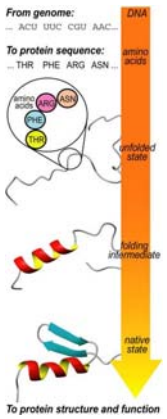
Protein Folding Purification and Myoglobin

Lecture 11
(29 September 2009)

Protein Folding

Protein folding problem

- Levinthal paradox
  - 100aa protein three conformations
    => 3^{100} possible orientations
    => random search for native structure would take longer than the age of the universe
- Prediction of three dimensional structure from its amino acid sequence
- Translate "Linear" DNA Sequence data to spatial information



Sidechain locations in proteins

- Non-polar sidechains (Val, Leu, Ile, Met, and Phe) occur mostly in the interior of a protein keeping them out of the water (hydro-phobic effect)
- Charged polar residues (Arg, His, Lys, Asp, and Glu) are normally located on the surface of the protein in contact with water.
- Uncharged polar residues (Ser, Thr, Asn, Gln, and Tyr) are usually on the protein surface but also occur in the interior of the protein.

Protein Stability

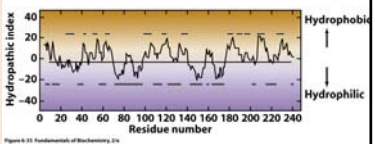
Forces that stabilize protein structure: 1, 2, 3

1. The Hydrophobic Effect

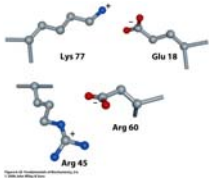
Table 6-2 Hydropathy Scale for Amino Acid Side Chains

Side Chain	Hydropathy
Ile	4.5
Val	4.2
Leu	3.8
Phe	2.8
Cys	2.5
Met	1.9
Ala	1.8
Gly	−0.4
Thr	−0.7
Ser	−0.8
Trp	−0.9
Tyr	−1.3
Pro	−1.6
His	−3.2
Glu	−3.5
Gln	−3.5
Asp	−3.5
Asn	−3.5
Lys	−3.9
Arg	−4.5

Source: Kyte, J. and Doolittle, R.F., *J. Mol. Biol.* **157**, 110 (1982).

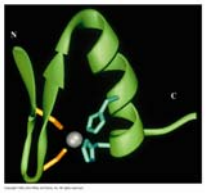


2. Electrostatic Interactions



Ion pair (salt bridge) of myoglobin

3. Chemical Cross-links



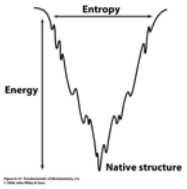
Zinc finger:
Nucleic acid-binding proteins

Protein Folding Pathways

Proteins can be unfolded/denatured.

Denatured proteins can be refolded, sometimes requiring helper proteins, and this refolding takes place via preferred pathways.

Common thought is that secondary structures form first, eventually collapsing due to the formation of hydrophobic cores.



Folding funnel
Energy-entropy relationship for protein folding

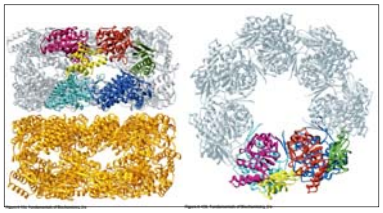
Molecular chaperons

Molecular chaperones:

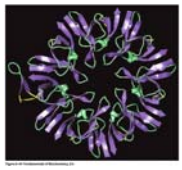
(1) Hsp70 proteins function as monomer

(2) Chaperonins, large multisubunit proteins

(3) Hsp90 proteins for the folding of proteins involved with signal transduction

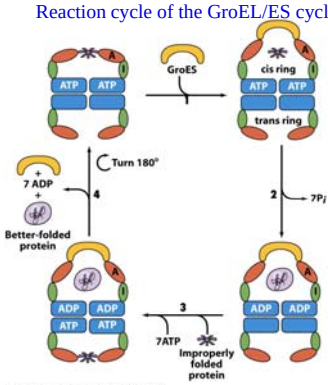


GroEL



GroES

Reaction cycle of the GroEL/ES cycle



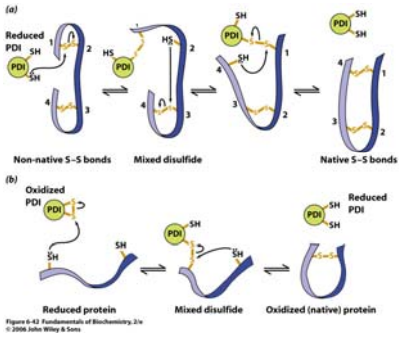
1. GroEL ring binding 7 ATP and a substrate (improperly folded protein). Then it binds a GroES cap to become the cis ring.

2. The cis ring catalyzes the hydrolysis of its 7 ATP.

3. A 2nd substrate binds to the trans ring followed by 7 ATP.

4. The binding of substrate and ATP to the trabs ring conformationally induces the cis ring to release its bound GroES, 7 ADP, and the better folded substrate.The trans ring becomes the cis ring.

Protein disulfide Isomerase

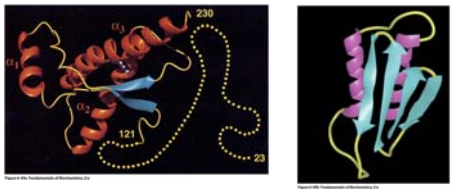


Diseases Caused by Protein Misfolding

Alzheimer's disease

Transmissible spongiform encephalopathies (TSE)

Amyloidoses



Prion protein conformation

A model of an amyloid fibril

Once it has formed, an amyloid fibril is virtually indestructible (interchain H- bonds).

It seems likely that protein folding pathways have evolved not only to allow polypeptides to assume stable native structures but also to avoid forming interchain H-bonds that would lead to fibril formation .

The factors that trigger amyloid formation remain obscure, even when mutation (hereditary amyloidoses) or infection (TSEs) appear to be the cause.

Protein Purification and Analysis

General approach to purifying proteins

Protein solubility

Chromatography

Electrophoresis

Ultracentrifugation

Strategy of Purification

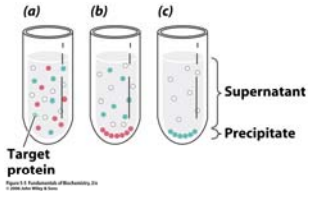
Fractionation procedures or steps to isolate protein based on physical/chemical characteristics.

Characteristic	Procedure
Charge	1. Ion exchange, 2. Electrophoresis, 3. Isoelectric focusing
Polarity	1. Adsorption chromatography 2. Paper chromatography 3. Reverse phase chromatography 4. Hydrophobic interaction
Size	1. Dialysis and ultrafiltration, 2. Gel electrophoresis, 3. Gel filtration, 4. Ultracentrifugation
Specificity	1. Affinity chromatography 2. Immunopurification
Solubility	1.Salt precipitation 2. Detergent solubilization

Protein Solubility

- Since proteins contain a number of charged groups, its solubility depends on the concentration of dissolved ions
- Salting in
  - At low ionic strength, increases in the concentration of dissolved ions leads to an increase in solubility by weakening the interaction between individual protein molecules. Interactions between protein molecules leads to aggregation (i.e. insolubility of proteins.

- Salting out
  - As the ionic strength increases, they out compete the proteins for water molecules and the proteins become less soluble, aggregate, and fall out of solution.



- Proteins are least soluble when they are neutral, so these salting out experiments are usually carried out at the pI of the protein (i.e. the isoelectric point where pH=pI, and the net charge on the protein is 0)

- a). At low ionic strength, all of the proteins are soluble
- b). As the ionic strength increases, the least soluble protein precipitates
- c). At even higher ionic strengths, further proteins precipitate. This process is continued until the desired protein is precipitated.
- This process not only allows you to obtain the desired protein, it removes many unwanted proteins in the process

Salting out

Use $(NH_4)_2SO_4$: it is a Very Soluble salt that does not harm proteins.

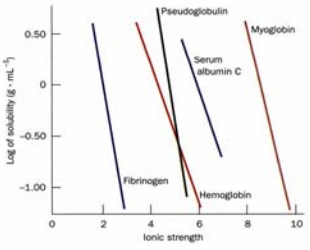
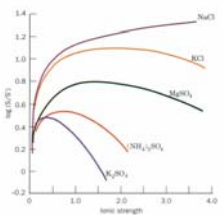


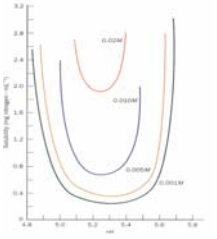
Table 5-2 Isoelectric Points of Several Common Proteins

Protein	pI
Pepsin	<1.0
Ovalbumin (hen)	4.6
Serum albumin (human)	4.9
Tropomyosin	5.1
Insulin (bovine)	5.4
Fibrinogen (human)	5.8
γ-Globulin (human)	6.6
Collagen	6.6
Myoglobin (horse)	7.0
Hemoglobin (human)	7.1
Ribonuclease A (bovine)	9.4
Cytochrome *c* (horse)	10.6
Histone (bovine)	10.8
Lysozyme (hen)	11.0
Salmine (salmon)	12.1

Solubility of carboxy-hemoglobin at its isoelectric point

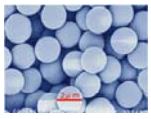


Solubility of β-lactoglobulin as a function of pH



Chromatography

Analytical methods used to separate molecules. Involves a mobile and a stationary phase.



•Mobile phase is what the material to be separated is dissolved in.

•Stationary phase is a porous solid matrix which the mobile phase surrounds.

•Separation occurs because of the differing binding/ interactions each molecule has with both the mobile and stationary phase.

•Interactions are different depending on the specific method.

Types of chromatography

- Gas - liquid: Mobile phase is gaseous, stationary phase is liquid usually bound to a solid matrix.
- Liquid - Liquid: Mobile phase is liquid, stationary phase is liquid usually bound to a solid matrix.
- If separation is based on ionic interaction the method is called Ion Exchange Chromatography.
- If separation is based on solubility differences between the phases the method is called Adsorption Chromatography.
- If the separation is base on size of molecule the method is called Gel Filtration or Size Exclusion.
- If the separation is base on ligand affinity the method is called Affinity Chromatography.

Ion Exchange Chromatography

A solid matrix with a positive charge, i.e., R^+ can bind different anions with different affinities.

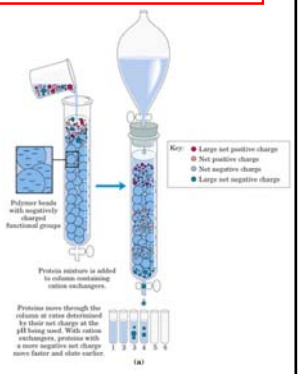
- We can swap one counter ion for another

$(R^+A^-) + B^- \Leftrightarrow (R^+B^-) + A^-$

R = Resin and exchanges Anions (-)

- This is an *anion exchange* resin – the stationary phase is decorated with positively charged groups which bind anions in the mobile phase
- There are also *cation exchange* resins. The type of an R group can determine the strength of interaction between the matrix, R and the counter ion.
- If R is R^-

$(R^-A^+) + B^+ \Leftrightarrow (R^-B^+) + A^+$

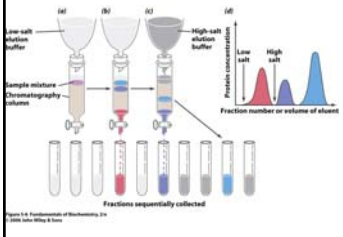


Proteins have a net charge

★ The charge is positive below pI, ★ while the charge is negative above pI

- The choice of exchange resin depends on the charge of the protein and the pH at which you want to do the purification.
- Once the protein binds, all unbound proteins are washed off the column. Bound proteins are eluted by increasing the ionic strength, changing the counter ion or changing the pH altering the charge on the protein or the column.

Ion Exchange Chromatography



- The most frequently used anion exchanger is: diethylaminoethyl (DEAE)

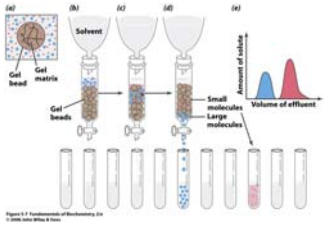
Matrix-CH_2-CH_2-$NH(CH_2CH_3)_2^+$

- The most frequently used cation exchanger is: carboxymethly

Matrix-CH_2-COO^-

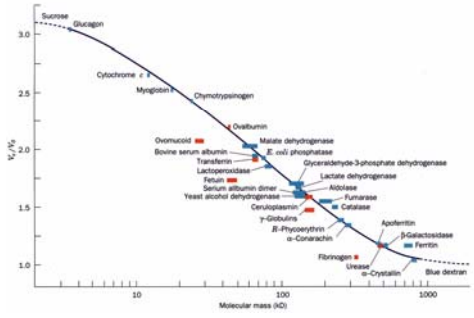
- The tan region is the ion exchange resin
- The mixture of proteins is the purple disc in a).
- The salt concentration is low at the beginning so the proteins with the lowest affinity for the column go through first (red protein)
- The salt concentration is then increased, washing off the proteins that interact more strongly with the ion exchange medium in the column

Gel Filtration Chromatography

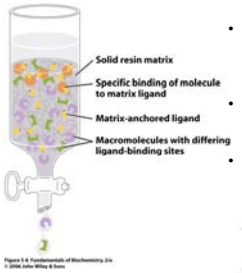


- Each gel bead consists of a gel matrix (wavy lines in the brown spheres)
- Small molecules (red dots) can fit into the internal spaces in the beads and get stuck
- Larger molecules (blue dots) cannot fit into the internal spaces in the beads and they come through the column faster

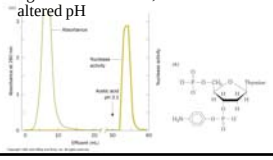
Gel filtration can be used to determine the molecular mass of proteins



Affinity Chromatography

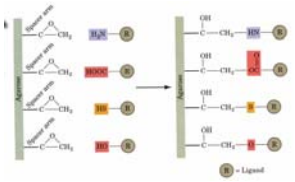


- Ligands (yellow in the figure to the left) are attached to the solid resin matrix
- The proteins in the eluant have ligand binding sites, however, only one of them will have the binding site for the ligand attached to the solid resin matrix
- The proteins that do not have the proper ligand binding site will flow through the column fastest
- The desired protein (i.e. the one with the proper ligand binding site) is then recovered from the column by washing with a solution with high ligand concentration, altered ionic strength, or altered pH



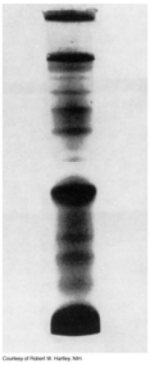
Affinity Chromatography

Based on molecular complementary between an enzyme and substrate. The substrate (R) is linked to a matrix with a spacer arm



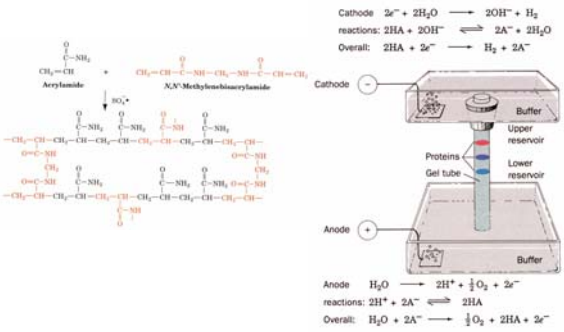
Only protein that binds R will stick to column. Put citrate on column citrate dehydrogenase will specifically bind. Add excess citrate and the enzyme will be released.

Electrophoresis

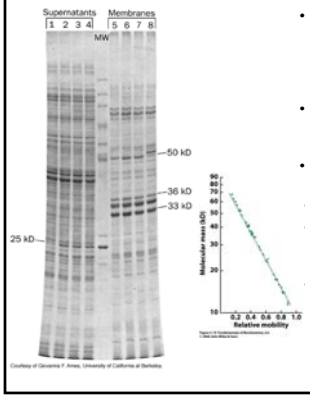


- Electrophoresis is a method for separating proteins based on how they move in an electric field
- Image to the left is an electrophoretogram of serum, stained with amido black
- The sample starts at the top, an electric field is applied, and proteins migrate
- The molecules at the bottom are the lightest
- Molecules of similar charge and size move through the gel as a band
- The pH is typically 9 in these experiments so most proteins have a net negative charge and move toward the positive electrode (i.e. the one attached to the bottom of the gel)
- Gels are typically made of polyacrylamide and so the experiment is called polyacrylamide gel electrophoresis (PAGE)

Polyacrylamide Gel electrophoresis (PAGE)



SDS-PAGE



- Sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (PAGE), SDS-PAGE is used to separate protein mixtures in a protein denaturing environment (SDS – soap)
- That is, the SDS causes proteins to denature and take on a rodlike shape and have similar charge to mass ratios
- Therefore, proteins are separated by molecular mass
- Again, the lighter proteins travel further
- In the figure, several (8) protein mixtures are run at the same time, some are controls and the others are samples
- Each sample is in a separate column, called a "lane"

Ultracentrifugation

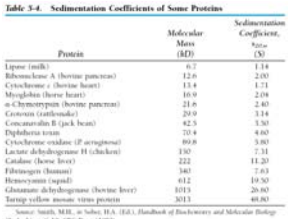


Table 3-4. Sedimentation Coefficients of Some Proteins

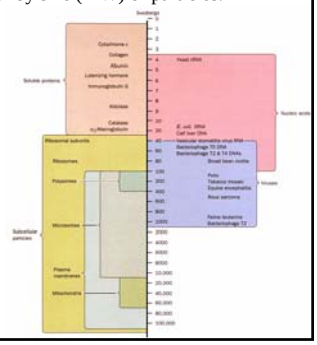
Protein	Molecular Mass (kD)	Sedimentation Coefficient, $s_{20,w}$ (S)
Lysine (milk)	6.7	1.14
Ribonuclease A (bovine pancreas)	12.6	2.00
Cytochrome c (bovine heart)	13.4	1.71
Myoglobin (horse heart)	16.9	2.04
α-Chymotrypsin (bovine pancreas)	21.6	2.40
Ceruvein (earthworm)	29.0	3.14
Concanavalin B (jack bean)	42.5	3.50
Diphtheria toxin	70.4	4.60
Cytochrome oxidase (P. aeruginosa)	89.8	3.80
Lactate dehydrogenase H (chicken)	130	7.31
Catalase (horse liver)	222	11.20
Fibrinogen (human)	340	7.63
Hemocyanin (squid)	612	19.50
Glutamate dehydrogenase (bovine liver)	1013	26.80
Turnip yellow mosaic virus protein	3013	48.80

Source: Smith, M.H., in Sober, H.A. (Ed.), *Handbook of Biochemistry and Molecular Biology* (2nd ed.), p. C-30, CRC Press (1970).

- A centrifuge is an instrument that rotates, generating centrifugal fields in excess of 600,000 times that of gravity
- This causes molecules in solution to undergo sedimentation at different rates, which are related to their masses
- The rate of sedimentation is measured in "s" which is the sedimentation velocity per unit of centrifugal force
- They are normally expressed in units of "S" (Svedbergs). One Svedberg is 10^{-13}s
- Proteins: 1-50S
- Viruses: 40-1000S
- Organelles: tens of thousands of S

Lysate - broken (lysed) cells- can be separated using
differential centrifugation
↓ RPM - "spun down"
separates by density differences or by size (MW) of particles.

Cellular fractionation
can separate
•mitochondria
•microsomes
•ribosomes
•soluble proteins



Myoglobin and Hemoglobin

Because of its red color, the red blood pigment has been of interest since antiquity.

•First protein to be crystallized - 1849.

•First protein to have its mass accurately measured.

•First protein to be studied by ultracentrifugation.

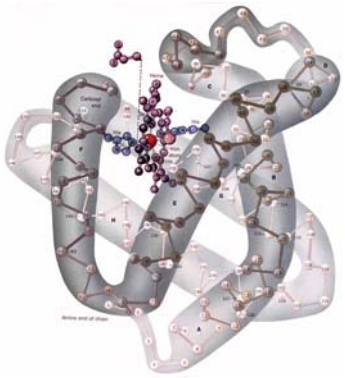
•First protein to associated with a physiological condition.

•First protein to show that a point mutation can cause problems.

•First proteins to have X-ray structures determined.

•Theories of cooperativity and control explain hemoglobin function

The Backbone structure of Myoglobin



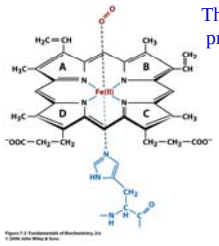
Myoglobin: 44 x 44 x 25 Å^3 single subunit 153 amino acid residues

121 residues are in an a helix. Helices are named A, B, C, …F. The heme pocket is surrounded by E and F but not B, C, G, also H is near the heme.

Amino acids are identified by the helix and position in the helix or by the absolute numbering of the residue.

The Heme group

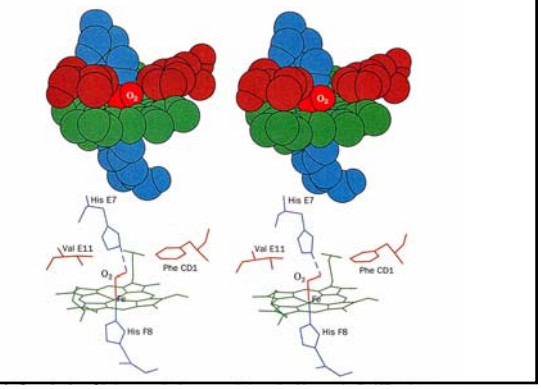
Each subunit of hemoglobin or myoglobin contains a heme.
- Binds one molecule of oxygen
- Heterocyclic porphyrin derivative
- Specifically protoporphyrin IX



The heme **prosthetic group** in Mb ad Hb: protoporphyrin IX + Fe(II)

The iron must be in the **Fe(II)** form or **reduced** form (ferrous oxidation) state.

The Heme complex in myoglobin

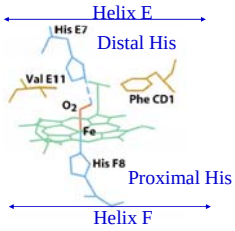


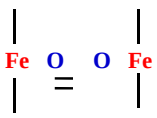
Role of the Globin

•Modulate oxygen binding affinity

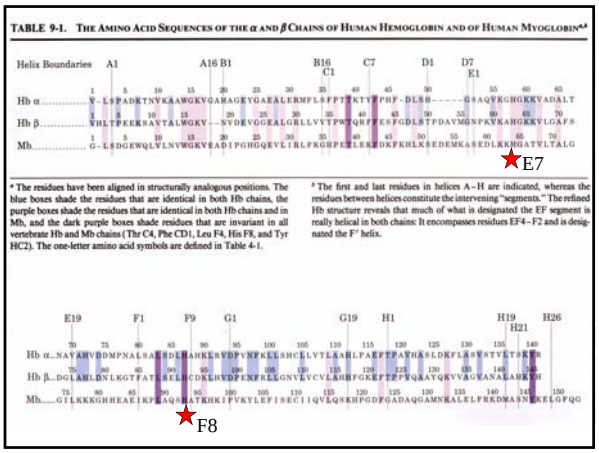
•Make reversible oxygen binding possible

By introducing steric hindrance on one side of the heme plane interaction can be prevented and oxygen binding can occur.

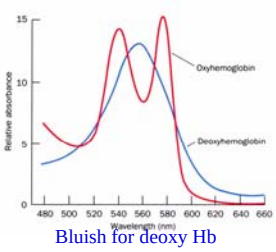




A heme dimer is formed which leads to the formation of Fe(III)



The visible absorption spectra for hemoglobin



Bluish for deoxy Hb
Redish for Oxy Hb

The red color arises from the differences between the energy levels of the *d* orbitals around the ferrous atom.

Fe(II) = *d6* electron configuration low spin state ↓↑ ↓↑ ↓↑

Binding of oxygen rearranges the electronic distribution and alters the *d* orbital energy.

This causes a difference in the absorption spectra.

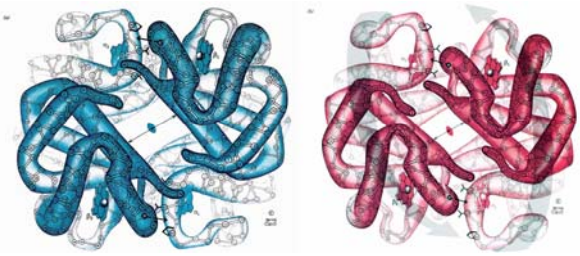
Measuring the absorption at 578 nm allows an easy method to determine the percent of O_2 bound to Hb

Hemoglobin

Spherical 64 x 55 x 50 Å two fold rotation of symmetry α and β subunits are similar and are placed on the vertices of a tetrahedron. There is no D helix in the α chain of hemoglobin. **Extensive interactions between unlike subunits** α2-β2 or α1-β1 interface has 35 residues while α1-β2 and α2-β1 have 19 residue contact.

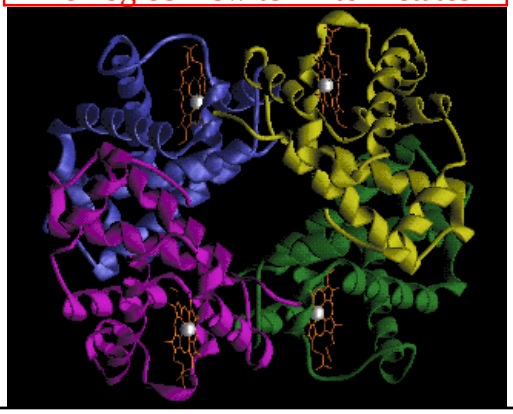
Oxygenation causes a considerable structural conformational change

Quaternary structure of deoxy- and oxyhemoglobin



T-state R-state

Hemoglobin switch T to R states



Lecture 12
Thursday 10/01/09
Protein Function - Globins