PROTEIN FOLDING and MISFOLDING, DISEASES ASSOCIATED WITH PROTEIN MISFOLDING & AGGREGATION

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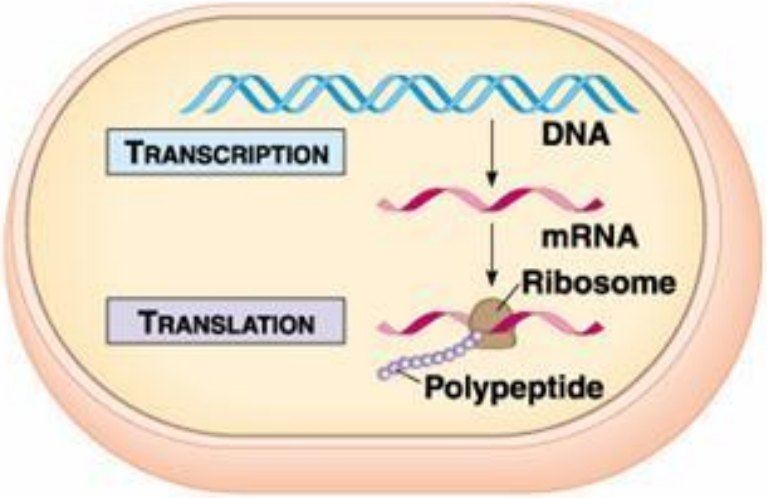
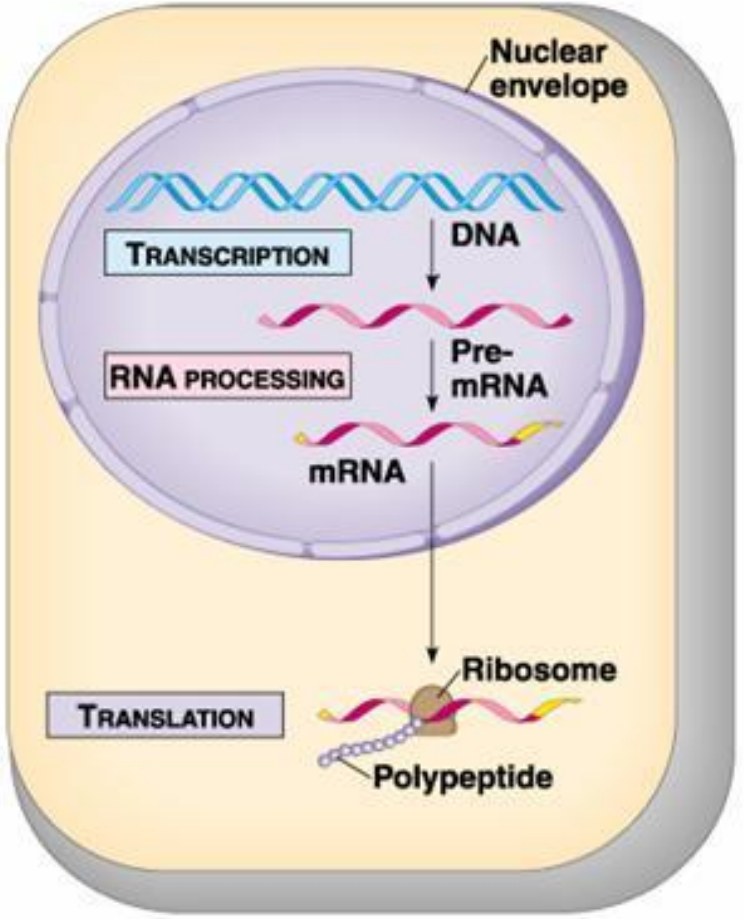
CENTRAL DOGMA IN MOLECULAR BIOLOGY

GENE EXPRESSION pathway



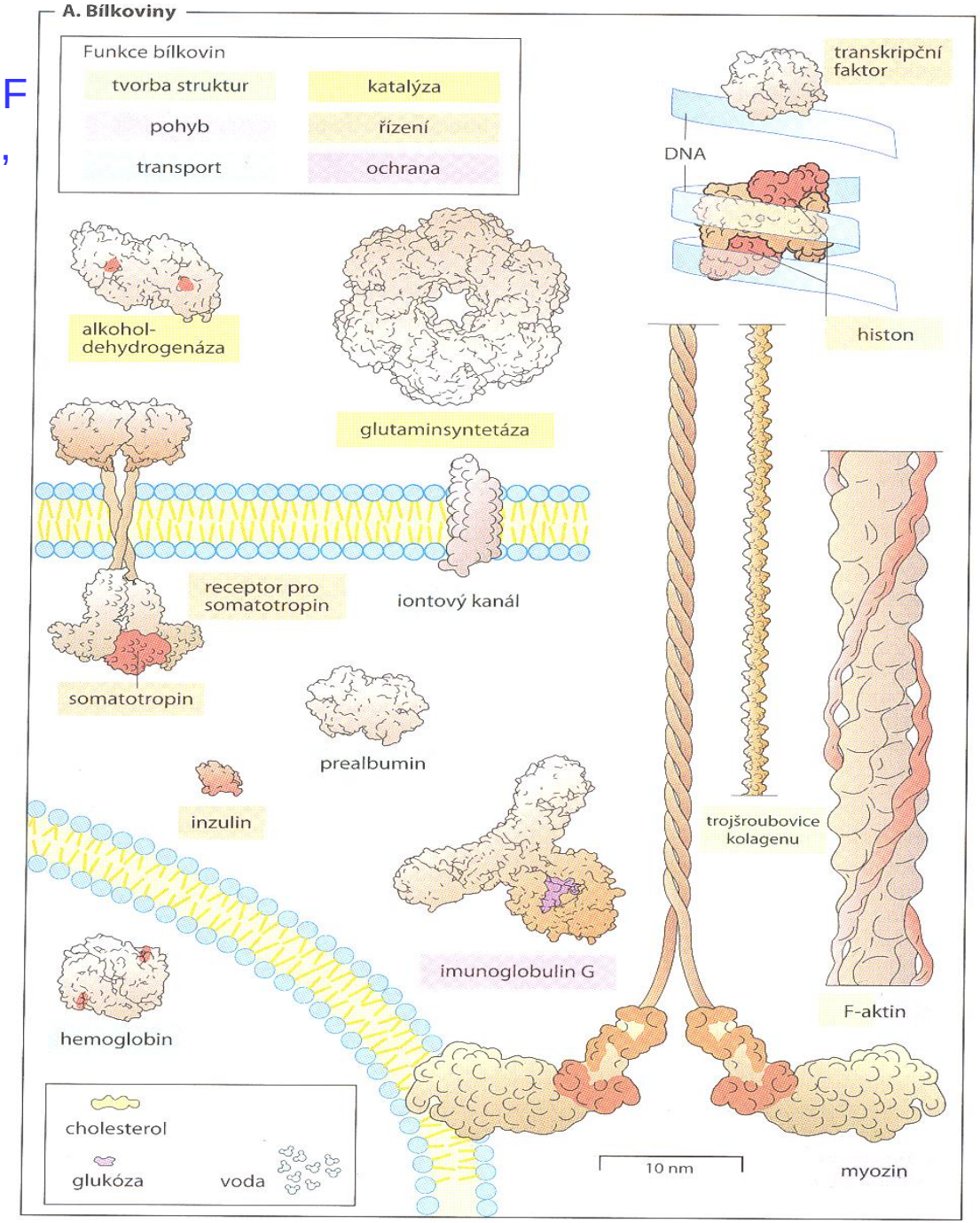
(a) Prokaryotic cell



(b) Eukaryotic cell

= Linear polypeptide chain :
posttranslational modification:
PROTEIN FOLDING

GREAT VARIETY OF CONFORMATIONS, SHAPES OF PROTEINS



PART I. Protein Folding

PART II. Abnormalities in Protein Folding and Clinical Consequences

I. Protein folding:

1. **WHY**

2. **HOW**

DOES THE PROTEIN REACH ITS FINAL CONFORMATION?

Under physiological conditions the folding of proteins into their native conformation is a **spontaneous,** self-assembling process, with no need of external templates to guide their formation.

Ch. Anfinsen (1957): **Thermodynamic hypothesis**.

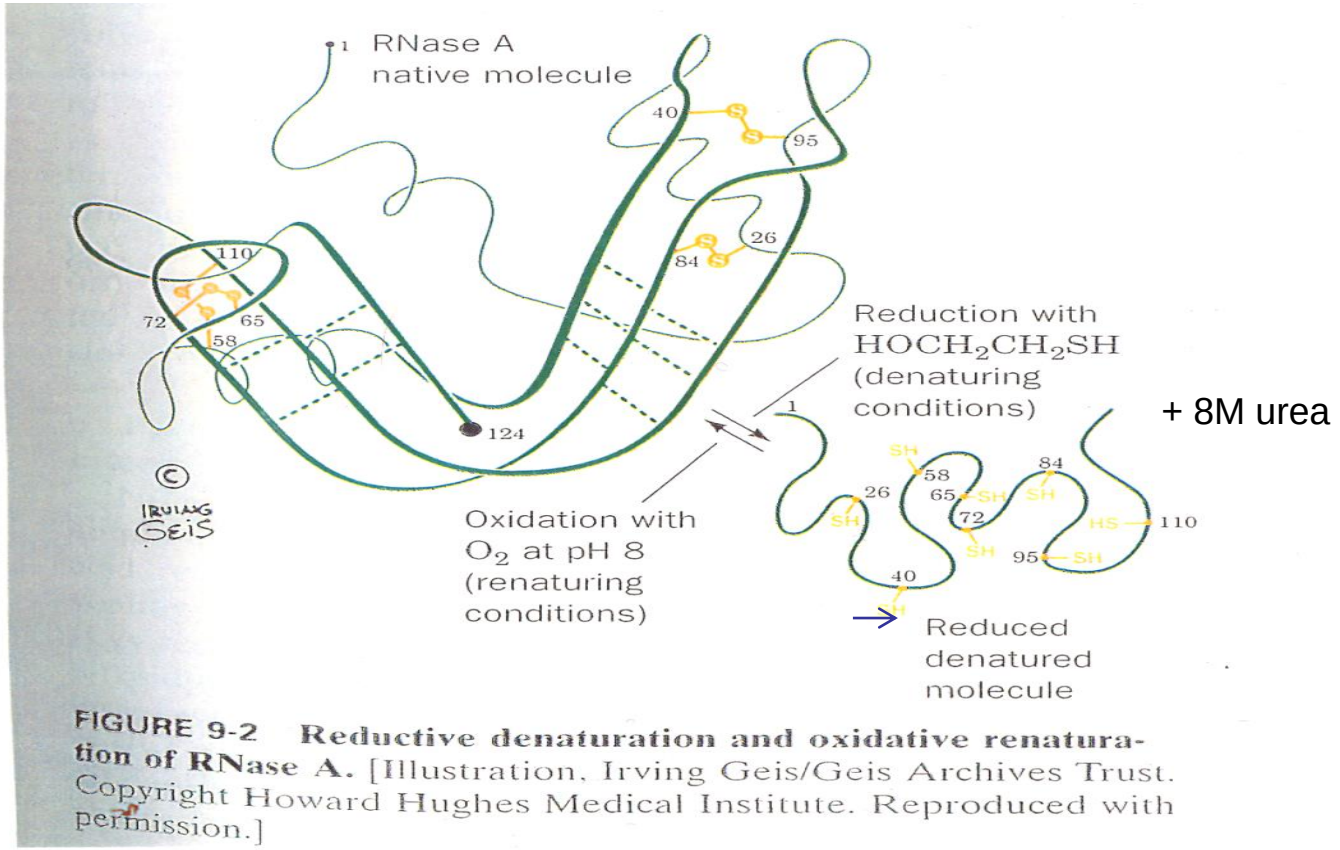


FIGURE 9-2 Reductive denaturation and oxidative renaturation of RNase A. [Illustration, Irving Geis/Geis Archives Trust. Copyright Howard Hughes Medical Institute. Reproduced with permission.]

The help of protein folding facilitating enzymes is often required. Mainly to prevent incomplete protein aggregation and to increase the speed of correct protein folding.

1.*WHY* is the folding taking place?

- Spontaneous = driven thermodynamically = the folding obeys laws of thermodynamics: 1. to achieve the lowest energetic state for the given primary structure of the polypeptide chain under present (physiological) conditions, 2. to proceed in directions that increase the overall disorder (entropy) of the system and its surroundings => the folding is accompanied by the reduction of free energy and/or increase in entropy of the system:

  UNFOLDED =>>> FOLDED + energy (heat, entropy)

A protein primary structure dictates protein's 3D structure.

- Living matter: To attain the shape evolved by natural selection for the particular biological function

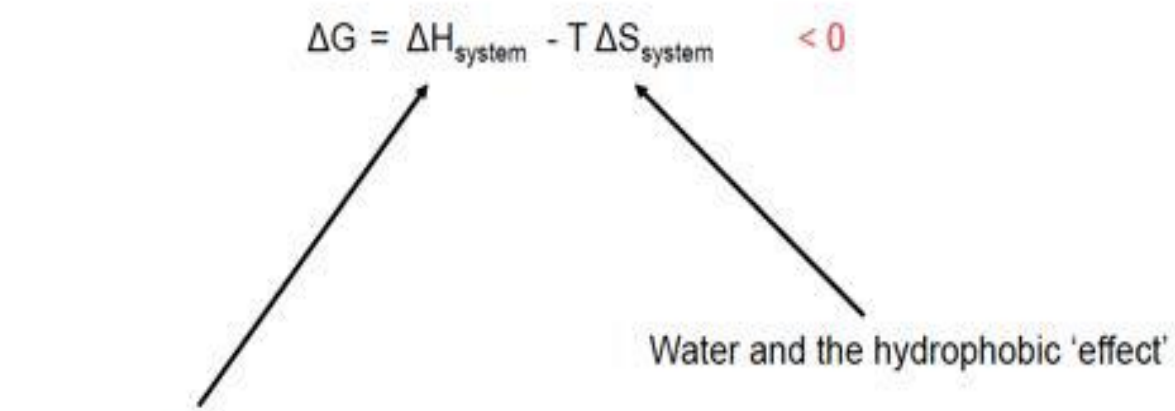
Gibbs-Helmholtz Equation

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

- **G**, **change in the free energy**. It is a measure of the driving force of the (chemical) reaction. It depends on three parameters: H, T and S.
  - **H**, **enthalpy change**. It is the heat that the process generates/releases (exothermic) or absorbs (endothermic). **T**, absolute temperature. **S**, **entropy change** (chain conformational entropy change), change in disorder
- **G < 0**, the free energy of the products is smaller than the free energy of the reactants; the reaction **can proceed spontaneously**, it is **exergonic**.

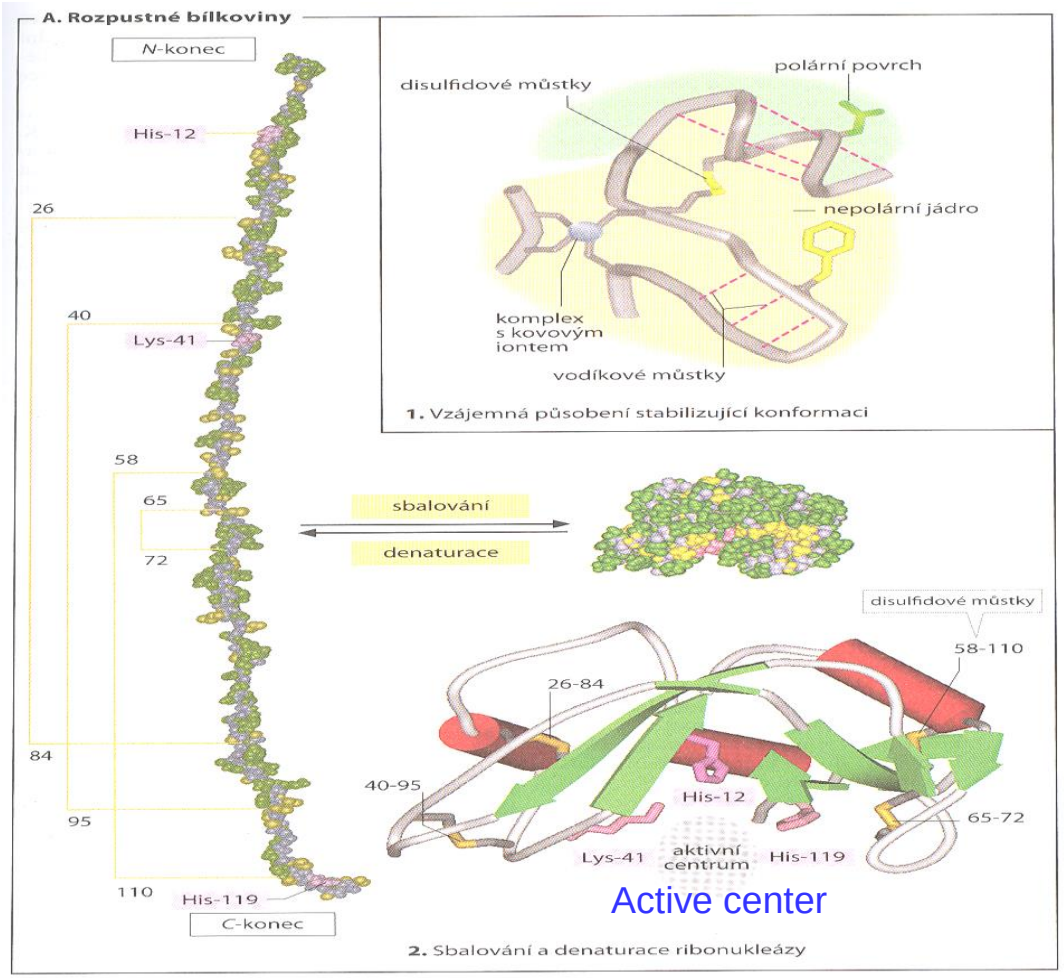
- **G** > 0, the free energy of the products is larger than the free energy of the reactants; the reaction cannot proceed spontaneously, it is **endergonic**.

Free Energy + Protein Folding



The polypeptide is folded in three dimensions by non-bonding interactions between its individual atoms

RNase A Folding and Denaturation



Free Energy + Protein Folding

- Protein chain folding (energetic summary):
- $\Delta H < 0$
- $\Delta S < 0$!
- $\Delta G = \Delta H - T.\Delta S$ ……> 0 !!!

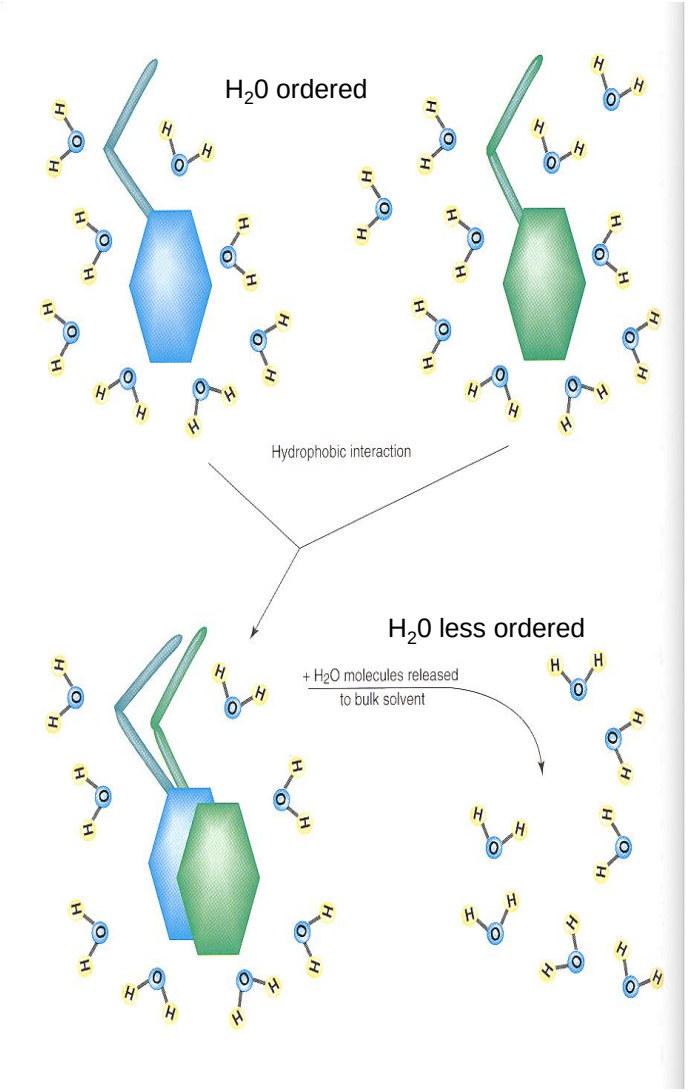
Free Energy + Protein Folding

THE PHYSIOLOGICAL FOLDING OF PROTEINS TAKES PLACE IN WATER ENVIROMENT

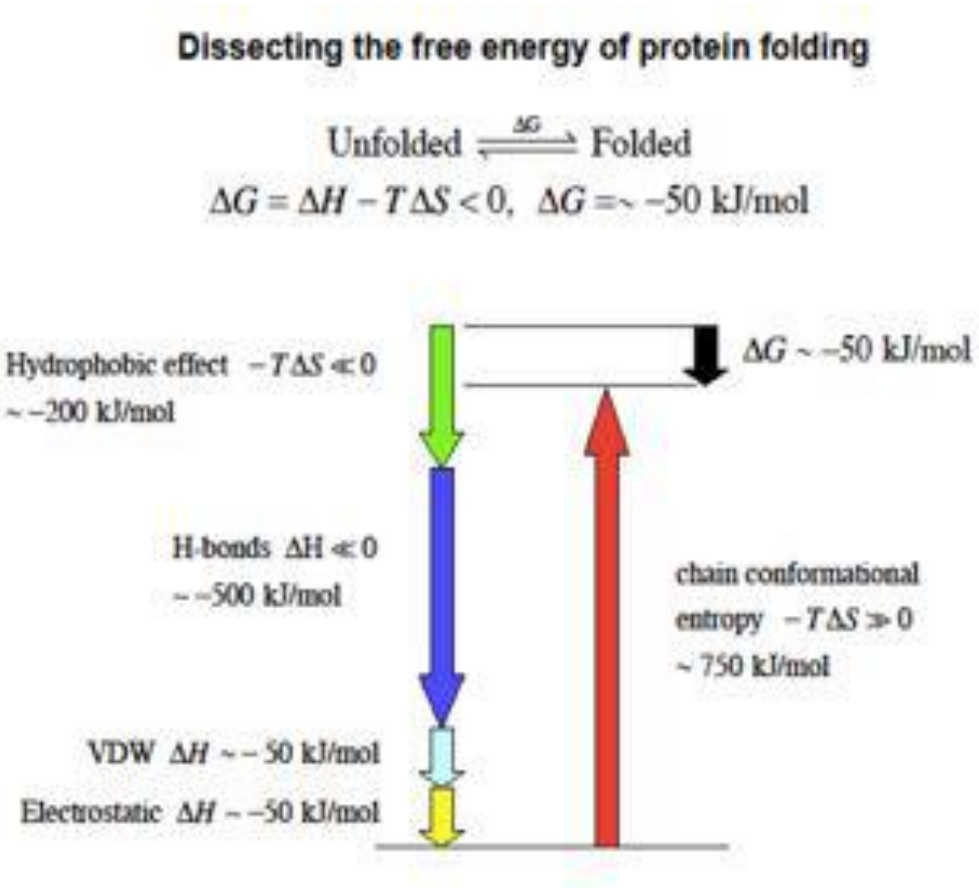
The hydrophobic effect

When two nonpolar side chains of Phe residues come together, their surface area exposed to solvent is reduced and some of the **ordered water molecules** (they have a lower degree of freedom), in the Phe solvation shells**,** are **released** to bulk solvent = the net **disorder of water molecules in the system is increased**. This **INCREASE IN WATER ENTROPY, $\Delta S>0$,** is thermodynamically favorable and it is THE DRIVING FORCE CAUSING NONPOLAR MOIETIES TO COME TOGETHER in aqueous solvent. The proteins, upon folding bury, on average, ~85% of their nonpolar side chains into their interiors.

This **hydrophobic effect** is the **MAIN DRIVING FORCE OF PROTEIN FOLDING**



Free Energy + Protein Folding



2. *HOW* do proteins build their native 3D conformation from their primary structure?

Starting points:

A) PEPTIDE BOND: O=C-N-H

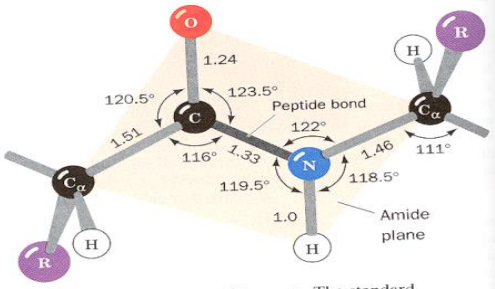


FIGURE 8-1 **The trans-peptide group.** The standard dimensions (in angstroms, Å, and degrees, °) of this planar group were derived by averaging the corresponding quantities in the X-ray crystal structures of amino acids and peptides. [After Marsh, R.E. and Donohue, J., *Adv. Protein Chem.* **22,** 249 (1967).] See Kinemage Exercise 3-1

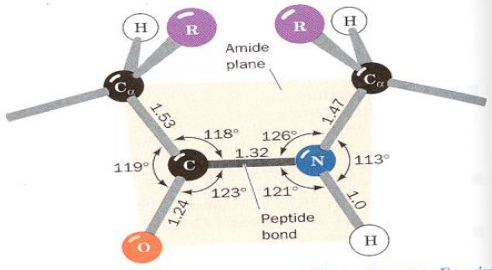


FIGURE 8-2 **The cis-peptide group.** See Kinemage Exercise 3-1

fully extended (all-trans) conformation and increase for a clockwise rotation when viewed from C_α (Fig. 8-4).

There are several steric constraints on the torsion angles, ϕ and ψ, of a polypeptide backbone that limit its conformational range. The electronic structure of a single (σ) bond, such as a C—C bond, is cylindrically symmetrical

Two conformations:

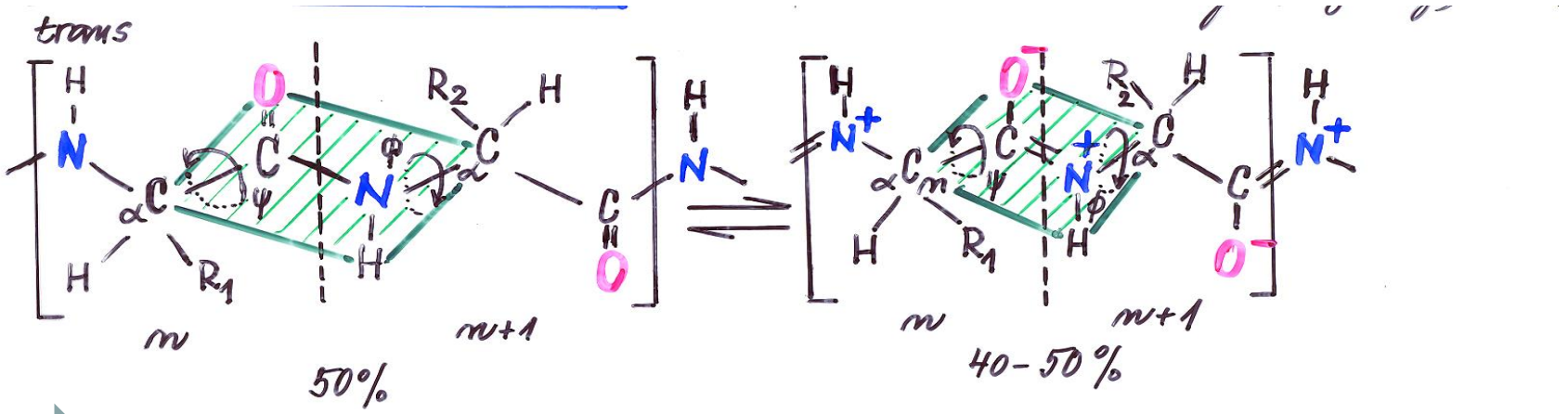
(i) TRANS-

Peptide bonds (groups), with few exceptions, assume the trans-conformation: that, in which successive Cα atoms are on opposite sides of the peptide bond joining them.

(ii) CIS-

~10% of Xaa-Pro peptide bonds

(iii) PEPTIDE BOND assumes a rigid, planar structure



➡ because it has about **40% double-bond character**

(exists in two resonance isomeric states)

Peptide Bond Length: 0.133 nm (C-N ~ 0.145 nm, C=N ~ 0.125 nm; RTG)

No rotation allowed around the peptide C-N bond, all atoms linked to C or N are in **one plane** (in green)

B) However: the backbone N-Cα single bonds can rotate (ϕ torsion angels)

the backbone C-Cα single bonds can rotate (ψ torsion angels)

The rotations are not unlimited, they are greatly restricted by collision of molecular orbitals of neighbouring atoms of the polypeptide chain – Ramachandran diagrams: sterically allowed values of ϕ and ψ

What folding shapes/structures may a polypeptide chain attain that would be optimally compatible with the sterically allowed values of ϕ and ψ ?

Optimal (and typical) folding shapes of secondary structural elements of proteins: Repetitive Regular Secondary Structure Elements of Proteins (A, B)

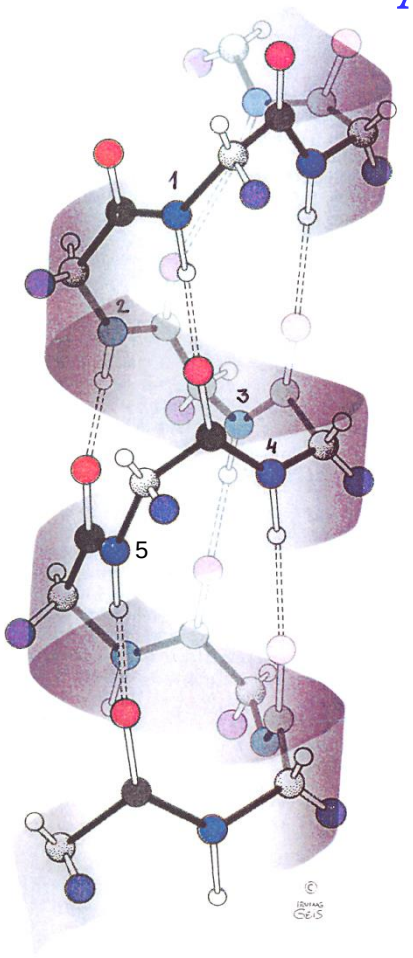


FIGURE 8-11 **The right-handed α helix.** Hydrogen bonds between the N—H groups and the C=O groups that are four residues back along the polypeptide chain are indicated by dashed lines. [Illustration. Irving Geis/Geis Archives Trust. Copyright Howard Hughes Medical Institute. Reproduced with permission.] See Kinemage Exercise 3-2 and the Animated Figures

A) α–helix

(right-handed, α_R)

3.6 aa res./turn

In order to exist, the polypeptide chain conformation angles (ϕ, ψ) must not only fall within the allowed regions but the particular conformation must be also **stabilized**. The "glue" that holds the secondary structural elements is, in part, **intra-chain hydrogen bonds**:

e.g. 5CO…1NH

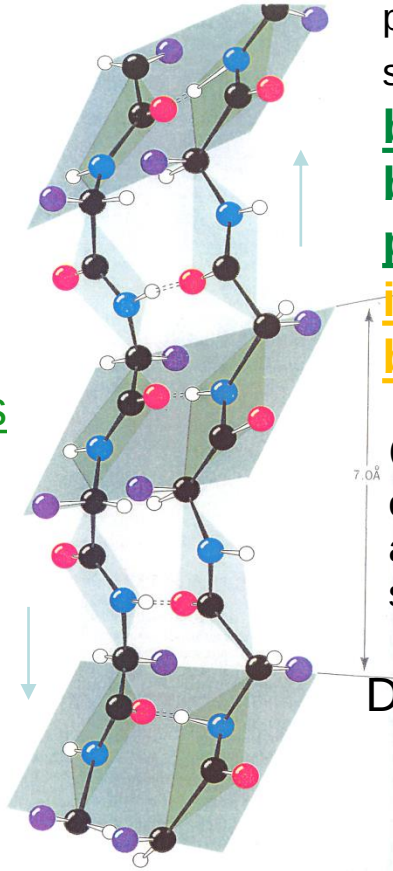


FIGURE 8-17 **A two-stranded β antiparallel pleated sheet drawn to emphasize its pleated appearance.** Dashed lines indicate hydrogen bonds. Note that the R groups (*purple balls*) on each polypeptide chain alternately extend to opposite sides of the sheet and that they are in register on adjacent chains. [Illustration, Irving Geis/Geis Archives Trust. Copyright Howard Hughes Medical Institute. Reproduced with permission.] See Kinemage Exercise 3-3

B) β–sheet

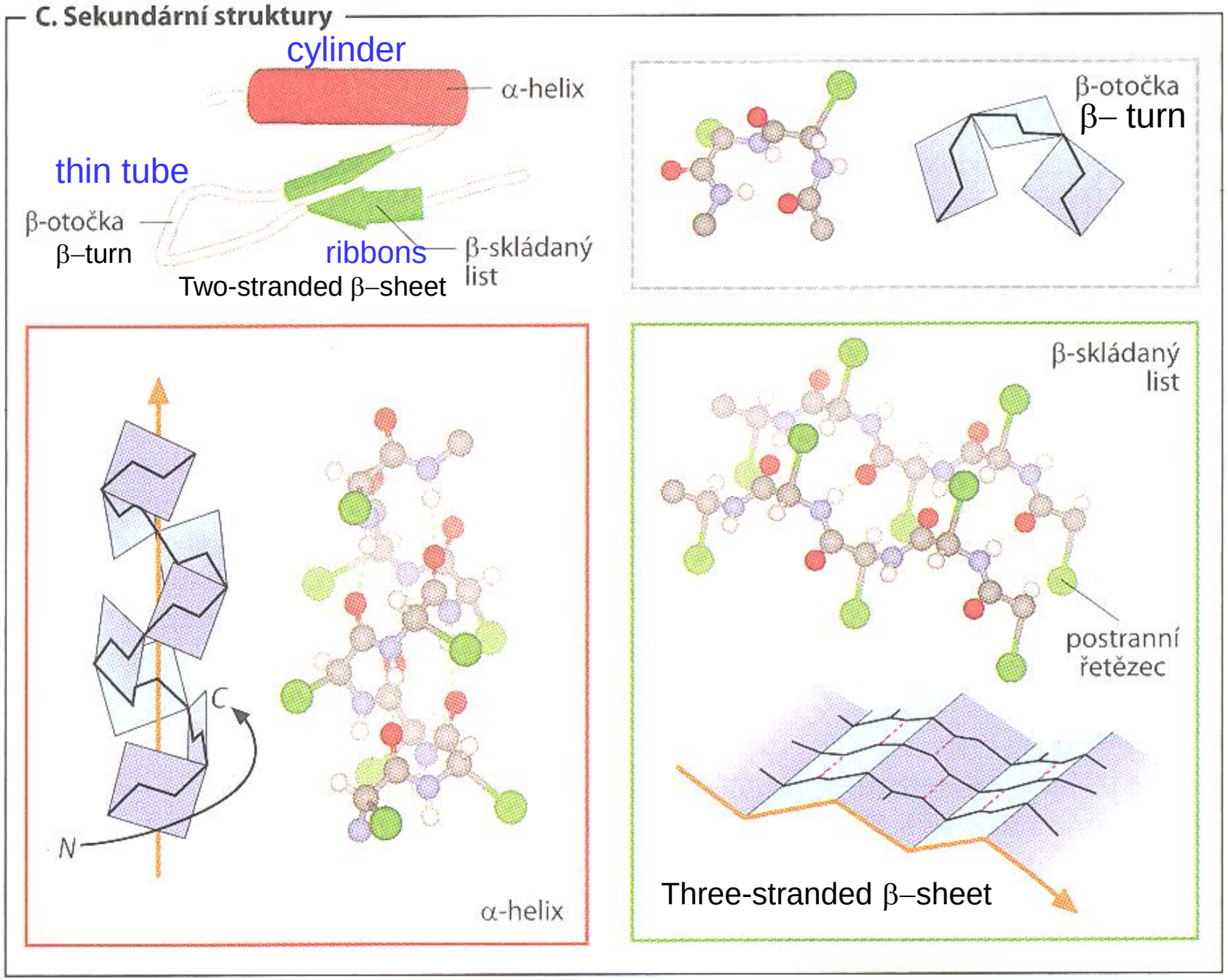
(Here a **two stranded** β anti-parallel pleated sheet is shown) **Hydrogen bonding** NH….CO **between neighboring polypeptide chains, inter-chain hydrogen bonds**

C) **Turns** (coil or loop conformation). Almost always occur at protein surfaces-direction change

D) **Disordered regions**

Helices and sheets constitute ~ 60% of the average protein.

Richardson's diagram



SUPERSECONDARY PROTEIN STRUCTURES

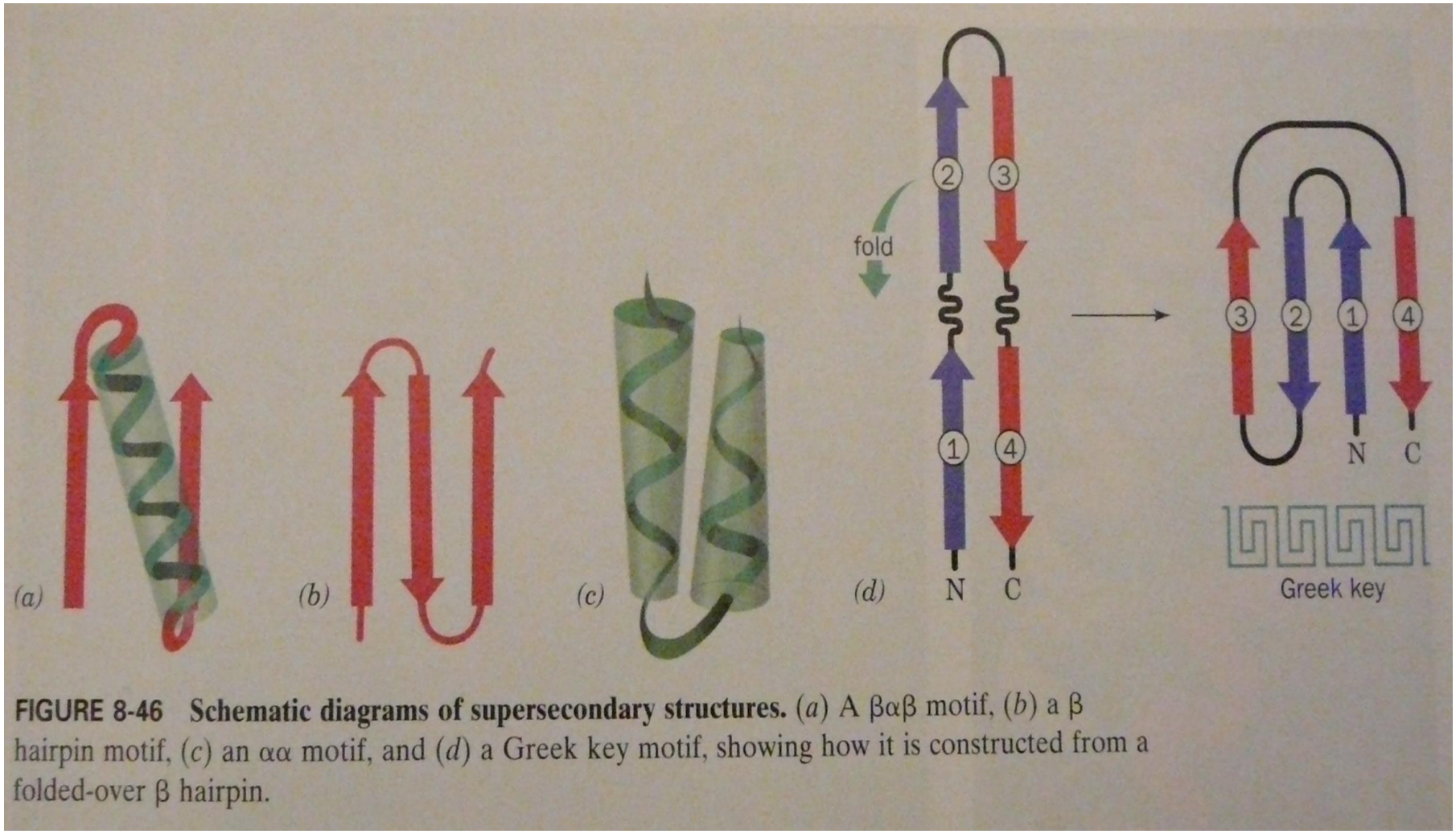


FIGURE 8-46 Schematic diagrams of supersecondary structures. (*a*) A βαβ motif, (*b*) a β hairpin motif, (*c*) an αα motif, and (*d*) a Greek key motif, showing how it is constructed from a folded-over β hairpin.

Noncovalent forces leading to protein folding into secondary structure elements and 3D conformation and contributing to protein's stability

NONCOVALENT ("WEAK") INTERACTIONS: 4-29 kJ/mol

1) **Electrostatic forces**

a) ionic, ionic or salt linkages, 4-25 kJ/mol. Between charged groups. Even though comparatively strong, their contribution to the stability of proteins in aqueous solutions is low due to a **high dielectric constant (D=78,5) of water.** Those on the surface of a protein do not usually interact with other charged groups of the protein but instead are stabilized by hydrogen bonding and polar interactions with water molecules.

Asp-COO^- ……$^+NH_3$-Lys *etc*

Strength of electrostatic interactions

$$U = \frac{kq_1q_2}{Dr}$$

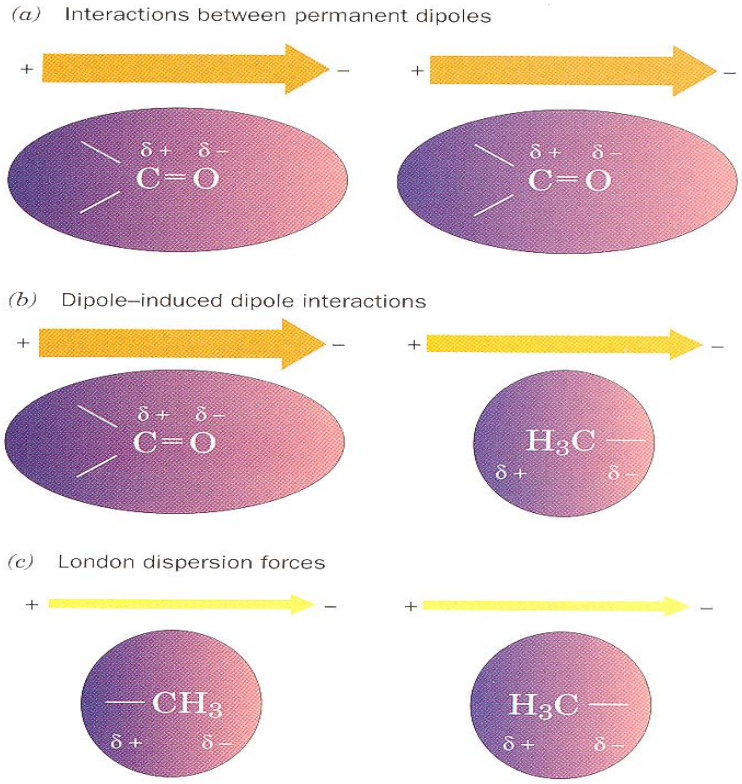


FIGURE 8-57 Dipole–dipole interactions. The strength of each dipole is represented by the thickness of the accompanying arrow. (*a*) Interactions between permanent dipoles. These interactions, here represented by carbonyl groups lined up head to tail, may be attractive, as shown, or repulsive, depending on the relative orientations of the dipoles. (*b*) Dipole–induced dipole interactions. A permanent dipole (here shown as a carbonyl group) induces a dipole in a nearby group (here represented by a methyl group) by electrostatically distorting its electron distribution (*shading*). This always results in an attractive interaction. (*c*) London dispersion forces. The instantaneous charge imbalance (*shading*) resulting from the motions of the electrons in a molecule (*left*) induces a dipole in a nearby group (*right*); that is, the motions of the electrons in neighboring groups are correlated. This always results in an attractive interaction.

Electrostatic forces (cont.)

b) dipole-dipole interactions, van der Waals forces. <4kJ/mol. Between electrically neutral molecules, arise from electrostatic interactions among permanent and/or induced **dipoles**. They may be attractive, as shown, or repulsive, dependent on the relative orientations of the dipoles. They maximally act at short distinct distances, which are the sum of van der Waals radii (electron orbitals) for two interacting atoms. However, as the atoms come even closer the repulsion, steric hindrance, results.
Although the weakest of the noncovalent forces they may be numerous and in the low dielectric constant core of the protein, dipole-dipole interactions significantly influence protein folding and determine its conformation.

Electrostatic forces (cont.)

c) hydrogen bonds, H bonds. 12-40 kJ/mol, (4-29 kJ/mol), with ~10% covalent character. Between
a weakly acidic **donor group** (**D-H**) and
an **acceptor** (**:A**) that bears a lone pair of electrons:

D-**H**…:A; ~-**H (~"proton" character) is "shared"** by the two atoms
~0.27-0.31 nm

In biology donors "D": weakly acidic **O**-H, **N**-H; **S**-H; rel. acidic **C**$_\alpha$-H;
acceptors "A": highly electronegative **:O**, **:N**; **:S**;
E.g. H bonds in α-helices and antiparallel β pleated sheets in proteins:
N-H…:O=C (atoms of peptide bonds!)
(in ideal α-helices between D and A at residues *n* and *n-4, (n-3),* respectively)

Many of the H bonds in proteins are members of **networks** in which each donor D is H bonded to two acceptors (a bifurcated hydrogen bond) and each acceptor A is H bonded to two donors.

On average, 68% of the H bonds in proteins are between **backbone atoms**: α-helices (1/3), 3^{10} helices (1/3), β pleated sheets (1/3). Only ~5% of the H bonds between backbone atoms are not wholly within a helix, sheet or turn.

Electrostatic forces (cont.)
c) hydrogen bonds, H bonds (cont).

The strength of a hydrogen bond depends on the distance between donor and acceptor atoms (the highest is at a distance between 0.27 and 0.31 nm).
Although the H bonds contribute to thermodynamic stability of a protein's conformation, their formation may not be a major driving force for folding. This is because peptide bonds and other hydrogen-bonding groups form H-bonds to the water solvent (water is a strong H bonding donor and acceptor) from the protein before its folding (in a denatured state) and the energy required to brake them must be subtracted from that gained from formation of new H-bonds in the protein. As the H-bonds are electrostatic they are likely to be stronger in the low polarity protein interior than on the high polarity aqueous surface.

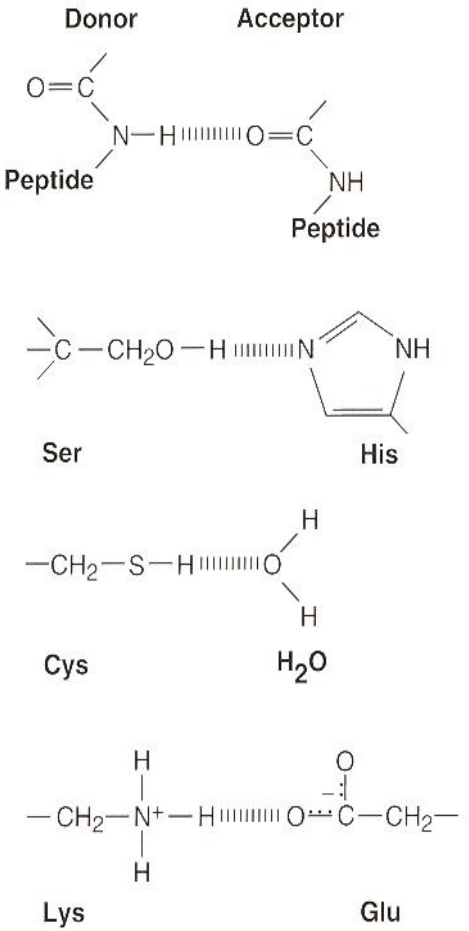


FIGURE 3.48
Some common hydrogen bonds found in proteins.

NONCOVALENT ("WEAK") INTERACTIONS (cont.)

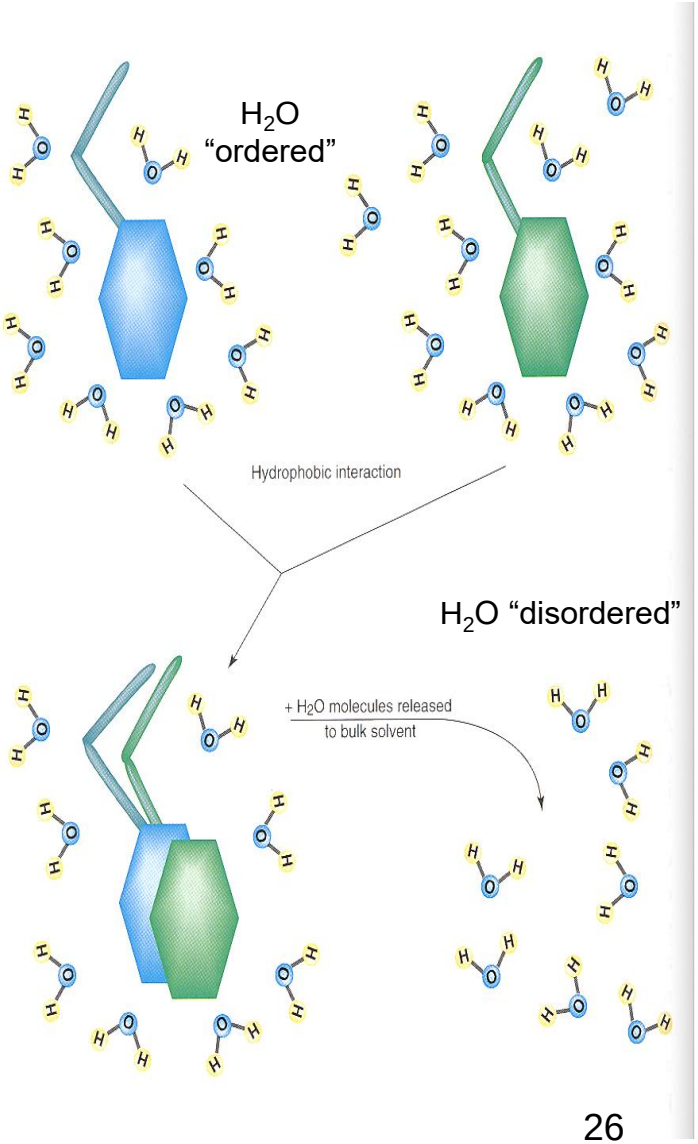
2) Hydrophobic forces. 8/12,5 kJ/mol.

The hydrophobic effect causes nonpolar substances to minimize their contact with water. It is not due to any intrinsic attraction between nonpolar groups. Rather:

When two nonpolar side chains come together, the surface area exposed to solvent is reduced and some of the highly **ordered water molecules** in the solvation shell are **released** to bulk solvent. The entropy of the system, i.e. net **disorder of water molecules in the system is increased**. This **INCREASE IN ENTROPY ΔS** is thermodynamically favorable and IT **IS THE DRIVING FORCE** CAUSING NONPOLAR MOIETIES TO COME TOGETHER in aqueous solvent. A favourable Gibbs free energy change ΔG~-8.5 kJ/mol for association of two Phe side chains in water is due to this gain-increase in entropy (see the Fig.).

($\Delta G = H - T.\Delta S$)

Formation of hydrophobic interactions between two Phe side-chain groups



2) Hydrophobic forces (cont.)
Individual amino acids can be scaled according to the hydropathies (hydropathic and hydrophilic tendencies) of their side chains; they are good predictors of which portions of the polypeptide chain are inside of the protein, and which outside in contact with the aqueous solvent.

W. Kauzmann (1958):

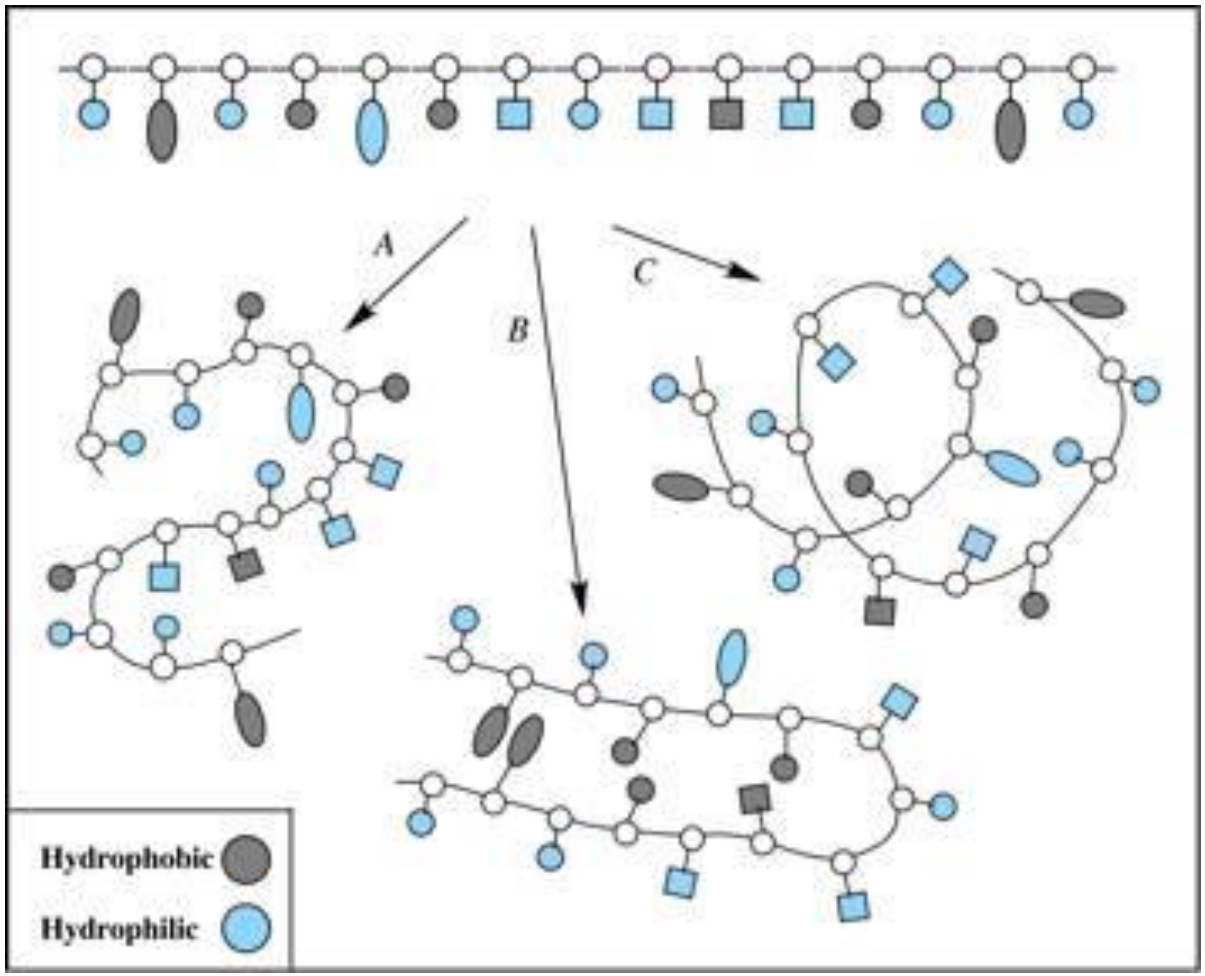
HYDROPHOBIC FORCES ARE A MAJOR INFLUENCE IN CAUSING PROTEINS TO FOLD IN AQUEOUS ENVIROMENT INTO THEIR NATIVE 3D-CONFORMATION. WATER IS THE KEY !

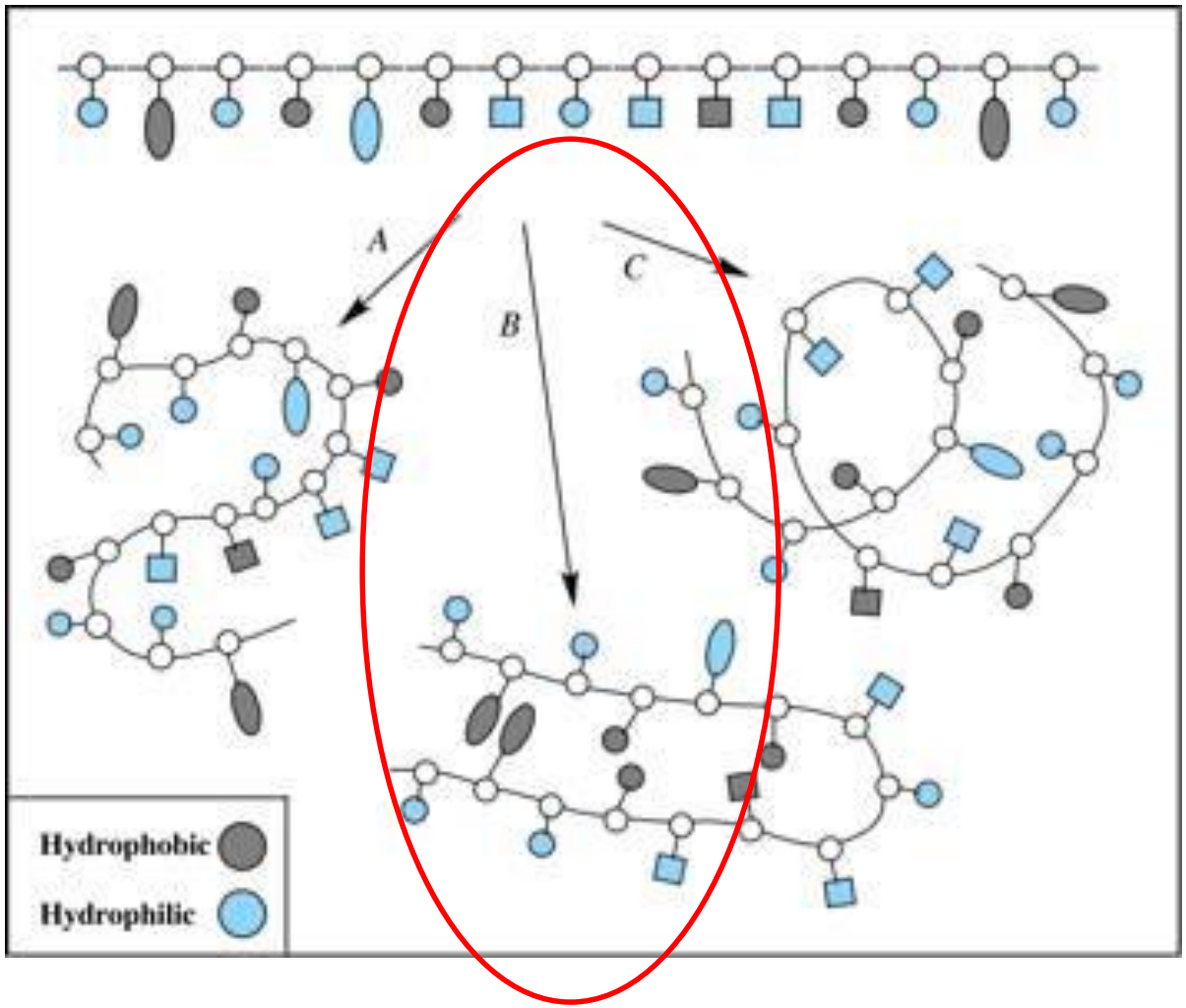
Protein folding is directed mainly by internal residues.

TABLE 8-6 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 Doolitle, R.F., *J. Mol. Biol.* **157,** 110 (1982).


A
B
C
Hydrophobic
Hydrophilic



http://www.indiana.edu/~oso/lessons/prot/folding1.htm

COVALENT PROTEIN STABILIZATION - **DISULFIDE BONDS**

Formation of disulfide bonds Cys-S – S-Cys is not efficient in the cytoplasm due to its relatively reducing character. They are formed in ER, which has an oxidizing environment, as a protein folds into its native conformation. Disulfide bonds give **additional 3D structural stability** to proteins destined primarily for secretion into more oxidized, "hostile", extracellular destinations with e.g. uncontrolled temperatures or pH and before further protein processing/maturation, e.g. removal of a prosequence structure (Fig.)

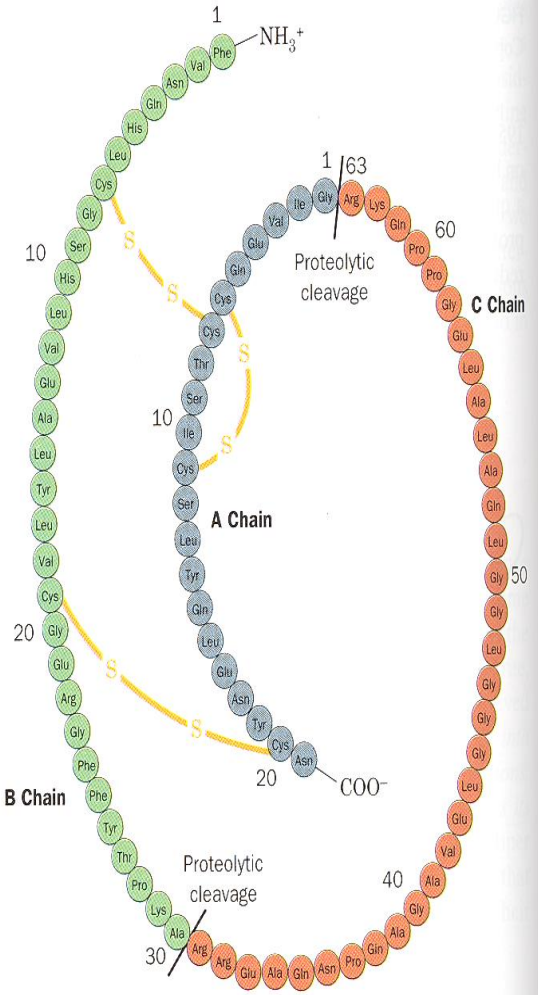


FIGURE 9-4 Primary structure of porcine proinsulin. Its C chain (*brown*) is proteolytically excised from between its A and B chains to form the mature hormone. [After Chance, R.E., Ellis, R.M., and Brommer, W.W., *Science* **161,** 165 (1968).]

Four levels of protein structure – Three levels of protein conformational folding

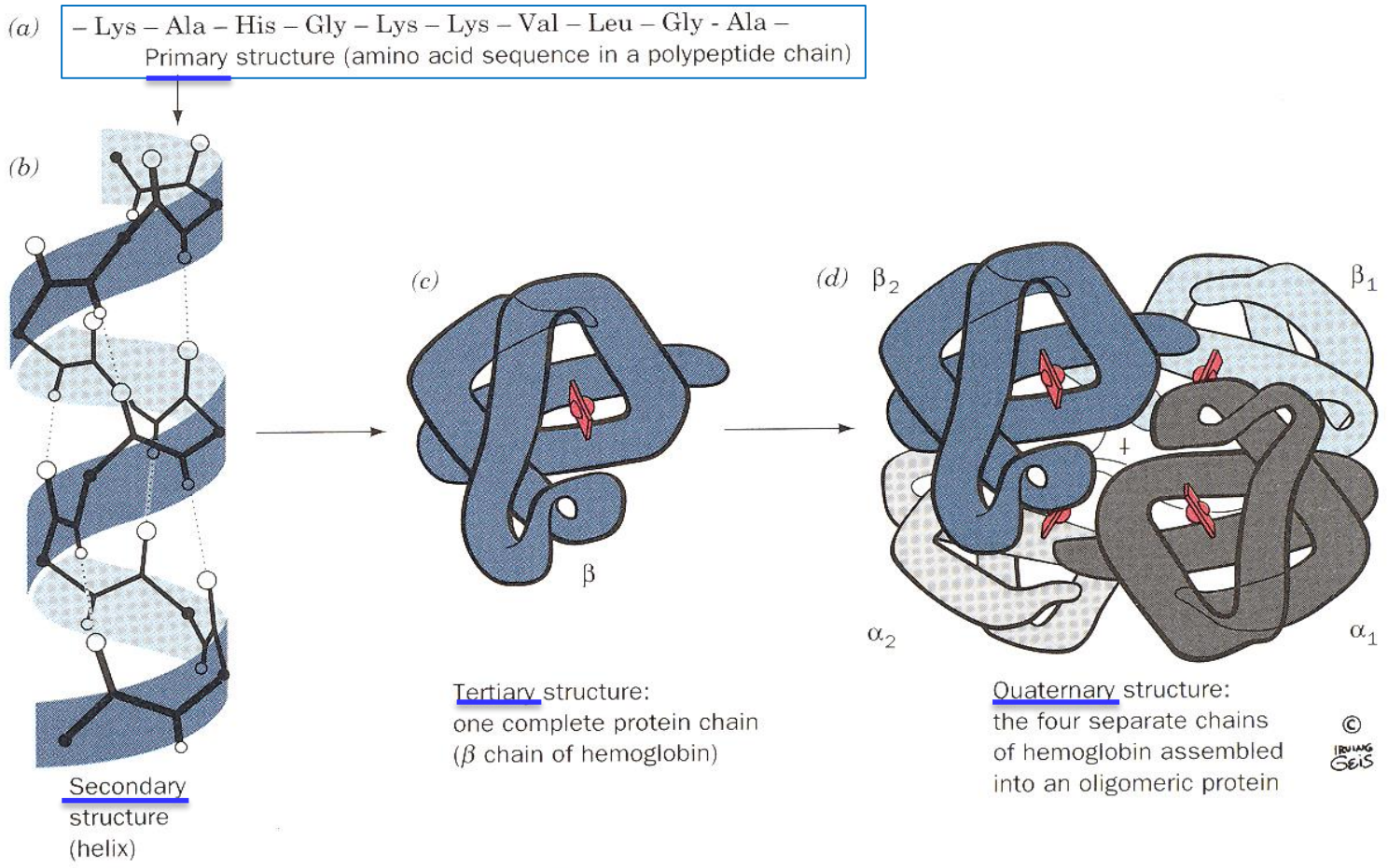


FIGURE 7-1 The structural hierarchy in proteins. (*a*) Primary structure, (*b*) secondary structure, (*c*) tertiary structure, and (*d*) quaternary structure. [Illustration, Irving Geis/Geis Archives Trust. Copyright Howard Hughes Medical Institute. Reproduced with permission.]

S. str.: regions of the polypept. chain with defined conformation stabilized by hydrogen bonds. **T. str.:** 3D conformation of a **protein** composed from **elements of second. structure** and unstructured regions. **Q. str.:** complexes from **protein oligomers**

Folding pathways -Levinthal paradox

What is the way a protein takes to fold to its native conformation?

Does a protein randomly explores all of the available, "allowed", conformations to it until it eventually "stumbles" onto its native conformation?

Calculations of C. Leventhal: **this cannot be the case!**

Assume that the 2*n* backbone torsional angles, ϕ and ψ, of an *n*-residue protein each have (only) three stable conformations. This yields $3^{2n} \sim 10^{n}$ possible conformations for the protein, (which is a gross underestimate, if only because the side chains are ignored). If a protein can explore new conformations at the rate at which single bonds can reorient, it can find ~ 10^{13} conformations per second (an overestimate).

For a **small protein of *n* = 100 residues** the time *t*, in seconds, required to explore all the conformations available to it is:

$t = 10^{100} : 10^{13} . sec^{-1} = 10^{87}$ s !!!!

This is immensely more than the apparent age of the Universe (~14 billion years = 4.4 x 10^{17} s) !

Proteins must fold in an ordered manner rather than via a random search of all their possible conformations. Each protein species appears to have evolved a conserved and simplified pathway of folding.

“PROTEIN FOLDING PATHWAY”

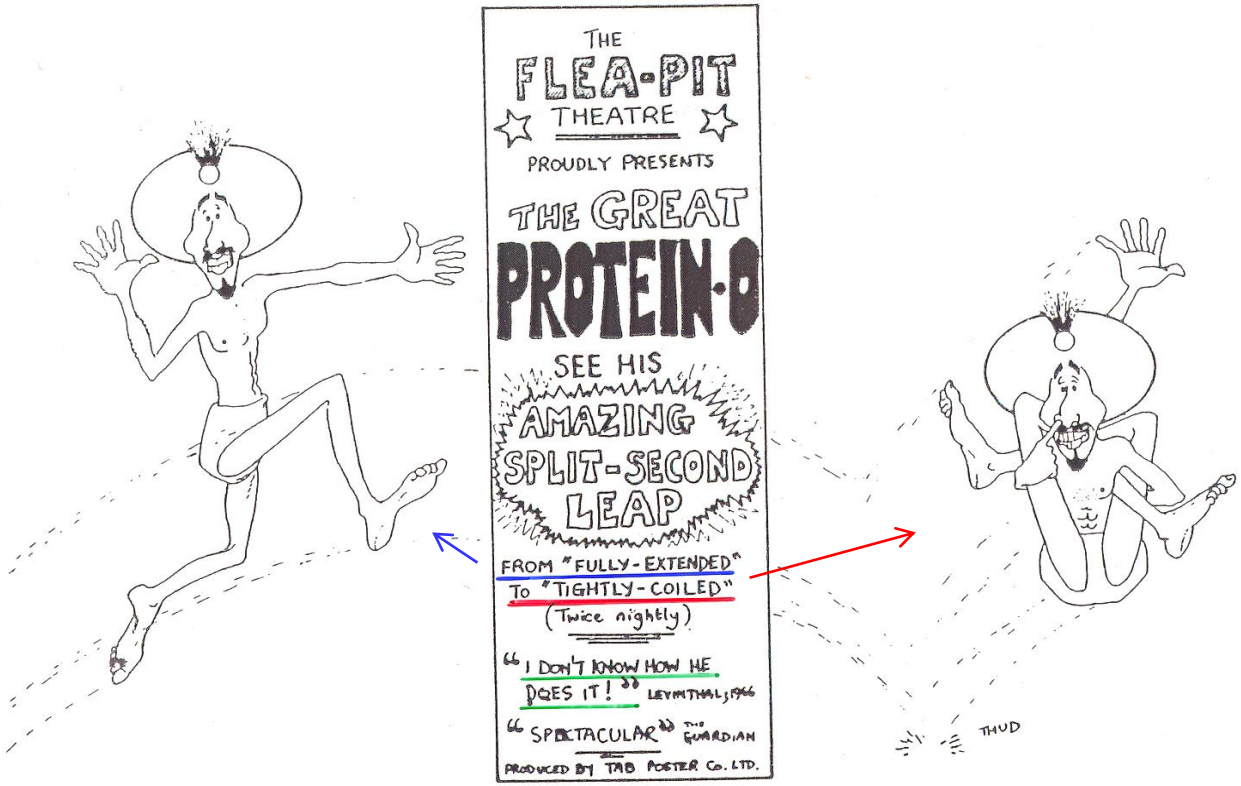
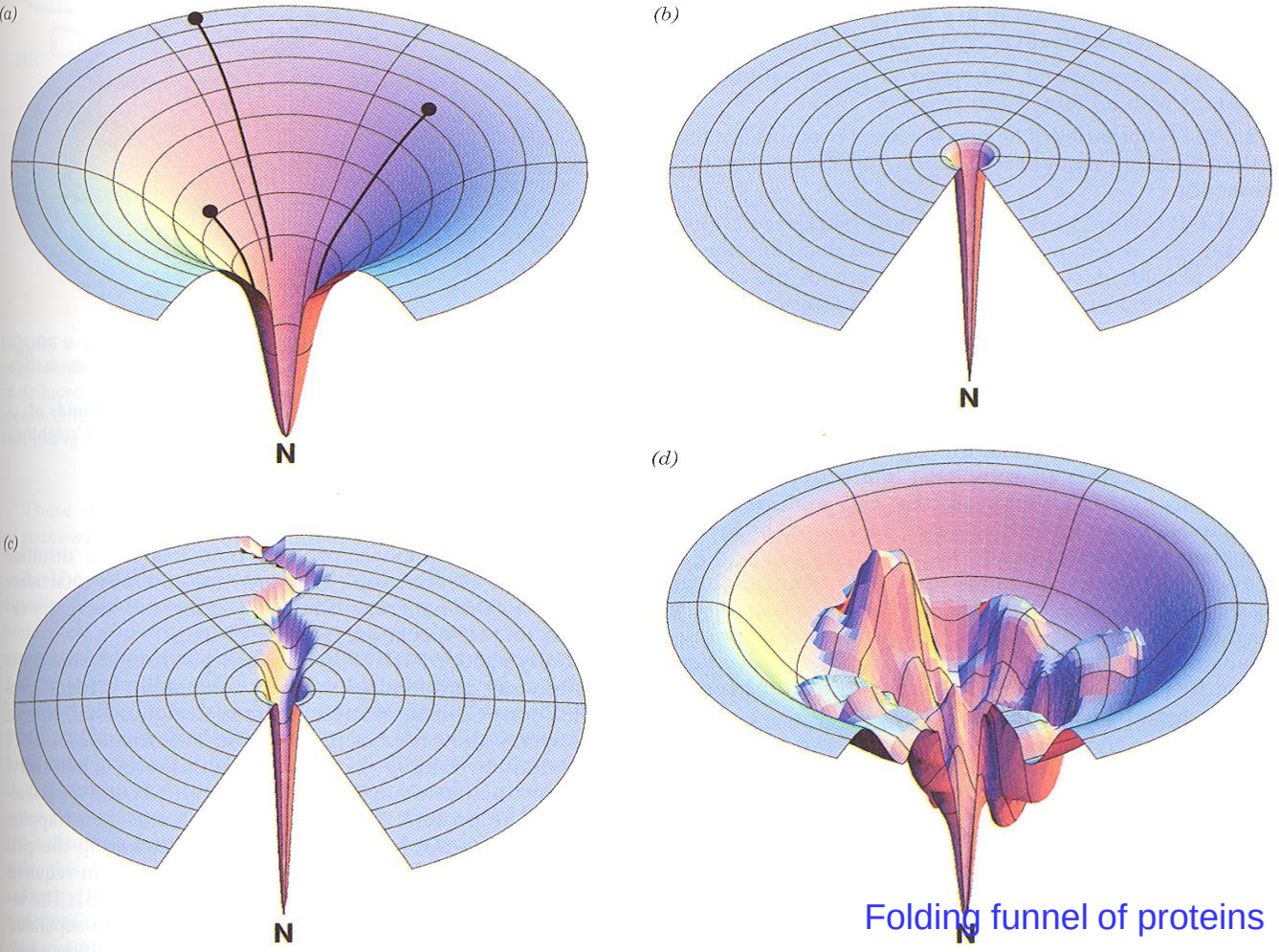


FIGURE 9-1 [Drawing by T.A. Bramley, *in* Robson, B., *Trends Biochem. Sci.* **1**, 50 (1976).

= Non-random + highly ordered +
+ ALL or NOTHING

ENERGETIC STEPS IN POLYPEPTIDE FOLDING

The folding follows an **energy surface or landscape-folding funnel** that represents the energy states available to a polypeptide under the prevailing conditions. Polypeptides fold via a series of conformational adjustments that reduce their free energy and/or increase entropy of the system until the native state is reached.



Folding funnel of proteins

FIGURE 9-11 **Folding funnels.** (*a*) An idealized funnel landscape. As the chain forms increasing numbers of intrachain contacts, its internal free energy (its height above the native state, N) decreases together with its conformational freedom (the width of the funnel). Polypeptides with differing conformations (*black dots*) follow different pathways (*black lines*) in achieving the native fold. (*b*) The Levinthal "golf course" landscape in which the chain must search for the native fold (the hole) randomly, that is, on a level energy surface. (*c*) The classic folding landscape in which the chain must search at random on a level energy surface until it encounters the canyon that leads it to the native state. (*d*) A rugged energy surface containing local minima in which a folding polypeptide can become transiently trapped. The folding funnels of real proteins are thought to have such topographies. [Courtesy of Ken Dill, University of California at San Francisco.]

3D-FOLDING PATHWAY STEPS (very complex, still not fully understood)

1) **"Hydrophobic collapse"** – the protein's hydrophobic groups coalesce so to expel most of their surrounding water molecules.

~ 5 ms. This initial state is known as the molten globule.

+ formation of local secondary structure elements

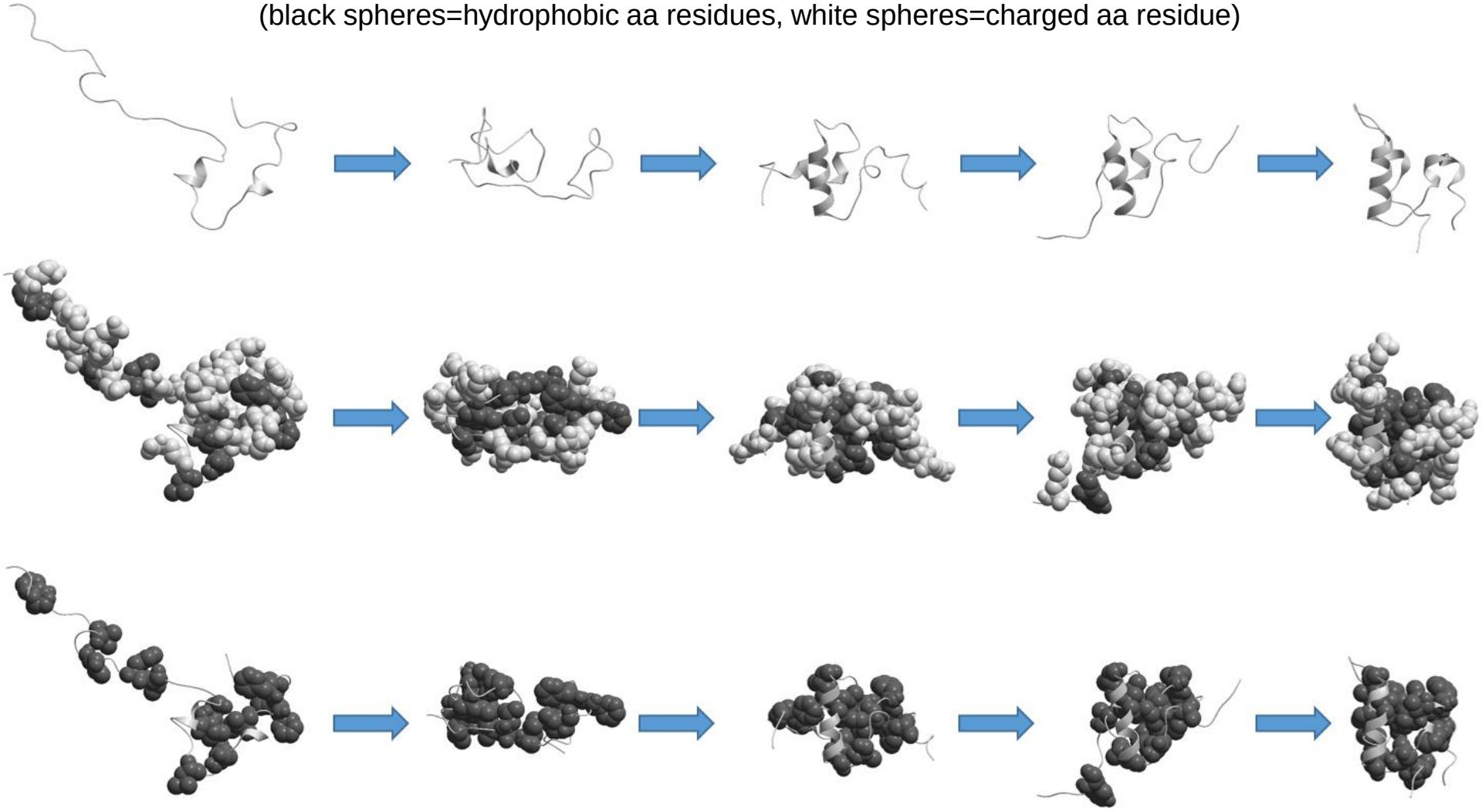
Smaller regions fold separately and more completely than larger regions.

2) Intermediate folding events. The native-like elements are thought to take the form of subdomains, etc. 5 – 1000 msec. Cooperativity.

3) Final folding events. Complex. Several seconds.

A computer-simulated model of folding of a 52 amino acid residues long polypeptide chain

(black spheres=hydrophobic aa residues, white spheres=charged aa residue)



Hydrophobic aa residues only

I. Barvík

POLYPEPTIDE CHAIN FOLDING SUMMARY

1) WHY?

THERMODYNAMICS

2) HOW?

STRUCTURE BUILDING RULES and SPACE RESTRICTIONS; INTERACTION STABILIZATION

Summary – protein folding and stabilization

A protein molecule in aqueous environment assumes its native conformation largely in response to the tendency of its surrounding water structure to be maximally disordered. The **tendency to maximize the entropy of the protein-water solvent system** is considered to be the main driving factor in protein folding. This involves the **hydrophobic effect**, in which proteins, upon folding bury, on average, ~85% of their nonpolar side chains into their interiors.
By this way the native conformation of a protein reaches the one of the lowest Gibbs free energy accessible to its sequence.

Despite that all, the conformational stabilities of native proteins are low making them easily susceptible to denaturation by altering the balance of weak stabilizing forces (e.g. by heat, chemicals-detergents, pH). This marginal stability of most proteins under physiol. conditions is an essential property that has arisen through evolution of function – to be flexible enough to carry out their physiological functions. [Average kinetic energy of thermal movement is ~-2.5 kJ/mol at 37^{o}C. Association energy of the covalent bond ~>-250 kJ/mol]

Some proteins may have two (more?) different stable conformations. Alternative folding. **Conformational diversity of proteins**. Their certain regions **("chameleon r.")** may adapt either α-helical or β-sheet structure dependent on the context, surrounding. Prions. (See later).

Folding into a compact conformation seems to be the main selective pressure against misfolding

ENZYMES OF THE CELL NATURALLY FACILITATING CORRECT PROTEIN FOLDING

A)Protein disulfide isomerases: S-S bridges

B)Peptidyl prolyl cis-trans isomerases (Rotamases). Xaa-Pro peptide bonds: *trans* conformation ~ 90%, *cis* conformation ~ 10%. **Immunophilins:** *Immunosuppressive effect when in complex with Cyclosporin A.*

Protein disulfide isomerases

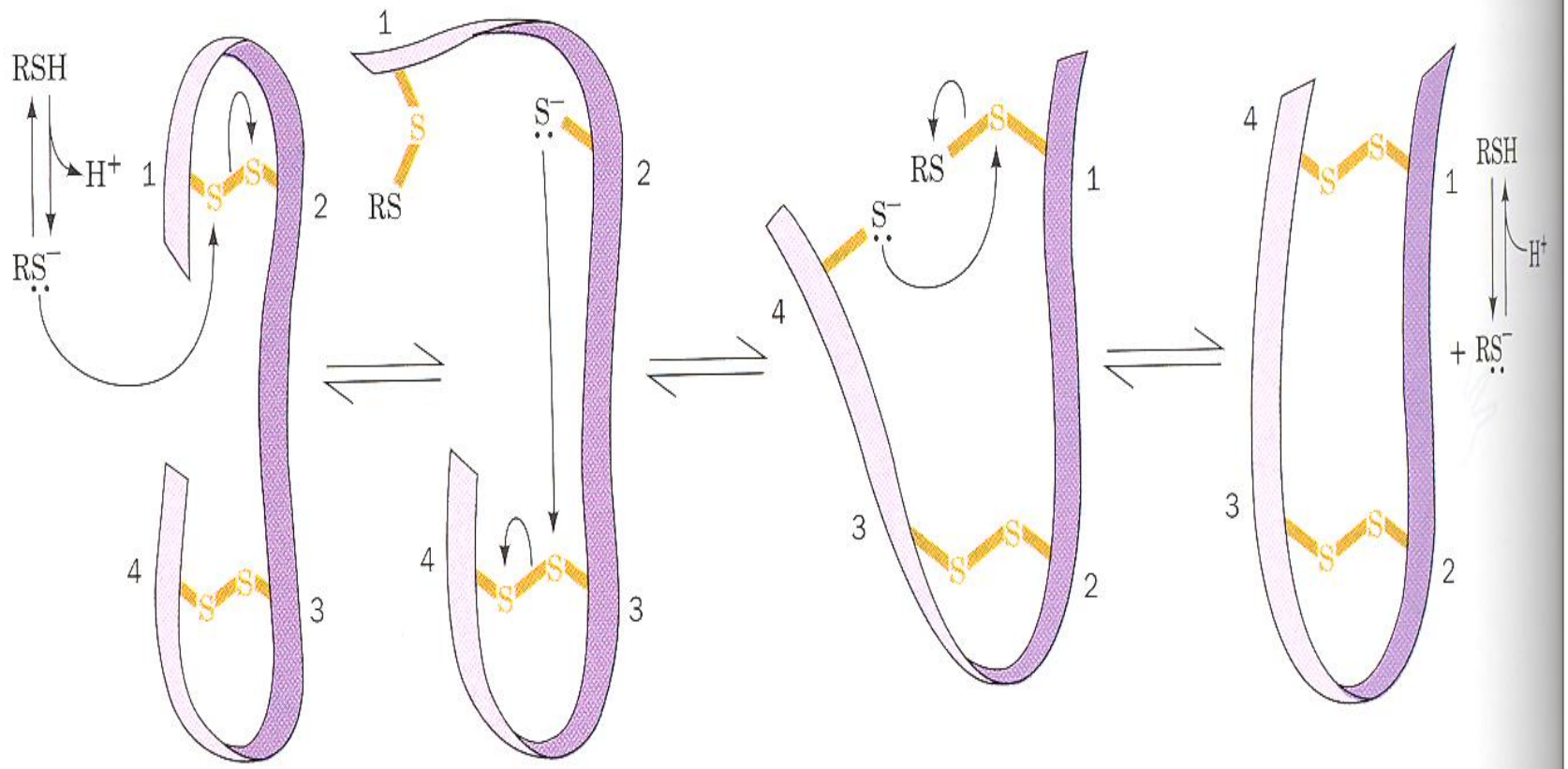


FIGURE 9-3 Plausible mechanism for the thiol- or enzyme-catalyzed disulfide interchange reaction in a protein. The purple ribbon represents the polypeptide backbone of the protein. The attacking thiol group must be in its ionized thiolate form.

ENZYMES OF THE CELL NATURALLY FACILITATING CORRECT PROTEIN FOLDING

C) Molecular chaperones: they function to **prevent or reverse improper associations/aggregations** of polypeptide chain regions, particularly in multidomain and multisubunit proteins. They do so by binding to unfolded or aggregated polypeptide's **solvent-exposed hydrophobic surfaces** and subsequently releasing them, in a manner that **facilitates their proper folding and/or 4D assembly**. Pathological associations *in vivo* are promoted by the fact that the folding takes place in the presence of extremely high concentrations of other macromolecules (~300g/L, which occupy ~25% of the available volume). Consequently, **unfolded proteins *in vivo* have a great tendency to form both intramolecular and intermolecular aggregates.** This condition is very carefully monitored by the organism and, if it gets out of control, it may trigger an overall **shock reaction** called **UNFOLDED PROTEIN RESPONSE (UPR,** Fig.). MAINTENANCE OF INTRACELLULAR PROTEIN HOMEOSTASIS.

Many molecular chaperones are **ATPases**, requiring ATP for its function. Energy-dependent process.

= Heat shock proteins, Hsp, chaperonines etc., stress proteins:

Cell's apparatus for handling of unfolded or misfolded proteins:

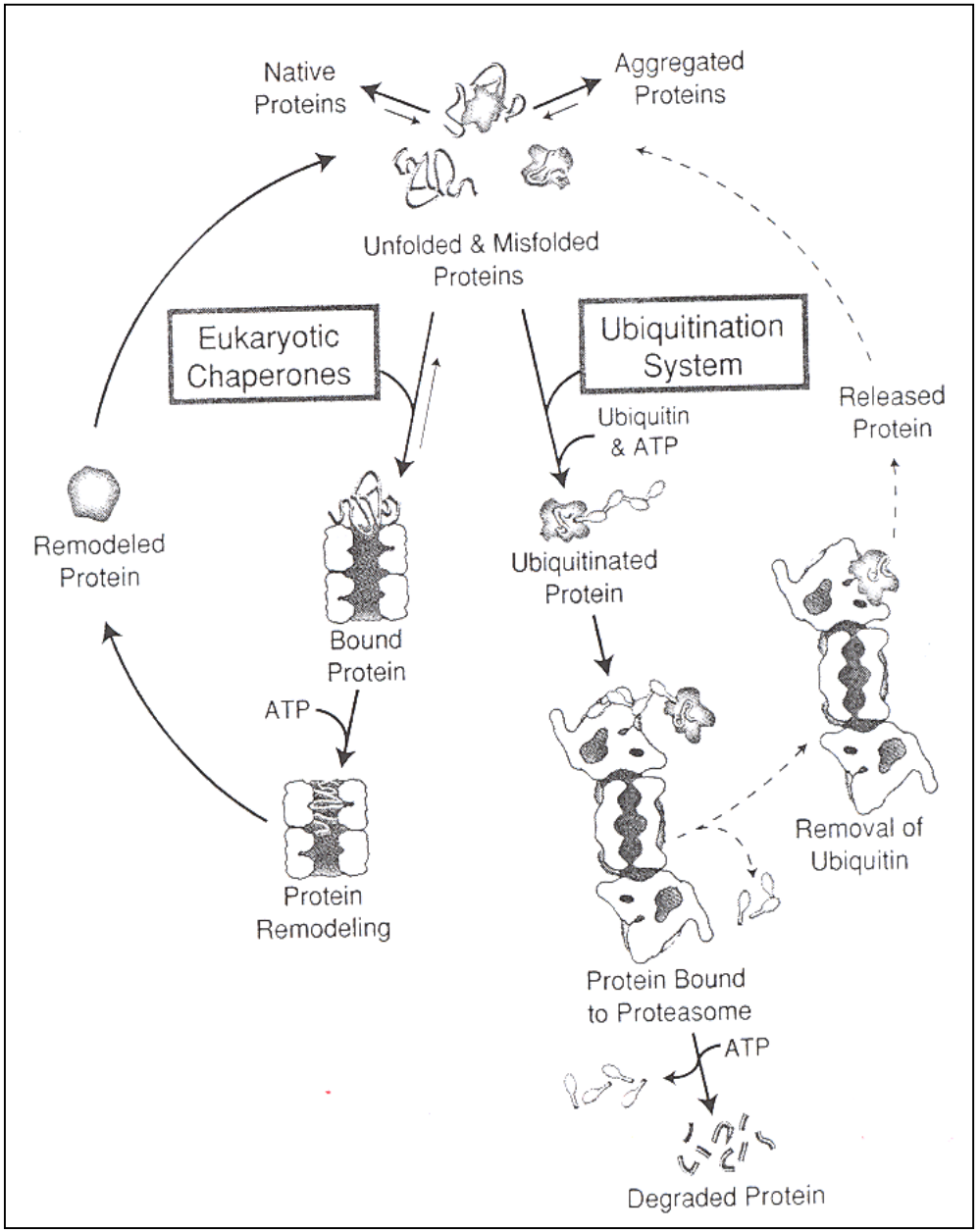
CHAPERONES and PROTEASOMES:

to catalyze removal of unfolded proteins either by

- renaturation/remodeling

or by

- degradation



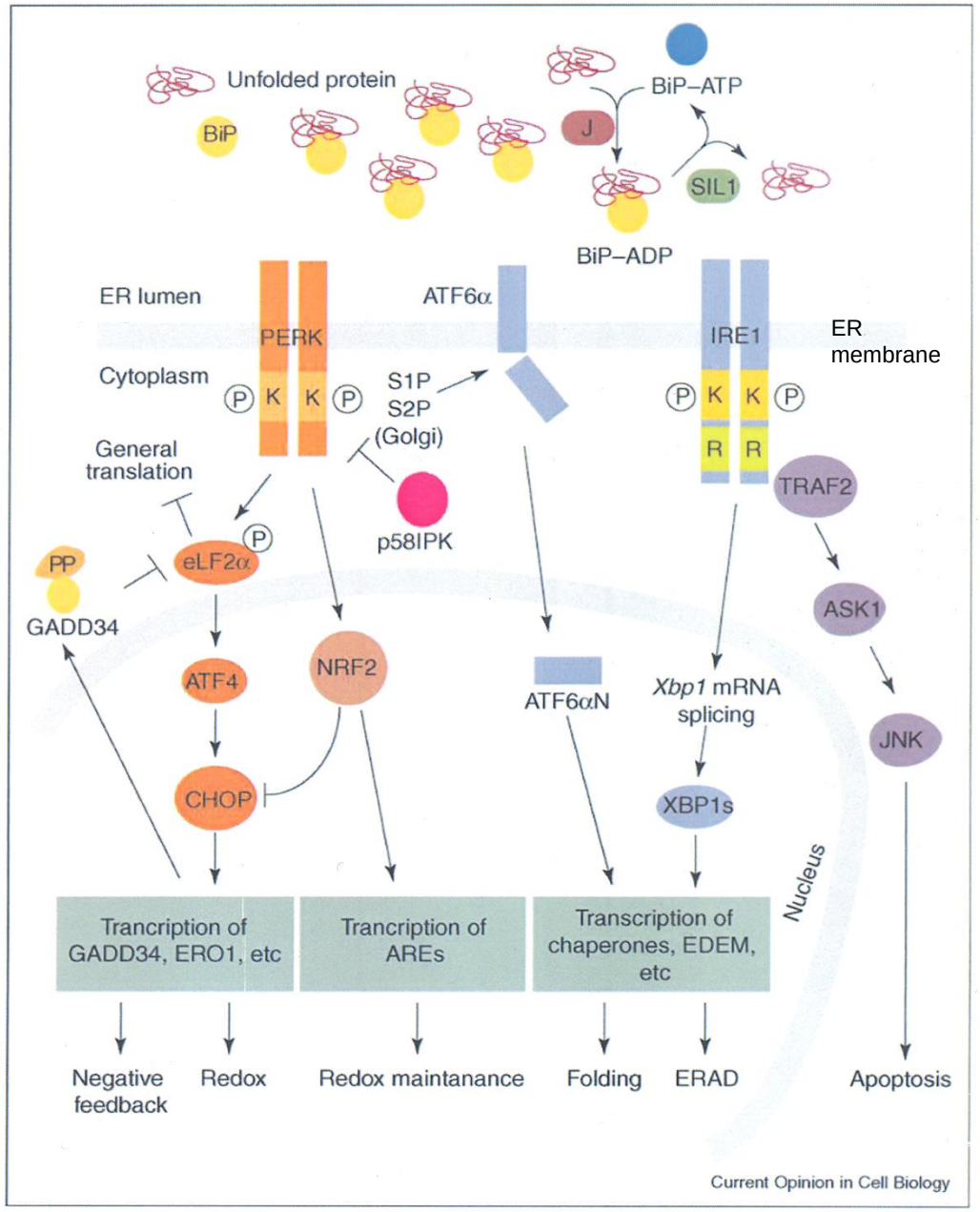
Pathology: UNFOLDED PROTEIN RESPONSE (UPR):

The capacity of the cell's unfolded protein control systems was exceeded:

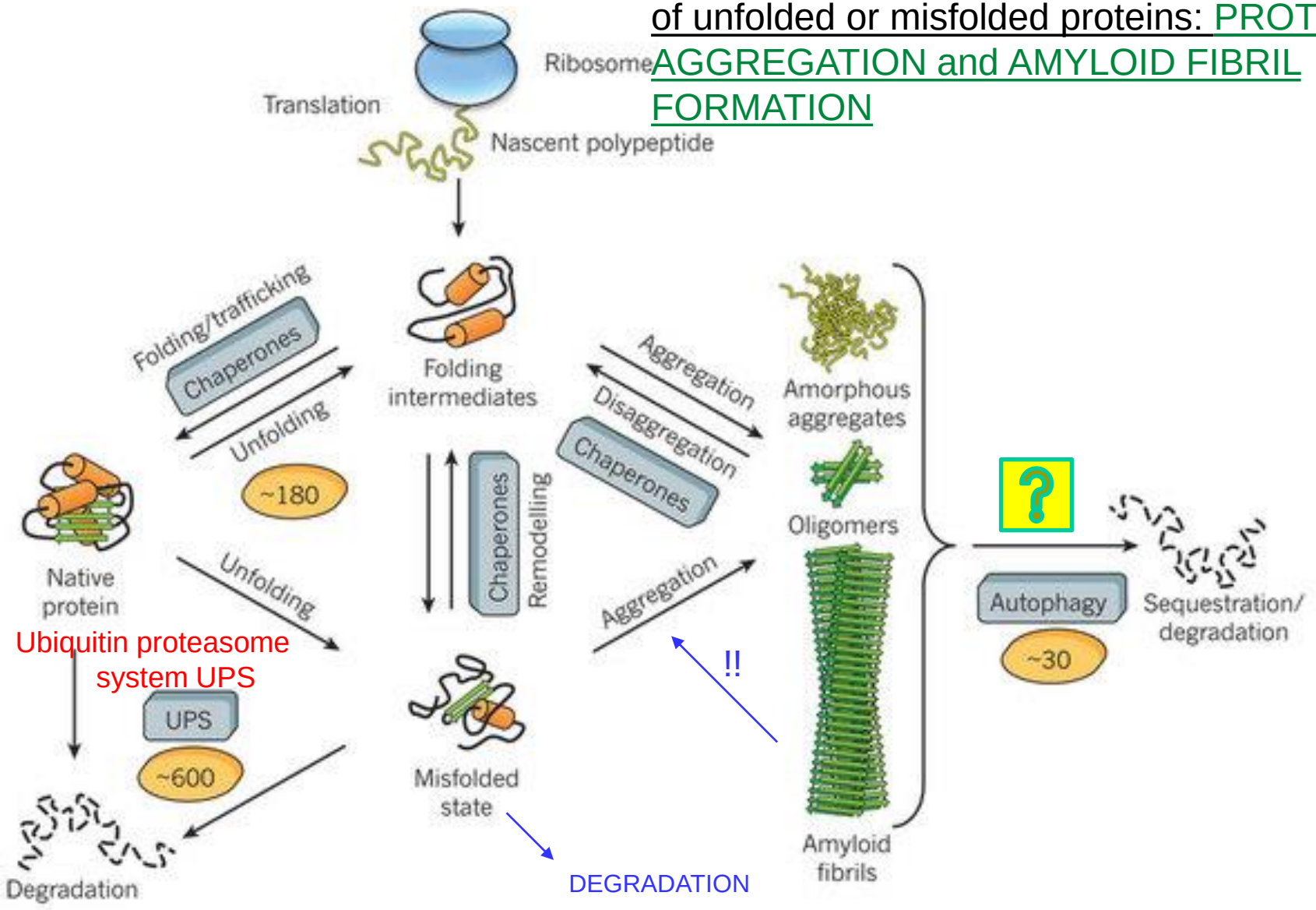
GENE SIGNALLING PATHWAYS ACTIVATED BY UNFOLDED PROTEIN ACCUMULATION

= to cope with this risky condition by activating an effective response

BiP = chaperone
ERAD = endoplasmic reticulum-associated protein degradation



Escape from the cell's apparatus for handling of unfolded or misfolded proteins: PROTEIN AGGREGATION and AMYLOID FIBRIL FORMATION



http://www.nature.com/nature/journal/v475/n7356/full/nature10317.html

PART II. ABNORMALITIES IN PROTEIN FOLDING AND ASSEMBLY MAY BE IMPORTANT MECHANISMS OF DISEASES – CONFORMATIONAL DISEASES

A) *Protein **degradation** due to **incorrect folding** caused **by mutation(s)***

CYSTIC FIBROSIS - the most common recessive genetic disease in the Caucasian population (1:2000)

Defective CFTR (*C*ystic *F*ibrosis *T*ransmembrane Conductance *R*egulator): glycoprotein, 1480 aa, Cl^- and HCO_3^- membrane transporter in epithelial cells; DEFECT: deletion of Phe 508 (in ~70% of cases)

Pathogenesis: deletion of Phe 508 results in an improper posttranslational processing of oligosaccharide chains in ER => improper conformation of CFTR => UPR degradation by proteolytic surveillance system (proteasomes). DESPITE the fact that the MUTATED CFTR still retains some biological activity!

Clinic: highly viscous mucus that blocks the small airways in the lungs – persistent infections, death at ~30y. (Meconium ileus in newborns!).

(Treatment? **Corrector drugs**-pharmacological chaperons, **potentiators**. Gene repair technology.)

CYSTIC FIBROSIS

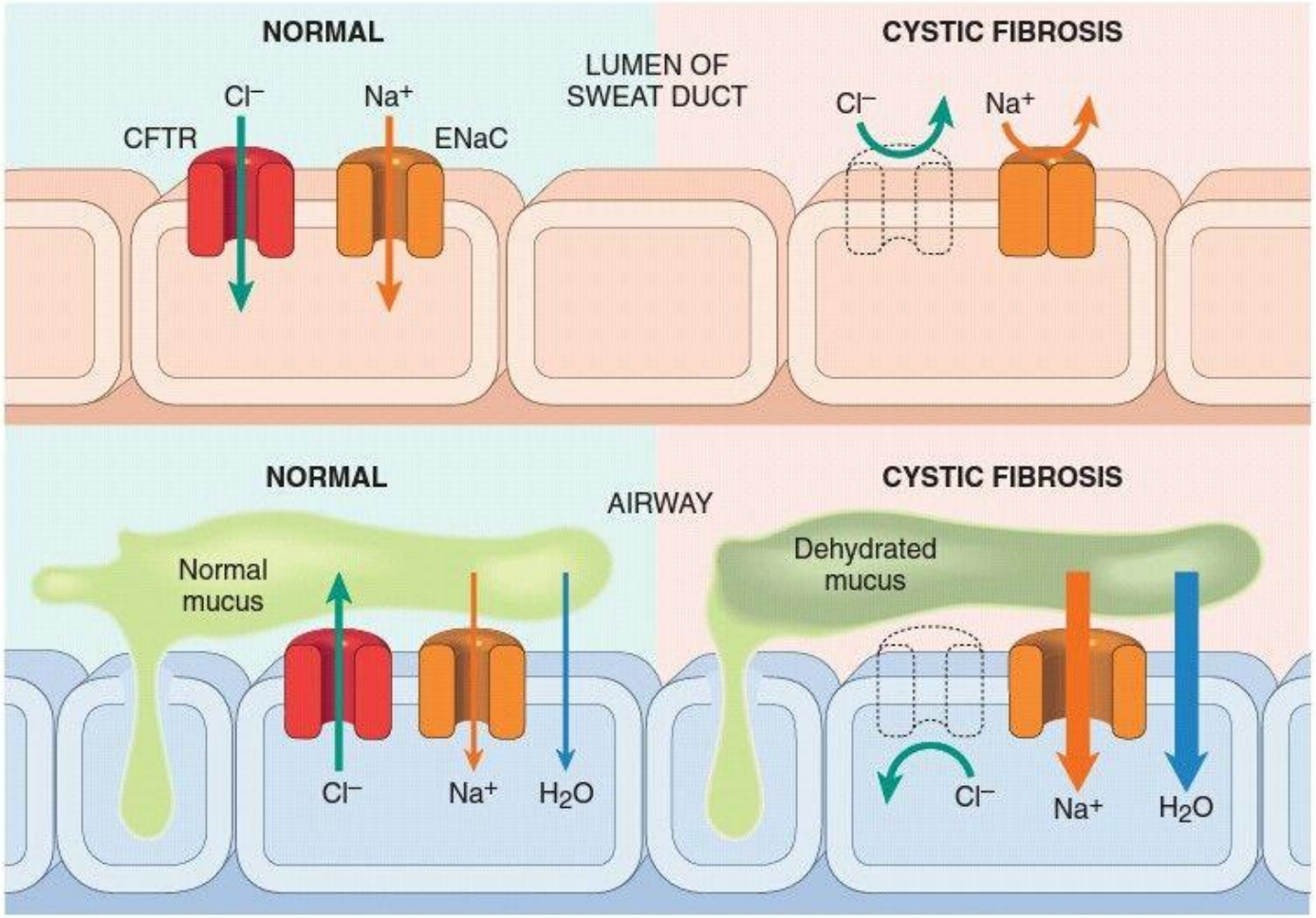
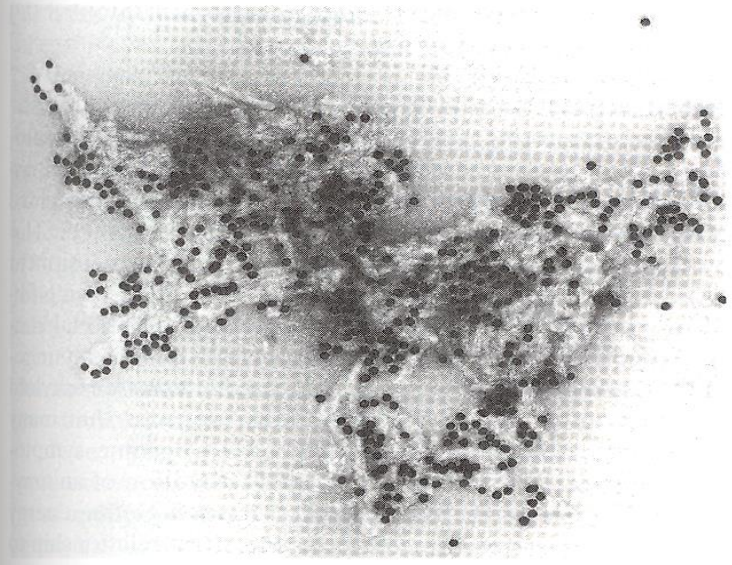


Figure 6–4 Top, In cystic fibrosis (CF), a chloride channel defect in the sweat duct causes increased chloride and sodium concentration in sweat. **Bottom,** Patients with CF have decreased chloride secretion and increased sodium and water reabsorption in the airways, leading to dehydration of the mucus layer coating epithelial cells, defective mucociliary action, and mucous plugging. CFTR, cystic fibrosis transmembrane conductance regulator; ENaC, epithelial sodium channel responsible for intracellular sodium conduction.

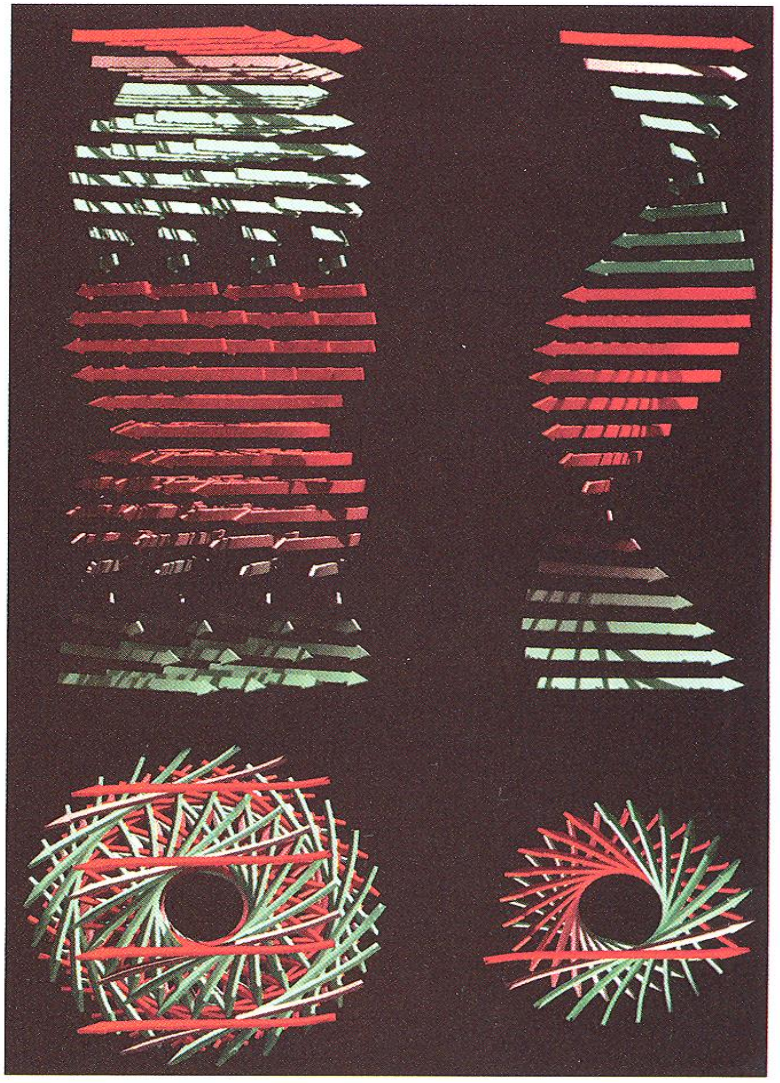
Uncontrolled protein aggregation due to protein's conformational change is a constant challenge in all compartments of living organisms. The failure of a peptide or protein to remain soluble may result in pathology.

So far, more than **50 human diseases, often fatal,** have been associated with **AGGREGATION** of certain peptides/proteins, first into still **soluble protein oligomers** and then into insoluble **amyloid fibrils**. Amyloid fibrils form deposits extracellularly (amyloid plaques) and /or intracellularly (tangles).

- **Amyloid** (= starch-like; a misnomer because it was originally thought that this material resembled starch)**:** protein fibrils exhibiting a cross-β-core/spine structure and specific staining characteristics with diagnostic dyes (thioflavins S and T, Congo red)



(a)



(b) (c)

FIGURE 9-32 Amyloid fibrils. (*a*) An electron micrograph of amyloid fibrils of the protein PrP 27–30 (Section 9-5C). These amyloid fibrils are visually indistinguishable from those of other proteins. The black dots are colloidal gold beads that are coupled to anti-PrP antibodies that are adhering to the PrP 27–30. (*b*) A model, based on X-ray fiber diffraction measurements, of an amyloid fibril protofilament viewed normal to the filament axis (*above*) and along the filament axis (*below*). The arrows indicate the paths but not necessarily the directions of the β strands. The β strands form four β sheets that are parallel to the filament axis. In a given β sheet, adjacent β strands are related by a twist of 15° about the filament axis to form a continuous β sheet helix. (*c*) An isolated β sheet, which is shown for clarity. The loop regions connecting the β strands have unknown structures. Two amyloid protofilaments wrap around each other in a left-handed helix to form an amyloid fibril. [Part *a* courtesy of Stanley Prusiner, University of California at San Francisco Medical Center; Parts *b* and *c* courtesy of Colin Blake, Oxford University, U.K. and Louise Serpell, University of Cambridge, U.K.]

B) *Protein/peptide* ***AGGREGATION DISEASES - AMYLOIDOSES***

(i) ***non-neuropathic;*** *due to* ***mutation:***
1. nonsystemic (**Sickle cell anemia);**
2. systemic

neuropathic:
(ii) due to ***increased production*** *of amyloidogenic peptides*
(iii) due to intrinsic ***susceptibility to alternative folding (prions)***

AGGREGATION DISEASES

- ***Ad (i) 2.*** **Amyloidoses *systemic*** **–** ***non-neuropathic*** **-** deposition of fibrilar form of often **mutant** proteins in heart, liver, or kidney interferes with cellular function, resulting in cell death or organ failure (type 2 diabetes)

Ad B (i): AMYLOIDOSES SYSTEMIC
Aggregation due to protein mutation

Many aggregation-prone/amyloidogenic proteins are mutant forms of normally occurring proteins. These include e.g.
lysozyme in the disease *familial visceral amyloidosis*,
transthyretin in *familial amyloid polyneuropathy*,
fibrinogen in *hereditary renal amyloidosis,*
immunoglobulin light chain AL in *primary myeloma*,
cystathion β–synthase in *homocysteinuria,* etc.

Most such diseases do not become symptomatic until the 3rd to 7th decades of life and typically progress over 5 to 15 years ending in death.

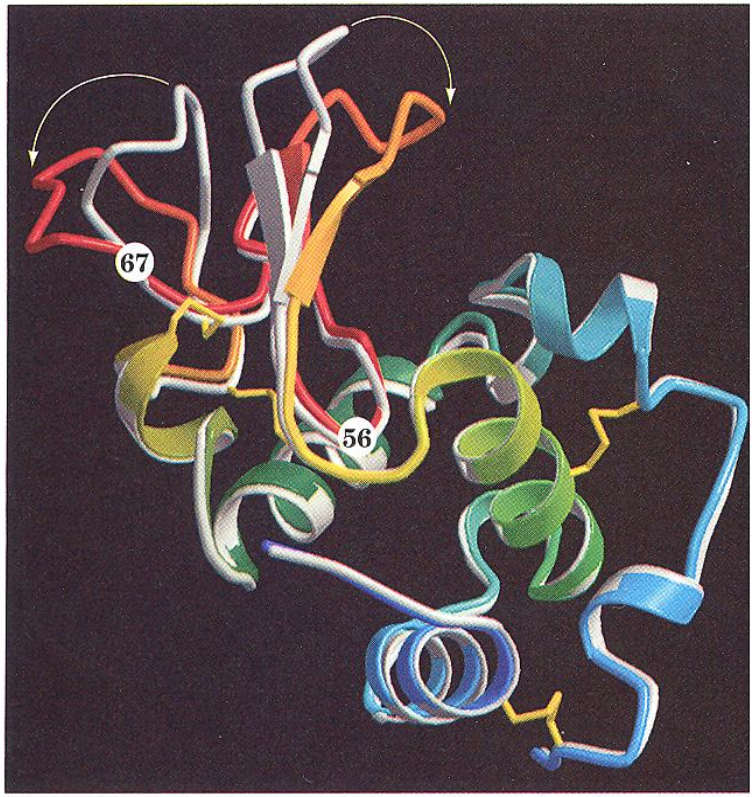


FIGURE 9-33 Superpositions of wild-type human lysozyme and its D67H mutant. Wild-type lysozyme is gray and its D76H mutant is color-ramped in rainbow order from blue at its N-terminus to red and back to blue at its C-terminus. The white arrows indicate the conformational shifts of residues 45 to 54 and 67 to 75 in the D67H mutant relative to those in the wild-type protein. The four disulfide bonds present in both the wild-type and mutant protein are shown in yellow. The positions of residues 56 and 67 are indicated. [Courtesy of Colin Blake, Oxford University, U.K., and Louise Serpell, University of Cambridge, U.K.]

- ***Ad B (ii) + (iii)*** ***AGGREGATION DISEASES-NEUROPATHIC***

- **Neurodegenerative diseases** – aggregation & damage occurs **in the brain**:

- **Alzheimer d.,**
- **Parkinson d.,**
- **Huntington d.,**
- **prion diseases = transmissible spongiform encephalopathies (TSEs)**,

  etc

Ad B (ii): NEURODEGENERATIVE DISEASES: ALZHEIMER DISEASE (AD)

Aggregation due to an increased production? of an amyloidogenic peptide

Clinic: mainly elderly: 10% over 65y, 50% over 85y; progressing dementia, death after 4 – 8 years after diagnosis. The *most prevalent neurodegenerative disease.* Mostly sporadic, multifactorial (polygenic & environment)

Pathology: brain hippocampus, cortex, entorhinal cortex (primary site of AD manifestation):
1. Extracellularly: amyloid plaques in the brain surrounded by dead and dying neurons. A. plaques consist of amyloid fibrils of a hydrophobic 40-42-res. long peptide called **amyloid-β peptide** (**$A\beta_{40}$, $A\beta_{42}$**). Aβ is pathologically excised from a big precursor, 695/770-res. long transmembrane Amyloid Precursor Protein (APP; the gene on chromosome 21) by the action of **β- and γ- secretases** (physiologically by α– and γ- secretases). APP appears to be neuroprotective.
2. Intracellularly: neurofibrilary tangles consisting of paired helical filaments containing the microtubule associated hyperphosphorylated **protein Tau**.

Schematic diagram of APP processing

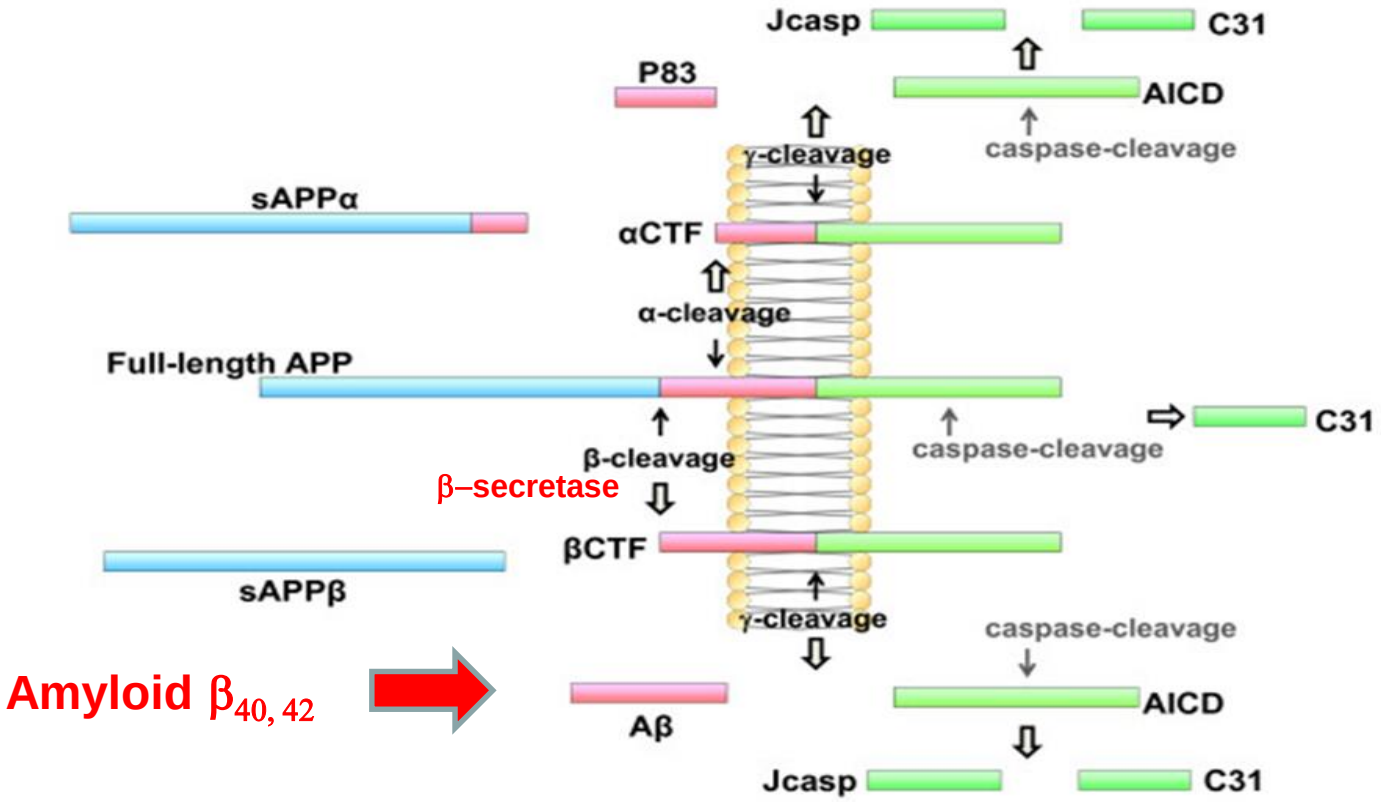
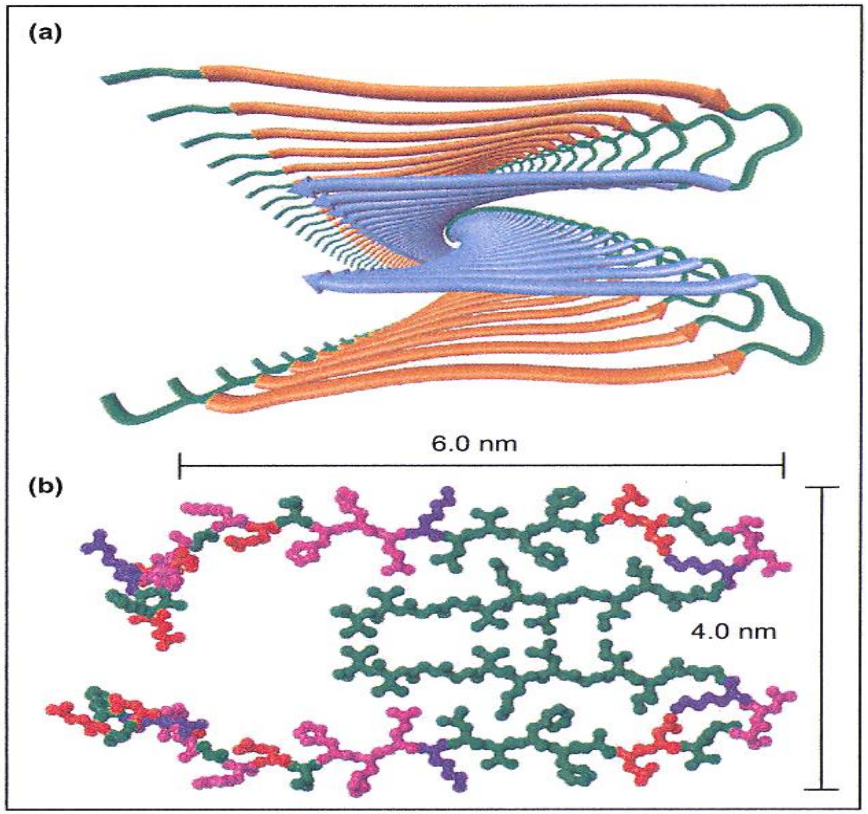


Figure 2

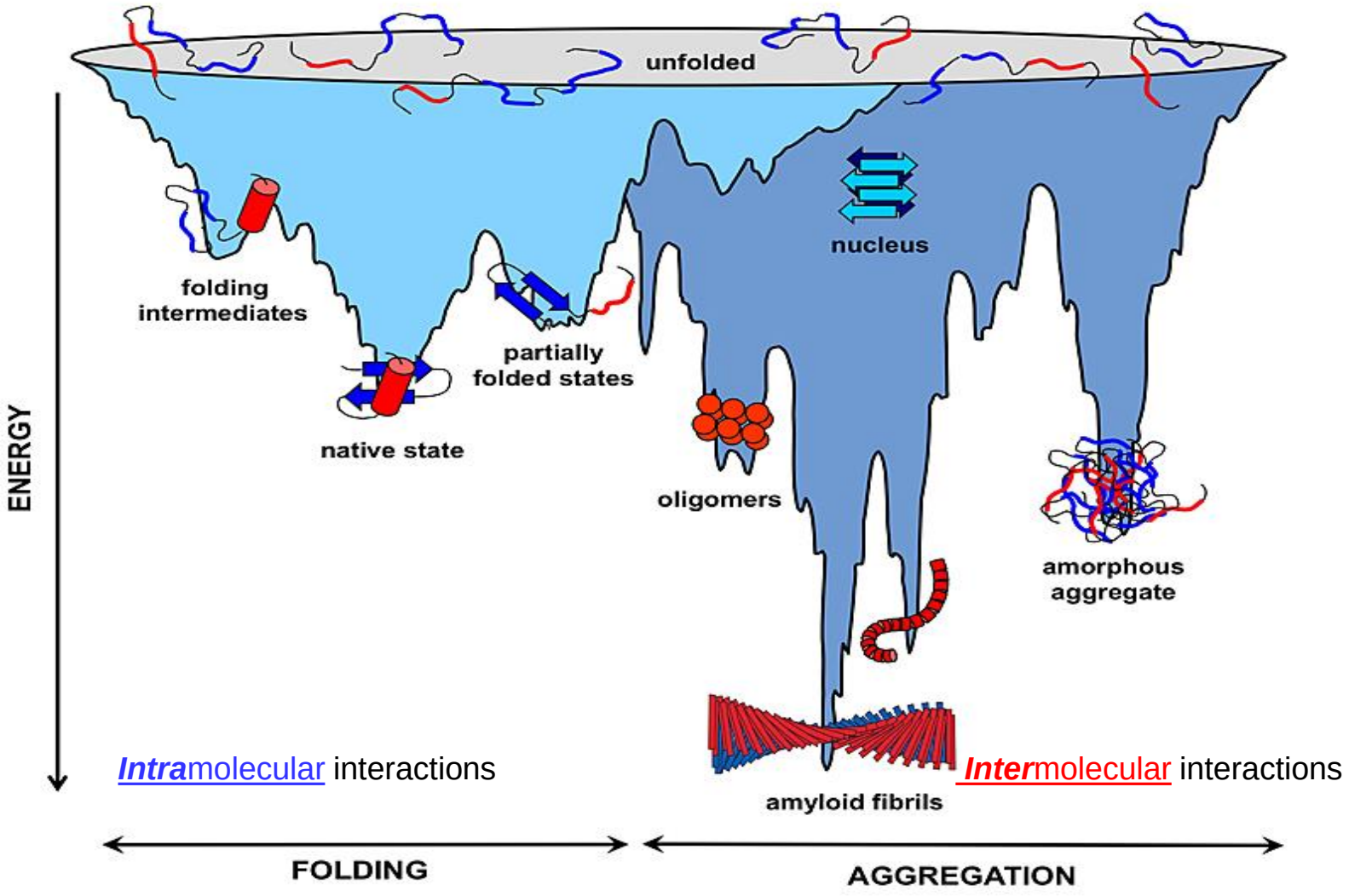


Structural model of an $A\beta_{1-40}$ protofilament, the minimal structural unit of $A\beta_{1-40}$ fibrils, based primarily on solid-state NMR data and consistent with constraints from EM, X-ray scattering, EPR and biochemical techniques [7••]. **(a)** Ribbon diagram of residues 9–40, showing two β-strands per molecule and parallel β-sheets in the cross-β motif. The long axis of the fibril extends out of the page. **(b)** Atomic representation, with residues colored according to sidechain type (green, hydrophobic; magenta, polar; red, negatively charged; blue, positively charged). The N terminus is disordered. The oppositely charged sidechains of D23 and K28 form salt bridges within the otherwise hydrophobic core. Reprinted from [12•]. Figure created with MOLMOL [63].

Green =
hydrophobic residues

Various types of amyloidogenic proteins are unrelated and their native forms have widely different folds but their **amyloid forms-fibrils** **have remarkably similar core structures:** Each consists of an array of self-assembled filaments, 0.1-10μm long and 10 nm wide, formed from limited portions of proteins/peptides in the β-sheet forms with extensive hydrophobic interactions, their β-strand chains running perpendicular to the long axis of the fibril: the **cross-β structure, cross-β spine.**

Folding energetic funnel of proteins and their aggregated forms



The protein oligomers, aggregates, amyloid fibrils and deposits arise from **intermolecular interactions**, intermolecular contacting between the same protein molecules.

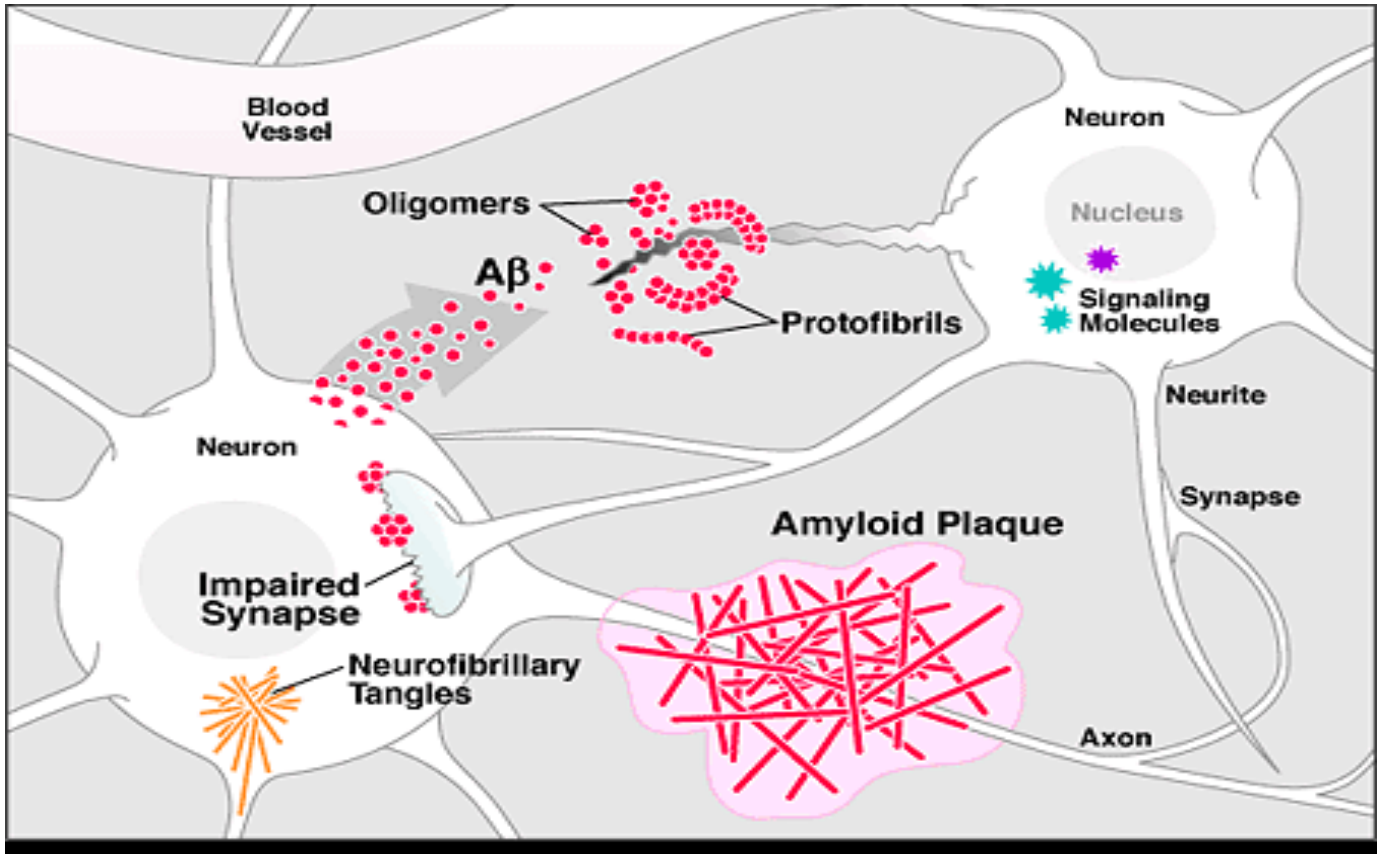
AD pathogenesis: **neurotoxic agents in AD are most likely soluble prefibrillar aggregates/oligomers of Aβ** before their deposition in amyloid plaques (microinjection exp.- rhesus monkey).

- Aβ oligomers bind to neuronal synapses.
- Aβ oligomers stimulate Tau phosphorylation => collapse of the microtubule network?

However, Aβ plaques do not correlate with cognitive decay, whereas **the tau neurofibrilary tangles do**. Memory deficit is usually diagnosed only after the plaques had already reached their plateau!
S. Prusiner (2017): A two-component disease: At least one conformer of the Aβ peptide becomes toxic when it stimulates the tau protein to polymerize into multimers and then into amyloid filaments that spread from cell to cell and coalesce into neurofibrillary tangles. The tangles or smaller tau multimers then cause brain dysfunction.

Aβ has a vastly different organization in oligomers than in plaques.
Enhanced expression of α-1-antichymotrypsin promotes Aβ polymerization.

Alzheimer disease pathogenesis



Early onset of AD:

- *e4* allele of *ApoE*: the main risk factor
- Down syndrome: chromosome 21 trisomy
- Familial AD forms (10-20%): mutations in APP (within Aβ sequence)
  mutations in genes for γ- secretase

Although mutant proteins that cause familial AD and also other familial neurodegenerative diseases (see later) are expressed early in embryogenesis**, signs of neurological dysfunction are generally delayed for decades**.

Parkinson's disease (PD) exists both in a sporadic (85 – 90% cases) and an inherited (10 - 15% cases) form. It is the second most common neurodegenerative disorder and one of the most common movement disorders. It is characterized with progressive loss of dopaminergic neurons in substantia nigra pars compacta of basal ganglia of the midbrain and accumulation of pathological, β sheet rich **α-synuclein** amyloid fibrils on the surface of **Lewy bodies** in neurons.

Deposition of Aβ is not limited to AD. Aβ plaques have been observed in vascular dementias, Parkinson disease with dementia, Levy body dementia as well as in the brains of aged individuals without any cognitive deficit.

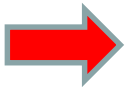
Neuronal proteins such as tau, CE-B1-synuclein, and polyglutamine aggregates,
which are causally implicated in the neurodegenerative disorders Alzheimer's disease, Parkinson's disease, and Huntington's disease, respectively, can be released from donor cells and taken up by neighboring acceptor cells.

Ad B (iii): PRION DISEASES - infectious, transmissible

•*origin of the protein misfolding and aggregation is its*

intrinsic susceptibility to alternative folding

Two stable conformational variants of the prion protein PrP:

Cellular normal conformation = PrP^{c}  Infectious conformation = PrP^{Sc}

a) Scrapie – 1732, sheep, goats; ataxia; transmission by ingestion of the feed from the diseased sheep; wild animals

b) BSE – 1987, *b*ovine spongiform *e*ncephalopathy, GB, mad cow disease, >180 000; transmission through feeding cattle by meat & bone meal from scrapie sheep

c) Chronic wasting diseases - deer

d) HUMAN PRION DISEASES

Creutzfeld-Jacob disease (CJD), rapid dementia (less than 2 years)

- **Sporadic** – 1920, 85%, (1:1 million/y: 5-10 cases/y in the Czech rep.), progressive, loss of memory & skill, depression, gait stability problems, speech problems, paralysis, + 0,5-(5y)
- **Familial –** 10-15%, hereditary mutation, "Oravian kuru"; Fatal familiar insomnia; Gerstmann-Sträussler-Schinkler sy
- **Infectious: i) iatrogenic –** cornea transplantation, surgery instruments, growth hormone, gonadotropin therapy (~100 cases); **ii) ingestion** of tissues from BSE cows = **new variant of CJD (**vCJD), interspecies transfer, young people, GB; **iii) kuru** – Papua, New Guinea – Fore people (cannibalism, brain); weakness, cerebellar ataxia, loss of coordination, trembles. Daniel Carleton Gajdusek:

1966 – **infectious disease! (long incubation period, death in 3–9 months)**

(exp. transmission to chimpanzees)

1976 Nobel Prize

Prion diseases are thus the only diseases that may have **three different ways of origin**:

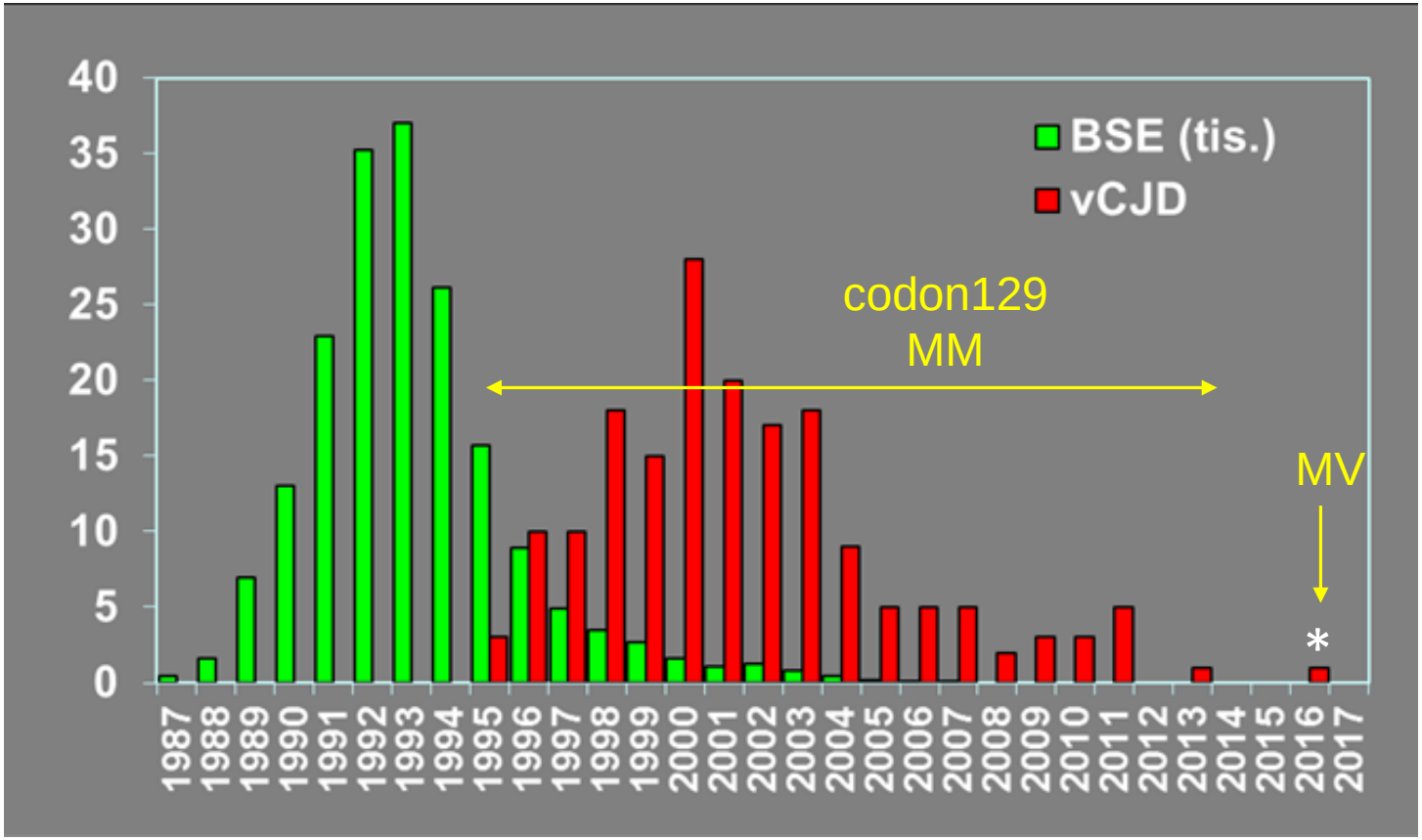
1) sporadic, 2) inherited, and 3) infectious.

Prion Diseases of Humans

- **Sporadic:** Creutzfeldt-Jakob disease (sCJD)

- **Hereditary:** (inherited germline mutations in the *PrPN* gene)
  - Familial CJD (fCJD)
  - Gerstmann-Straussler-Scheinker Disease (GSS)
  - Fatal Familial Insomnia (FFI)
- **Infectious:**
  - Kuru
  - Iatrogenic CJD
  - Variant CJD (vCJD)

Epidemiology of BSE and vCJD

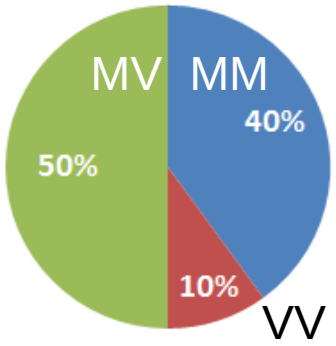


vCJD outside UK

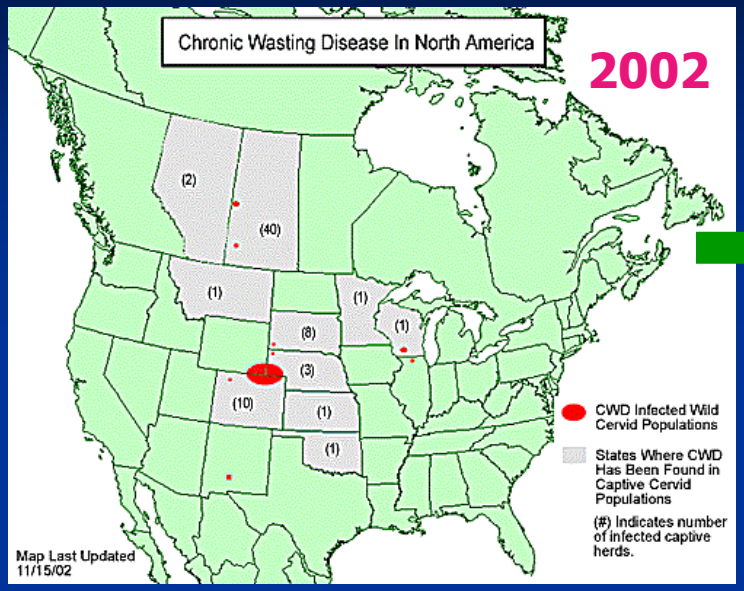
France	-	27
Spain	-	5
Irland	-	4
USA	-	4
Netherl.	-	3
Portug.	-	2
Saud. A.	-	1
Italy	-	2
Canada	-	2
Japan	-	1
Taiwan	-	1
All	-	**52**

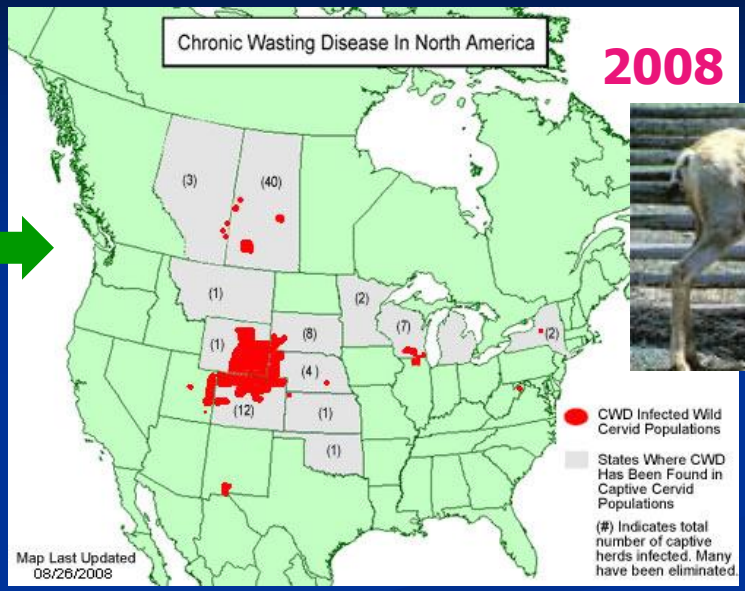
Identified cases of vCJD in UK **178** (* 25.5.2016).

BSE in UK: 2011 – **7**; 2012 – **3**; 2013 – **3**; 2014 – **0**.



Chronic wasting disease - CWD

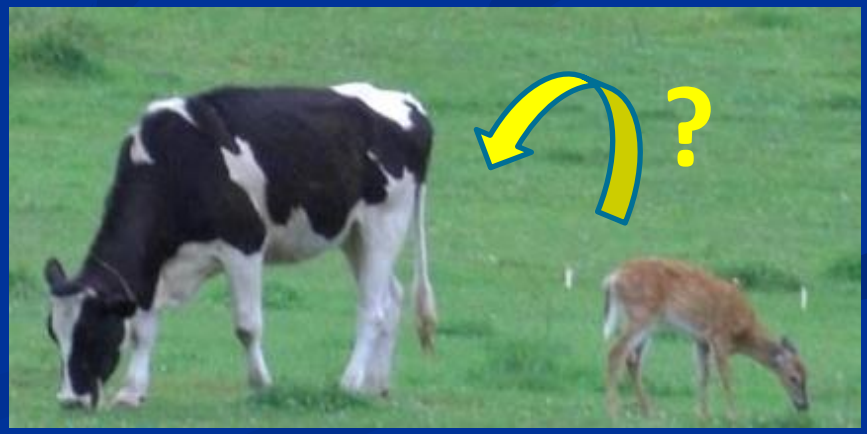


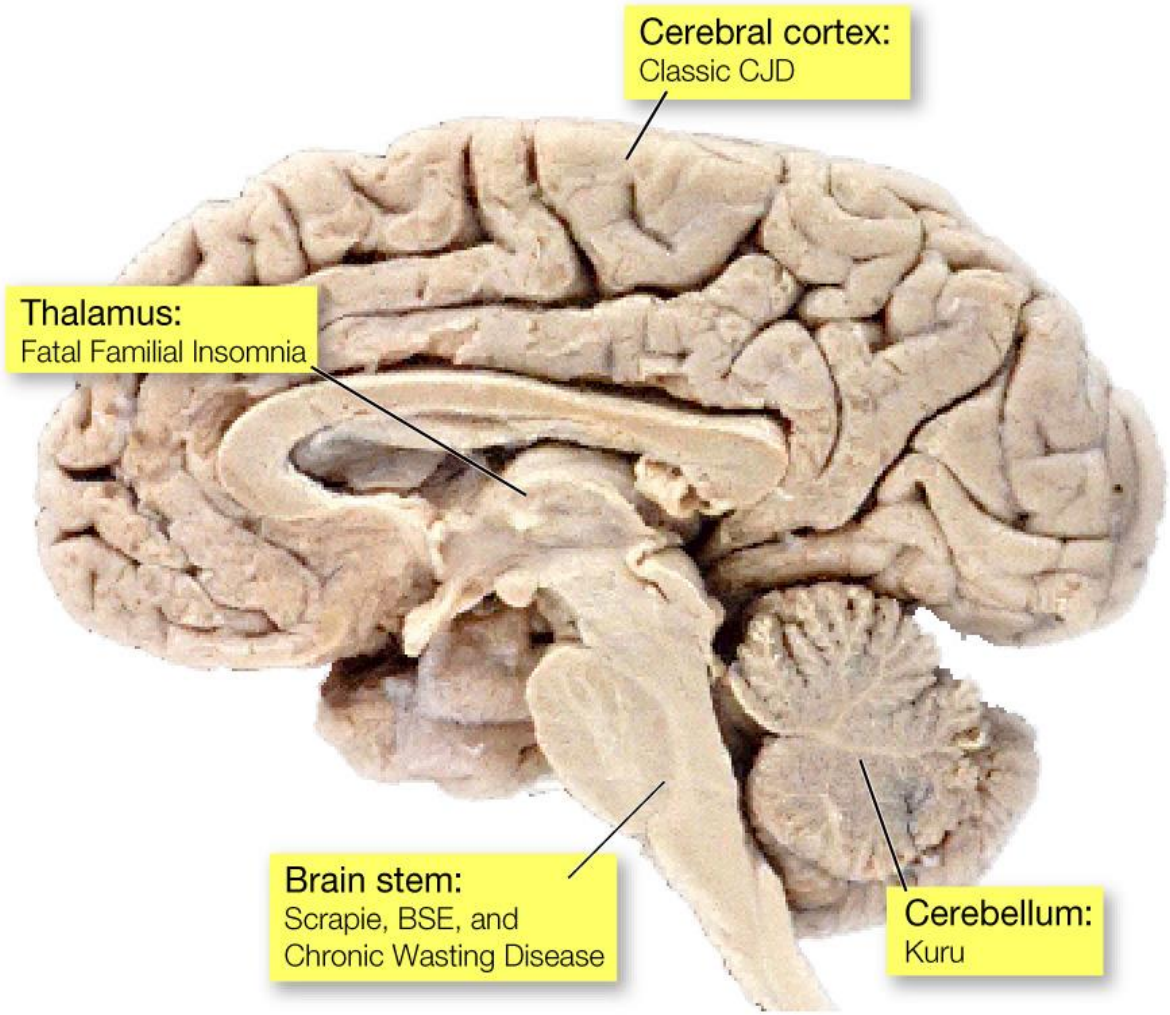


Colorado & Wyoming (1970)

www.cwd-info.org

- Deer, elk, moose, reindeer..
- Penetration up to 90 %
- Species barrier (?!)
- Contaminated environment
- **2016 Norway, 2018 Finland, 2019 Sweden**




Cerebral cortex:
Classic CJD
Thalamus:
Fatal Familial Insomnia
Brain stem:
Scrapie, BSE, and
Chronic Wasting Disease
Cerebellum:
Kuru

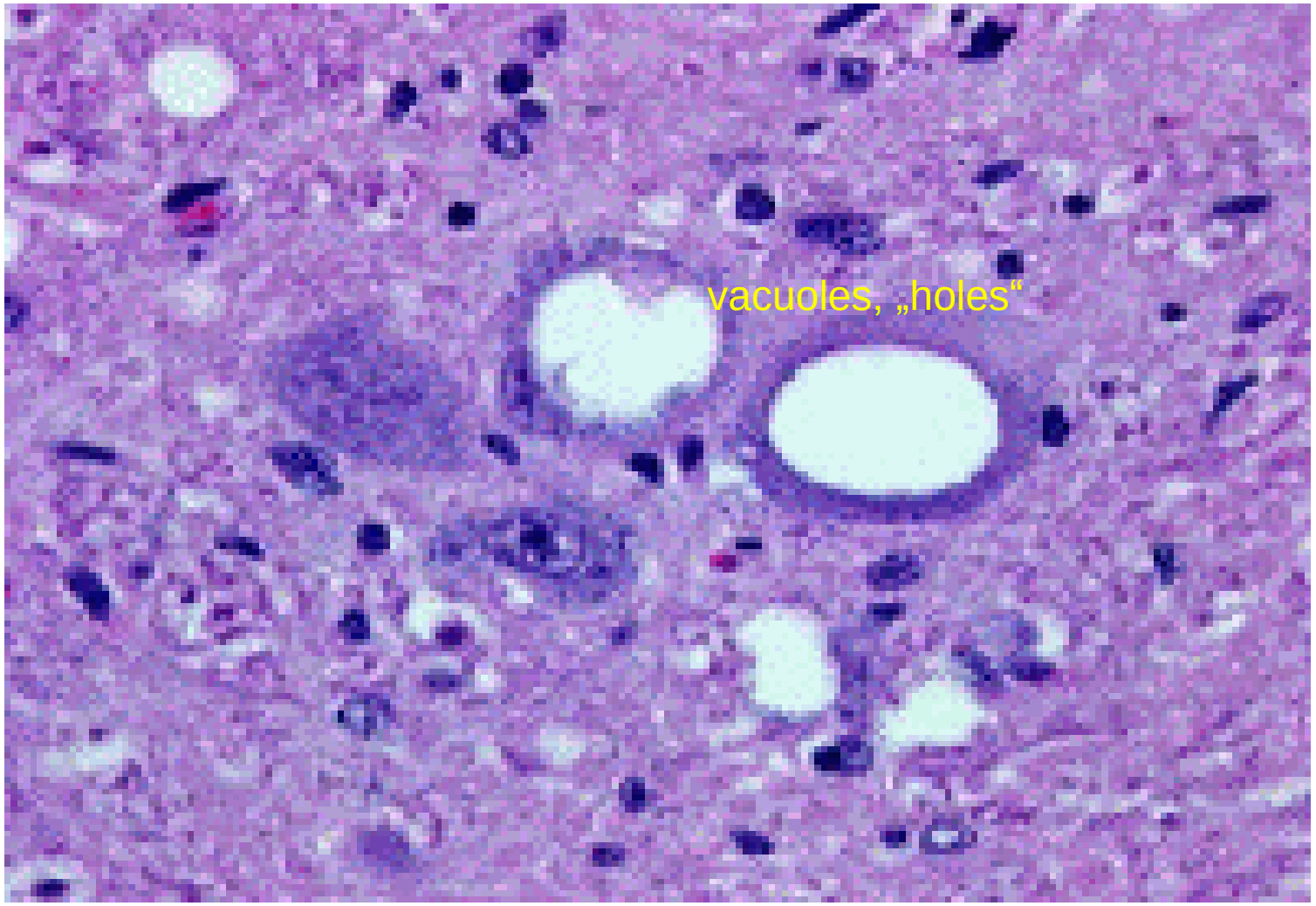
ALL of the diseases are fatal, (have similar symptoms), there is no cure.

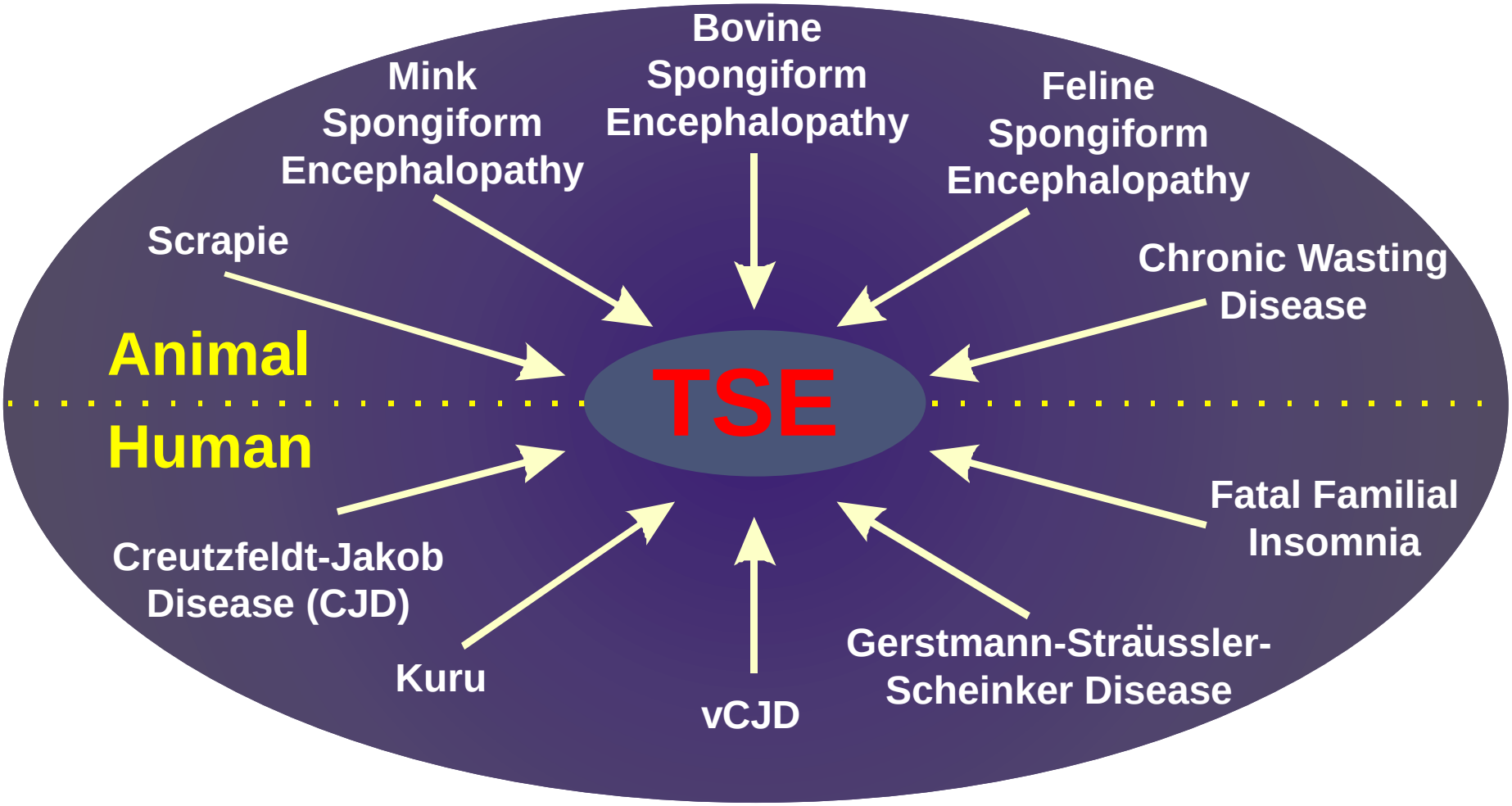
Neurons develop large vacuoles that gives brain tissue a spongelike microscopic appearance:

TRANSMISSIBLE SPONGIFORM ENCEPHALOPATHIES (TSE).

Spongiform encephalopathy

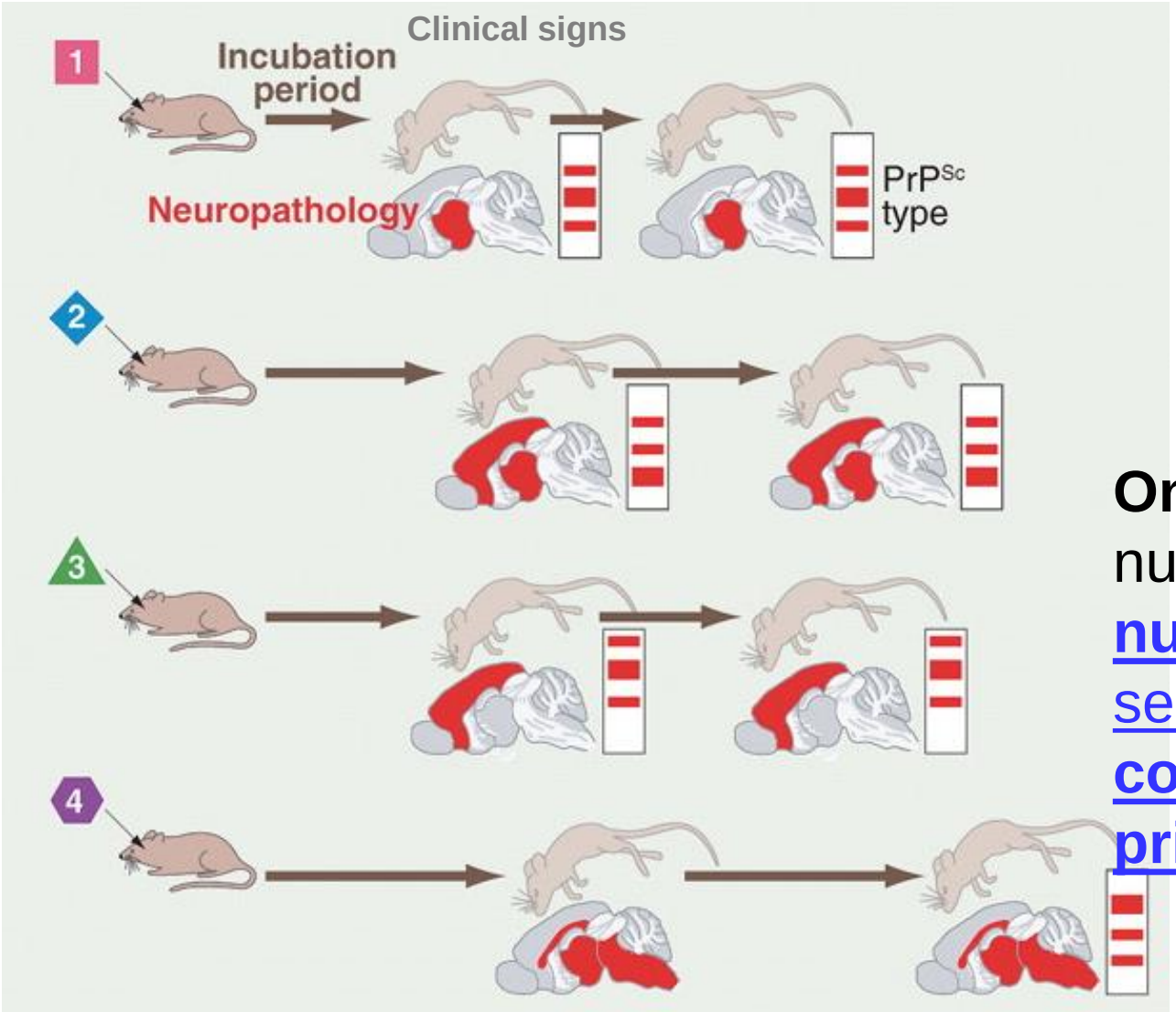
= Loss of neurons





- All fatal and transmissible to laboratory animals
- All - accumulation of misfolded prion protein (PrP^{TSE} = PrP^{Sc}) (TSE = transmissible spongiform encephalopathy)

Prion strains



Collinge J and Clarke AR, Science 2007;318:930-6

PrP^C

Rudd PM et al.
PNAS 1999;96:13044-9

One protein - number of diseases, **number of** self-propagating **conformations** (!) = **prion strains**

1984: Stanley Prusiner isolated the infectious particle of scrapie disease and called it **prion** (for *pr*oteinaceous *i*nfectious *on*ly): **PrP^{Sc}**

Nobel Prize 1997

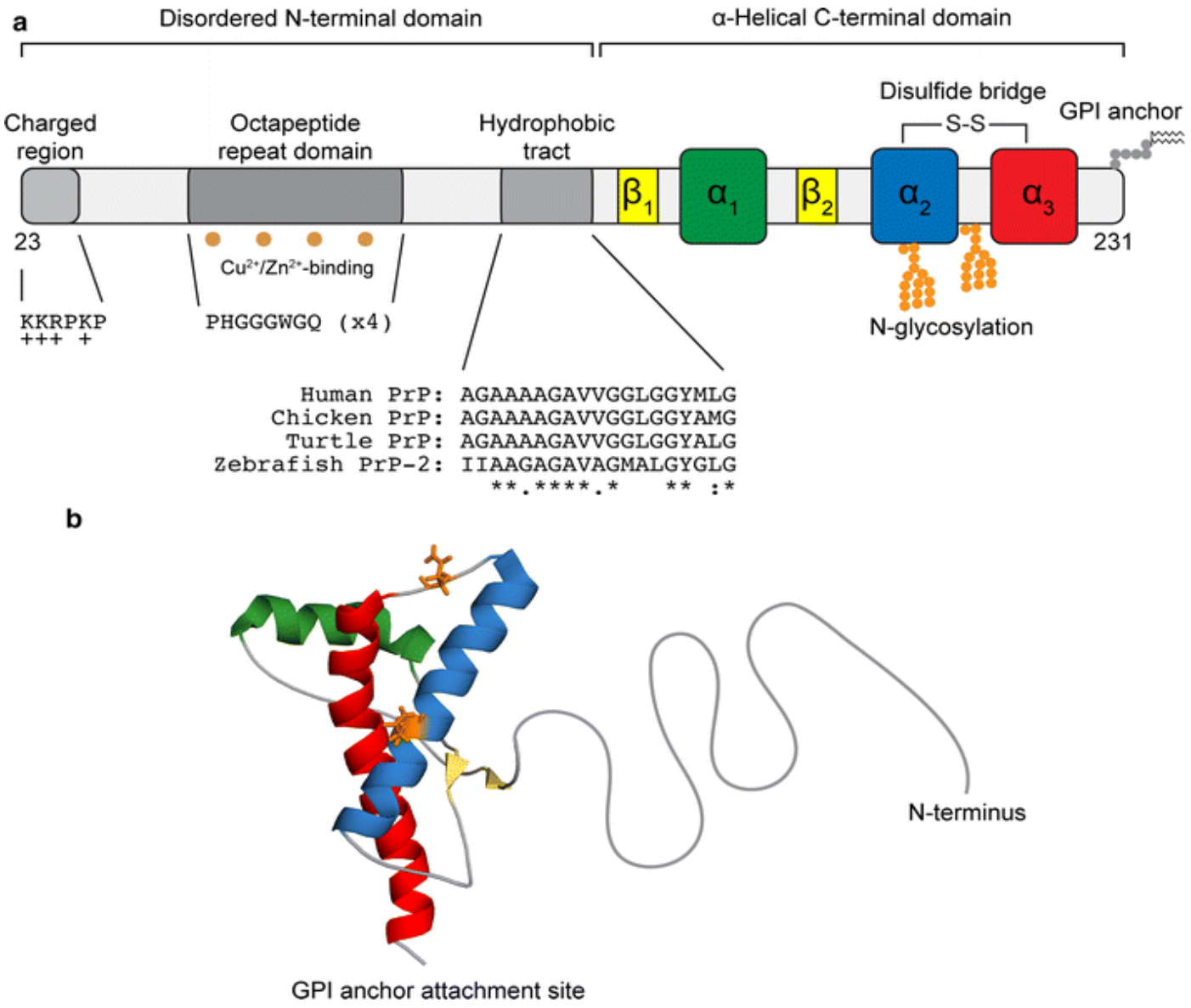
The scrapie particle is a **new kind of infectious agent, one that does not need a nucleic acid to replicate. It is a single species of a**

MISFOLDED BODY PROTEIN called **PRION (PrP^{Sc})**

NEW PARADIGM IN MOLECULAR BIOLOGY!

(Now we know that we can die from an infection by a misfolded protein only, no genes are required !?)

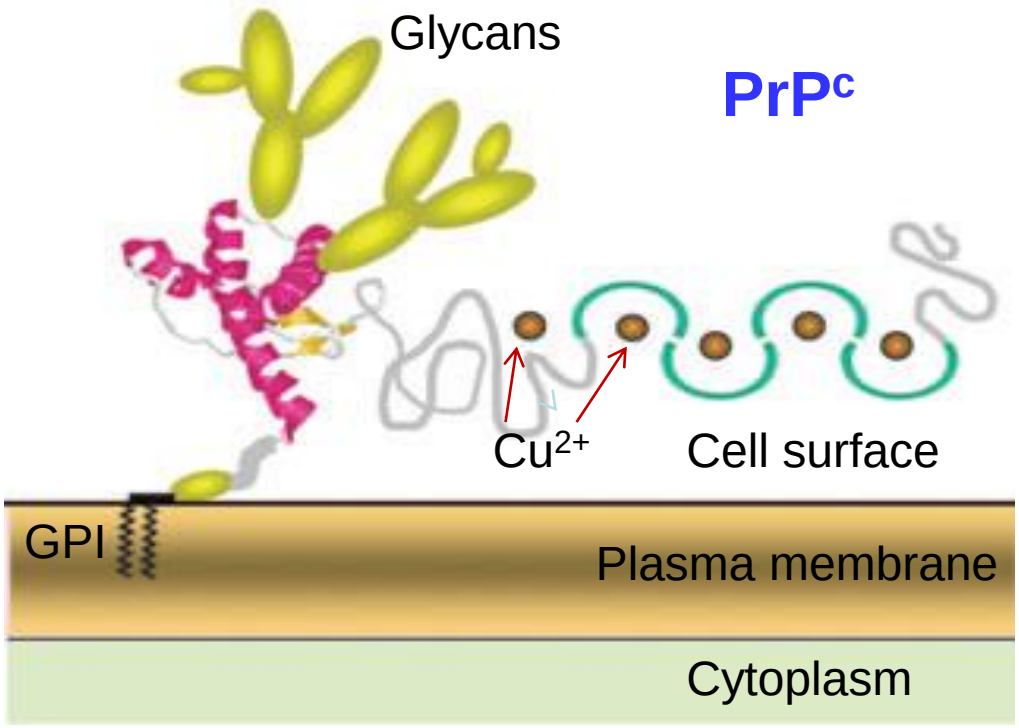
Domain structure of human PrP^{C}



- **PrP^C** is a normal protein (prion protein) found on the membranes of cells. It is an expression product of a *prp*^{c} gene. PrP^C has 208/209 amino acids (in humans) -after posttranslational modification (from 253 aa), one disulfide bond, a molecular mass of 35–36 kDa and a mainly alpha-helical structure. PrP^C is readily digested by proteases (incl.proteinase K) and can be degraded into amino acids.

  It can also be liberated from the cell surface *in vitro* by the enzyme phospho-inositide phospholipase C (PI-PLC), which cleaves the glycosylphosphatidyl-inositol (GPI) glycolipid anchor.

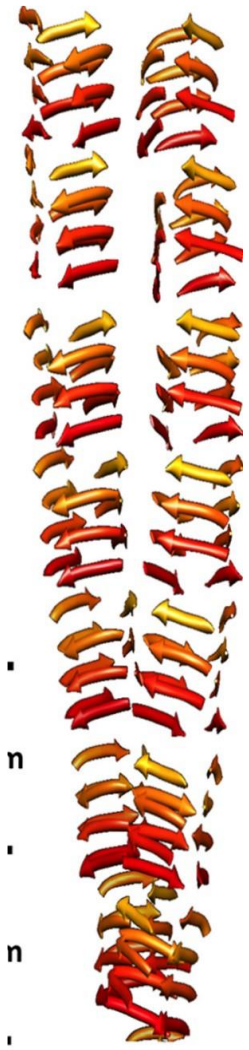
- PrP^C function is not fully known: roles in cell-cell adhesion and intracellular signaling *in vivo?*, cell-cell communication in the brain?, biological clock?, memory?
- PrP^C is highly conserved through mammals and expressed in all vertebrates as well as invertebrates.



Synthesized glycoprotein PrP^c is attached to the plasma membrane through the glycosylphosphatidylinositol (GPI) anchor

- **Infectious prions = PrP^{Sc}** are the infectious isoforms of the prion protein PrP^{C}. PrP^{Sc} are not considered living organisms but are misfolded protein molecules which may propagate by transmitting a misfolded protein state.
- PrP^{Sc} has a different secondary and tertiary structure from PrP^{C} but identical primary sequence. The exact 3D structure of PrP^{Sc} is not known but it contains **predominantly β-sheet structure**.
- Aggregations-oligomers of these abnormal isoforms form highly structured amyloid fibers, which accumulate to form plaques. **The oligomers** before deposition into plaques are considered to be the cause of cell damage (vacuolar degeneration of neurons).
- Prions are species specific (have different strains) with different stable conformations.

Cartoon depicting the proposed configuration of the polypeptide chains in the infectious prion PrP^{Sc} fibril.



Transmissible spongiform encephalopathies (TSE) are diseases of prion protein conformation.
PROTEINOPATHIES

Prions PrP^{Sc}

are (due to 3D-conformation different from that of PrP^{C}):

resistant to

proteases (proteinase K, **body** proteases) – only partial hydrolysis, heat, boiling, UV radiation, H_2O_2, I_2, ethanol, lysol, peracetic acid and formalin treatments.

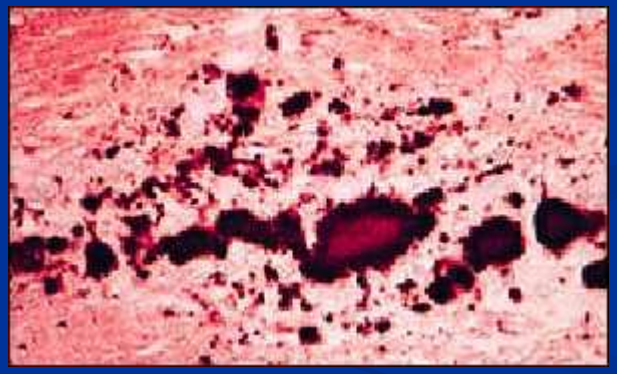
degraded by

concentrated bases (NaOH, KOH), NaClO (SAVO), water steam (for 20 min., 3 bar, 135°C)

keratinase

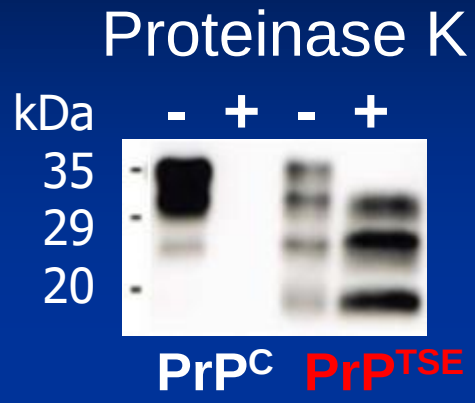
Prion PrP^{Sc} detection

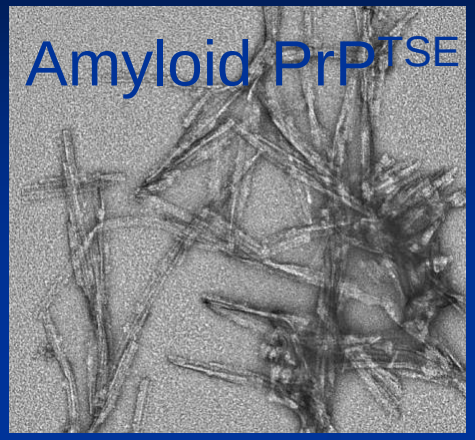
- Reaction of prion-containing tissue digested with proteinase K with an antibody against PrP^{Sc} (immuno-elfo)
- Histological examination of the brain
- Biological tests on mice, (+genetically modified mice). Syrian hamster. PMCA



Tests: with blood, urine, brain, meat and lymphatic tissues

Properties of PrP^{C} and $PrP^{TSE(Sc)}$



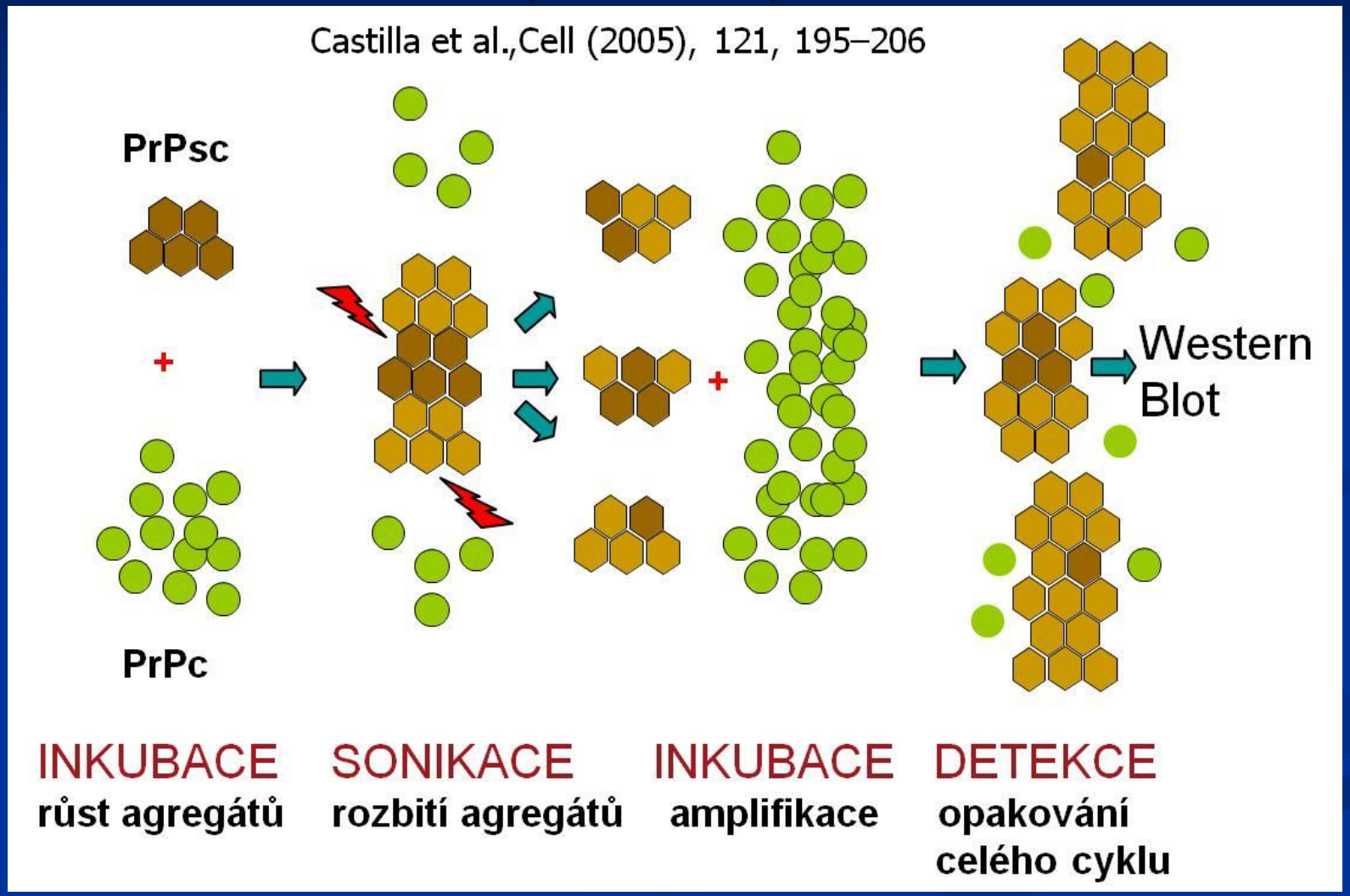


https://pines.berkeley.edu/

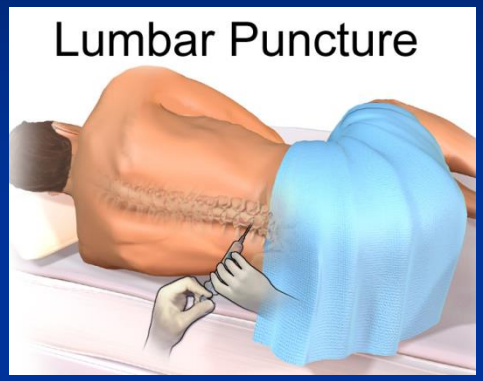
- Normal cellular **PrP^{C}**
- Sensitive, soluble
- Half-life **2-4** hours

- Pathological **PrP^{TSE}** (=PrP^{Sc})
- Partially resistant, insoluble, aggregates
- Half-life **16-20** hours

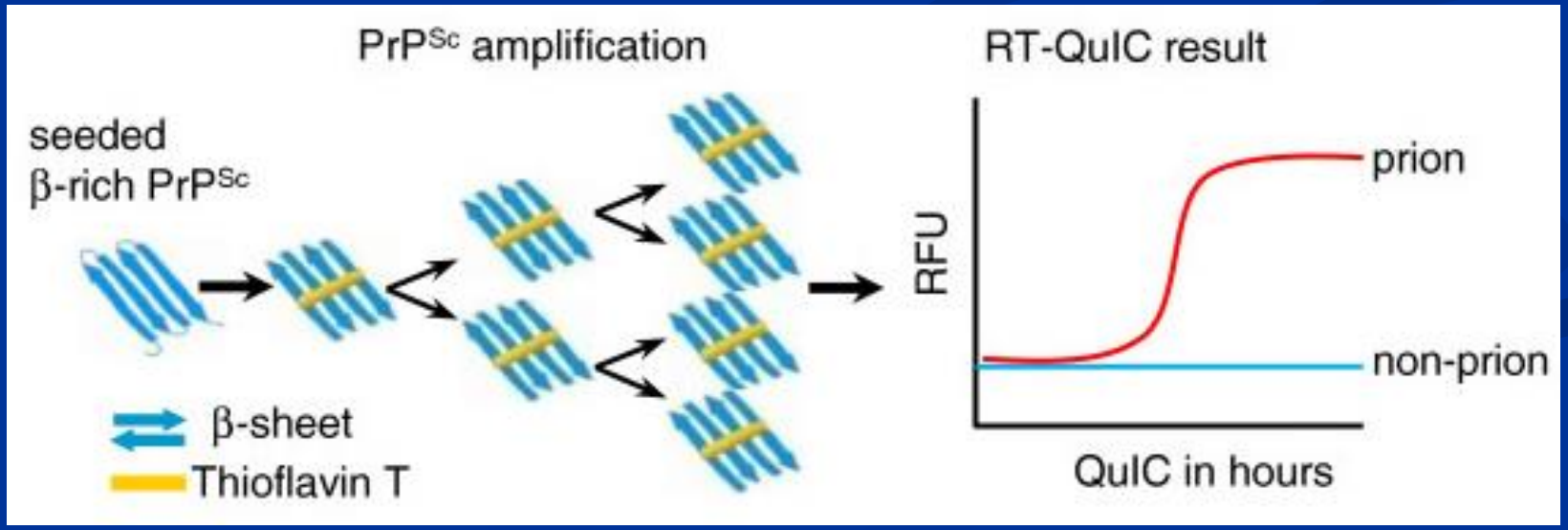
Protein misfolding cyclic amplification (PMCA)



Real-time quaking-induced conversion (RT-QuIC) assays



CSF + rPrP + Thioflavin T + shaking



HYPOTHETICAL PATHOGENESIS OF PRION DISEASES – PRION HYPOTHESIS

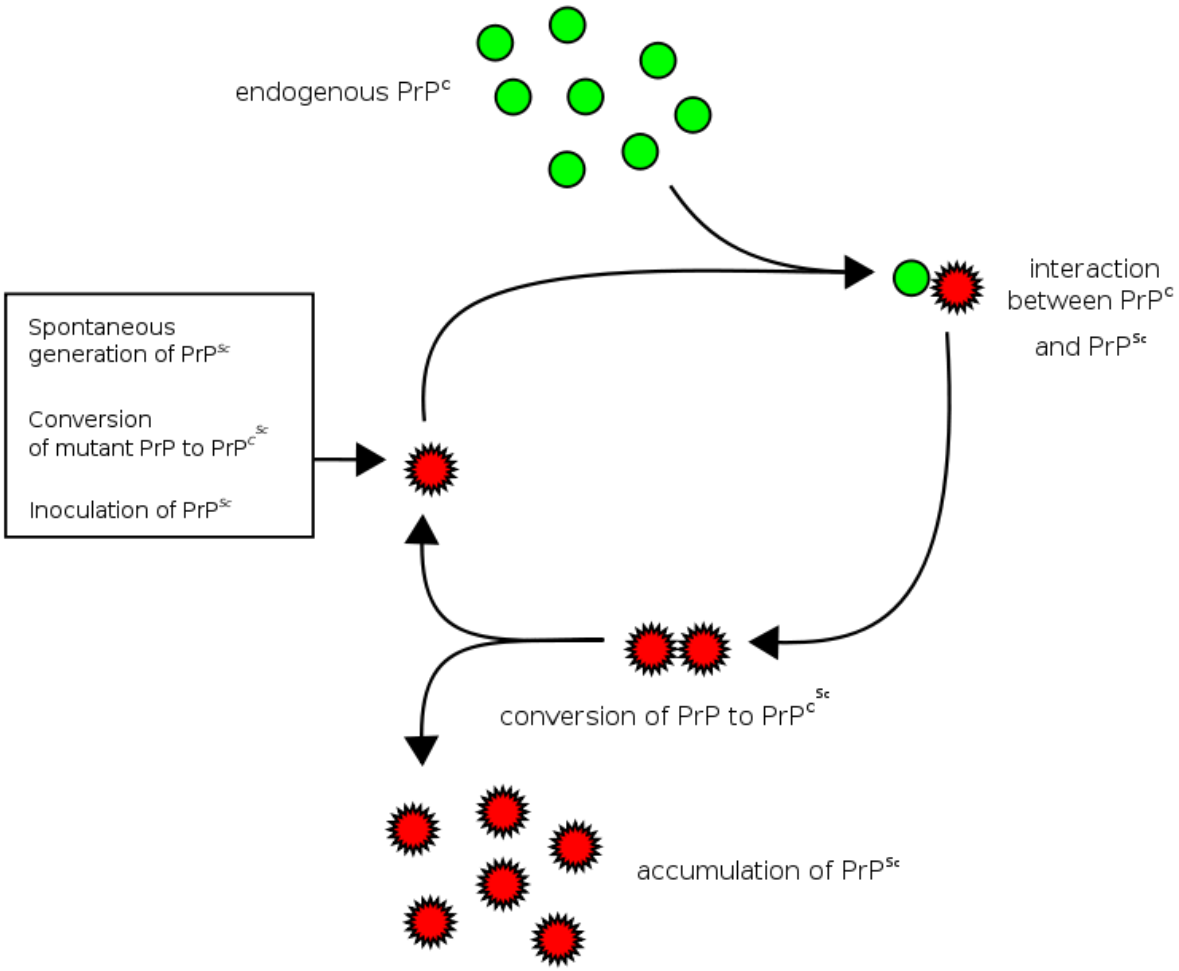
1) **Prion protein PrP^c** converts into **infection prion PrP^{Sc}** due to sporadic spontaneous? change of the PrP^c conformation. (Under normal conditions the spontaneously converted forms of PrP^c are rare and apparently eliminated by the protein folding quality control system of the cell).

2) **The PrP^c** --> **PrP^{Sc}** conversion becomes highly accelerated by **exogenous PrP^{Sc} /mutated PrP^c with a PrP^{Sc} character**("chain reaction", Figs). **PrP^{Sc}** is a **stable conformational variant** of normal **PrP^c.**

3) To develop into a disease the expression of the corresponding normal **PrP^c prion protein is required.** Animals that do not express the normal form of the prion protein can neither develop nor transmit the disease.

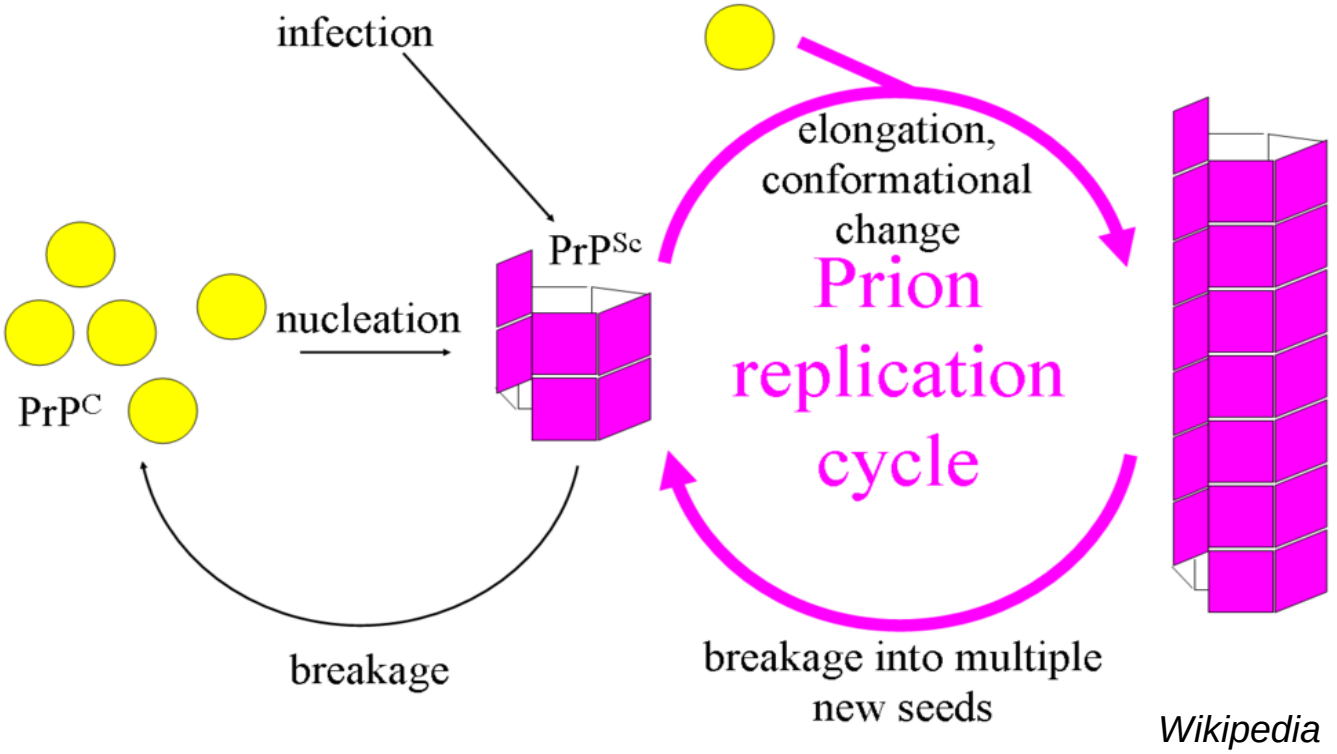
4) All known prions induce the formation of an amyloid fold, in which the protein polymerizes into an aggregate, oligomer consisting of tightly packed beta sheets. **Amyloid aggregates** are fibrils, growing at their ends, and replicating when breakage causes two growing ends to become four growing ends. **PrP^{Sc} oligomerization and oligomer-mediated cytotoxicity appear to be a key issue in the TSE** similarly as in as in the AD, PD and HD pathologies described above.

Heterodimer model of prion propagation



Wikipedia

Fibril model of prion propagation.



The end of each fiber acts as a template onto which free protein molecules may attach, allowing the fiber to grow. Under most circumstances, *only PrP^{c} molecules with an identical amino acid sequence to the infectious PrP^{Sc} are incorporated* into the growing fiber. However, rare cross-species transmission is also possible (see the text).

The inherited prion diseases can be diagnosed by finding mutation in the prion protein gene. No such pathogenic mutations are present in sporadic and acquired prion disease.

Treatment?

- **Heterologous PrP molecules**, which differed by as little as one residue, **interfere** with the generation of PrP^{Sc} in scrapie-infected mouse cells (Priola et al. 1994)

  => interaction between dissimilar PrP^{Sc} and PrP^{c} molecules (= have different aa sequence) might slow the aggregation and accumulation of PrP^{Sc} by interfering with the interaction of similar PrP monomers.
- To design a molecule that binds to prion protein and stabilizes its normal shape, thereby preventing propagation of the disease.
- NO TREATMENT AVAILABLE YET!!!

Neurodegenerative proteinopathies

• *Alzheimer disease*	**amyloid β, Tau**	270
• *Parkinson disease*	**α – synuclein**	80
• *Amyotrophic lateral sclerosis*	**SOD1, TDP-43**	20
• *Frontotemporal dementia*	**SOD1, TDP-43**	14
• *Huntington disease*	**huntingtin**	11
• *Prion diseases*	**PrP**	1-2

Fibrilar aggregates of proteins in changed conformation.
Only *Prion diseases* proven transmissible.

TAR DNA-binding protein 43 is a transcriptional repressor that binds to chromosomally integrated TAR DNA and represses HIV-1 transcription.

SUMMARY **TSE = diseases of prion protein conformation**

Prions **PrP^{Sc}** are infectious, species specific strains of proteins, that are responsible for a number of fatal mammalian diseases. They are highly protease-resistant and thus easily transmissible by ingestion. **PrP^{Sc}** are toxic to neurons and as such causative agents of transmissible spongiform encephalopathies (**TSE**s).
TSEs have been described in a number of species such as mink (TME), cat (FSE), elk (CWD), sheep (scrapie), cattle (BSE) and humans (CJD).
Normally, interspecies transmission of different **PrP^{Sc} strains** doesn't induce the disease except the sheep-cattle (BSE) and the recent cattle-human (new variant CJD) transmission events.
To develop into a disease (sCJD) the spontaneously generated/ingested/inoculated **PrP^{Sc}** molecules must increase in number. They propagate themselves by converting cellular **PrP^{c}** into **PrP^{Sc}** . The conversion mechanism has not been elucidated.
Infectious **PrP^{Sc}** molecules originate from native, naturally occurring non-infectious cellular protein **PrP^{c}** by a **spontaneous change in its conformation or induced change** by an introduced **PrP^{Sc}** . Clearly, infectious **PrP^{Sc}** **is a misfolded β-sheet-rich form of** **PrP^{c}** . This conformational conversion of some regions of **PrP^{c}** gives the protein a **fibrillogenic/amyloidogenic character leading to its aggregation into insoluble plaques**. **PrP^{Sc} oligomerization and oligomer-mediated cytotoxicity appear to be a key issue in the TSE** similarly as in as in the AD, PD and HD pathologies described above. **PrP^{Sc}** can be transmitted from cell to cell.

SUMMARY (cont.)

Genetically well-defined prion proteins were also found **in yeast**. Critical for establishment and propagation of yeast prion aggregates are the prion-forming domains (PrD), which are rich in glutamines (Q) and asparagines (N). *In vitro*, the individual PrDs are able to spontaneously undergo conformational rearrangement ("**chameleon" regions**) in the absence of any other proteins or nucleic acids, to generate highly stable amyloid fibrils.

One such fibril forming motif in the N-terminal domain of the best documented yeast prion, Sup 35 (translation termination factor) is a seven residue peptide, GNNQQNY, (G=Gly; N=Asn; Q=Gln; Y=Tyr). It forms amyloid-like fibrils and also closely related microcrystals. The X-ray diffraction analysis of the microcrystals allowed to determine the first fully objective atomic structure of the cross-β spine. The structure consists of the peptide molecules, each an extended strand stacking on each other to form parallel, in register sheets. The strands within the sheet run perpendicular to the long axis of the microcrystals (fibrils). Each strand hydrogen bonds with the strands above and below it in its sheet, using both main chain and side-chain hydrogen bonds. A pair of such sheets mates across interfaces formed by N- and Q- side-chains to form a steric zipper with strong van der Waals contacts and, less firmly, at Y residue by bridging water molecules (Fig.). In the Aβ model protein (from AD) the strong interaction at the sheet interfaces is provided by hydrophobic amino acid residues (Fig.). The basic building block of the fibril is a pair of β-sheets. Yeast's prions do not appear to cause disease in their hosts. Investigations of the yeast prion system suggest that molecular chaperones may be involved in prion propagation. Functional amyloidogenic "good" prions also in humans: CPEB, TIA-1.

In conclusion:

C. Dobson: “Peptides or proteins convert under some conditions from their soluble forms into highly ordered fibrilar aggregates. The ability to form the amyloid structures is an inherent or generic property of polypeptide chain although the propensity to do so varies dramatically. Such transitions can give rise to pathological conditions ranging from neurodegenerative disorders to systematic amyloidosis”.

“Living organisms can take advantage of this ability to generate novel and diverse biological functions ”(e.g. a domain of the human Pmel17 protein: it forms, inside melanosomes, fibrous striations upon which melanin granules form; yeast’s Sup 35 prion enables the survival under changing conditions, simply by protein conformational change = the quick adaptation-epigenetic mechanism, before change in DNA).

“In light of the fact that cells have evolved protein folding quality control systems (chaperons and proteasomes), how prions managed to survive and replicate *in vivo* is puzzling”.

Acknowledgements

Some illustrations in this presentation were reproduced from D. Voet and J.G. Voet, Biochemistry, 3rd Edition, J.Wiley & Sons, Inc. 2004, from the Textbook of Biochemistry with Clinical Correlations (T.M. Devlin, ed.), 6th Edition, Wiley–Liss, Hoboken, 2006, from a presentation of K. Holada (1st Medical Faculty, Charles University, Prague) and from some other sources.