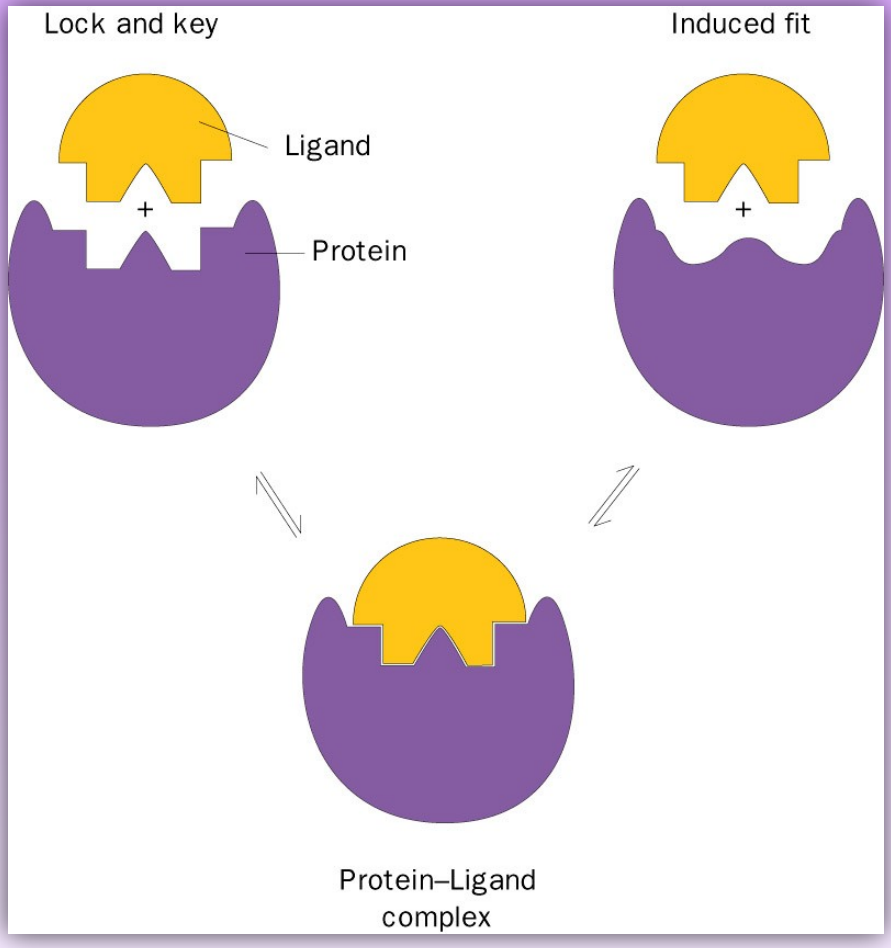
Ligand Binding, Allosterism and Cooperativity
Myoglobin & Hemoglobin

CHEM 420 – Principles of Biochemistry
Instructor – Anthony S. Serianni

Chapter 10: Voet/Voet, *Biochemistry*, 2011
Fall 2015

September 25 & 28



The two standard models of ligand binding: lock-and-key and induced fit

Problem: 1:1 A:P binding

Worked example

Mg^{2+} and ADP form a 1 : 1 complex. In an experiment, the concentration of ADP was kept constant at 80 μmol dm^{-3} and the concentration of Mg^{2+} varied. The following results were obtained.

Total Mg^{2+} (μmol dm^{-3})	20	50	100	150	200	400
Mg^{2+} bound to ADP (μmol dm^{-3})	11·6	26·0	42·7	52·8	59·0	69·5

Determine the dissociation constant for MgADP under these conditions.

Solution

At each value of the total Mg^{2+} concentration, the free Mg^{2+} concentration ([A] in the equations) can be evaluated simply by difference. The value of r is found by dividing the concentration of bound Mg^{2+} by the ADP concentration (i.e. 80 μmol dm^{-3}). We can convert the data into the correct form for graphical treatment.

Total Mg^{2+} (μmol dm^{-3})	20	50	100	150	200	400
Bound Mg^{2+} (μmol dm^{-3})	11·6	26·0	42·7	52·8	59·0	69·5
Free Mg^{2+} (μmol dm^{-3})	8·4	24·0	57·3	97·2	141·0	330·5
r	0·145	0·325	0·534	0·660	0·738	0·869
$\frac{1}{r}$	6·90	3·08	1·874	1·515	1·356	1·151
$\frac{1}{[Mg^{2+}]_{free}}$ (μmol dm^{-3})$^{-1}$	0·1190	0·0417	0·0175	0·0103	0·0071	0·0030
$\frac{r}{[Mg^{2+}]_{free}}$ (μmol dm^{-3})$^{-1}$	0·0173	0·0135	0·0093	0·0068	0·0052	0·0026

The appropriate plots ($1/r$ vs. $1/[Mg^{2+}]_{free}$ and $r/[Mg^{2+}]_{free}$ vs. r) are shown in Figs. 4.1 and 4.2 respectively.

Of course we would not normally do both, but this is done here for the sake of completeness.

From both plots we obtain the result that $K_d = 50\ \mu mol\ dm^{-3}$ or 50×10^{-6} (mol dm^{-3}).† It is noticeable that in the 'double reciprocal plot' (Fig. 4.1) the experimental points are much more unevenly spaced than in the alternative plot (Fig. 4.2). This has led many workers to prefer the type of plot shown in Fig. 4.2 for the analysis of binding data, since it is rather easier in this case to draw the best straight line through the experimental points. In any experiment it is important to make a proper analysis of the distribution of errors in the method of plotting the data. This is also true in the analogous plots which are used in the analysis of enzyme kinetic data (see Chapter 10) and is discussed in the books by Cornish-Bowden mentioned in the reading list.

It is possible to simplify the experiment considerably if one component is present in a considerable excess over the other. For instance, suppose that P is present at a concentration of 1 μmol dm^{-3} and [A] is varied from 50 μmol dm^{-3} up to 500 μmol dm^{-3}. Then, throughout the titration very little of the total A is actually bound to P and it is a very good approximation to write $[A]_{free} = [A]_{total}$. The equations would then become

$$\frac{1}{r} = 1 + \frac{K_d}{[A]_{total}} \quad \text{and} \quad \frac{r}{[A]_{total}} = \frac{1}{K_d} - \frac{r}{K_d}.$$

We often use this simplification in enzyme kinetic work. The substrate (S) of the enzyme is almost always greatly in excess over the enzyme concentration. (i.e. $[S]_{free} = [S]_{total}$.) In this type of work, we use the velocity of the enzyme catalysed reaction (v) to give a measure of r (the amount of S bound to E) in the equations, since only the ES complex shows enzyme activity. We shall see in Chapter 10 that we do in fact plot $1/v$ vs. $1/[S]_{total}$ or $v/[S]_{total}$ vs. v to obtain the *Michaelis constant* which characterizes the interaction of the enzyme with its substrate.‡

The simplification of the algebra which is achieved by setting $[S]_{free}$ equal to $[S]_{total}$ is illustrated in the following example.

Worked example

Consider the equilibrium $E + S \leftrightharpoons ES$, and let K_s be the dissociation constant of the ES complex.

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† Strictly speaking, K_d is dimensionless, as is pointed out earlier in Chapter 3. However, biochemists invariably quote units, i.e. $K_d = 50\ \mu mol\ dm^{-3}$. Referred to a 1 mol dm^{-3} standard state, we could say $K_d = 50 \times 10^{-6}$. We shall adopt the convention of writing dissociation constants as for example, 50×10^{-6} (μmol dm^{-3}) where the bracketed quantity refers to the standard state of a 1 mol dm^{-3} solution.

‡ *Cautionary note*: the Michaelis constant (K_m) is not generally a true dissociation constant (see Chapter 10).

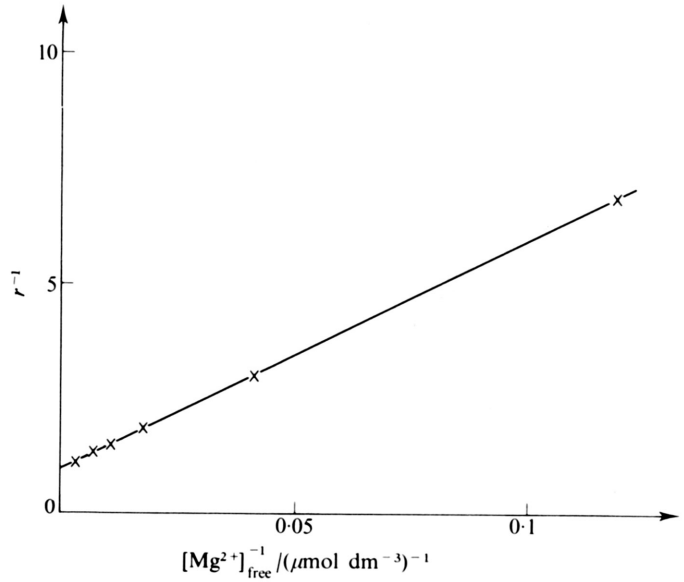


FIG. 4.1. Plot of binding data in 'Worked example' according to eqn (4.6).

double-reciprocal plot

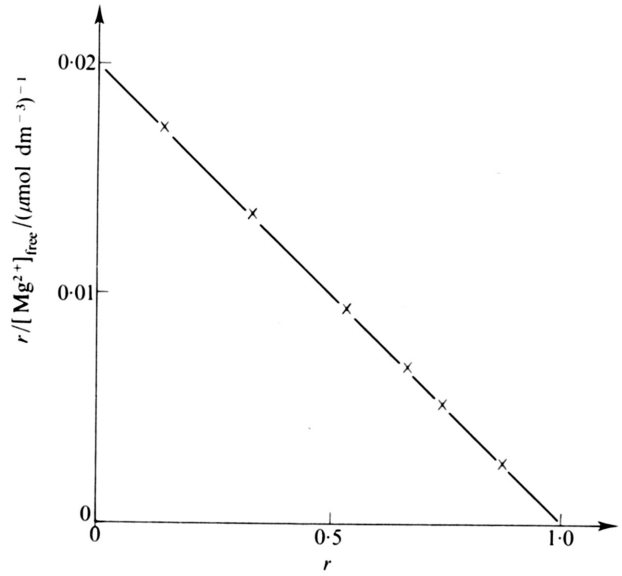


FIG. 4.2. Plot of binding data in 'Worked example' according to eqn (4.7).

single reciprocal plot

Worked example

In an experiment the concentration of an enzyme is kept constant at 11 μmol dm^{-3}, and the concentration of inhibitor [I] varied. The following results were obtained.

$[I]_{total}$(μmol dm^{-3})	5·2	10·4	15·6	20·8	31·2	41·6	62·4
$[I]_{free}$(μmol dm^{-3})	2·3	4·8	7·95	11·3	18·9	27·4	45·8

Determine the dissociation constant for the enzyme-inhibitor complex and the number of inhibitor binding sites on the enzyme.

Solution

At each value of $[I]_{total}$ we can evaluate $[I]_{bound}$ by subtraction; r is obtained by dividing $[I]_{bound}$ by the concentration of enzyme (i.e. 11 μmol dm^{-3}). The following table can be constructed:

$[I]_{total}$ (μmol dm^{-3})	5·2	10·4	15·6	20·8	31·2	41·6	62·4
$[I]_{free}$ (μmol dm^{-3})	2·3	4·8	7·95	11·3	18·9	27·4	45·8
$[I]_{bound}$ (μmol dm^{-3})	2·9	5·6	7·65	9·5	12·3	14·2	16·6
r	0·264	0·510	0·695	0·864	1·118	1·291	1·510
$\frac{1}{r}$	3·793	1·964	1·438	1·158	0·894	0·775	0·663
$\frac{r}{[I]_{free}}$ (μmol dm^{-3})$^{-1}$	0·115	0·106	0·087	0·076	0·059	0·047	0·033
$\frac{1}{[I]_{free}}$ (μmol dm^{-3})$^{-1}$	0·435	0·208	0·126	0·088	0·053	0·036	0·022

The two binding plots are shown in Figs. 4.3 and 4.4 respectively. From the 'double reciprocal plot' we find that the intercept on the y axis is 0·5, so that $n = 2$. The slope of the line is 7·6 so that $K_d = 15{\cdot}2 \times 10^{-6}$ (mol dm^{-3}).

From the 'Scatchard' plot (Fig. 4.4), again we find that $n = 2$ and the value of K_d is $15{\cdot}2 \times 10^{-6}$ (mol dm^{-3}). It is also clear that the sites are equivalent and independent, since, otherwise a curved plot would be expected.

As in the previous example (Mg^{2+} and ADP) we find that the Scatchard plot has a more even spacing of the experimental points, than does the 'double reciprocal'. (However, this need not always be the case.)

It is important to note that in order to determine the number of binding sites n accurately it is essential to cover as wide a range of the total saturation curve as possible. Roughly, the required range is the region

Problem:
Multiple equivalent binding sites;
nA and P

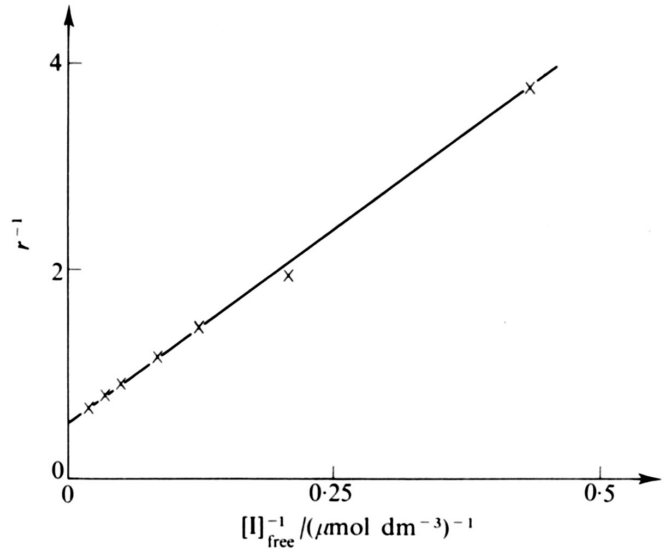


FIG. 4.3. Plot of binding data in 'Worked example' according to eqn (4.9).

double reciprocal plot
(Hughes-Klotz plot)

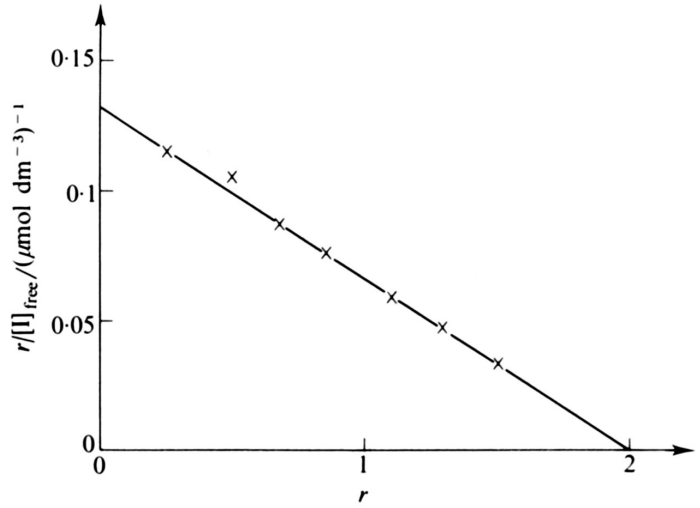


FIG 4.4. Plot of binding data in 'Worked example' according to eqn (4.10).

single reciprocal plot
(Scatchard plot)

Multiple binding site equilibria: Non-equivalent

(Non-equivalent ligand sites on a macromolecule)

Non-linear binding plots: ligand binding sites are not equivalent

e.g. O_2 binding to hemoglobin — tetrameric protein (4 subunits)

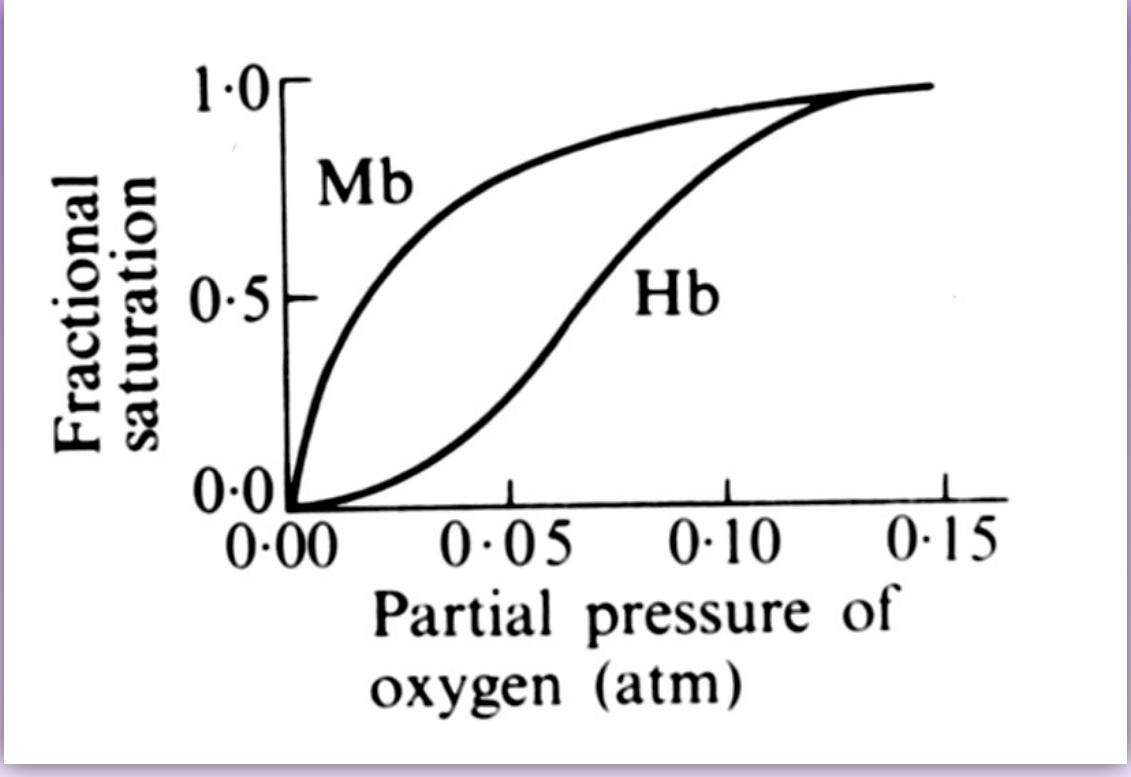
sigmoidal binding curve vs

hyperbolic for myoglobin — monomeric protein (one subunit)

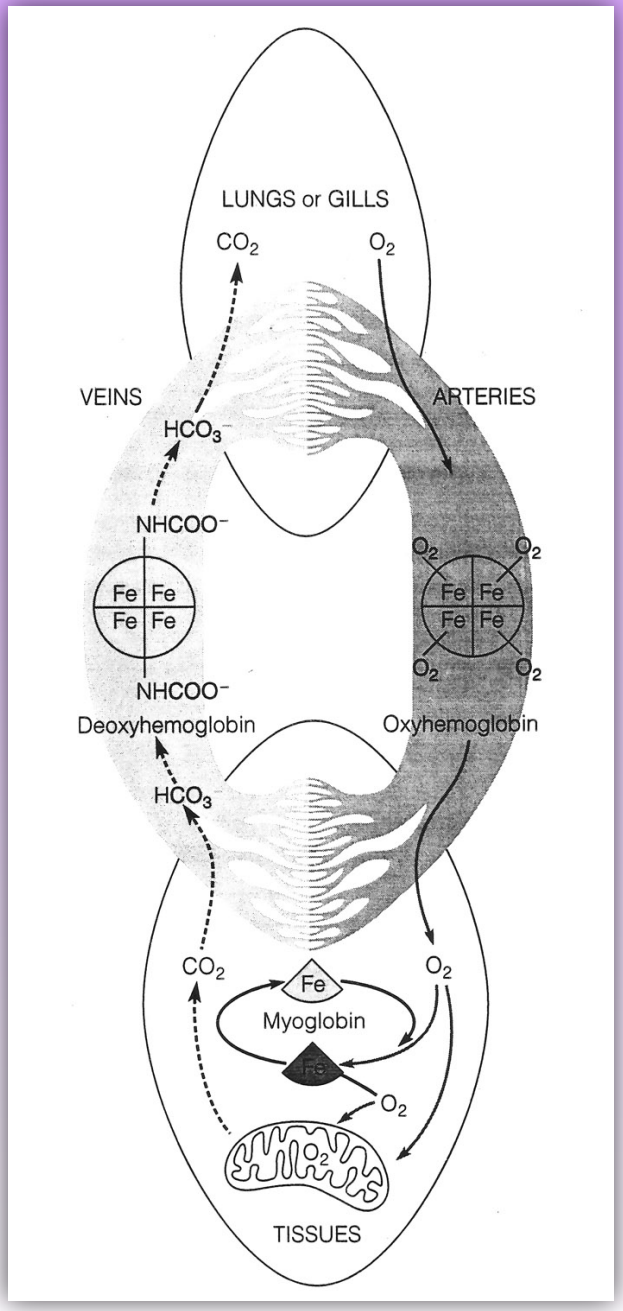
Hb displays positive cooperativity

(binding of first O_2 enhances the binding of subsequent O_2)

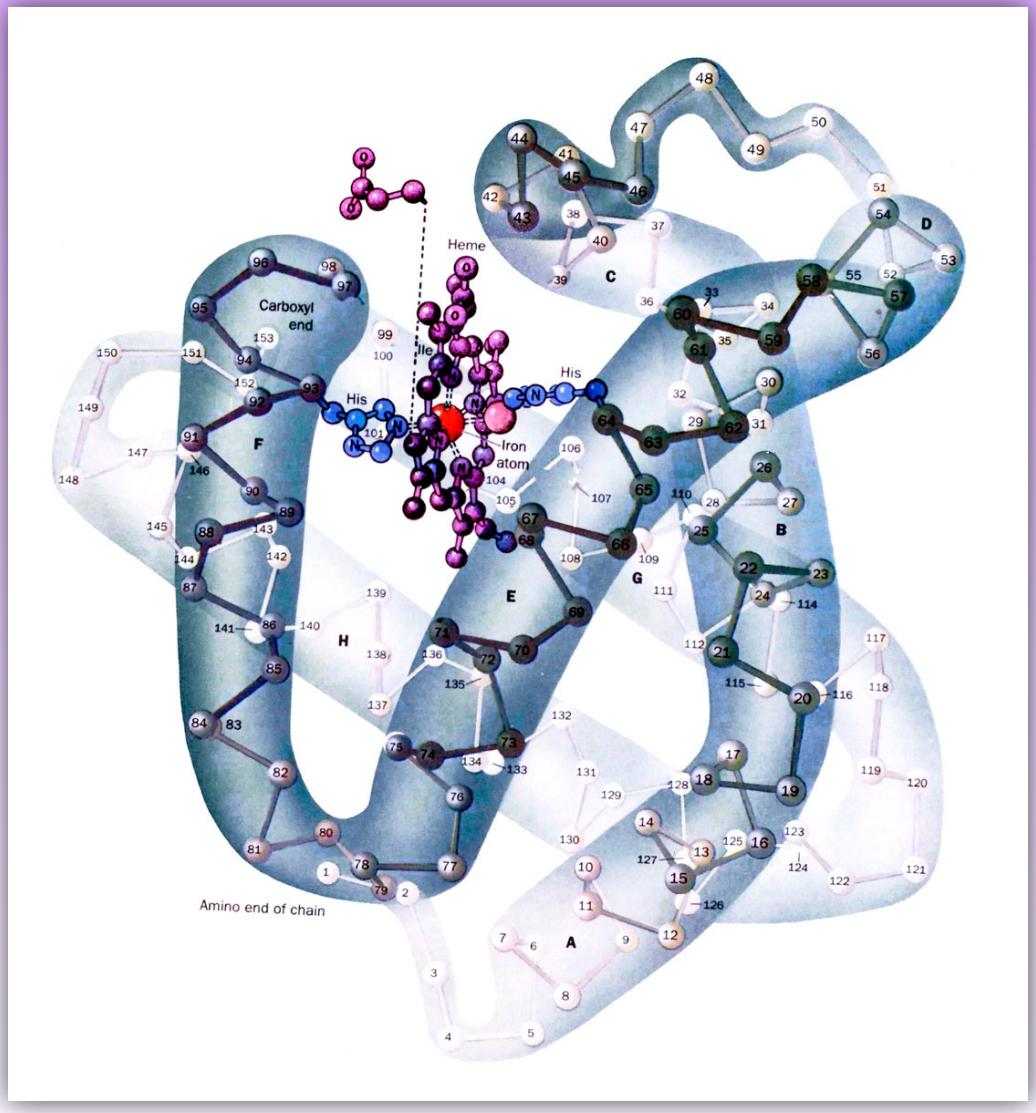
Type of binding can be recognized from inspection of saturation curve, double-reciprocal plot, or Scatchard plot.



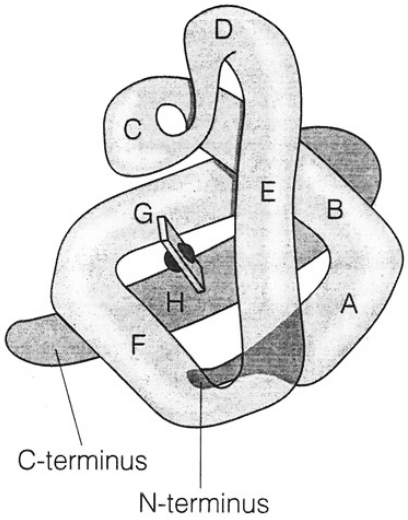
O_2 saturation curves for myoglobin (Mb) and hemoglobin (Hb)



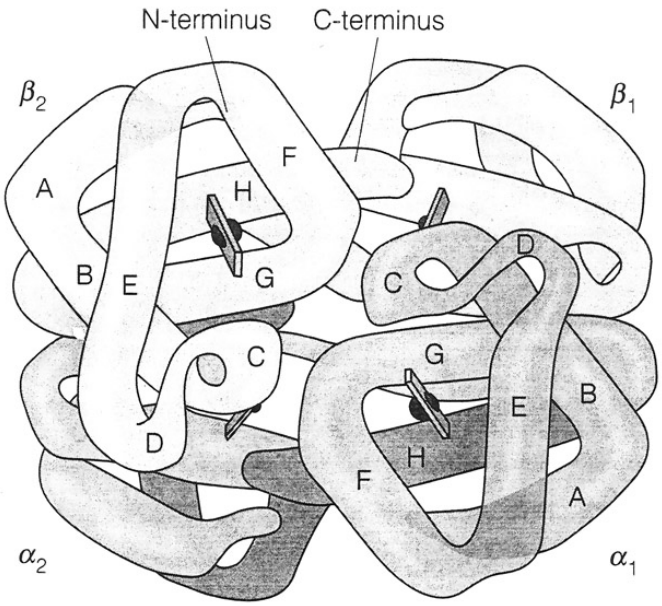
Biological functions of myoglobin and hemoglobin



3D structure of myoglobin showing bound heme group

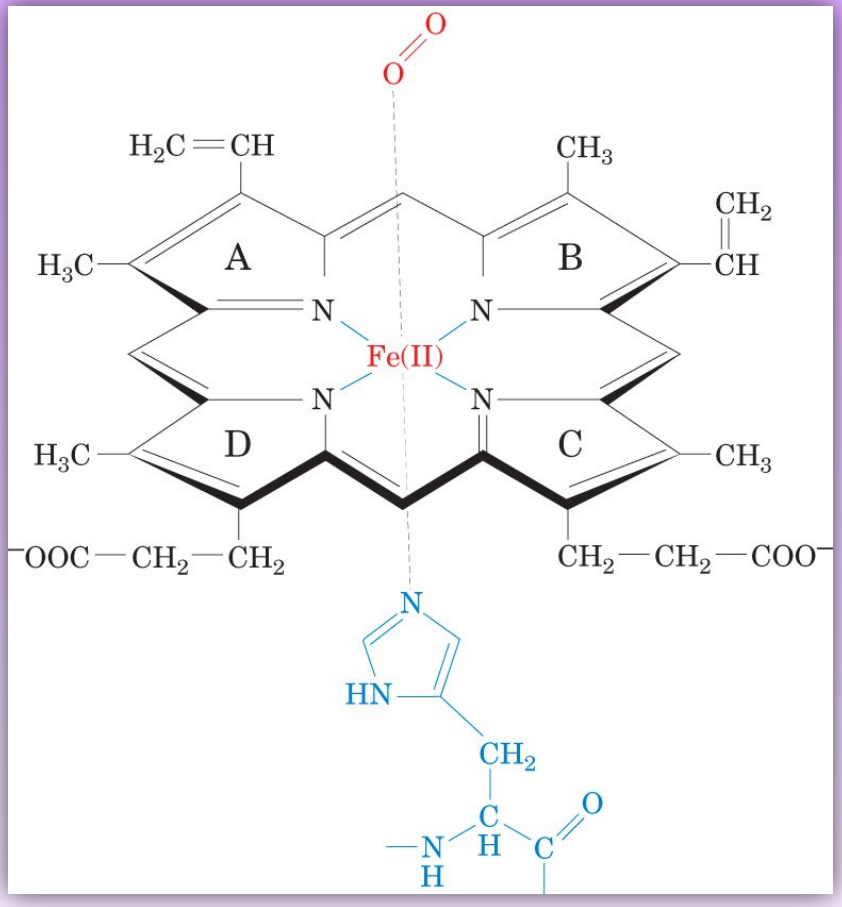


Myoglobin



Hemoglobin

Comparison of the functional forms of myoglobin (monomer) and hemoglobin (tetramer)



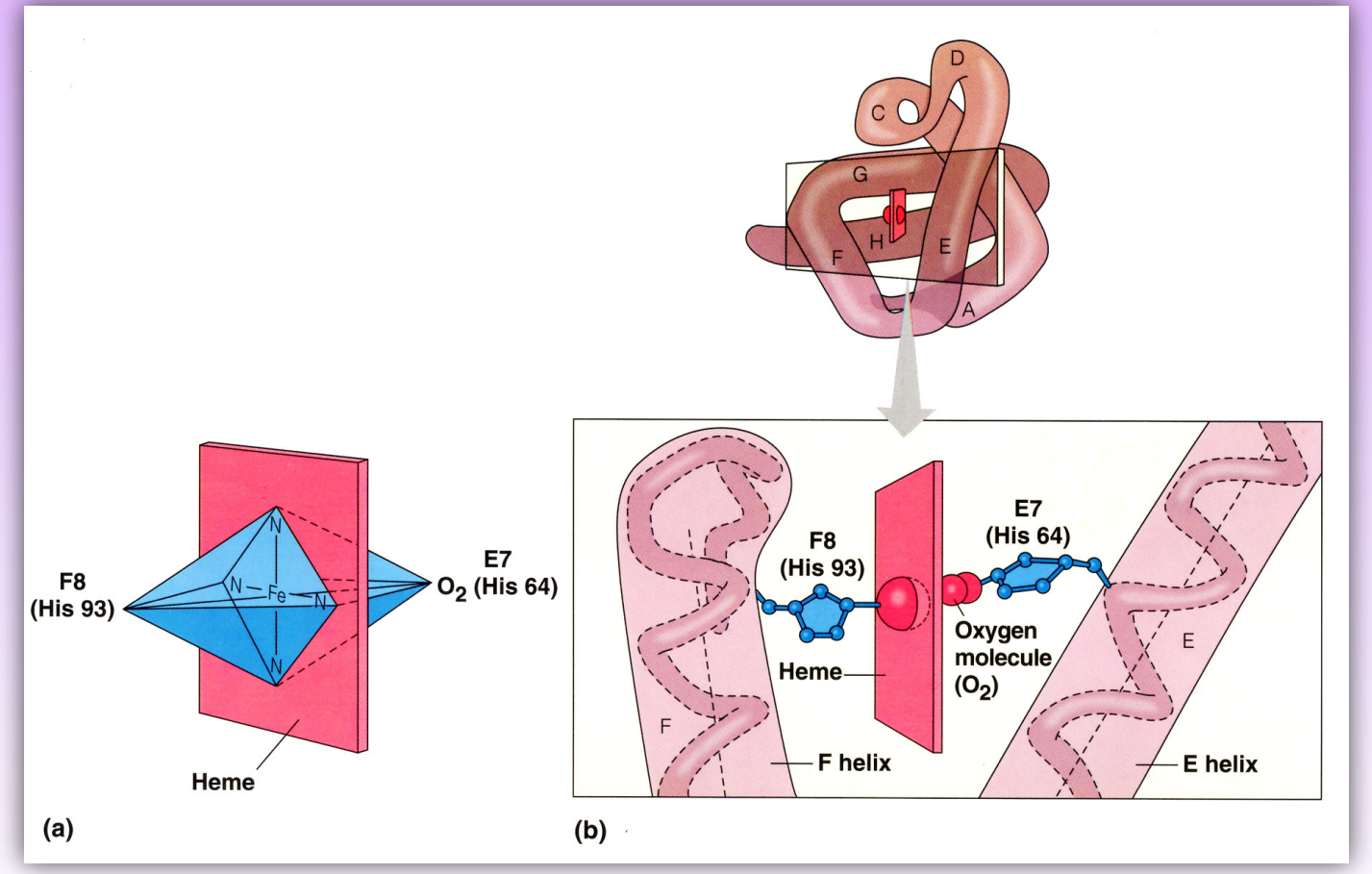
Iron(II) ligands (6):

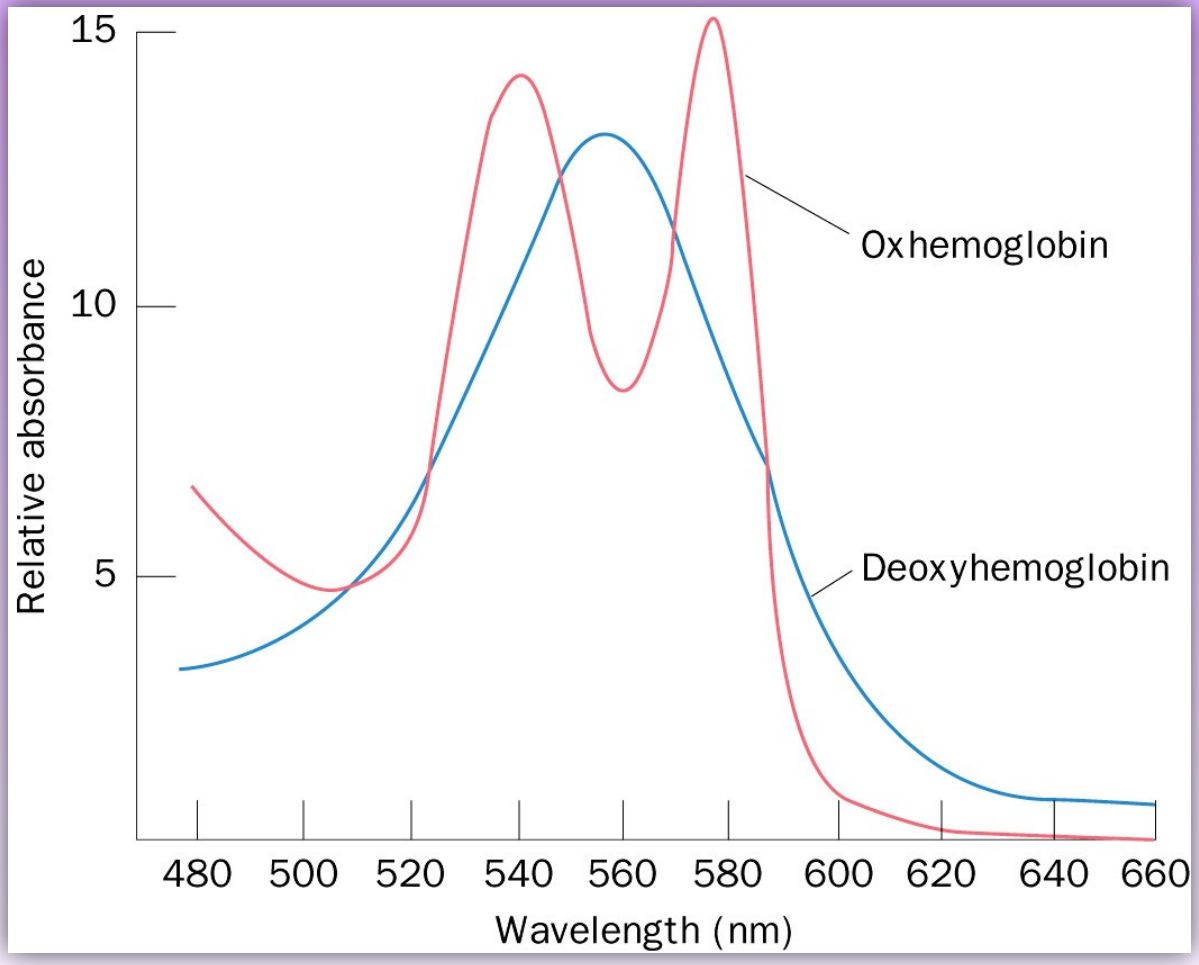
- 4 from heme
- proximal His93 (F helix)
- variable ligand (O_2, CO or H_2O)

Distal His64 (E-helix) influences variable ligand affinity; not directly bound to the iron center

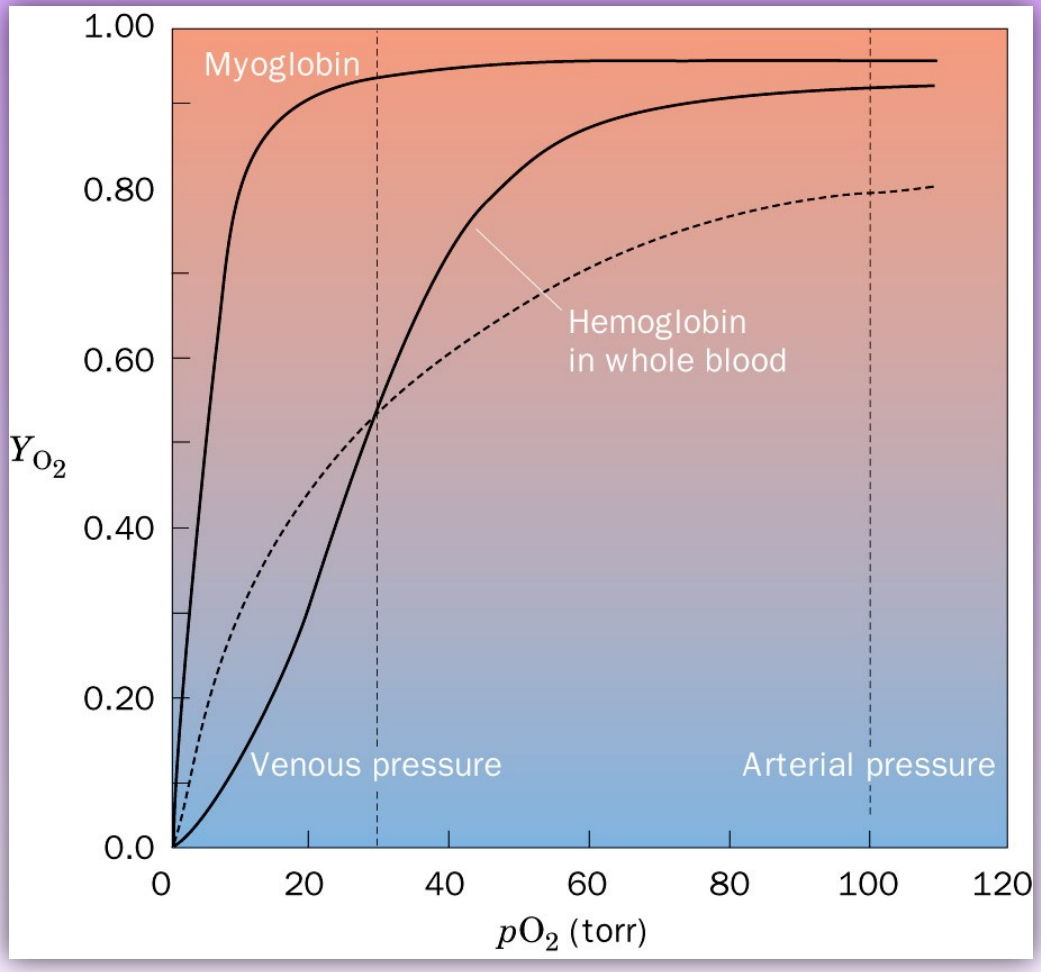
The heme O_2 binding site in myoglobin (Mb) and hemoglobin (Hb): prosthetic group tightly bound between Helix E and Helix F.

The heme group: Distal histidine 64 and proximal histidine 93





Visible absorption spectra of oxygenated (oxyHb) and deoxygenated (deoxyHb) hemoglobin



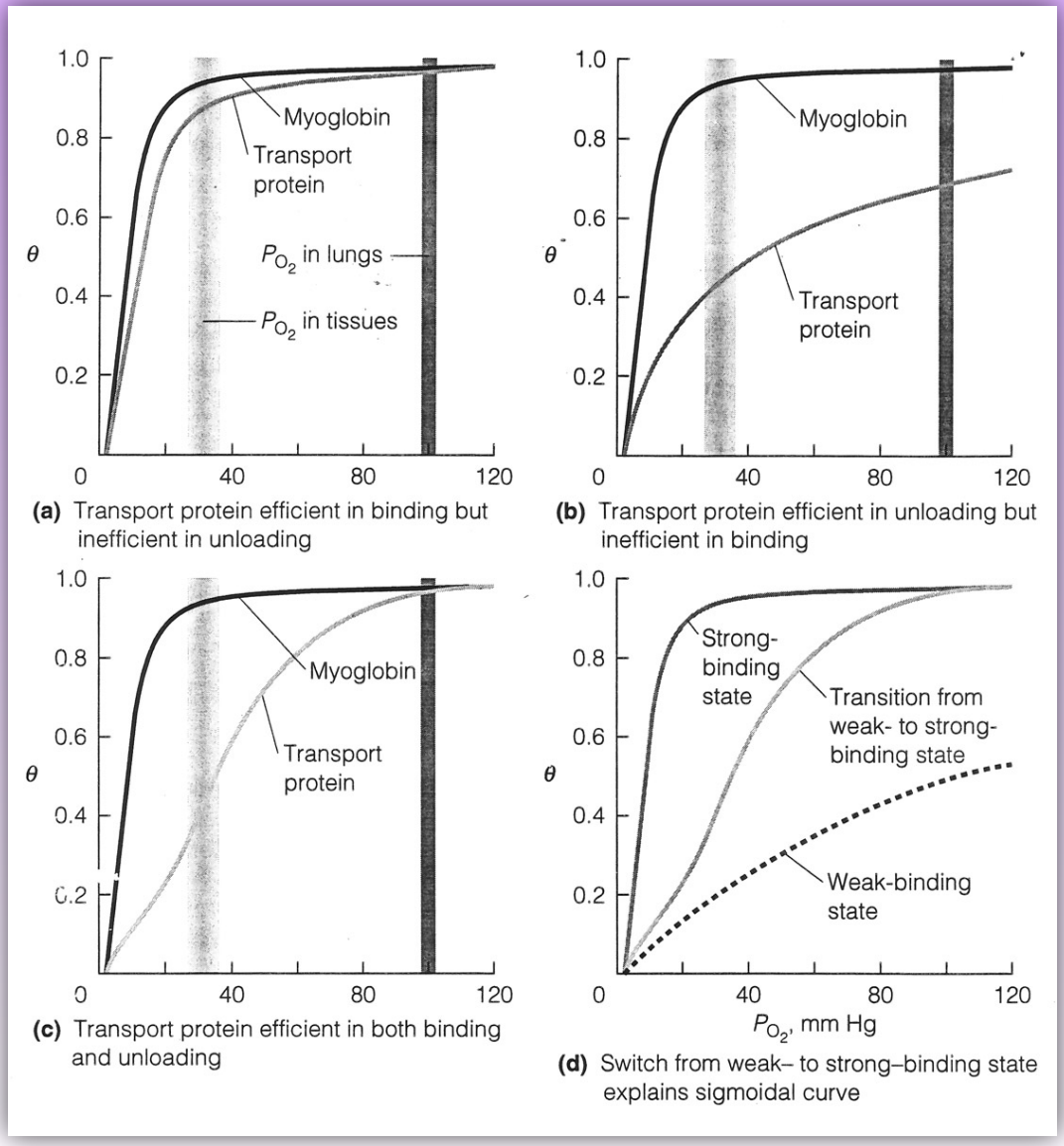
y_{O2} = **fractional saturation** = fraction of O_2-binding sites occupied by O_2

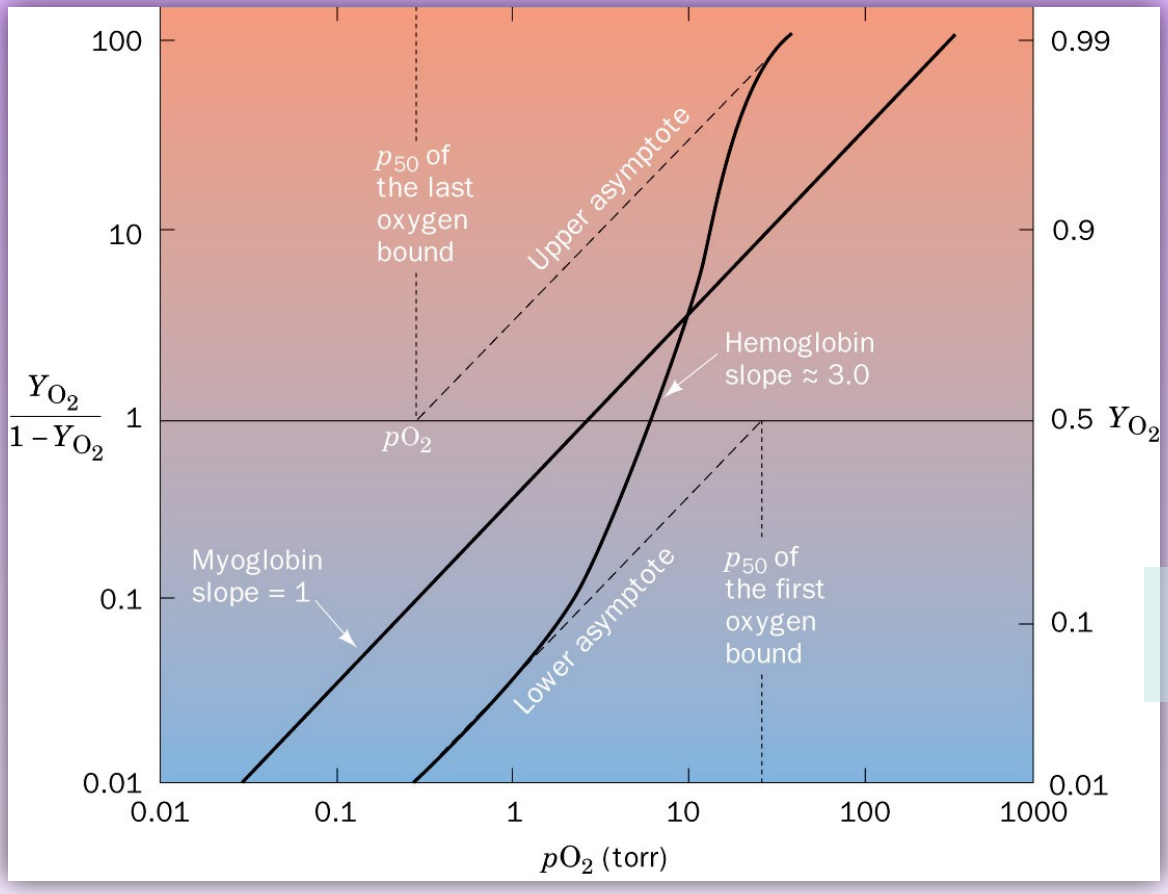
Mb = hyperbolic
Hb = sigmoidal

p_{50} (Mb) = 4 mm
p_{50} (Hb) = 30 mm

O_2 dissociation curves (binding isotherms) of Mb and of Hb in whole blood

Optimizing O_2 storage and O_2 transport proteins

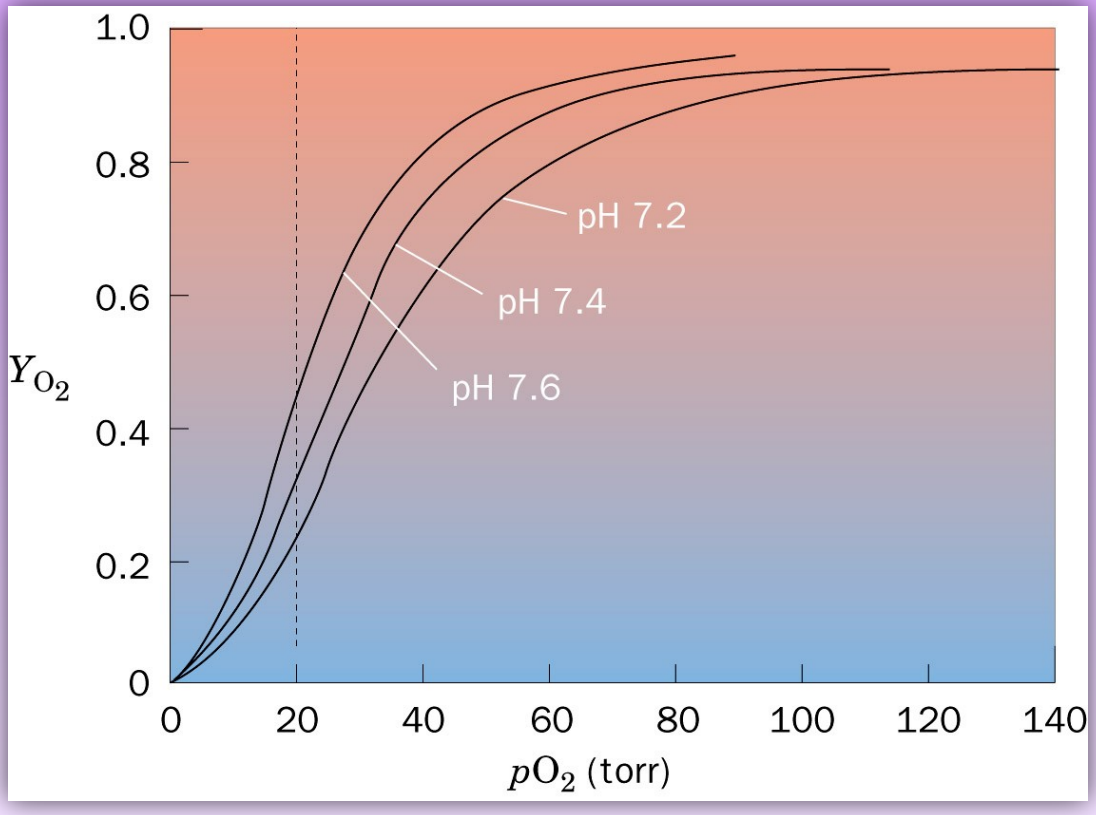




***n* = Hill coefficient** (slope): related to the degree of cooperativity among interacting ligand binding sites

n > 1: positive cooperativity
n = 1: non-cooperative

Hill plots for Mb and purified ("stripped") Hb



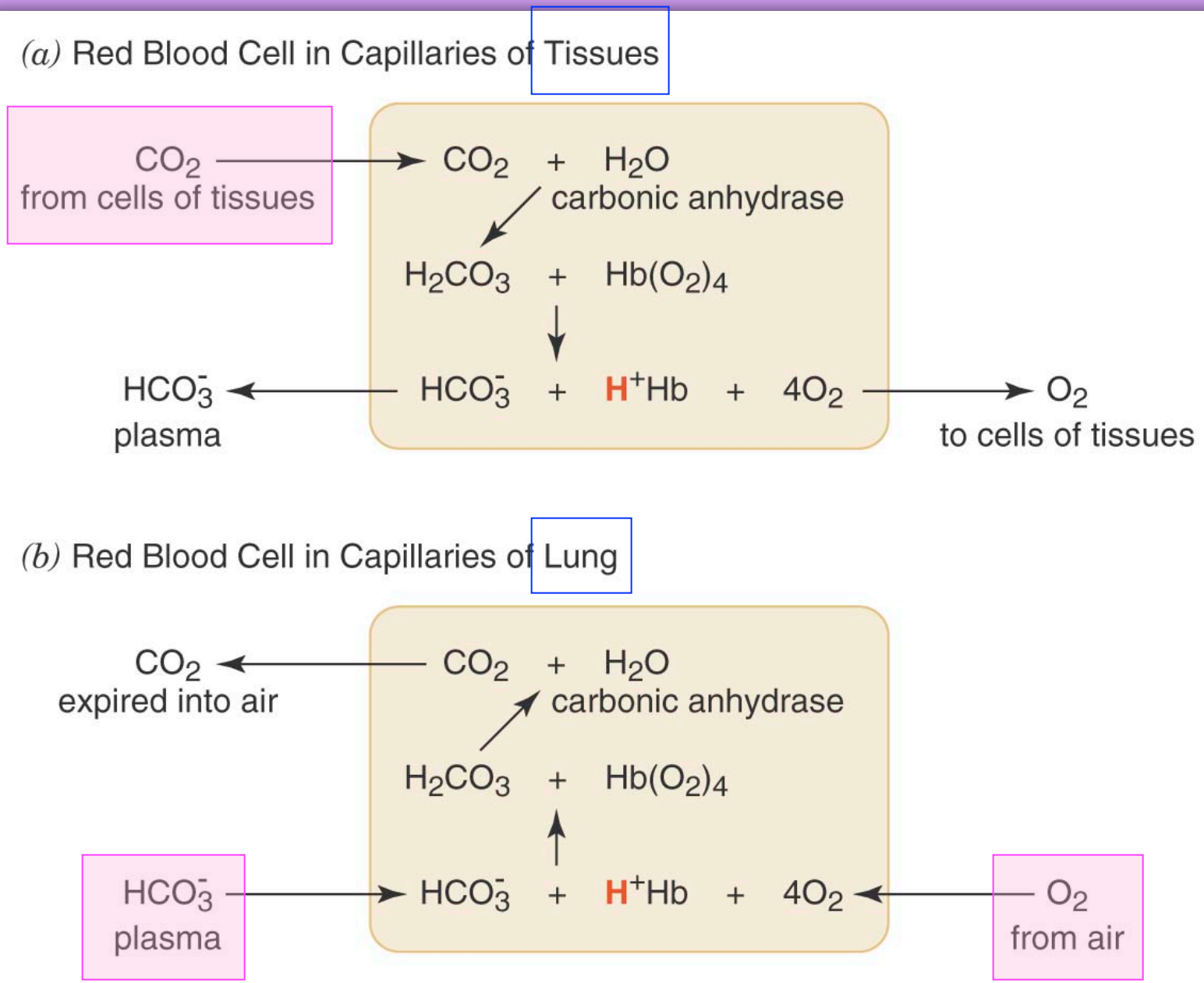
Effect of pH on the O_2 dissociation curve of Hb: **the Bohr effect**

$$Hb(O_2)_nH_x + O_2 \leftrightarrow Hb(O_2)_{n+1} + xH^+ \qquad x \approx 0.6$$

CO_2 removal from tissue: <u>dissolved bicarbonate in blood</u>; covalent transport via Hb

$$CO_2 + H_2O \leftrightarrow H^+ + HCO_3^-$$

Catalyzed by carbonic anhydrase in erythrocytes; decreased pH of actively metabolizing tissue results in enhanced release of O_2 from Hb


(a) Red Blood Cell in Capillaries of Tissues
CO2 from cells of tissues
CO2 + H2O
carbonic anhydrase
H2CO3 + Hb(O2)4
HCO3- + H+Hb + 4O2
HCO3- plasma
O2 to cells of tissues
(b) Red Blood Cell in Capillaries of Lung
CO2 expired into air
CO2 + H2O
carbonic anhydrase
H2CO3 + Hb(O2)4
HCO3- + H+Hb + 4O2
HCO3- plasma
O2 from air

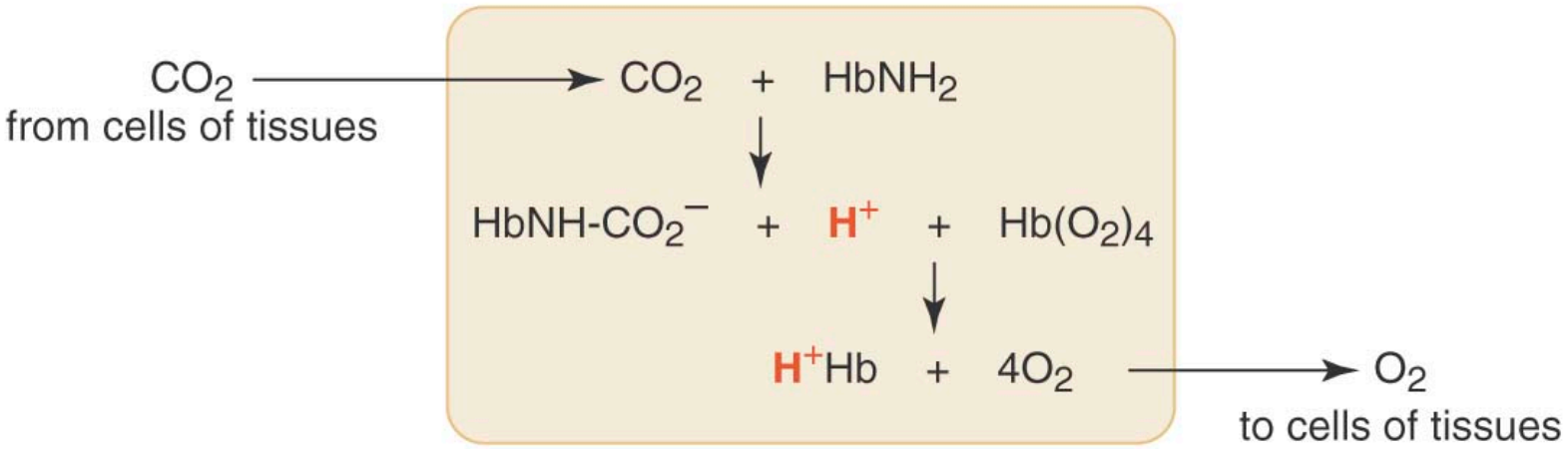
CO_2 removal from tissue: dissolved bicarbonate in blood; <u>covalent transport via Hb</u>

Carbamate Formation (*N*-termini)

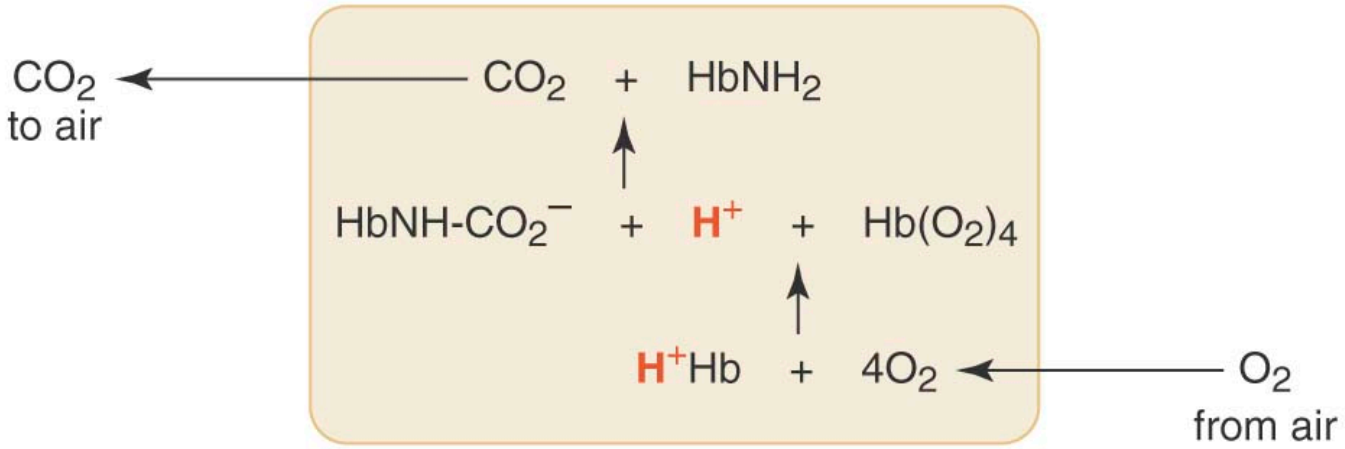
$$R\text{-}NH_2 + CO_2 \leftrightarrow R\text{-}NH\text{-}COO^- + H^+$$

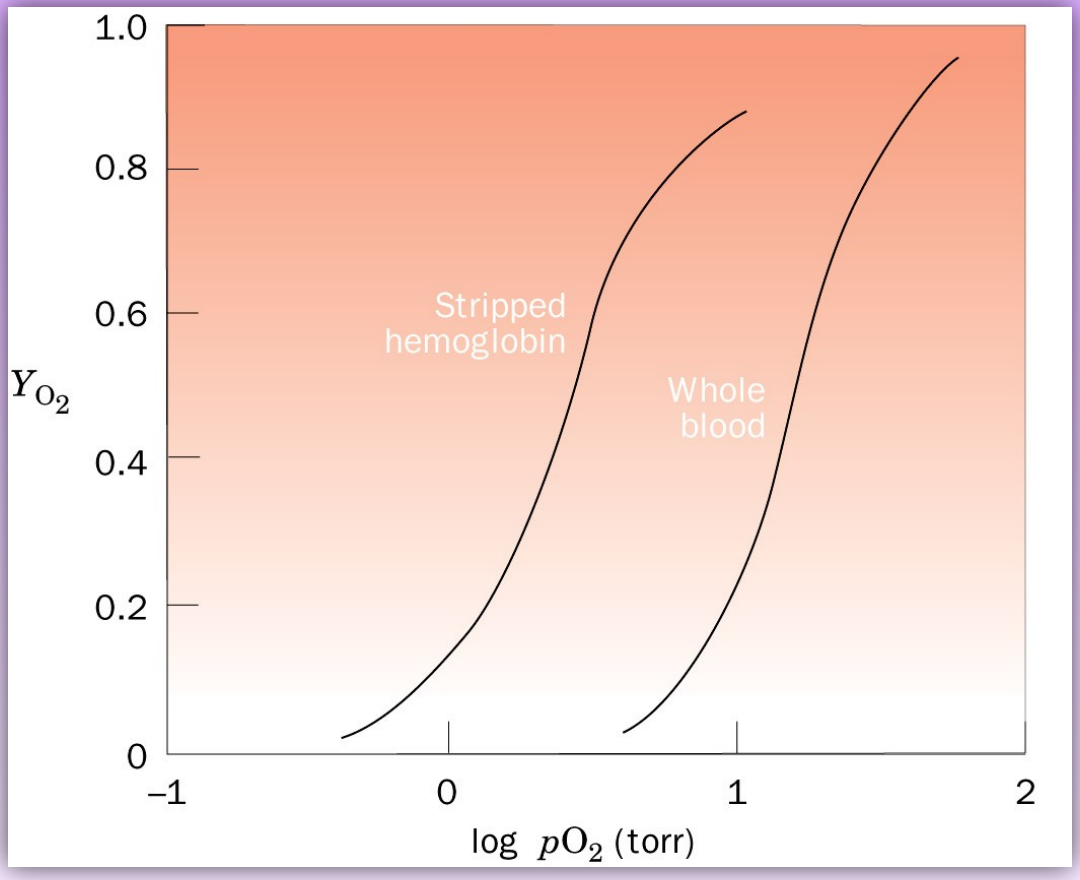
DeoxyHb binds more CO_2 as carbamate than does oxyHb

(a) Red Blood Cell in Capillaries of Tissues



(b) Red Blood Cell in Capillaries of Lung





Effect of 2,3-BPG present in whole blood on Hb oxygen affinity

One 2,3-BPG molecule binds per Hb tetramer

Comparison of the O_2 dissociation curves of "stripped" Hb and whole blood in 0.01*M* NaCl at pH 7.0

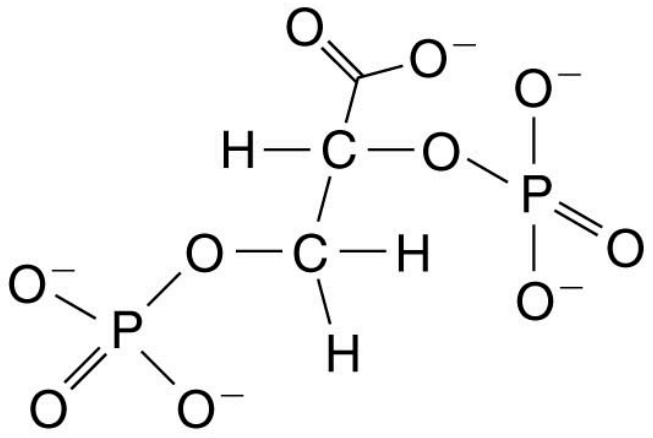
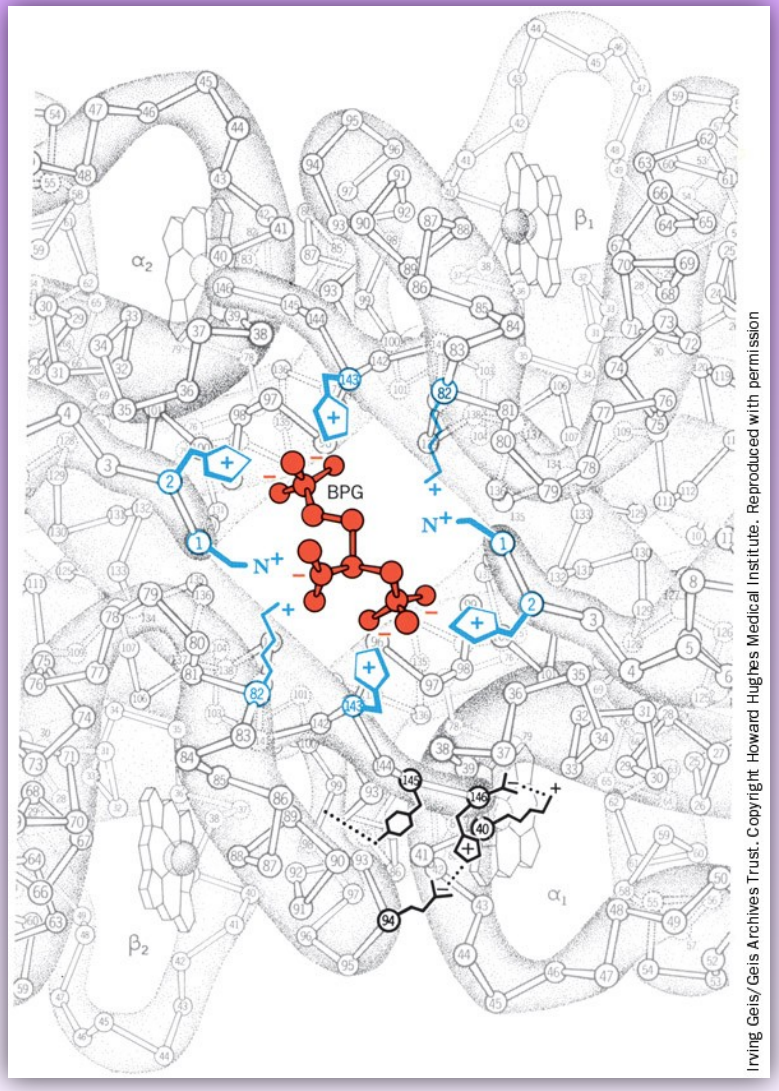


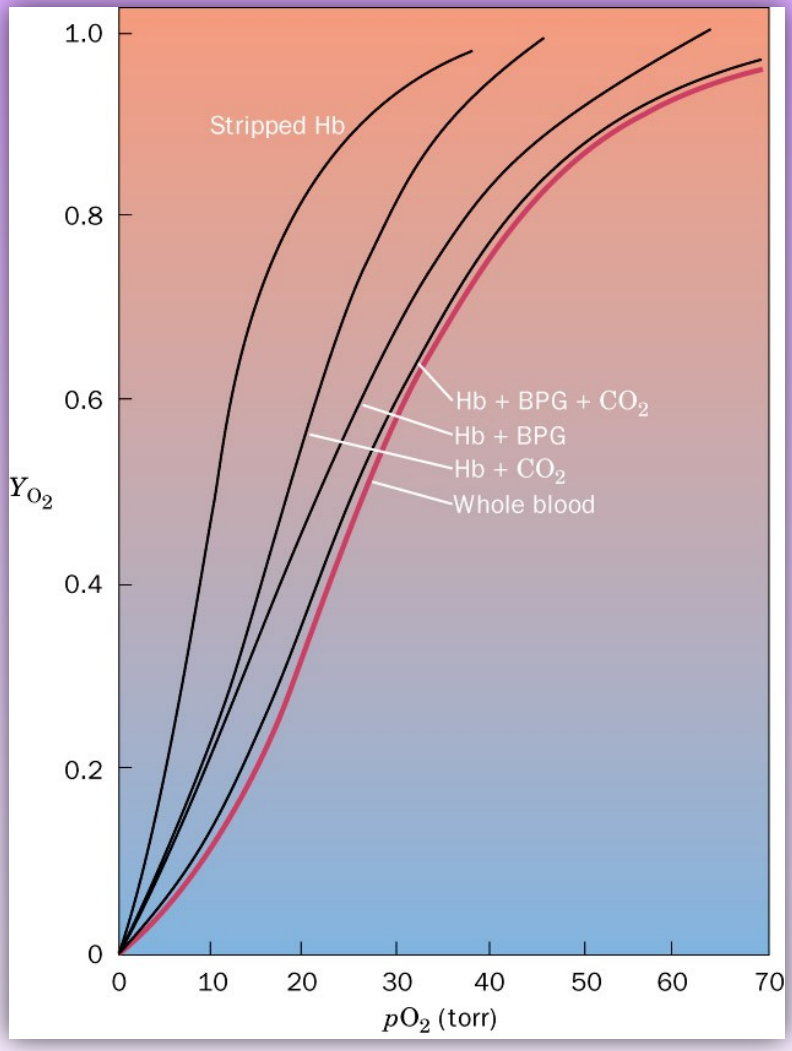
Figure 9.33. Structure of 2,3-bisphosphoglycerate (BPG). Molecule has a charge of –5 at pH 7.4.



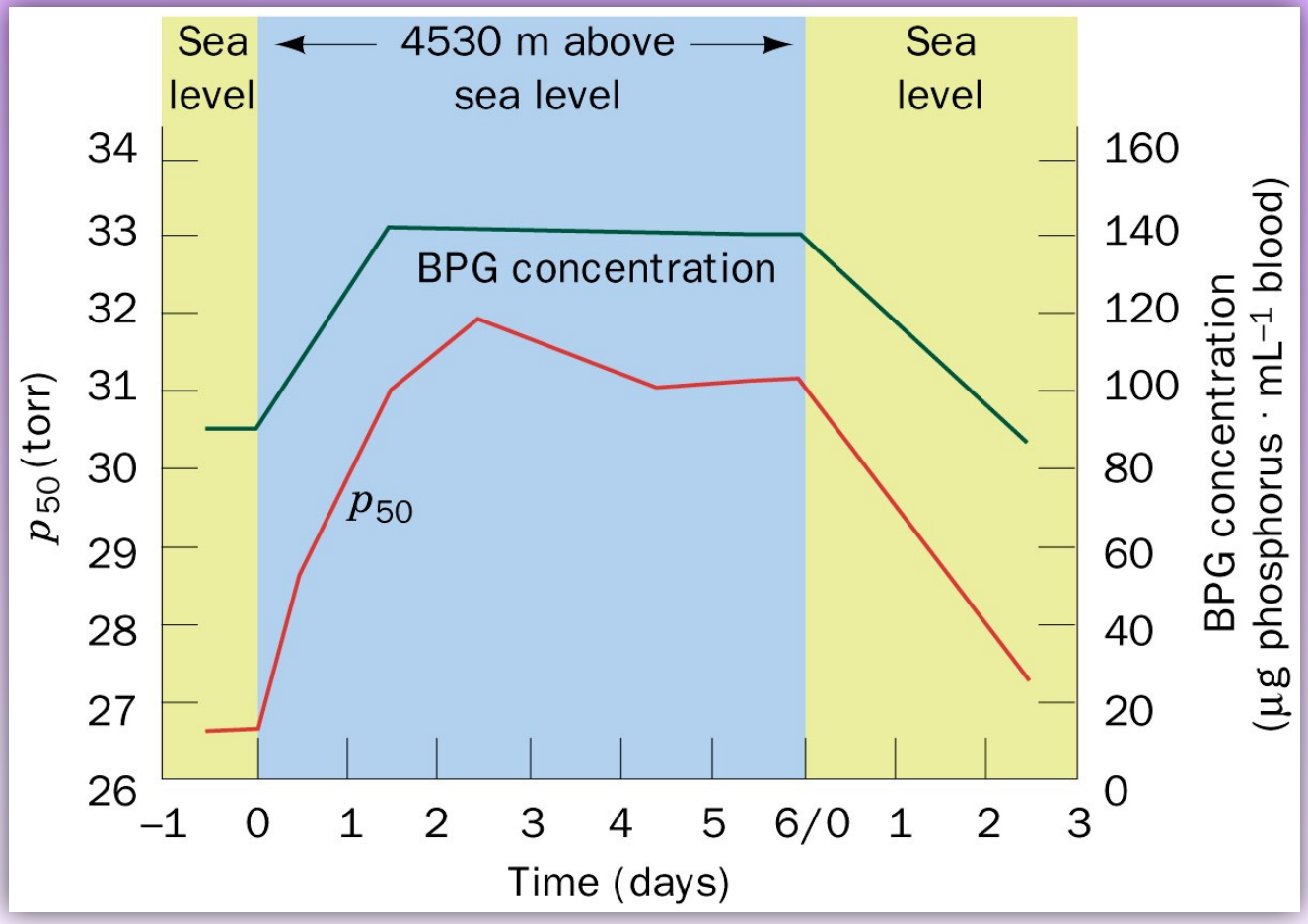
BPG binding pocket is lined with positive charge (Lys, His, *N*-termini): complementary to BPG's negative charge.

BPG preferentially binds to deoxyHb: central cavity is smaller in oxyHb

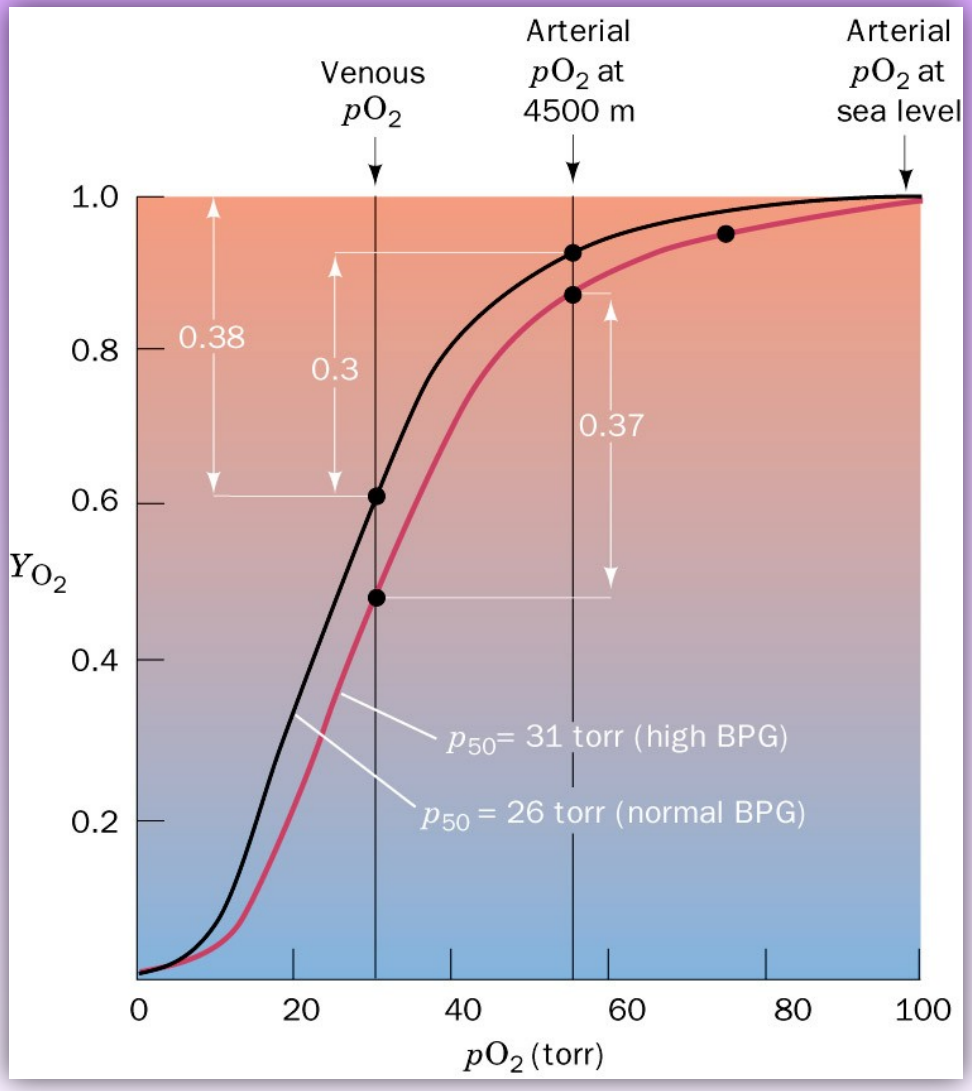
Binding of BPG to deoxyHb: selective stabilization of the T form



The effects of 2,3-BPG and CO_2, both separately and combined, on the O_2 dissociation curve of Hb compared with that of whole blood (*red curve*)



The effect of high-altitude exposure on the p_{50} and the 2,3-BPG concentration of blood in sea level–adapted individuals



The O_2 dissociation curves of blood adapted to sea level (*black curve*) and to high altitude (*red curve*).

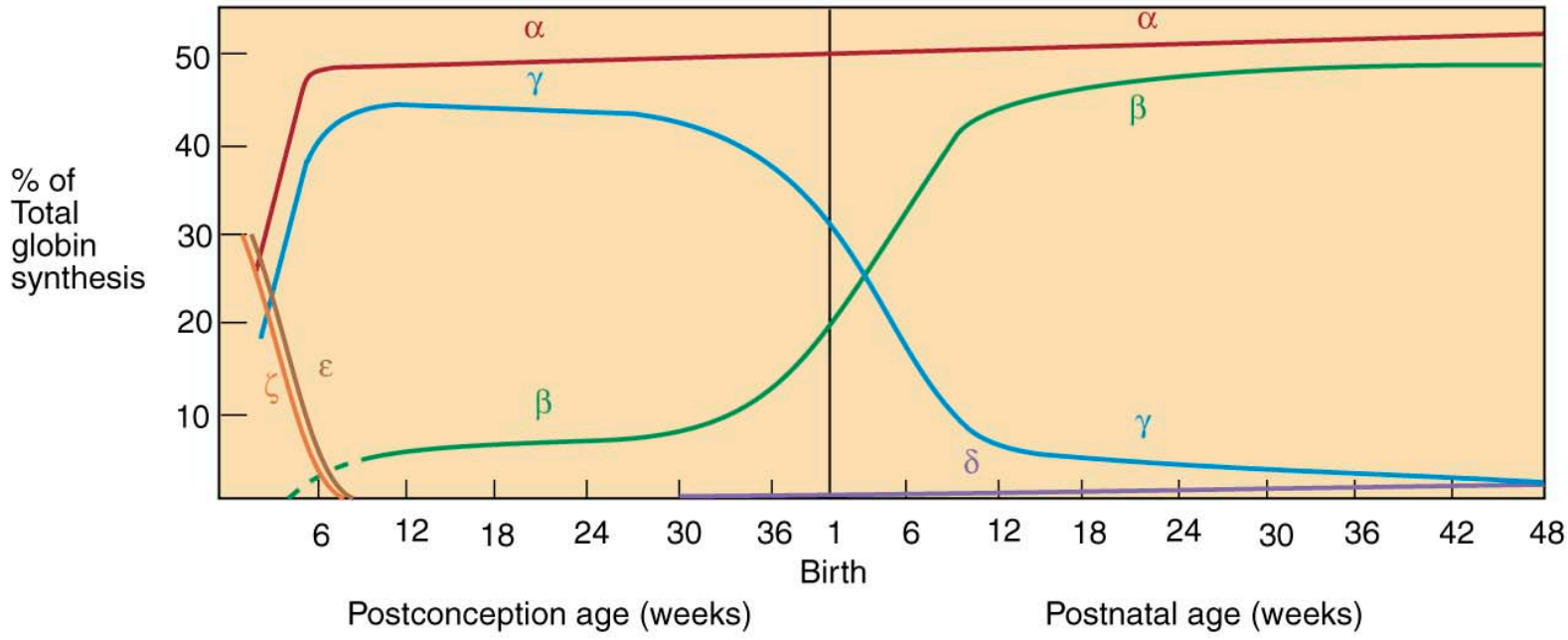
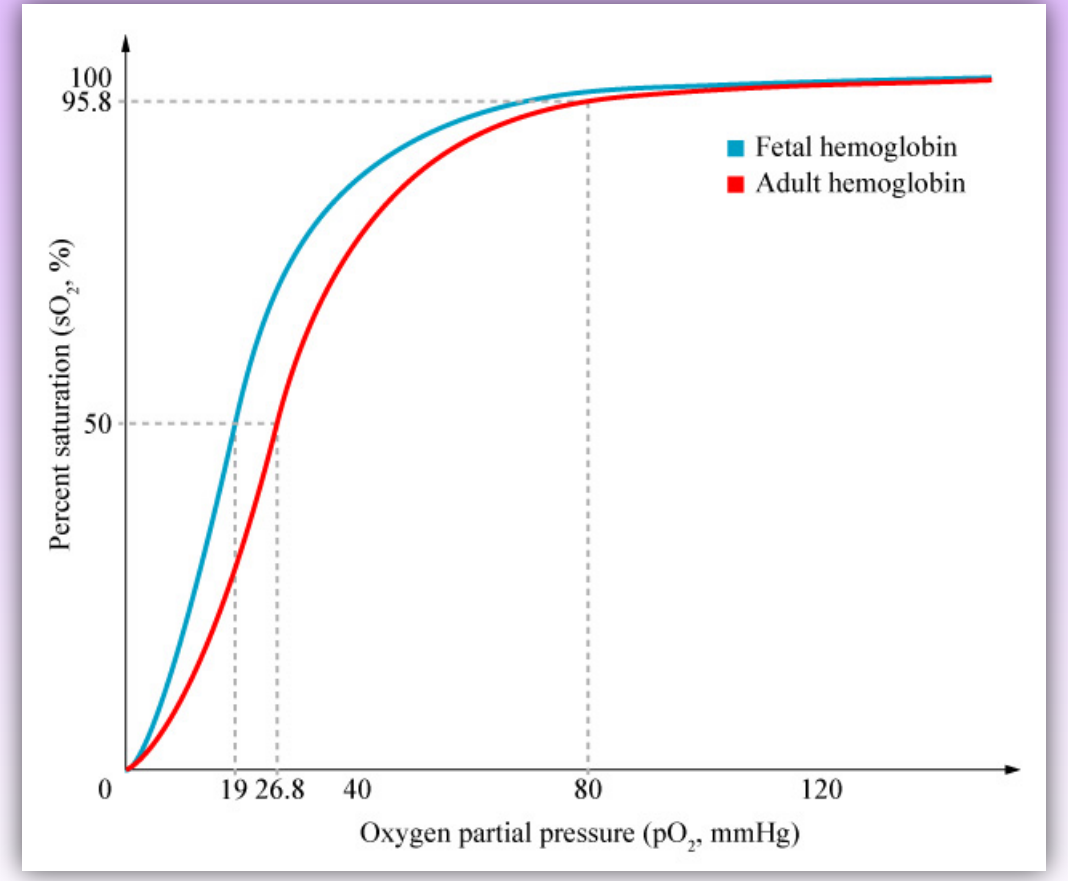


Figure 9.18. Changes in globin chain production during development. Redrawn from Nienhuis, A.W. and Maniatis, T. In: G. Stamatoyannopoulos, A. W. Nienhuis, P. Leder, and P. W. Majerus (Eds.), *The Molecular Basis of Blood Diseases.* Philadelphia: Saunders, 1987, p. 68, where the following reference is acknowledged: Weatherall, D. J. and Clegg, J. B., *The Thalassemia Syndromes,* 3rd ed., Oxford: Blackwell Scientific Publications, 1981.

HbF has a higher affinity for O_2 than HbA due to its reduced affinity for 2,3-BPG. The two γ-globin chains have Ser substituted for His at position 143, giving two fewer (+) charges in the 2,3-BPG binding pocket of HbF compared to HbA.



In erythrocytes, 15-25% of glucose that is converted to lactate goes by way of the BPG shunt for the synthesis of 2,3-BPG. No net production of ATP occurs when glucose is converted to lactate via this shunt because the 1,3-BPG kinase step of glycolysis is bypassed.

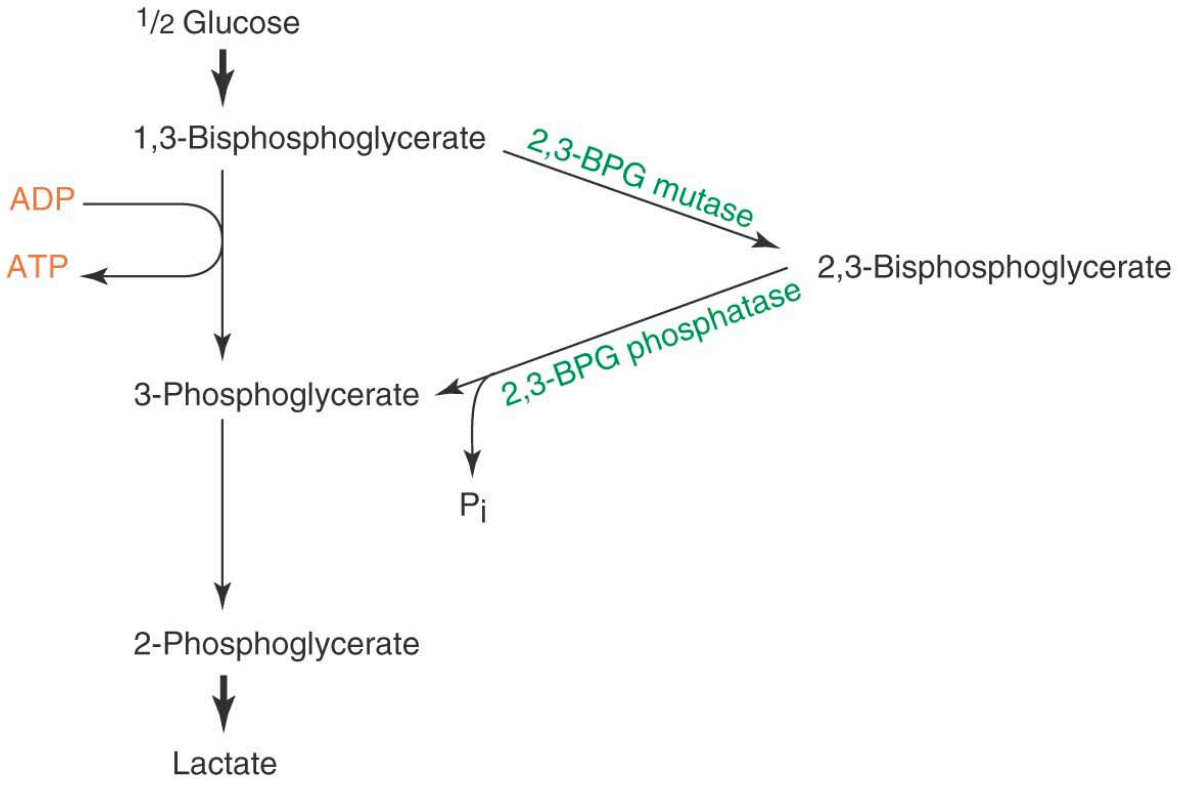
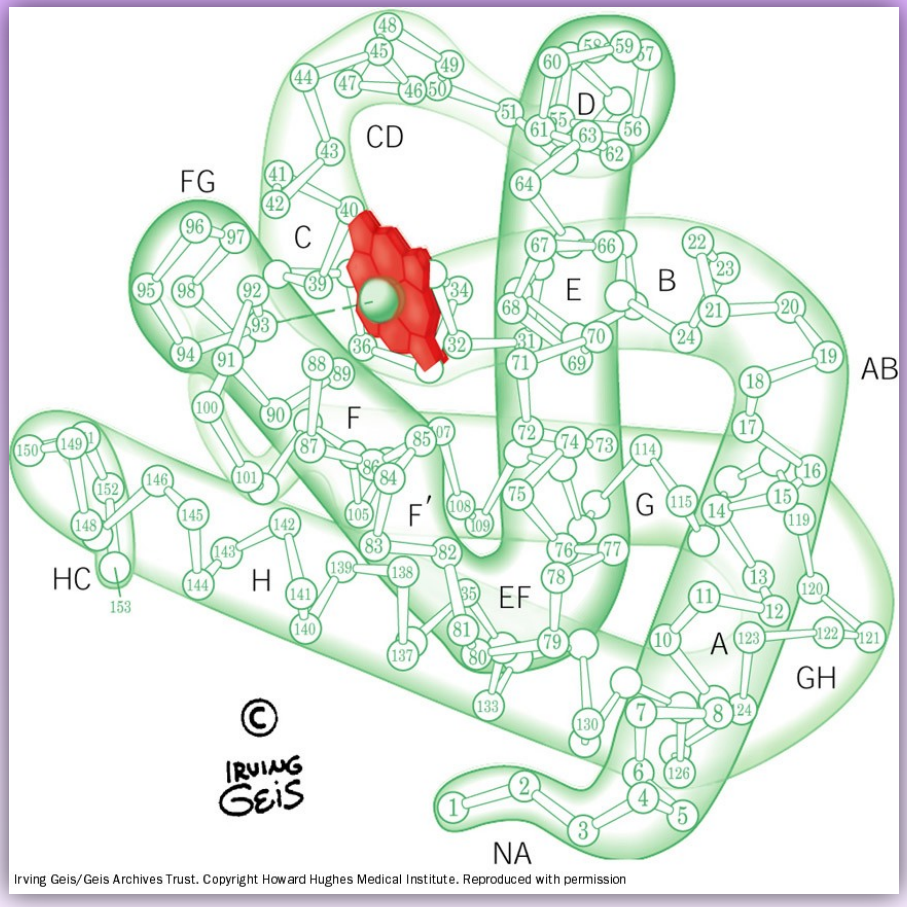


Figure 15.9. The reactions of 2,3-bisphosphoglycerate (2,3-BPG) shunt are catalyzed by the bifunctional enzyme, 2,3-BPG mutase/phosphatase.

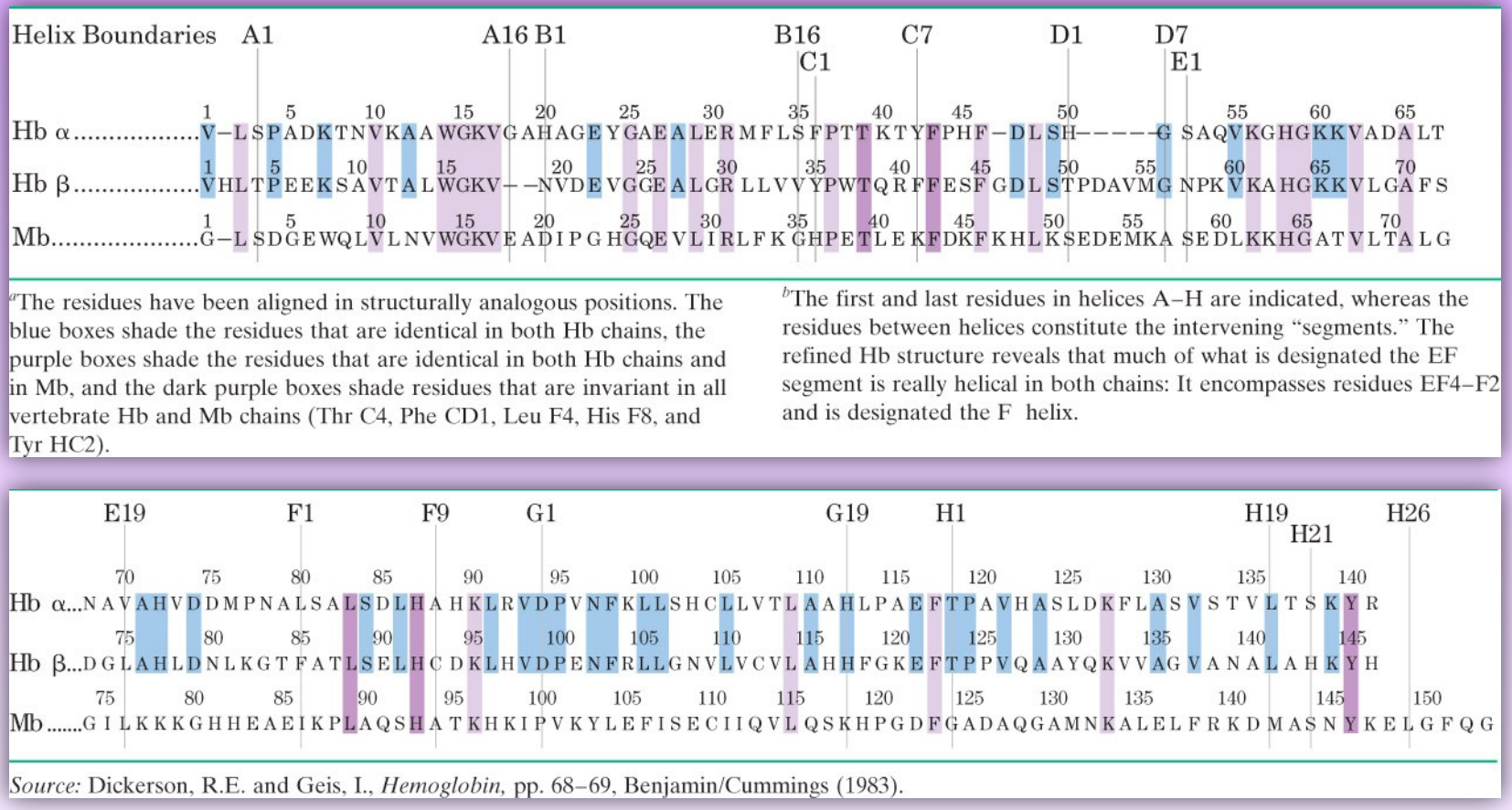


Contains 8 helices: A-H

Contains some 3_{10} helices

Subunits of Hb are similar to Mb

Structure of sperm whale myoglobin (Mb)

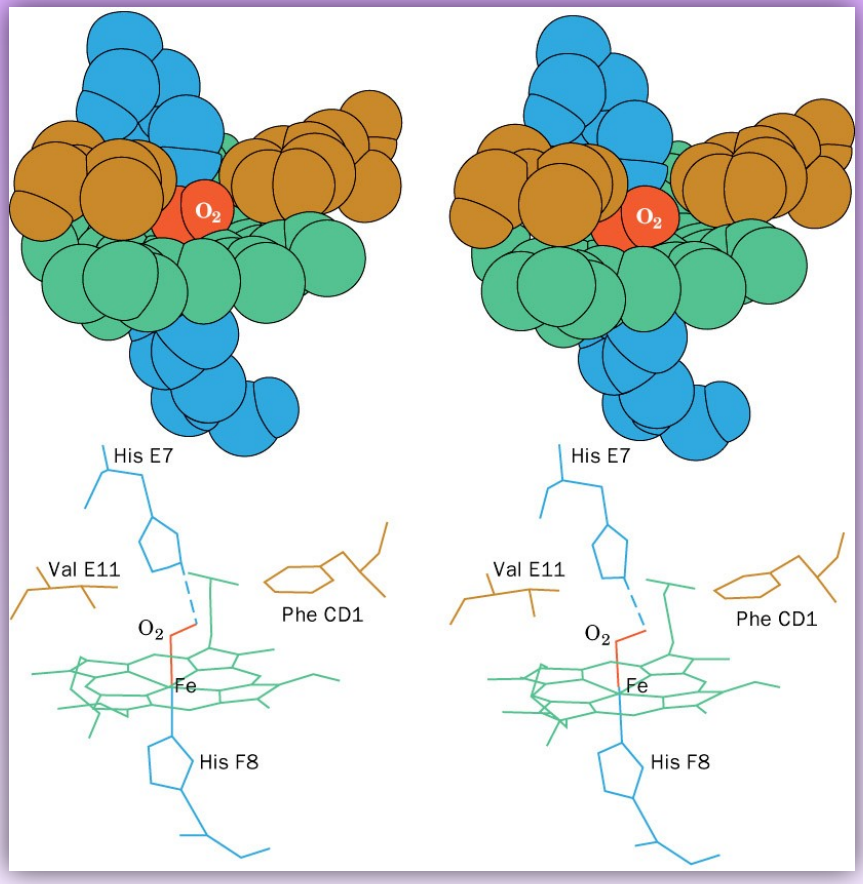


[a]The residues have been aligned in structurally analogous positions. The blue boxes shade the residues that are identical in both Hb chains, the purple boxes shade the residues that are identical in both Hb chains and in Mb, and the dark purple boxes shade residues that are invariant in all vertebrate Hb and Mb chains (Thr C4, Phe CD1, Leu F4, His F8, and Tyr HC2).

[b]The first and last residues in helices A–H are indicated, whereas the residues between helices constitute the intervening "segments." The refined Hb structure reveals that much of what is designated the EF segment is really helical in both chains: It encompasses residues EF4–F2 and is designated the F helix.

Source: Dickerson, R.E. and Geis, I., *Hemoglobin,* pp. 68–69, Benjamin/Cummings (1983).

The amino acid sequences of the α and β chains of human hemoglobin and of human myoglobin



Stereo drawings of the heme complex in oxyMb

The heme is located in a hydrophobic pocket formed mainly by helices E and F.

Fe(II) is 0.22 Å out of the heme plane in oxyMb on the proximal His93 side; O2 is bound in a bent geometry.

Fe(II) is 0.55 Å out of plane in deoxyMb.

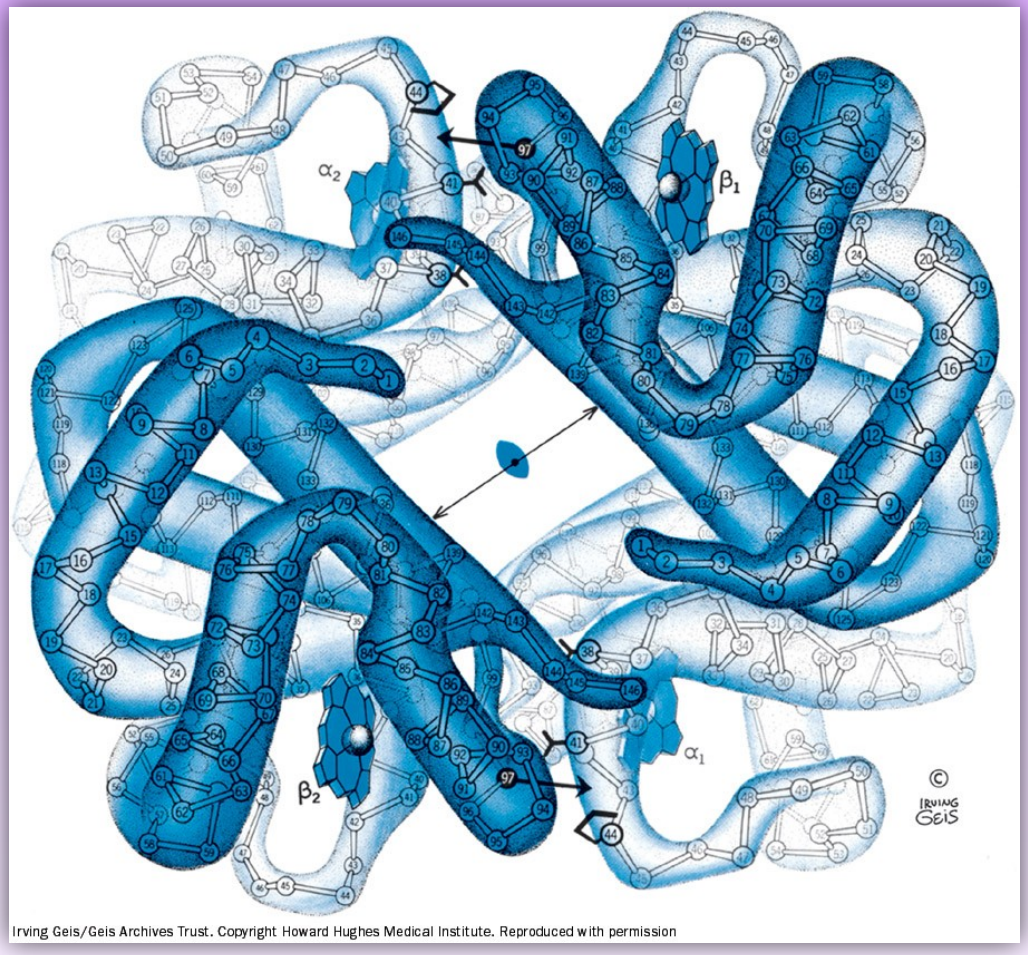
Structures of oxyMb and deoxyMb are largely superimposable.

Hb contains two $\alpha\beta$ protomers.

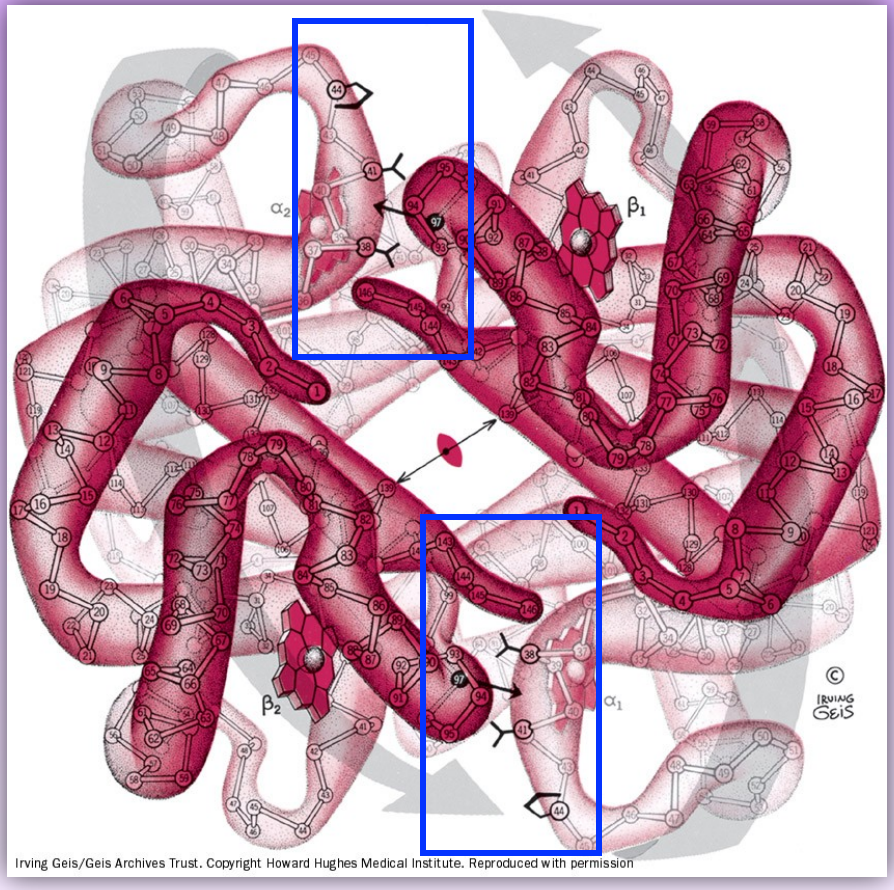
Tertiary structures of α and β subunits are similar to each other and to Mb.

There are extensive interactions between unlike subunits (α_1-β_1 and α_2-β_2); these interactions are **hydrophobic** in character. These interactions are abundant and relatively fixed.

Contacts between like subunits (α_1-β_2 and α_2-β_1) are few and **polar**.



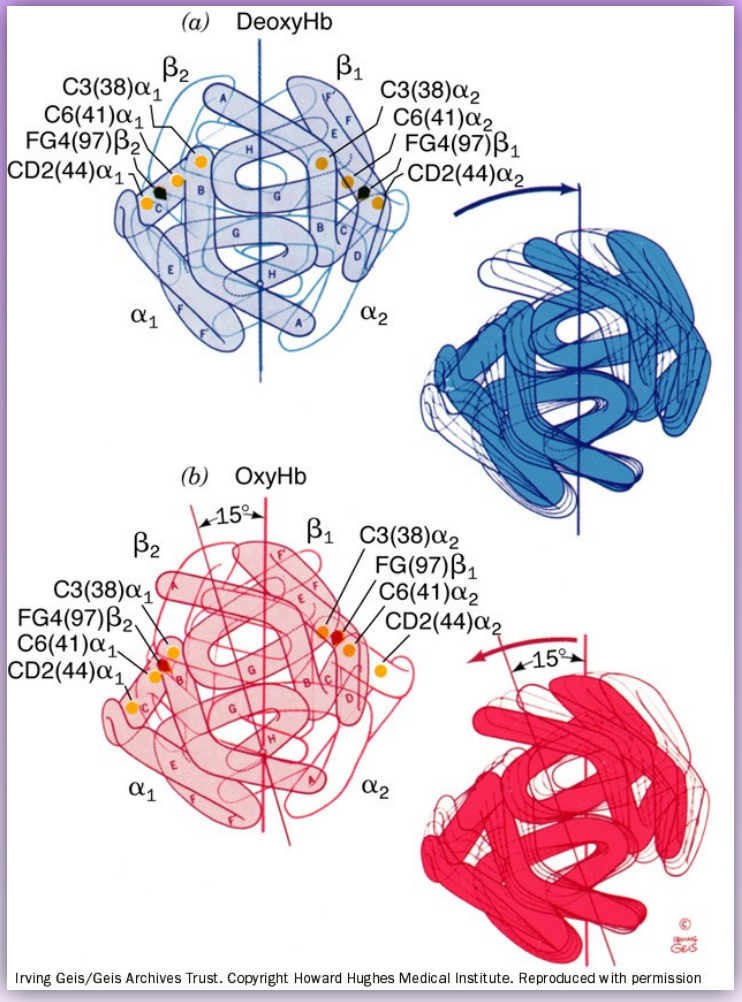
The X-ray structure of deoxyHb as viewed down its exact 2-fold axes



Extensive **quaternary** structural changes occur to the Hb tetramer upon oxgenation.

Structural changes occur at the α_1-β_2 **and** α_2-β_1 interfaces. The α_1-β_1 **and** α_2-β_2 interfaces remain unchanged.

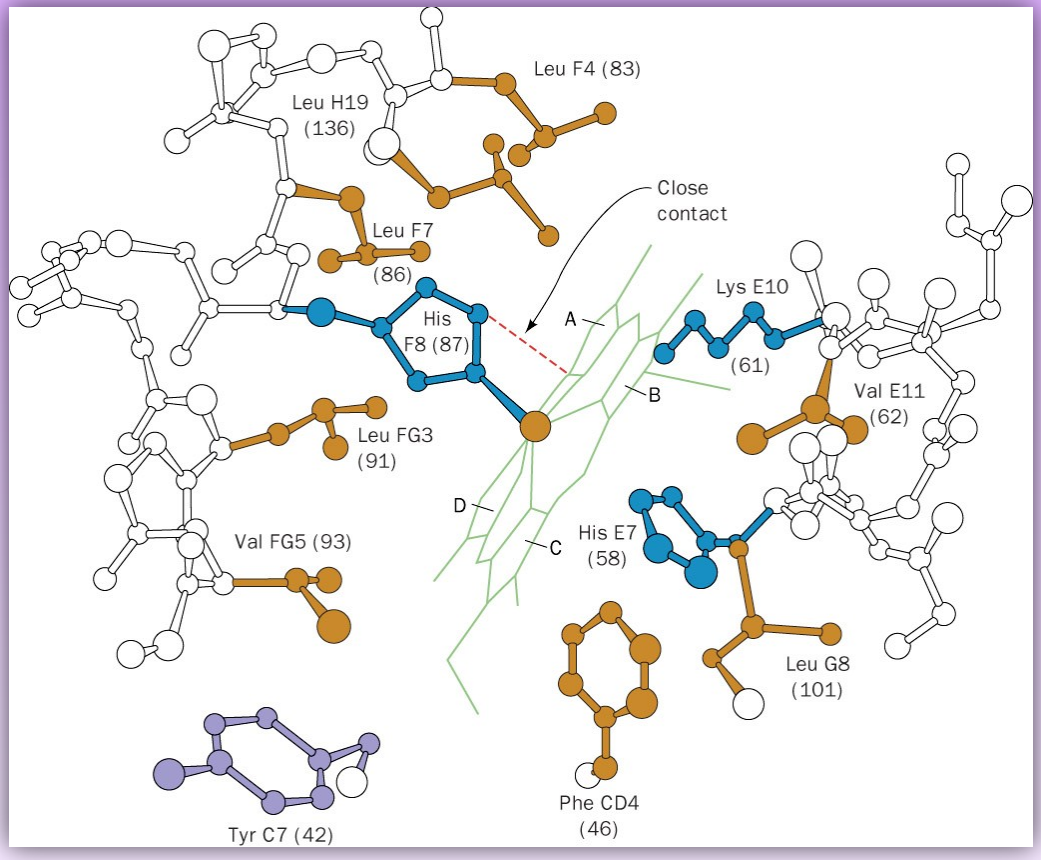
The X-ray structure of oxyHb as viewed down its exact 2-fold axes.



Oxygenation rotates the α_1-β_1 dimer by 15° with respect to the α_2-β_2 dimer; two-fold symmetry is maintained.

4° forms:
deoxyHb = T state (tense)
oxyHb = R state (relaxed)

The major structural differences between the quaternary conformations of (*a*) deoxyHb and (b) oxyHb

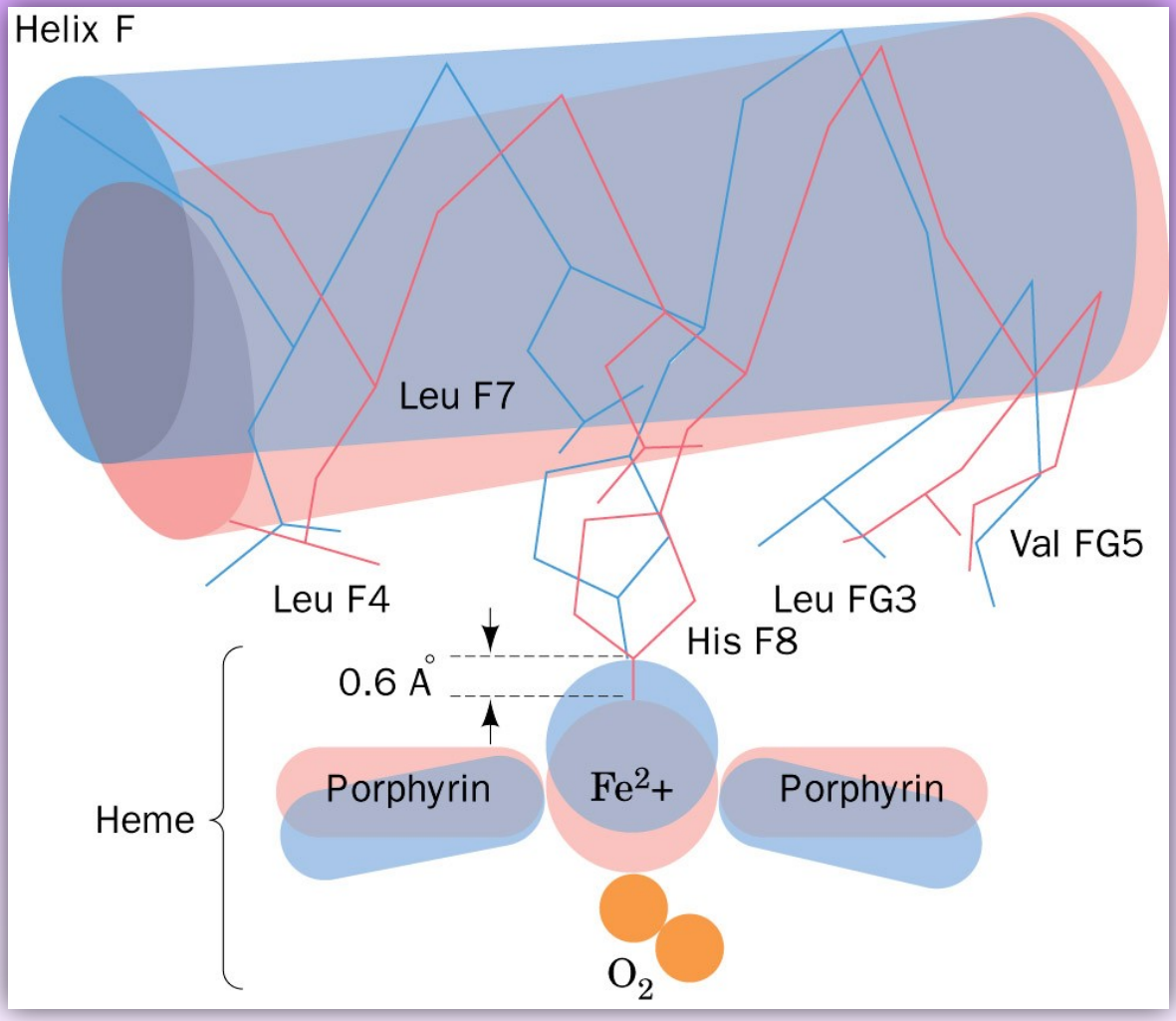


The heme group and its environment in the unliganded α chain of human Hb.

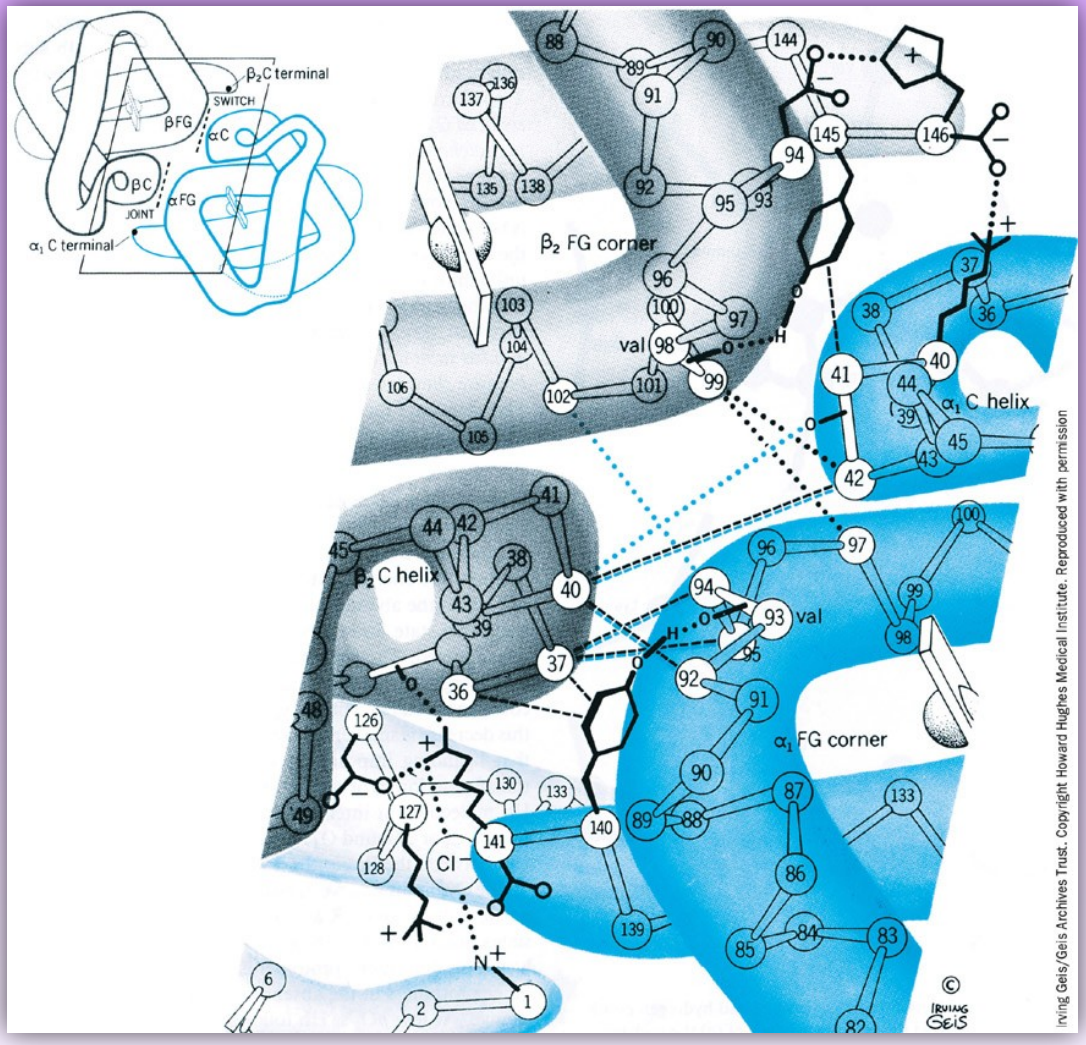
Explaining cooperativity:

Perutz mechanism
(based on X-ray analyses)

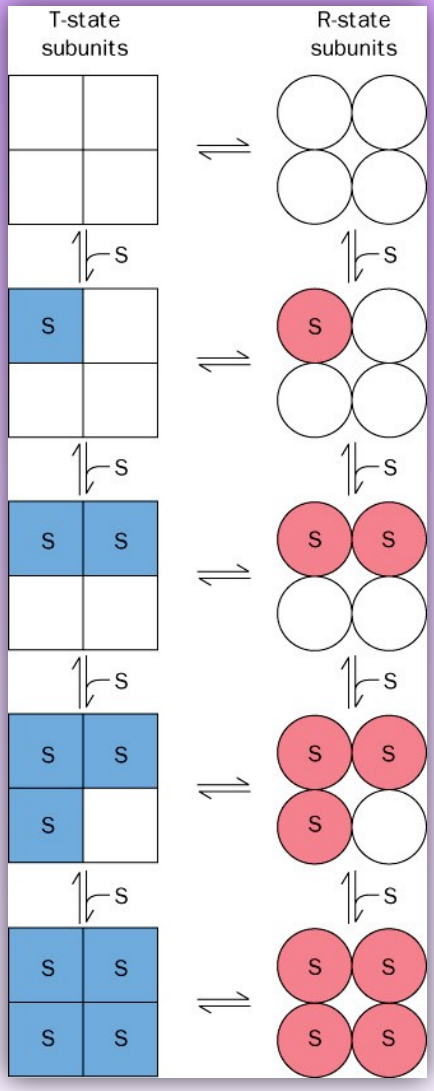
Note out-of-plane
Fe(II) in deoxyHb (0.55 Å);
ion moves nearly in-plane in
oxyHb (0.22 Å), and pulls
on the proximal
His93, thus moving the F helix.



Triggering mechanism for the T → R transition in Hb
(T = blue; R = pink)



The hemoglobin α_1-β_2 interface (there is an identical interface at α_2-β_1)

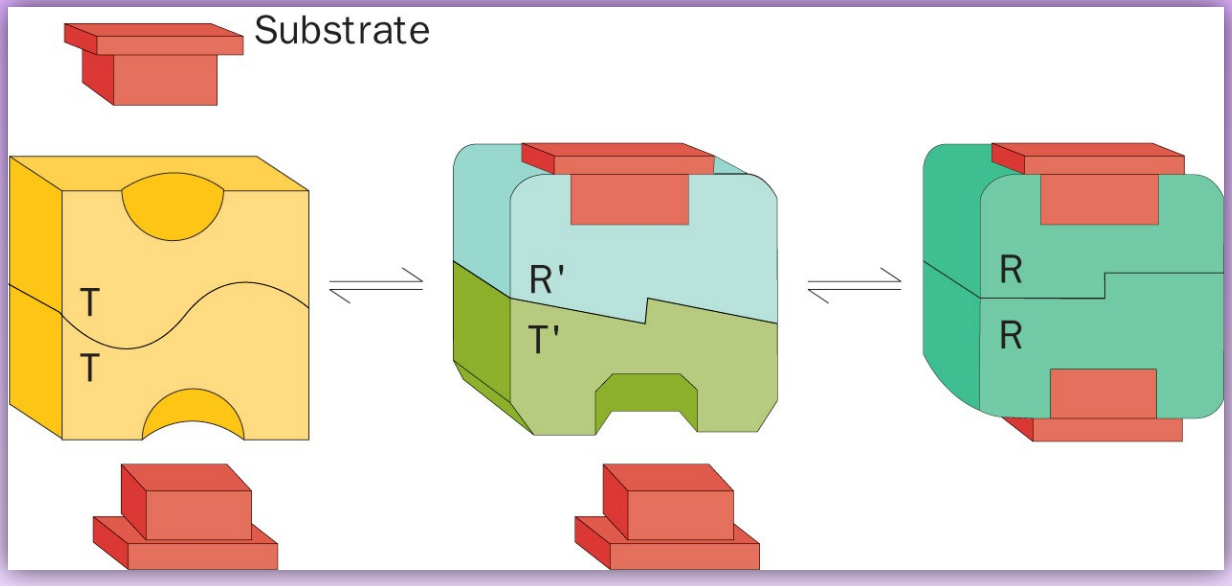


Allosteric regulation: two general models

Monod, Wyman, Changeux: **symmetry model**

Conformational change alters affinity for ligand: molecular symmetry conserved

Species and reactions permitted under the symmetry (MWC) model of allosterism

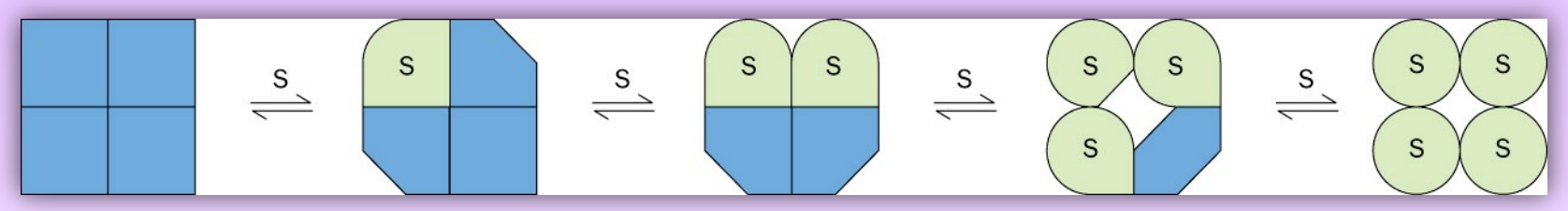


The sequential model of allosterism

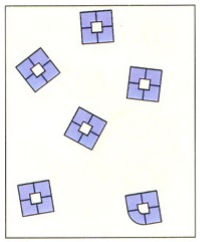
Koshland, Nemethy, Filmer (KNF)

Binding to T-state induces conformational changes in unliganded subunits (intermediate affinity between T and R)

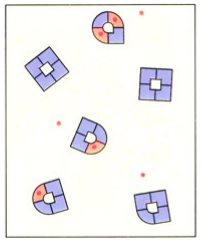
Ligand affinity varies with number of bound ligands; intermediate conformations: Hb



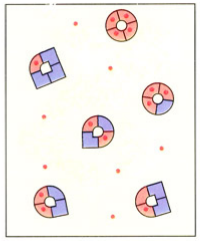
Sequential binding of ligand in the sequential model of allosterism; molecular symmetry is not maintained in intermediate states



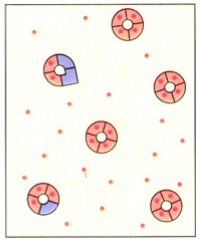
❶ No oxygen bound. Almost all subunits in all molecules are in weak-binding state. Only a few happen to be in the strong-binding state.



❷ Some oxygen bound. Each binding of an oxygen molecule favors the transition of adjacent subunits to the strong-binding state and promotes their binding of oxygen.

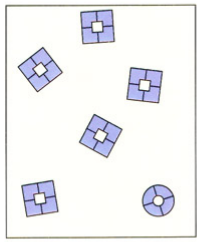


❸ More oxygen bound. More and more subunits next to oxygen-occupied sites are switching to the strong-binding state.

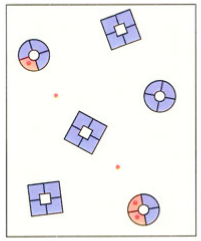


❹ Approaching saturation. Almost all sites are filled, and almost all subunits are now in the strong-binding state.

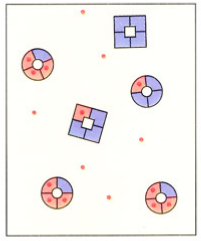
(a) KNF model



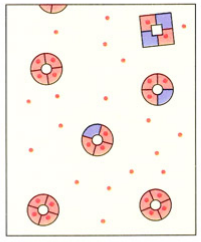
❶ No oxygen bound. Most tetramers are in the T state, with only a few in the R state.



❷ Some oxygen bound. Preference is for binding to molecules in R state so T ⇌ R equilibrium is shifted toward R.

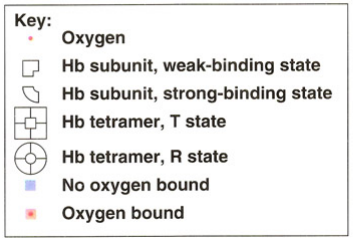


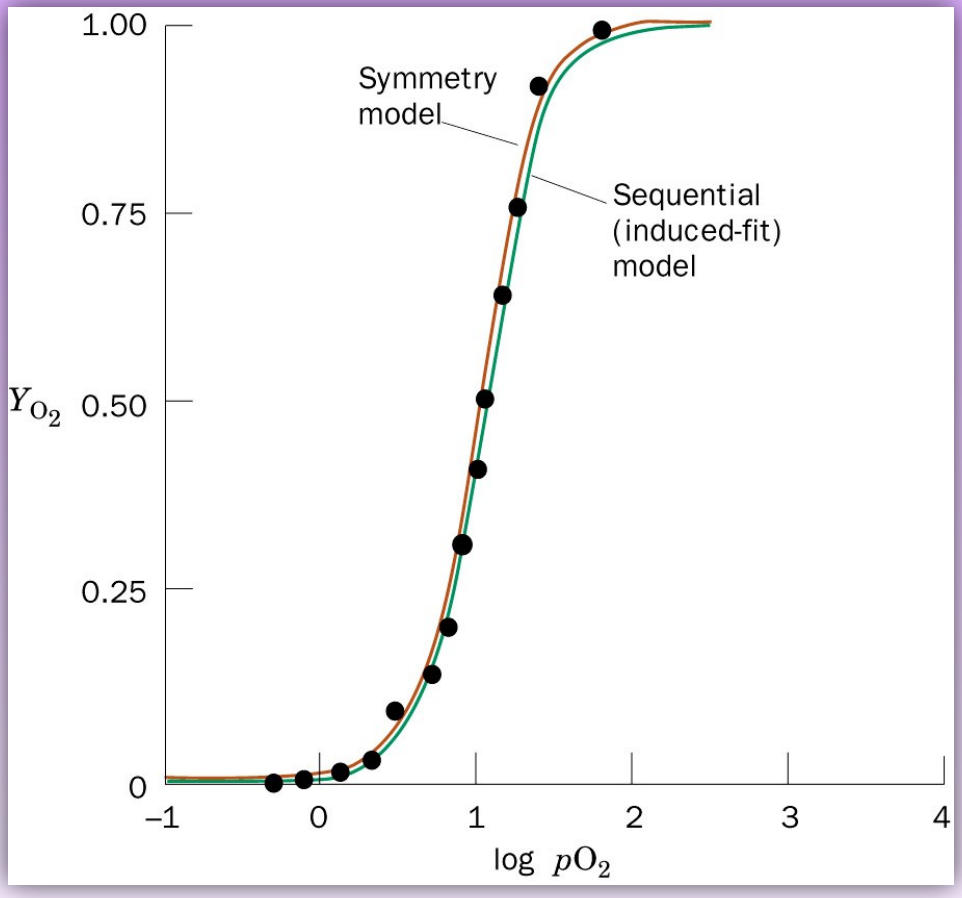
❸ More oxygen bound. Now most molecules are in R state. Note that T also binds oxygen, but more weakly.



❹ Approaching saturation. Almost all molecules have shifted to R state. Almost all sites are filled.

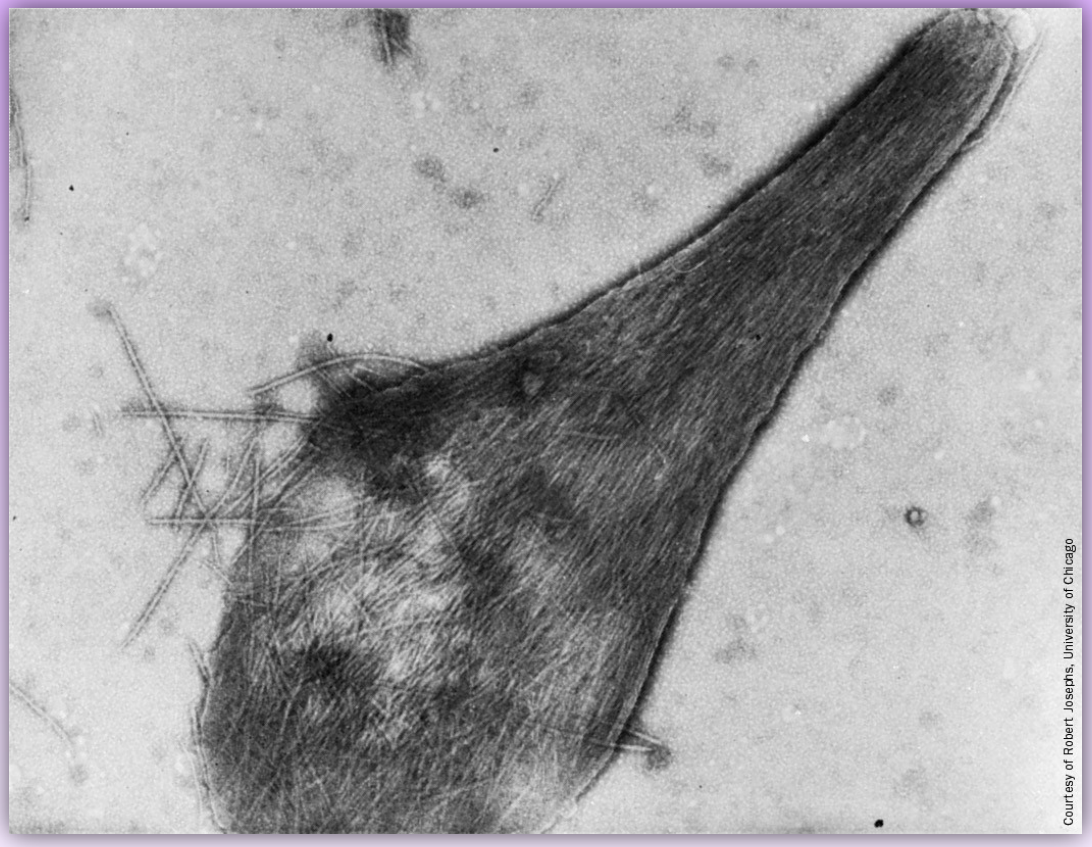
(b) MWC model





The sequential and symmetry models of allosterism provide equally good fits to the measured O_2-dissociation curve of Hb.

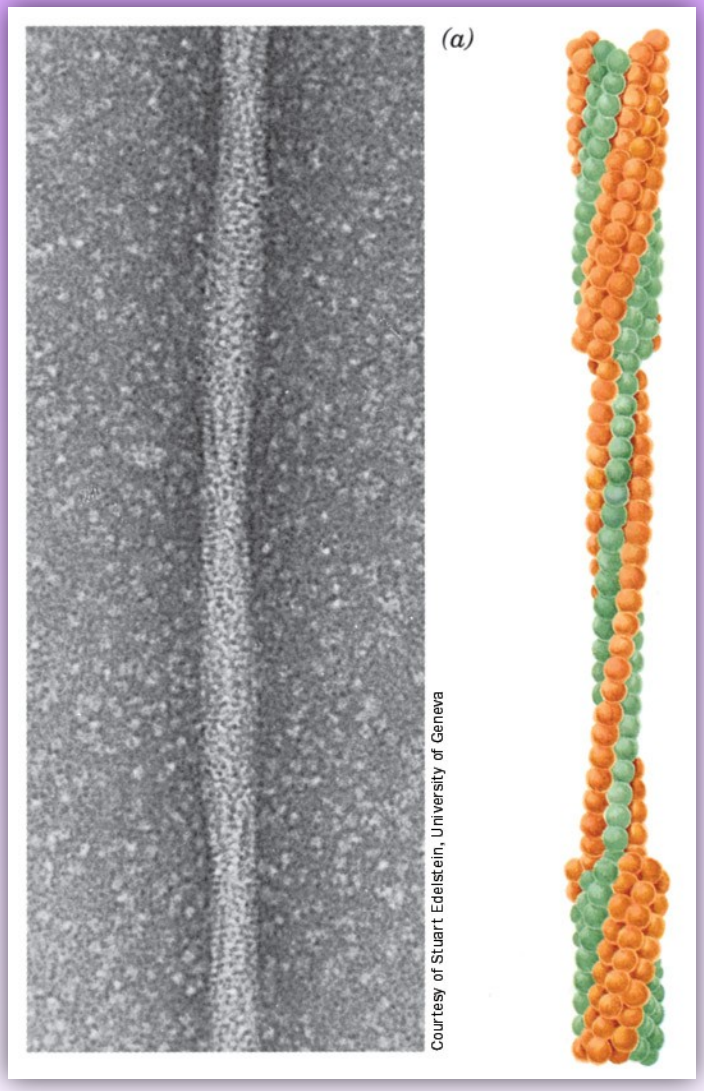
Abnormal Hemoglobins



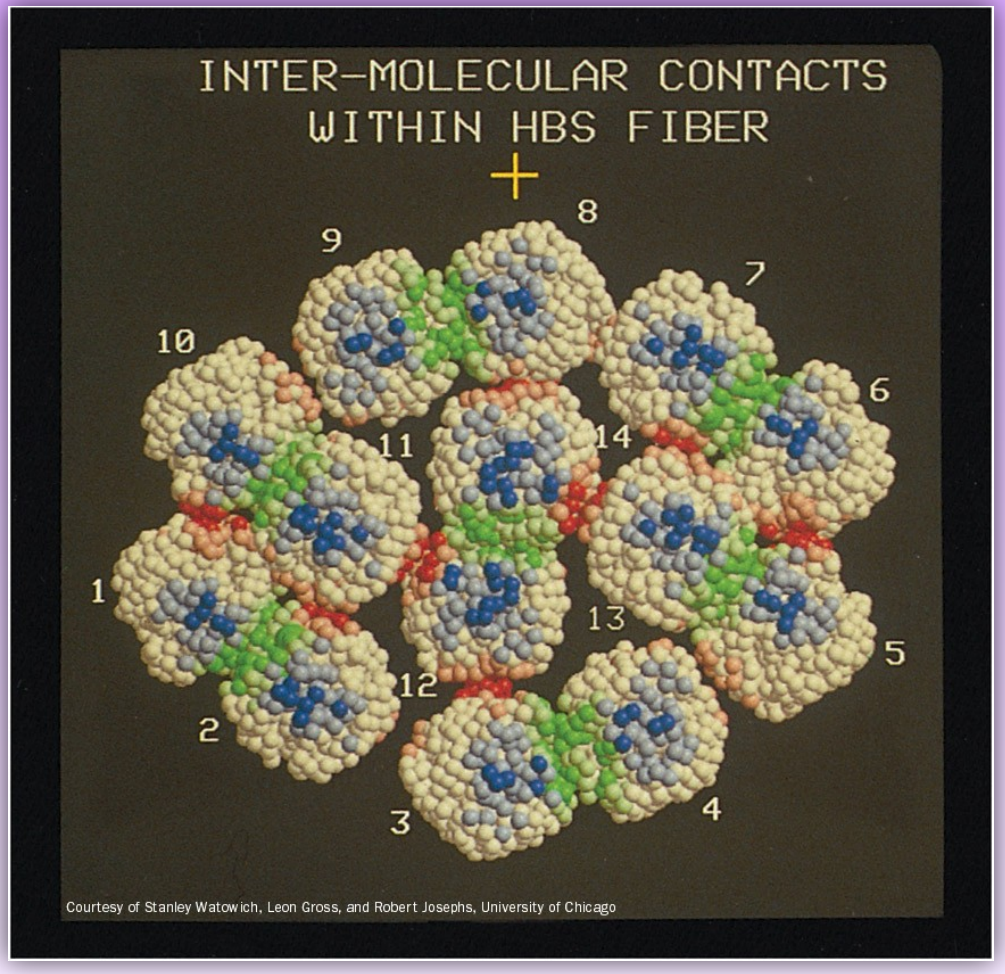
Sickle-cell anemia: HbS

Single-site mutation: Valine replaces Glu A3(6)β

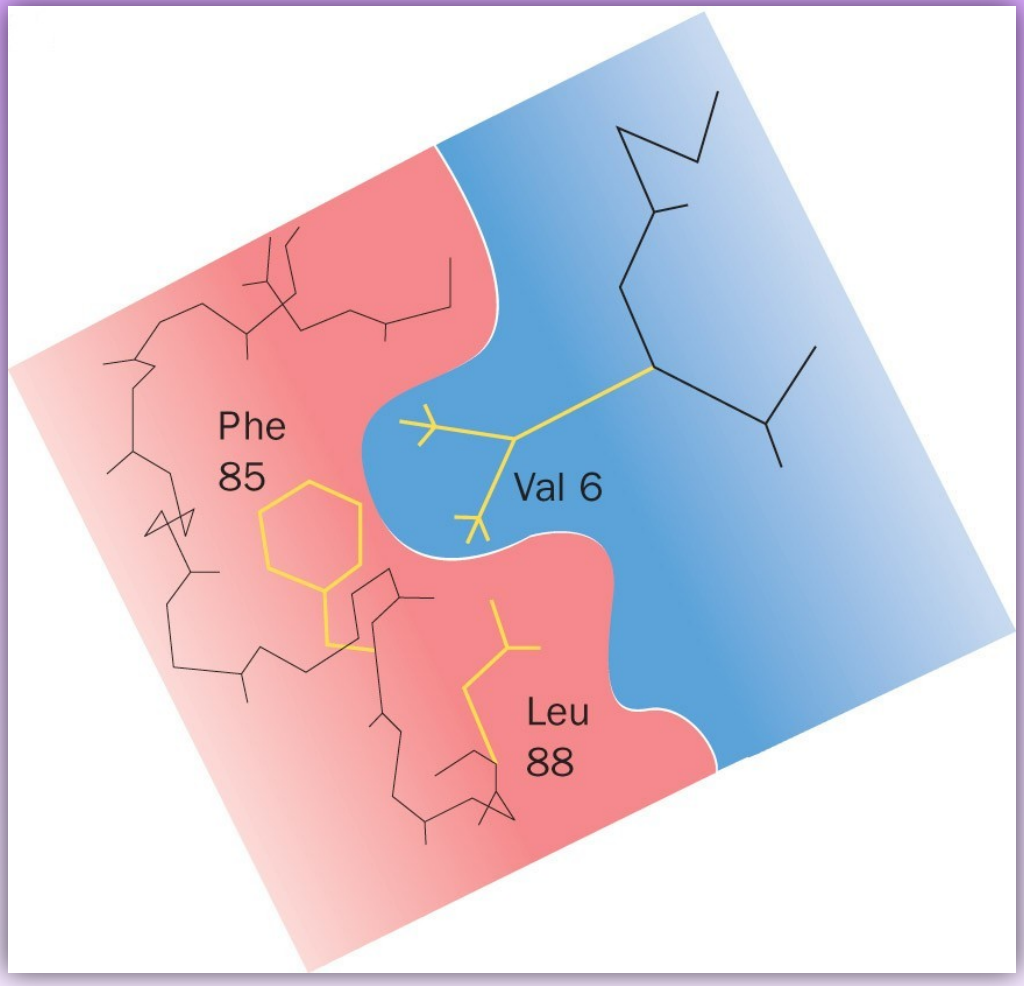
Electron micrograph of deoxyHbS fibers spilling out of a ruptured erythrocyte; an amyloid-like disease



220-Å in diameter fibers of deoxyHbS: an electron micrograph of a negatively stained fiber

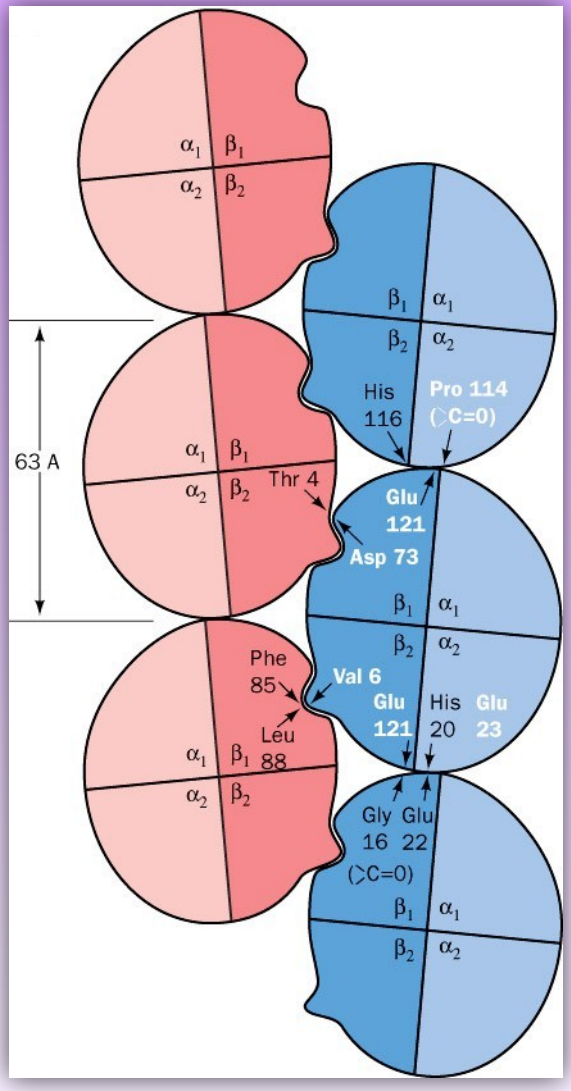


220-Å in diameter fibers of deoxyHbS: a model, viewed in cross section, of the HbS fiber.



Molecular basis for fibril formation in HbS

Structure of the deoxyHbS fiber: the mutant Val 6β_2 fits neatly into a hydrophobic pocket formed mainly by Phe 85 and Leu 88 of an adjacent β_1 subunit.



Intermolecular association
Val 6 of β_2 binding
in β_1 pocket

Structure of the deoxyHbS fiber: a schematic diagram indicating the intermolecular contacts in the crystal structure of deoxyHbS.

Table 7-1 Some Hemoglobin Variants

Name[a]	Mutation	Effect
Hammersmith	Phe CD1(42)β → Ser	Weakens heme binding
Bristol	Val E11(67)β → Asp	Weakens heme binding
Bibba	Leu H19(136)α → Pro	Disrupts the H helix
Savannah	Gly B6(24)β → Val	Disrupts the B–E helix interface
Philly	Tyr C1(35)β → Phe	Disrupts hydrogen bonding at the α_1–β_1 interface
Boston	His E7(58)α → Tyr	Promotes methemoglobin formation
Milwaukee	Val E11(67)β → Glu	Promotes methemoglobin formation
Iwate	His F8(87)α → Tyr	Promotes methemoglobin formation
Yakima	Asp G1(99)β → His	Disrupts a hydrogen bond that stabilizes the T conformation
Kansas	Asn G4(102)β → Thr	Disrupts a hydrogen bond that stabilizes the R conformation

[a]Hemoglobin variants are usually named after the place where they were discovered (e.g., hemoglobin Boston).