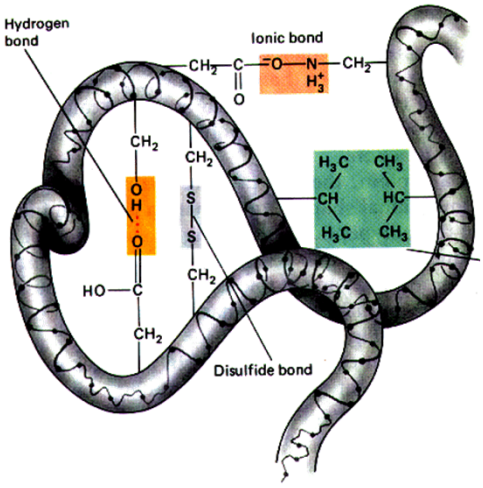
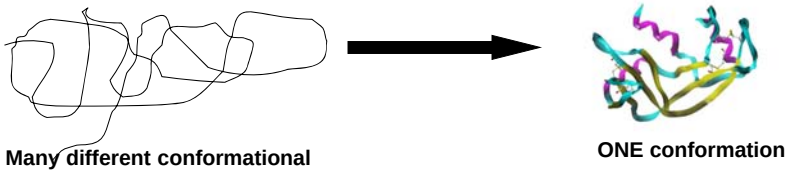
Tertiary Structure of Protein



How Does a Newly Synthesized Protein Go From a Random Coil to the Final Intricately Folded Protein?



Many different conformational species

ONE conformation

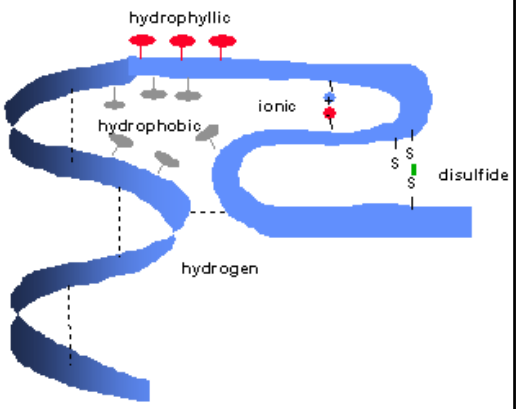
What are the Forces that Guide this Process?
What are the Steps Involved?
How Fast Can this Happen?

"The native, folded structure of a protein, under optimal conditions, is the most energetically stable conformation possible" Christian Anfinsen, 1972

Protein Tertiary and Quaternary Structures

Tertiary (3°) structure:
3-D arrangement of all atoms in a protein.
3-D folding of the secondary structural elements.

Forces that influence folding
(usually all are optimized):
1. Hydrogen bonding
2. Hydrophobic interactions
3. Ionic interactions
4. van der Waals forces
5. Disulfide bridges
6. Metal chelation
(cross linking)



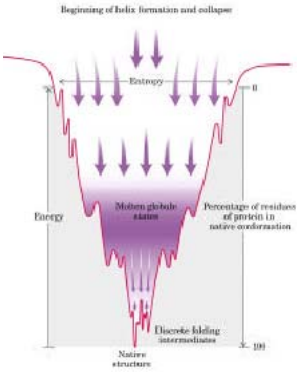
Forces driving protein folding

- It is believed that *hydrophobic collapse* is a key driving force for protein folding
  - Hydrophobic core
  - Polar surface interacting with solvent
- Minimum volume (no cavities)
- Disulfide bond formation stabilizes
- Hydrogen bonds
- Polar and electrostatic interactions

Native state is typically only 5 to 10 kcal/mole more stable than the unfolded form

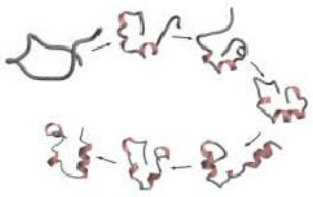
Thermodynamics of Protein Folding

- **Bond stretching:** 10^{-14} - 10^{-13} sec.
- **Elastic vibrations:** 10^{-12} - 10^{-11} sec.
- **Rotations of surface sidechains:** 10^{-11} - 10^{-10} sec.
- **Hinge bending:** 10^{-11} - 10^{-7} sec.
- **Rotation of buried side chains:** 10^{-4} - 1 sec.
- **Protein folding:** 10^{-6} - 10^{2} sec.



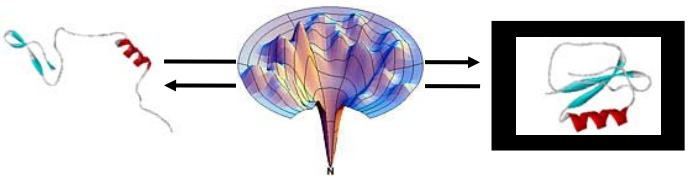
Free Energy Funnel

Simulated folding in 1 μsec; peptide in a box of water



Features of the native state

-well defined 3D structure
-Isoelectric point (pI)
-Some characterized molecular function

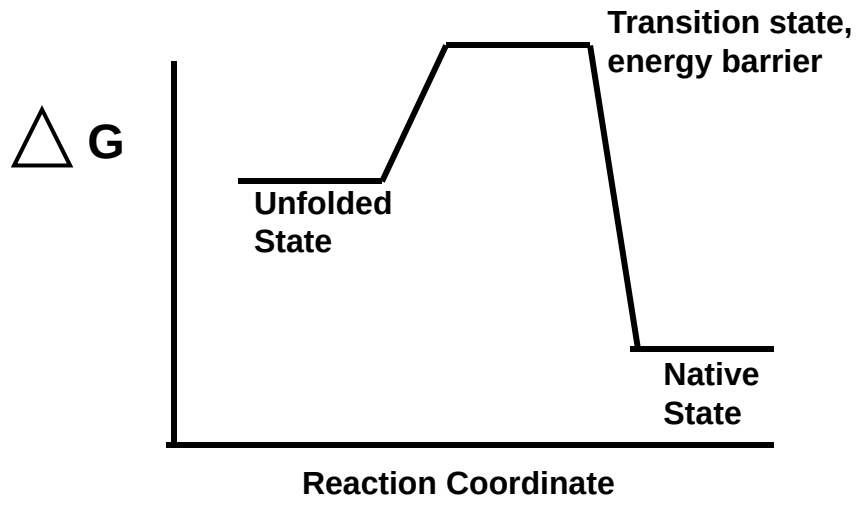


- Many proteins fold spontaneously to their native structure
- Protein folding is relatively fast
- Chaperones speed up folding, but do not alter the structure

The "native" structure of a protein is the form we find when we isolate that protein in an active state from a natural source.

If the protein loses that structure, by unfolding, or unwinding, it loses activity.
Therefore, native = folded
denatured = unfolded

The "native" structure is necessary to create the binding pockets that make up the active site of an enzyme.



△G, Gibbs Free Energy, the more negative, the more stable the system

Proteins are marginally stable

- As a protein folds, it must exchange the many noncovalent interactions it makes with water for other interactions that it makes within itself.
- Main forces that favor protein folding are hydrophobic interactions and H-bond. The main force opposing folding is conformational entropy.
- During the process of protein folding, the hydrophobic side chains are shielded from the solvent and pack against one another.
- Many of its H-bond donors and acceptors pair with each other, especially those in the backbone.

- Each of these interaction energy is small, but the number of interactions is very large. Hence, the total interaction energies in the native and denatured states is very large.
- Proteins only marginally stable, with a net stability difference (ΔG) between the native and unfolded state of 5-15 kcal/mol.

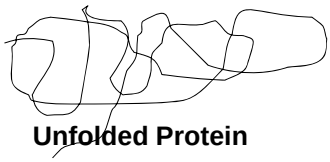
$$\Delta G_{folding} = \Delta S_{protein} + \Delta H_{protein} + \Delta S_{water} + \Delta H_{water} = 5\text{-}15 \text{ kcal/mol}$$

(-) (-) (++) (+)

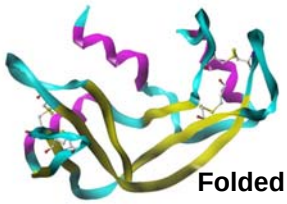
Entropy and Enthalpy in Protein Folding

$$\Delta G = \Delta H - T\Delta S$$

bonding flexibility



Unfolded Protein



Folded Protein

Δ H, small, negative
ΔS, large, positive

Δ H, large, negative
ΔS, small, positive

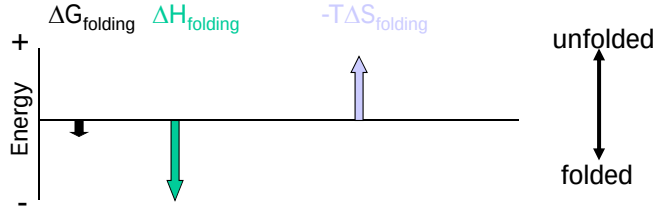
Compensation in entropy and enthalpy for protein
Contribution of entropy of water molecules released upon folding

ΔS of water is large and positive

Thermodynamics of Protein Folding

$$\Delta G_{folding} = G_{folded} - G_{unfolded} =$$

$$(H_{folded} - H_{unfolded}) - T(S_{folded} - S_{unfolded}) = \Delta H_{folding} - T\Delta S_{folding}$$



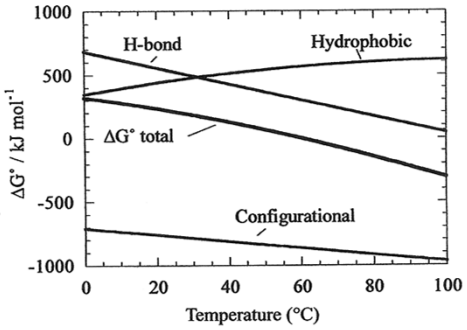
Folded proteins are highly ordered

$\therefore$ $\Delta S_{folding}$ negative, so $-T\Delta S_{folding}$ is a positive quantity

$\Delta H_{folding}$ is a negative quantity - enthalpy is favored in folded state.

Total Gibbs free energy difference is negative – folded state favoured

Representative contributions of interactions to the overall stabilization of a globular protein.



Configurational entropy - Backbone contributes between 9J / K.mol per residue (Val & Ile) and 27 J / K.mol per residue (Gly).

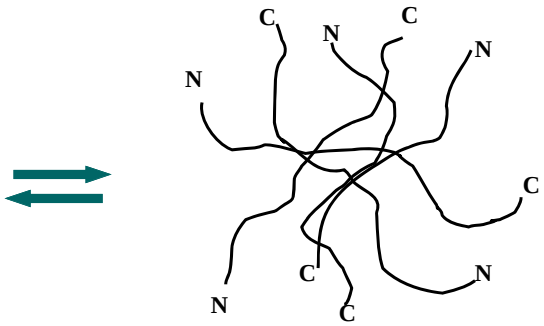
Therefore, to change a residue that is restricted in the native state for Gly results in a big cost in conformational entropy.

Side chain contribute between 0-30 J/K. mol

Below 100°C, the hydrophobic effect usually increases with temperature, but levels off at very high temperature because the structure of water decreases with increasing temperature.

Native state (N)

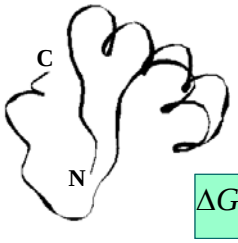
Denatured state (D)



$$\Delta G_D^N = -RT \ln[D]/[N]$$

Stability of the native state is defined as the difference in free energy between the native and denatured states

Native state (N)



$$\Delta G_D^N = \Delta H_D^N - T\Delta S_D^N$$

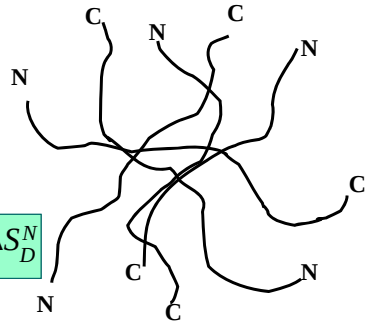
Size of cavity in solvent ~6500Å²

ΔS chain: **significantly decreased, due to the well defined conformation**

Non-bonded interactions: **intra-molecular**

Compact structure

Denatured state



Average size of cavity in solvent ~20,500Å²

ΔS chain: **large, due to the large number of different conformations**

Non-bonded interactions: **inter-molecular**

Non compact structure

Factors that disrupt the Native state

1) **ELECTROLYTE ADDITION**
   - **interference with the colloid state**

2) **INSOLUBLE SALT FORMATION**
   - **Protein+Trichloracetate**

3) **ORGANIC SOLVENTS**
   - **ETHANOL - interferes with the dielectric constant**

4) **HEAT DENATURATION**
   - **more energy in system (bonds break)**

5) **pH**
   - **destroys charge**
   - **destroys ability to interact with water**

6) **DESTRUCTION OF HYDROGEN BONDING**
   - **UREA - known H-bond disrupter**

Thermodynamic Description of Protein Folding

The native and unfolded states are in equilibrium, the folding reaction can be quantified in terms of thermodynamics.

The equilibrium ($N \leftrightarrow U$) between the native (N) and unfolded (U) states is defined by the equilibrium constant, K, as:

$$K = [U]/[N] = K_U$$

The difference in Gibbs free energy (ΔG) between the unfolded and native states is then:

$$\Delta G = -RT \ln K$$

For K_u, a positive ΔG indicates that the native state is more stable.

The free energy is composed of both enthalpic and entropic contributions:

$$\Delta G = \Delta H - T \Delta S$$

where ΔH and ΔS are the enthalpy and entropy change, respectively, upon unfolding.

Thermal Unfolding

Since ΔH and ΔS are strongly temperature-dependent, ΔG is better expressed as:

$$\Delta G = \Delta H_1 + \Delta C_P (T-T_1) - T [\Delta S_1 + \Delta C_P \ln(T/T_1)]$$

where the subscript "1" indicates the value of ΔH and ΔS at a reference temperature, T_1, and ΔC_p is the specific heat or heat-capacity change.

Most proteins denature reversibly allowing thermodynamic analysis.

Specific Heat (C_p)

•The specific heat is the energy required to raise by 1°C, the temperature of 1 mol of water.

•A large C_p is the hallmark of aqueous solutions of nonpolar solutes. Therefore, the unfolded state of proteins has a high C_p.

•As the temperature increases, the ordered shell around non polar solutes tend to melt, producing an increase in specific heat, C_p.

•The ΔCp of unfolding is roughly 12 cal/deg/mol per residue of the protein.

Interaction	***Approx. bond strength in kJ/mole***
Covalent bonds	**> 200 (ranging up to 900)**
Ionic	**20-40**
Hydrogen bond	**~5-10**
Hydrophobic	**~ 8**
van der Waals	**~ 4**

AMBER (*Assisted Model Building with Energy Refinement*) force field

$$E_{total} = \sum_{bonds} K_r (r - r_{eq})^2 + \sum_{angles} K_\theta (\theta - \theta_{eq})^2 + \sum_{dihedrals} \sum_{1}^{3} \frac{V_n}{2}[1 + \cos(n\omega)]$$
$$+ \sum_{i<j}^{atoms} \left(\frac{a_{ij}}{r_{ij}^{12}} - \frac{b_{ij}}{r_{ij}^{6}} \right) + \sum_{i<j}^{atoms} \frac{q_i q_j}{\varepsilon r_{ij}}$$

Techniques for Measuring Stability

Any method that can distinguish between U and F

- Absorbance (e.g. Trp, Tyr)
- Fluorescence (Trp)-difference in emission max & intensity.
- CD (far or near UV) - (2^o or 3^o)
- NMR
- DSC (calorimetry)
- Urea gradient gels - difference in the migrating rates between F and U.
- Catalytic activity
- Chromophoric or fluorophoric probes

Denaturation may not require complete unfolding of proteins. It might be still a folded structure but in random conformation.

Denaturation is cooperative - changes in one part of protein accelerate the unfolding of the other part.

Some proteins are resistant to denaturation by heat.

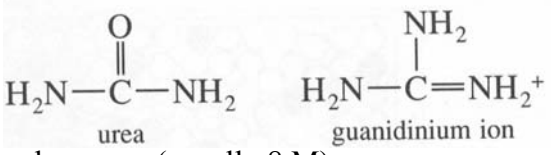
Heat: destabilizes H-bonding
Detergents, Urea, organic solvents: destabilize hydrophobic interactions
Extreme pH conditions: cause ionization of side chains resulting in electrostatic repulsion and collapse of structure.

Most proteins denature reversibly allowing thermodynamic analysis.

Denaturing Proteins at Extreme pHs

- High pH and low pH denature many, but not all proteins
- The basic idea is that the net charge on the protein due to the titration of all the ionizing groups leads to intramolecular charge-charge repulsion, which is sufficient to overcome the attractive forces (mostly hydrophobic and dispersive) resulting in at least partial unfolding of the protein.
- The presence of specific counterion binding leads to formation of compact intermediate states such as the molten globule (substantial secondary structure, little or no tertiary structure, relatively compact size compared to the native state).

Denaturants



- The effects of denaturants such as urea (usually 8 M) or Guanidinium Hydrochloride (usually 6 M GuHCl) are complex.
- involve preferential solvation of the denatured (unfolded) state, involving predominantly hydrophobic related properties, and to a lesser extent H-bonding (both side-chains and backbone appear to be more soluble in the presence of the denaturants).
- There is no good solvent because solvents that are good for the hydrophobic components are bad for the hydrophilic ones and vice versa.
- As in the case of pH-induced denaturation, not all proteins are unfolded by these denaturants.
- Protein stability: $SCN^- < Cl^- <$ Urea $< SO_4^{2-}$

  e. g. midpoints of unfolding transition for RNase: GuSCN = 0.3M, GuHCl = 0.8 M, and urea nearly 3 M.

Denaturants

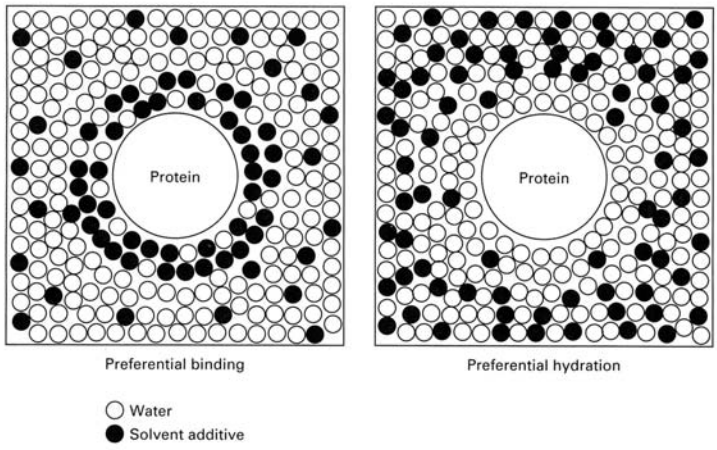


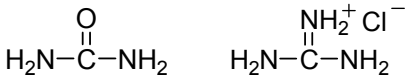
FIGURE 7.14
Schematic illustration of preferential binding and preferential hydration by solvent additives. In preferential binding, the additive occurs in the solvation shell of the protein at a greater local concentration than in the bulk solvent. Preferential hydration results from exclusion of the additive from the surface of the protein. (From S. N. Timasheff and T. Arakawa, in *Protein Structure: A Practical Approach*, T. E. Creighton, ed., pp. 331–345. IRL Press, Oxford, 1989.)

Protein Denaturation and Folding

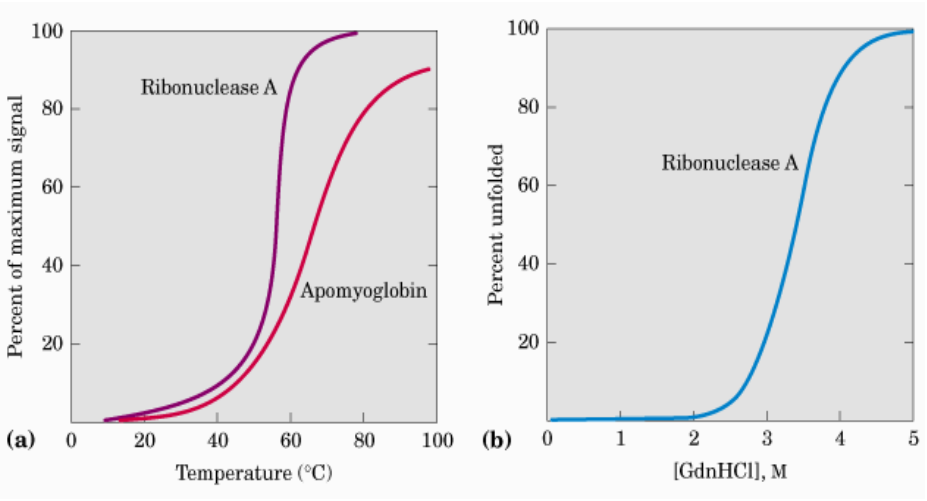
Denaturation:
Loss of 3-D structure sufficient to cause loss of function

- Unfolding is abrupt and a cooperative process.
- T_m: melting point

Denaturants: heat, pH, organic solvents, detergents
urea, guanidine chloride (GdnHCl)



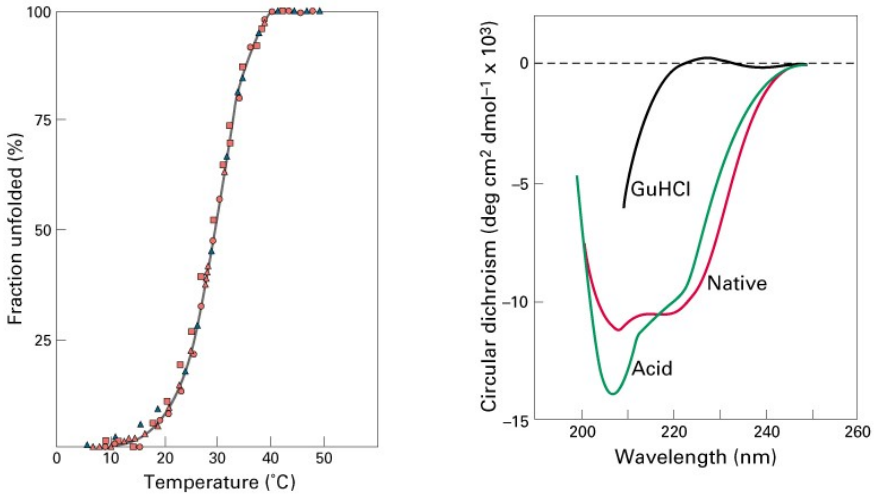
Denaturation of proteins by heat and guanidine hydrochloride



Two-state Unfolding of Protein

- $K_{eq}=[N]/[U]= ([\theta]_{obs}- [\theta]_D)/([\theta]_N- [\theta]_D) = F_N/(1- F_N)$

F_N = fraction folded

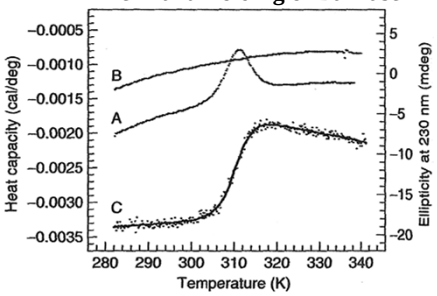


Measuring Thermal Denaturation

•Thermal unfolding of a protein is best measured by differential scanning calorimetry, which measures the heat absorbed by a protein as it is slowly heated through its melting transition.

•As the protein denatures, there is a large uptake of heat because the process is highly endothermic.

Thermal unfolding of Barnase



A. Heat capacity; B. Baseline trace of buffer; C. Transition measure by CD at 230 nm. T_m = 311 K

•The temperature at the maximum of the peak is the T_m, the melting temperature.

•The area under the curve, after subtracting the baselines, is the enthalpy of unfolding, ΔH.

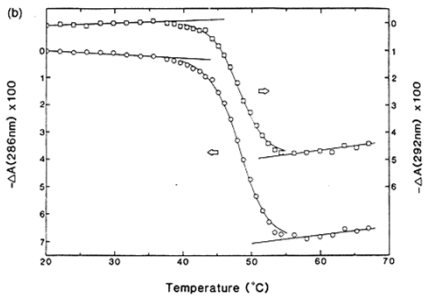
•The difference between the heat capacity before and after the transition yields the ΔC_p.

Circular dichroism (CD) can also be used to monitor thermal denaturation.

Denaturation Curve

Two important requirements for thermodynamic analysis:

- That unfolding is reversible
- That equilibrium has been reached before taking measurements.



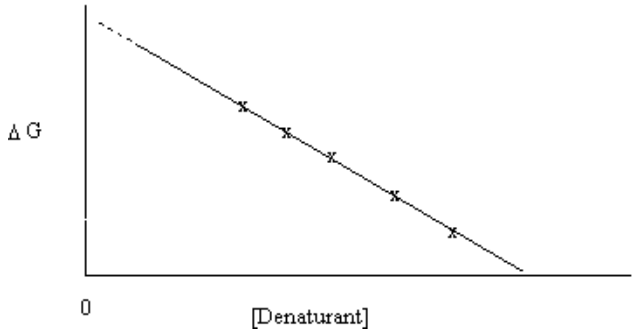
All unfolding curves can be conveniently divided into three regions:

a) Pre-transition region - shows how the signal for the folded protein depend on the denaturant.

b) Transition region - shows how the signal varies as unfolding occurs.

c) Post-transition region - shows how the signal for the unfolded protein depend on the denaturant.

Denaturants



- It is common to extrapolate the data for the unfolding transition as a function of denaturant to 0 M to give the value in water (e.g. $G(H_2O)$).

  $\Delta G_{D\text{-}N} = \Delta G^{H20}_{D\text{-}N} - m_{D\text{-}N}$ [denaturant]

  $\Delta G^{H20}_{D\text{-}N}$ is about –5 to –10 kcal/mol

- The extrapolation can have large errors.

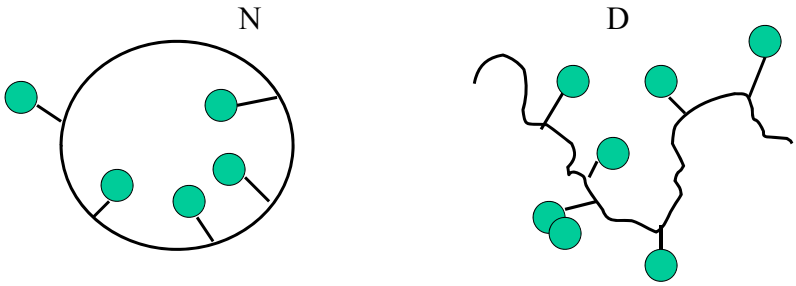
m - value

- m-value reflects the dependence of the free energy on denaturant concentration
  - Typically for urea m ~ 1 kcal/mol
  - For GuHCl m ~ 3 kcal/mol
- The variation in slope (m) is due to change in the solvent accessible area of hydrophobic residues.
- The m-value is related to how cooperative the transition is, how much structure remains in the denatured state, perhaps how much denaturant binds to the unfolded state, etc.

The hydrophobic effect

Non-polar solutes prefer to interact with each other than with water, because this reduced the contact surface of the solute with the solvent.

For a polypeptide, this means that hydrophobic sidechains such as Val, Ile, Leu,Phe, would tend to be buried in the protein interior.



The hydrophobic effect

The free energy gain from burying a hydrophobic group is proportional to the surface area buried

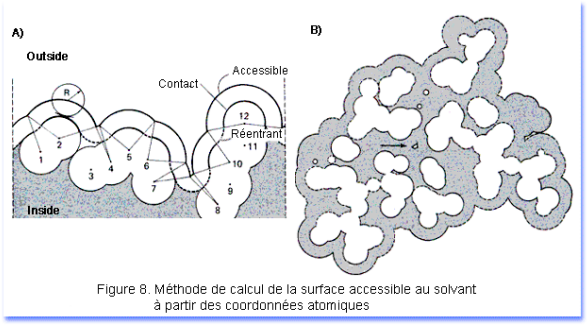
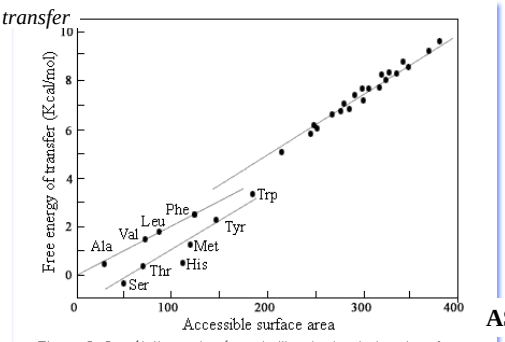


Figure 8. Méthode de calcul de la surface accessible au solvant à partir des coordonnées atomiques

Calculating the surface area in contact with solvent (Lee & Richards, 1971)

The hydrophobic effect

$\Delta G_{transfer}$



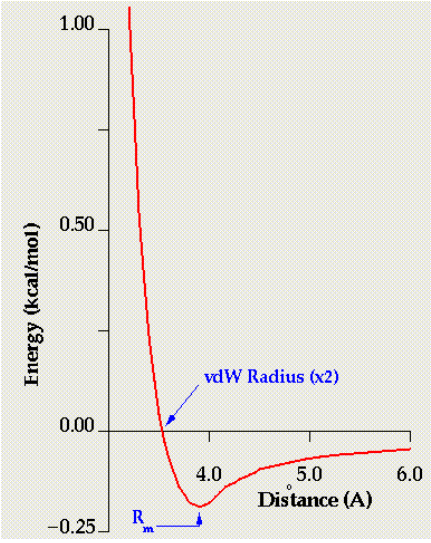
ASA(Å²)

Figure 9. Corrélation entre énergie libre hydrophobe et surface accessible au solvant des 20 acides aminés naturels.

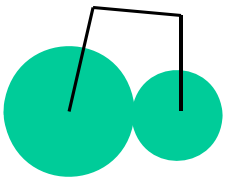
$\Delta G_{transfer} = -\gamma ASA$

$\gamma = 0.025\ cal/Å^2$

Linear relation between the solvent accessible surface area and the transfer free energy of amino acids (Chothia, 1972)

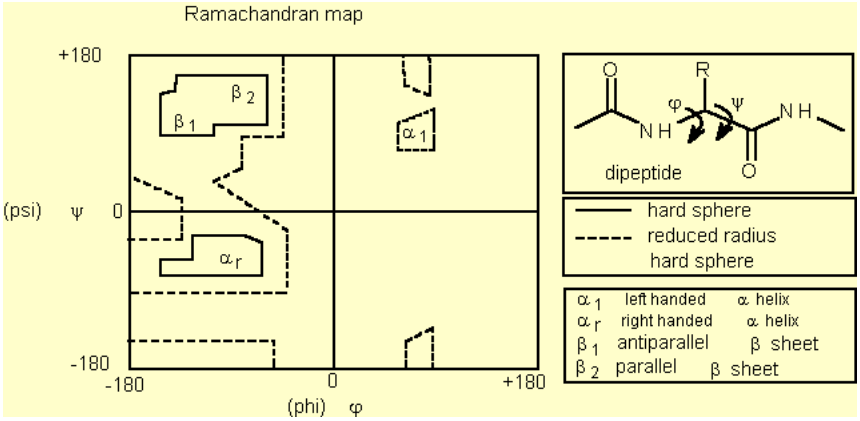


van der Waals interactions

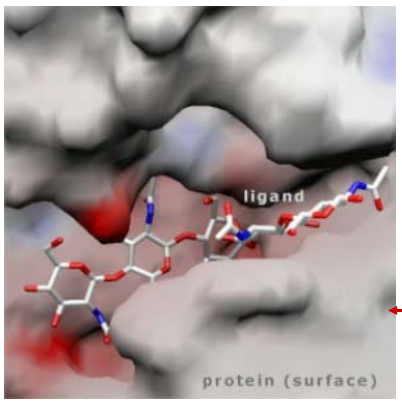


$$E = \frac{A}{r^{12}} - \frac{B}{r^{6}}$$

The Ramachandran map



Electrostatic interactions



Electrostatic potential displayed on molecular surface

Coulomb's law

$$U_{el} = \frac{1}{2}\sum_{i \neq j}\frac{q_i q_j}{r_{ij}}$$

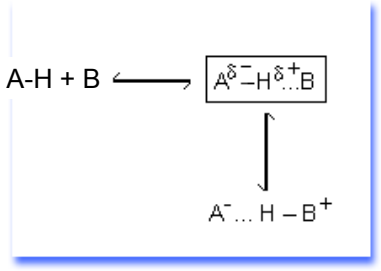
Electrostatic energy of interaction, between charged and polar groups

$$\nabla\Phi = \vec{E}$$

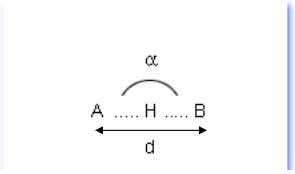
Electric field (vector)

Electrostatic potential (scalar), computed using a continuum model to represent the surrounding water

Hydrogen bonds: a special case of electrostatic interactions



H-bond geometry



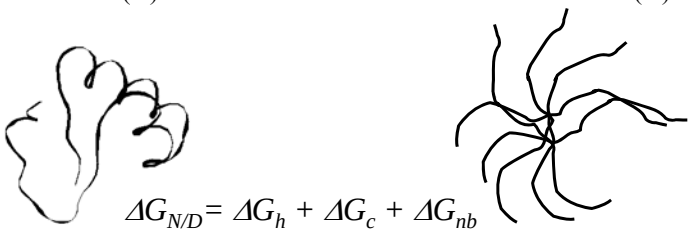
α is close to 180º (180 ±30º)

d depends on the donor-acceptor pair

N-H--O 2.55 - 3.04 Å
O-H--N 2.62 - 2.93 Å
O-H--O 2.65 - 2.93 Å

Contributions to the stabilisation free energy of a soluble monomeric protein of 100 residues

Native state (N) Denatured state (D)



$\Delta G_{N/D} = \Delta G_h + \Delta G_c + \Delta G_{nb}$

ΔG_h = *~25 cal/mol/Å² x (20,500 - 6,500)Å²* >>> ***-350 kcal/mol***

ΔG_c = *~3-3.5 kcal/mol/residue x 100 residues*>>> ***+350 kcal/mol***

ΔG_{nb}= *very small overall* >>>***~5-10kcal/mol***

$\Delta G_{N/D}$= >>>***~5-10 kcal/mol***

Gibbs free energy

The native and denatured forms of a protein are generally in equilibrium.

$$Native \longleftrightarrow Denatured$$

For a certain protein, (total conc 2.0 * 10^{-3} M) the concentration of the denatured and native forms at 50°C and 100°C is given in the table.

Temp	Denatured (M)	Native (M)
50	$5.1*10^{-6}$	$2.0*10^{-6}$
100	$2.8*10^{-4}$	$1.7*10^{-3}$

1. Determine ΔH and ΔS for the folding reaction (assuming they are independent of T)
2. Calculated ΔG for this protein at 25°C. Is this process spontaneous?
3. What is the denaturing temperature for this protein at standard conditions?