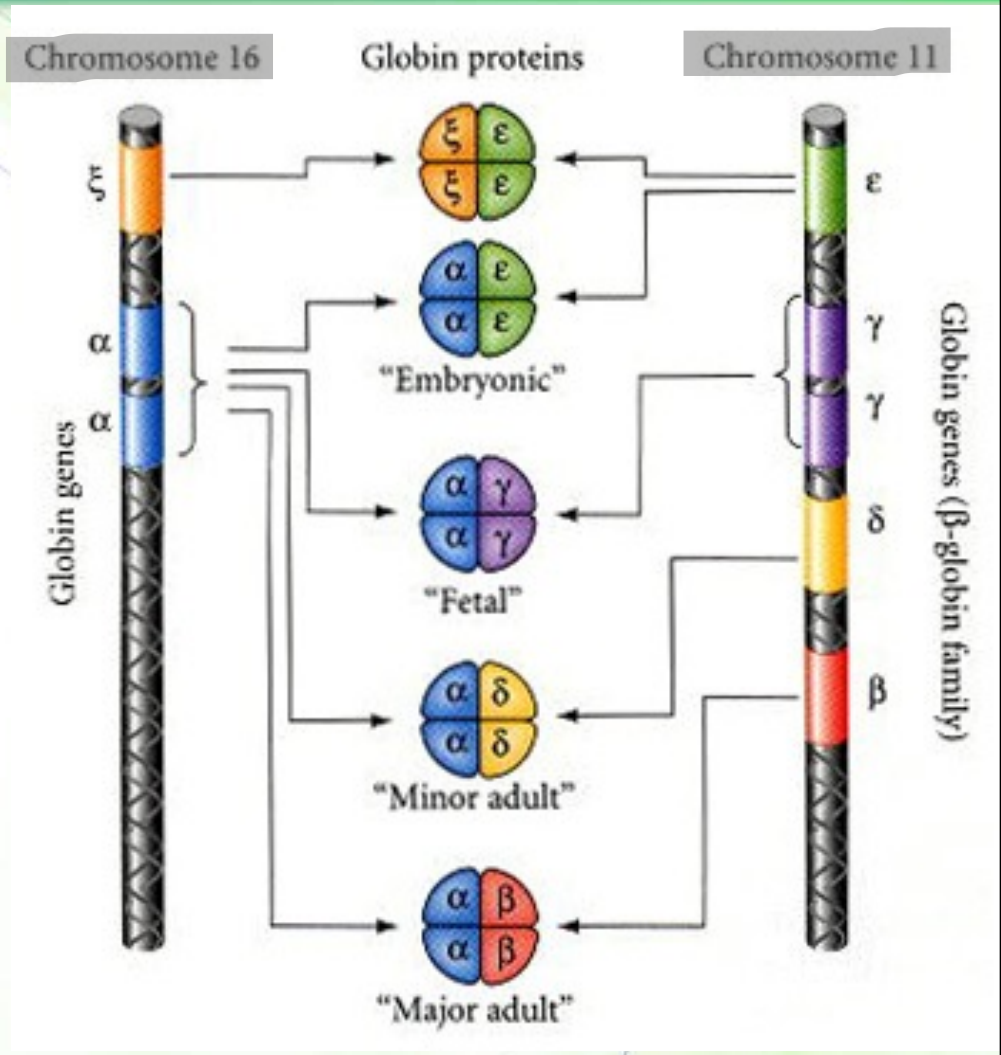
You have to memorize this table

Genetics of globin synthesis

The switch of zeta and epsilon → alpha and gamma → beta and delta

The genes

- The α gene cluster contains two α genes (α1 α2) and ζ (zeta) gene.
- The β gene cluster contains β gene in addition to ε (epsilon) gene, two γ (gamma) genes, and δ (delta) gene.
- The gene order parallels order of expression.
- Genetic switching is controlled by a transcription factor-dependent developmental clock, independent of the environment.
- Premature newborns follow their gestational age.
  If a switch takes place at birth (after 9 months) it will also take place in premature babies
  (In other words, premature babies produce HbF until 9 months of age regardless of when they are born)

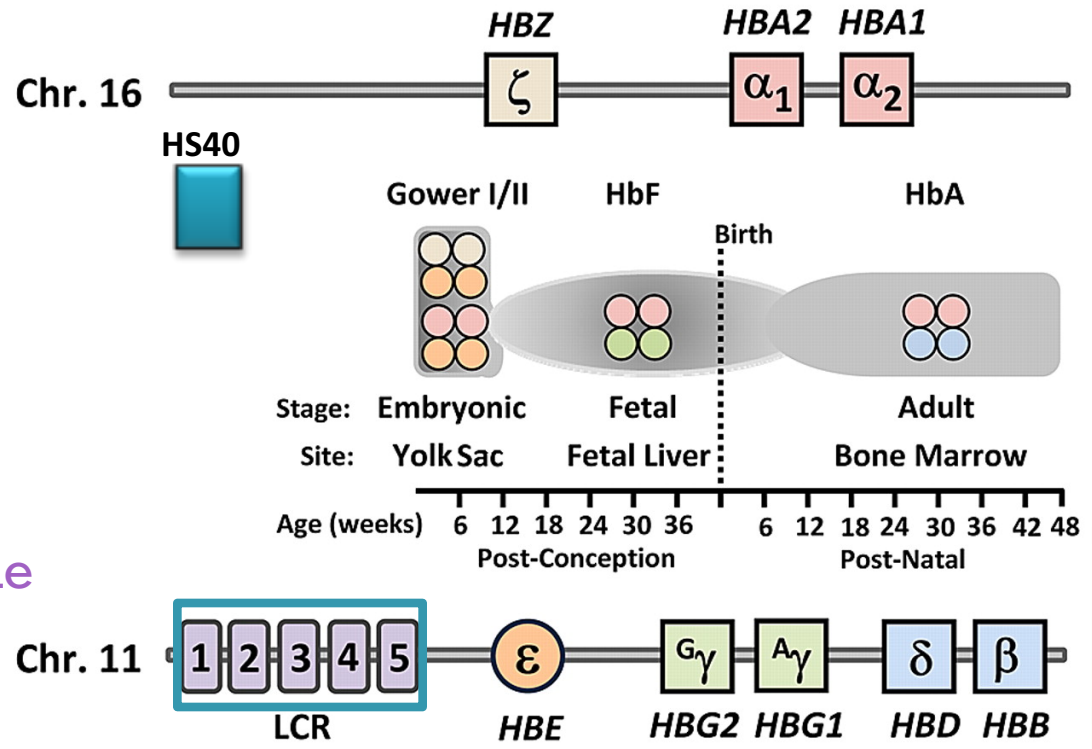


- In the alpha gene cluster, we have 2 genes (each chromosome contains 2 genes of alpha) whereas in the beta gene cluster, we have 1 beta gene.
  - We have one beta gene per one chromosome and we have 2 alpha genes per one chromosome

Locus structure

How is the switching of transcription and expression of genes from one to the other is done?

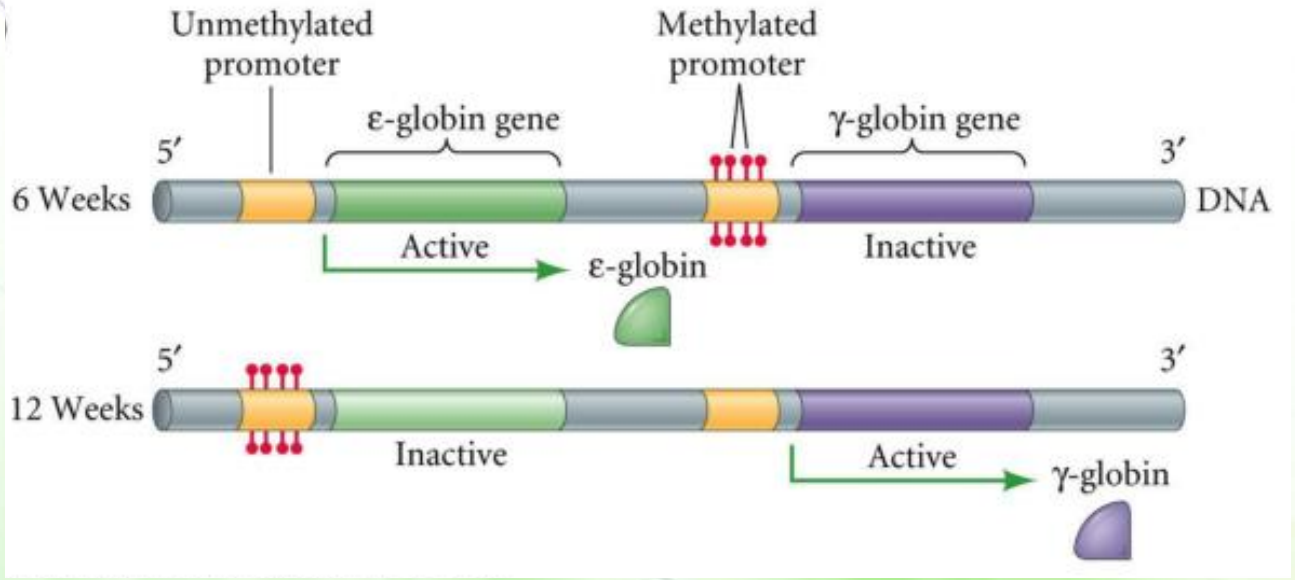
- This switching is controlled by a number of activators, silencers and repressors at regulatory sequences.
- Each gene has its promoter and regulatory sequences (activators, silencers).
- The α gene cluster is controlled by HS40 region (it's an enhancer region that lies upstream of the alpha gene cluster)
- With DNA looping, we have some proteins that bind to HS40 enhancer, these proteins can interact with regulatory elements present at the zeta gene, and different co-regulators would bind regulatory proteins on the promoter region of the alpha gene. So, we have different expression of these regulatory proteins that take place at different stages of development
- The β-globin cluster is controlled by a master enhancer called locus control region (LCR).

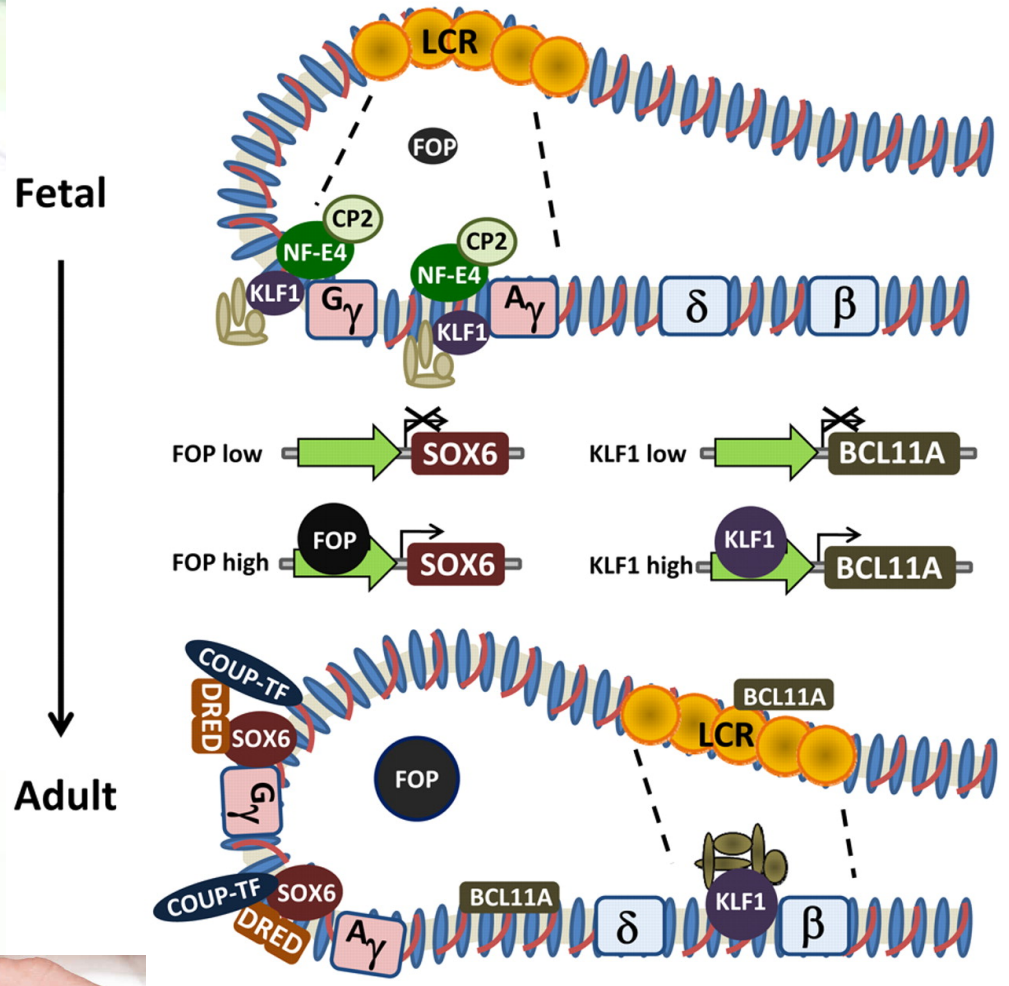


For the beta gene cluster, there is another regulatory protein known as locus control region, which also lies upstream of the beta gene cluster. It's the same idea though, you have proteins that bind to this region, then DNA loops around and they can interact with proteins that bind to the promoter region that lies upstream of different genes.

The mechanism of regulation

- In the fetal stage, there are interactions between proteins on the LCR region with regulators that control the expression of gamma genes, and as the switching from fetal to adult happens, there are interactions between proteins on the LCR region with other proteins that bind regulators of the beta gene and so on.
  The process is timed and this timing starts with fertilization.





The mechanism of regulation

- We have modification of DNA regions by epigenetic regulation.
- The mechanism requires *timed* expression of regulatory transcription factors for each gene, epigenetic regulation (e.g. acetylation, methylation), and chromatin looping (chromatin packaging → euchromatin VS heterchromatin).
- Note: treatment!! What if we treat people with defects in the beta gene (beta thalassemia) by inducing the expression of the gamma gene producing gamma polypeptide.