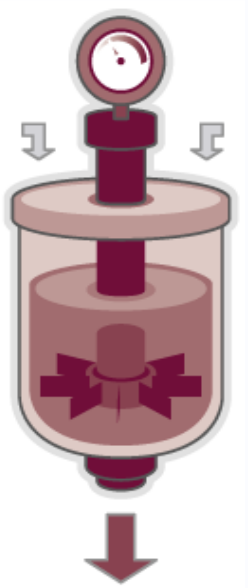
Evaluating a biocatalysts for given process:

1.Biocatalyst productivity

2.Reactor productivity

3.Product yield

4.Biocatalyst stability



1. Biocatalyst productivity: turnover number

,

In catalysis it refers to:

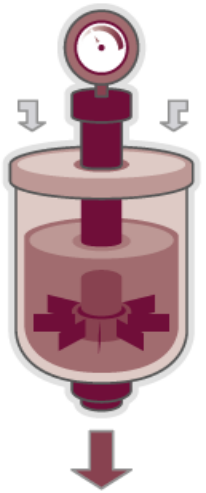
the number of moles of product formed per mole of catalyst over the reaction period.

dimensionless ratio:

(mol product) / (mol enzyme)

Evaluating a biocatalysts for given process:

1. Biocatalyst productivity
2. Reactor productivity
3. Product yield
4. Biocatalyst stability



- Important for larger-scale operation
- Presented as **"Space-Time Yield"** or "Volumetric productivity"

$$\frac{\textbf{Amount of product generated}}{\textbf{Reactor volume x time}}$$

Product yied « y»

$y = X \sigma$

σ = selectivity

X = conversion

Total yield

$y_{tot} = (y_{step})^{n}$

Biocatalyst stability: evaluating residual activity after exposure to denaturant factors (or even inactivation)

Thermal stability

Inactivation at high temperature:

•it should be specified whether the activity assays is carried out at the **preincubation temperature, or after cooling** to some standard assay temperature

Solvent stability

Inactivation by co-solvents:

•Enzyme incubated **before assay**, or **co-solvent still present in the essay medium**

Operational stability

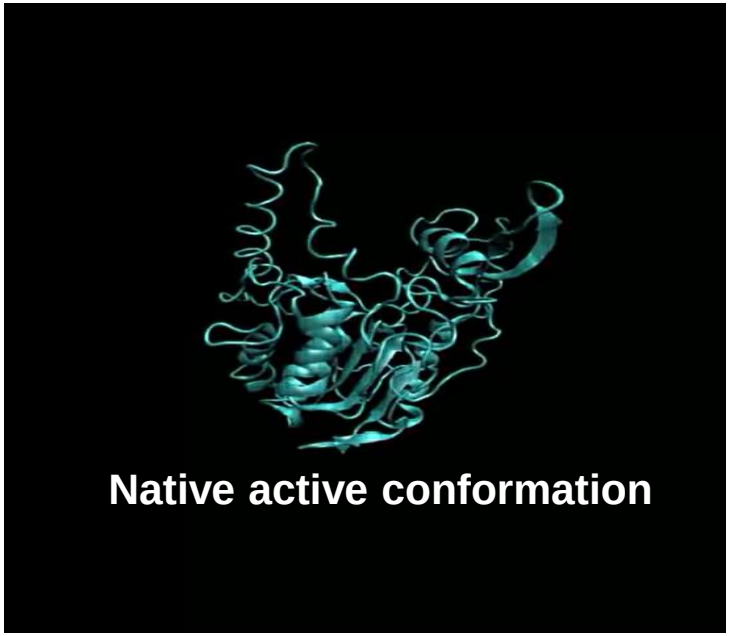
- Inactivation under operational conditions

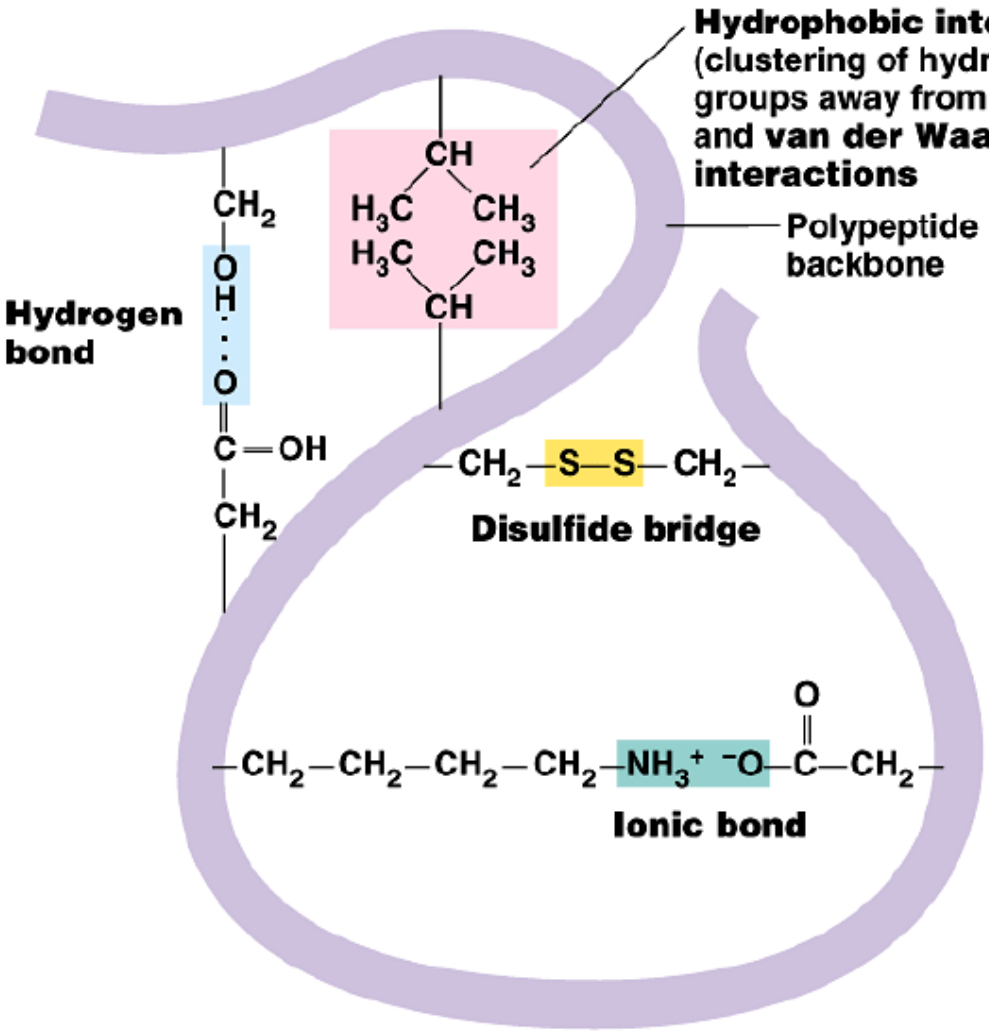
Possibly stability should be studied under conditions as closer as possible to the final operational system

Protein folding and active conformation: drivers

Protein folding is driven by hydrophobic interactions, due to the unfavourable entropy decrease of the forming surface area of non-polar groups with water.

Consider a water molecule next to a surface to which it cannot hydrogen bond. The incompatibility encourages the surface minimization that drives the proteins' tertiary structure formation.



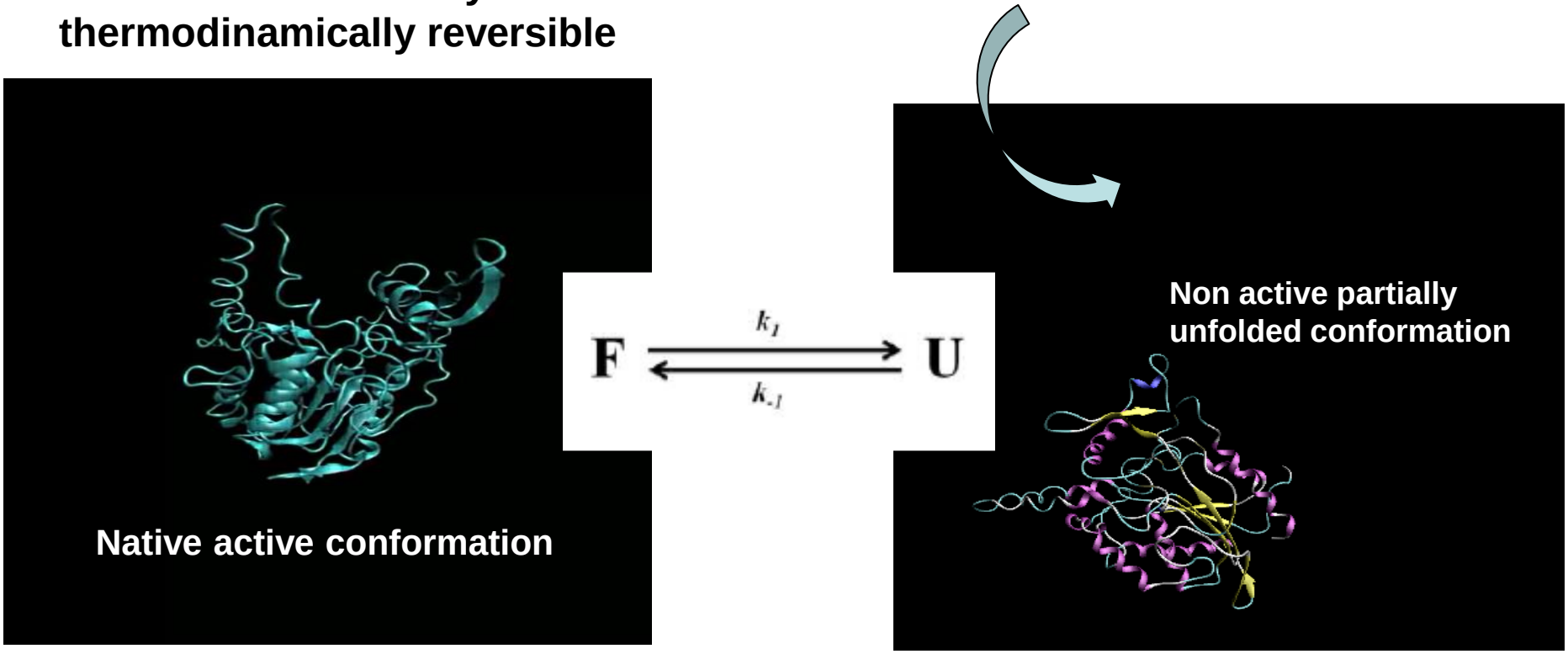


Such hydrophobic collapse is necessarily accompanied and guided by (secondary) structural hydrogen-bond formation between favorable peptide linkages in parallel with their desolvation .

A driving force for this is the release of water to be available for the hydration of other solutes and maximizing its entropy.

Active conformation corresponds to a minimum of energy referred to a set of environmental conditions: pH, T, p, μ (ionic strength)

When environmental conditions are changed the protein conformation changes and reaches a new minimum of energy that might correspond to an active or inactive enzyme: such conformation or «state» is thermodinamically reversible

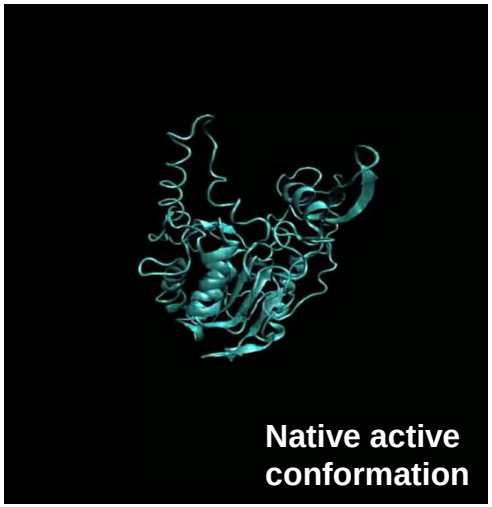


Thermodynamic stability

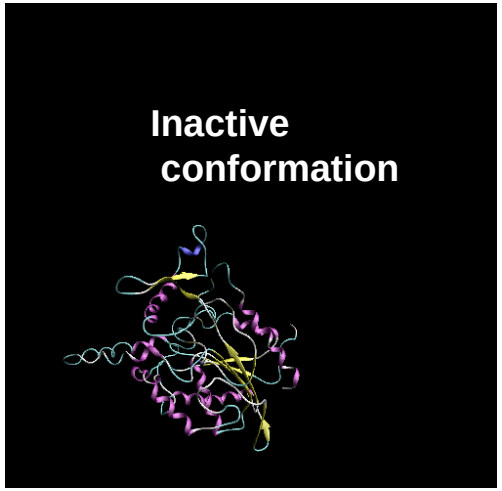
The Gibbs free energy changes between the folded state and unfolded state is described using the free energy equation:

$$\Delta G_f = G_f - G_u = \Delta H - T\Delta S$$

If folding of a globular protein is thermodynamically favorable, ΔG_f should have negative value.



$$F \rightleftharpoons U$$

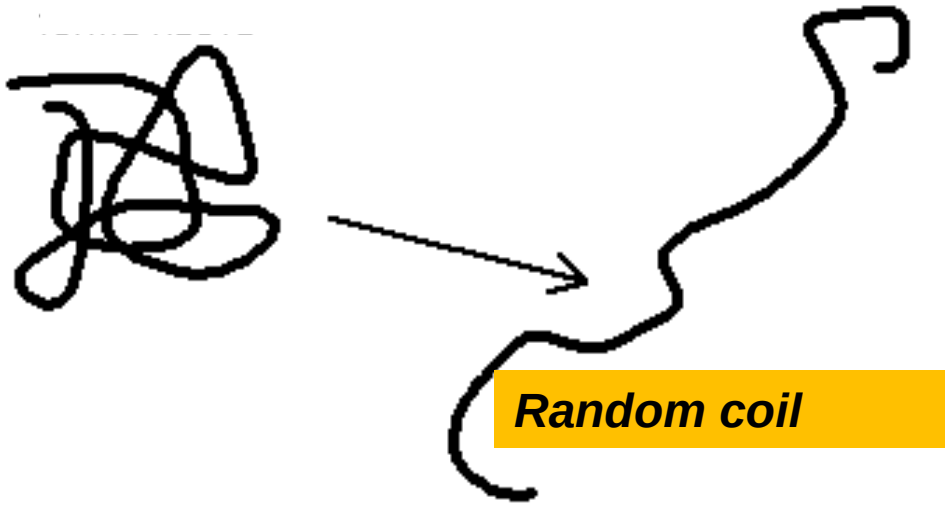


Thermodynamic stability

From the theoretical and experimental evidences, the folded state is only little more stable than the unfolded state and this means that small negative value, < 20 kcal/mol, can determine the stability of a protein.

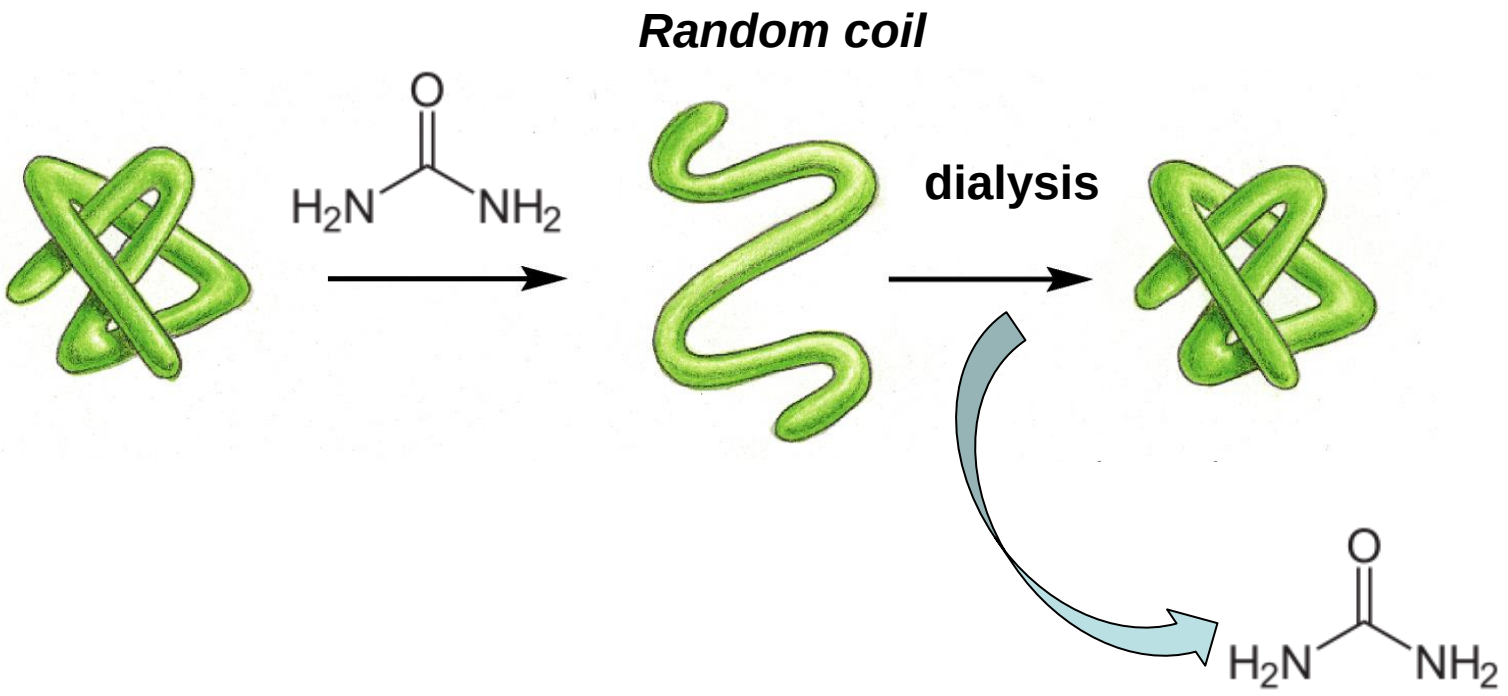
$$\Delta G_f = G_f - G_u = \Delta H - T\Delta S$$

This small value of Δ G_f for conformational stability results from the summation of contributions from several non-covalent interactions such as hydrophobic interaction, hydrogen bond, electrostatic interaction and conformational entropy.



Thermodynamic stability

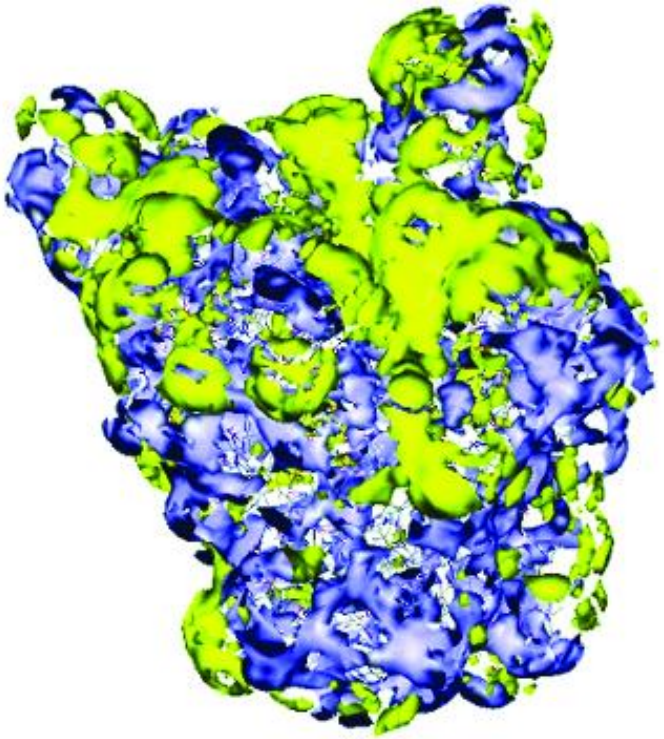
Example: reversible denaturation with urea



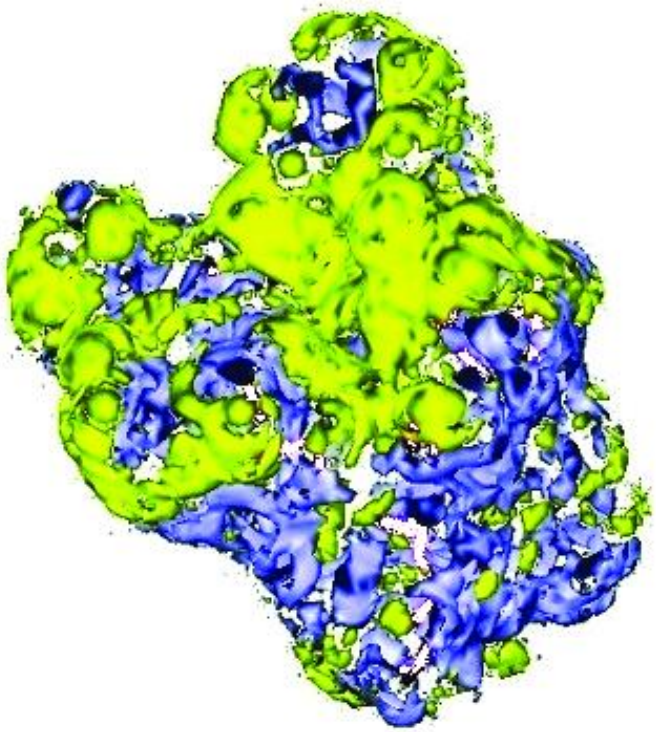
Conformational modifications sometimes are necessary for displaying enzyme activity

Lipases undergo activation at the water-lipid interface

Pseudomonas cepacia LIPASE



closed conformation in water

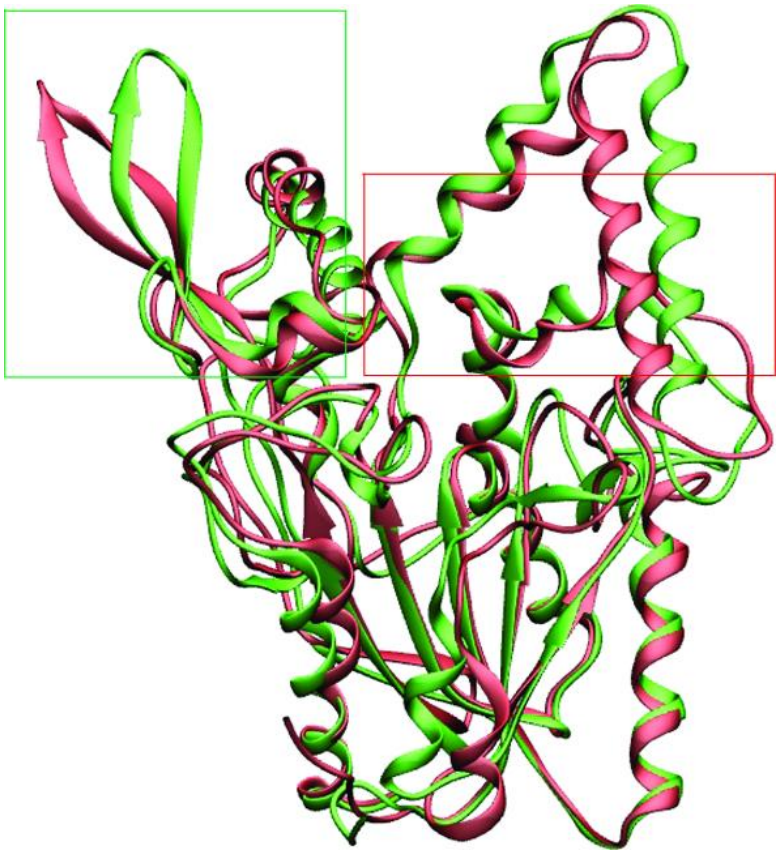


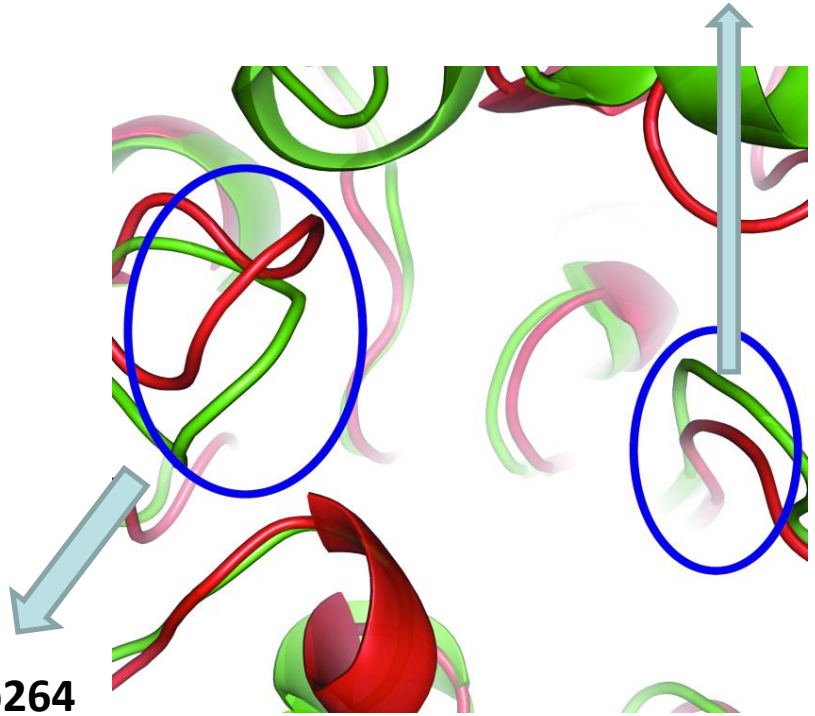
open conformation in hydrophobic media

HYDROPHILIC AREA

HYDROPHOBIC AREA

Molecular dynamic simulation of lipase from *Pseudomonas cepacia*





OPEN CONFORMATION in hydrophobic media

Closed conformation in water

Conformational changes affect the catalytic machinery

V. Ferrario & al., *Adv. Synth. Catal.,* 2011, 353, 2466.

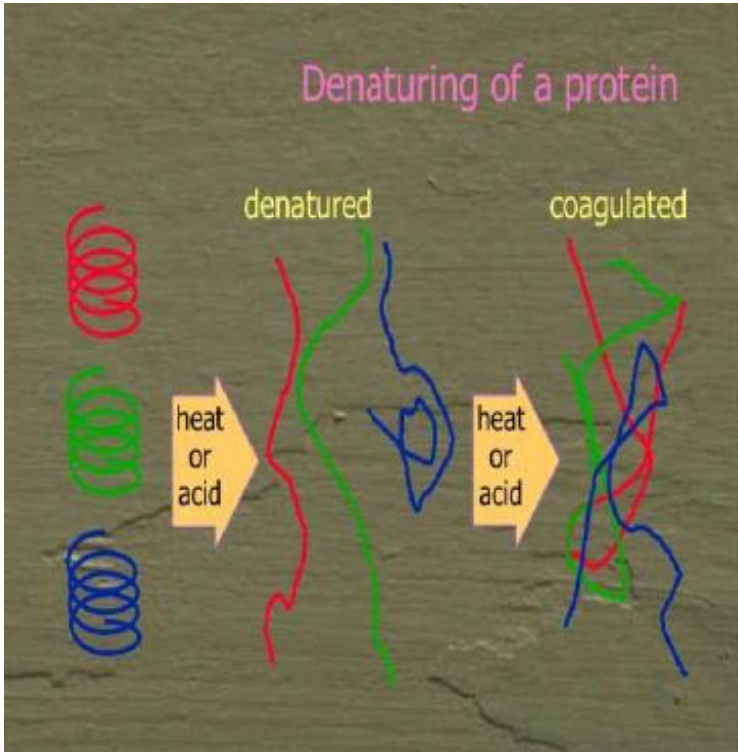
Conformational modifications of a lipase enable the access of hydrophobic substrates to the active site

Open in hydrophobic media; close in water

Kinetic stability

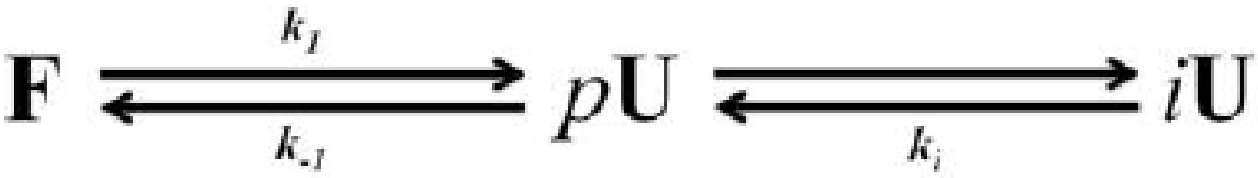
Most mesophilic proteins undergo irreversible unfolding upon extreme changes of the environment. They unfold into inactive structures (scrambled structures), and they often form aggregates (intermolecular mechanism).

During aggregation, the hydrophobic residues that are normally buried in the native protein become exposed to the solvent and interact with hydrophobic residues from other unfolding protein molecules to minimize their exposure to the solvent.



Kinetic stability

b) Irreversible folding process : kinetic stability



- **Protein initially undergoes partial unfolding**
- **Then it undergoes INACTIVATION, virtually irreversible**

Main physical factors affecting protein stability

- T
- pH
- Ionic species
- Oxidative metals
- Solvents
- Dehydration
- Surfactants
- Cold
- Pressure
- Mixing
- Interfaces
- Shear forces
- Denaturing agents (urea)

Effect of temperature on enzymatic reactions

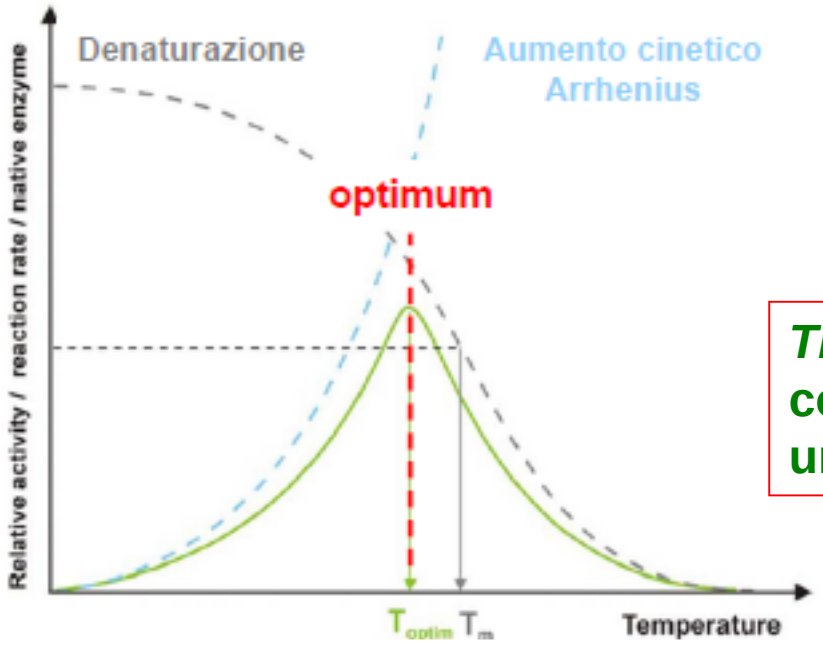
$$k = A \cdot e^{-E/RT}$$

Reaction rate generally increases with temperature
Rate increases by a factor of 1.2-2.5 for a 10°C increase

At the same time structure of enzyme undergoes conformational changes that might lead to unfoding and decrease of activity

Temperature also affects the reactivity of functional groups on the protein

e.g. pKa



***Tm*, temperature corresponding to 50% of unfolded protein**

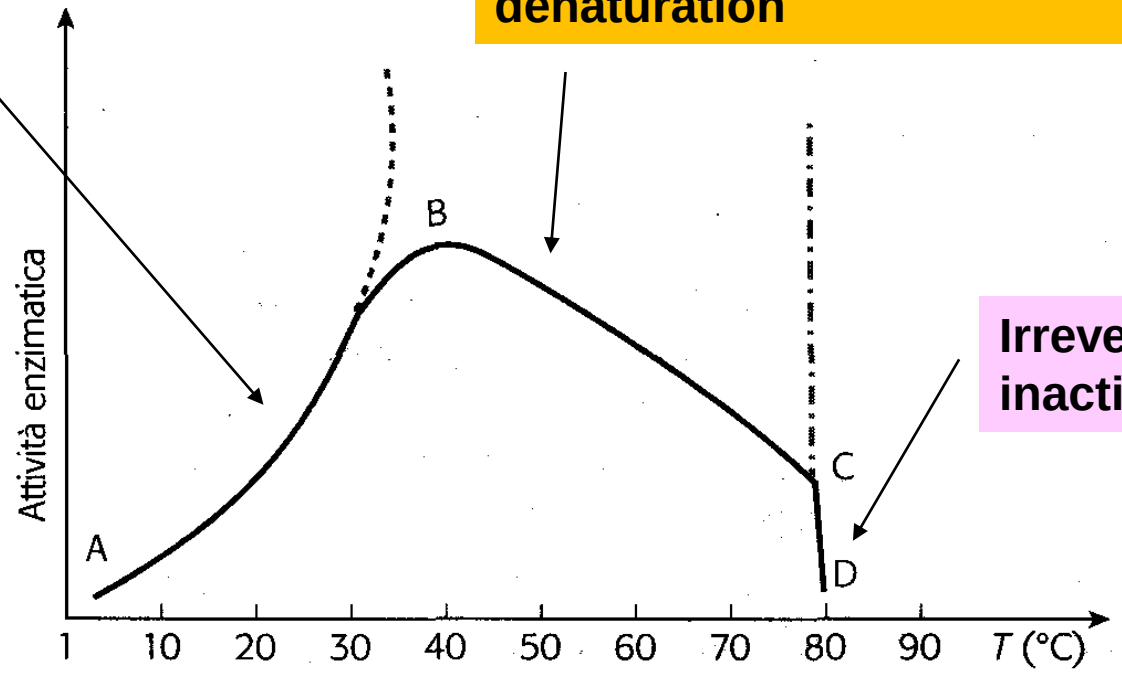
Different enzymatic reactions carried out at different temperatures. Reaction rates are used to build up the profile

Effect of temperature on an enzymatic reaction

Increase due to Arrhenius effect

combined effect of Arrhenius and denaturation

Irreversible inactivation.

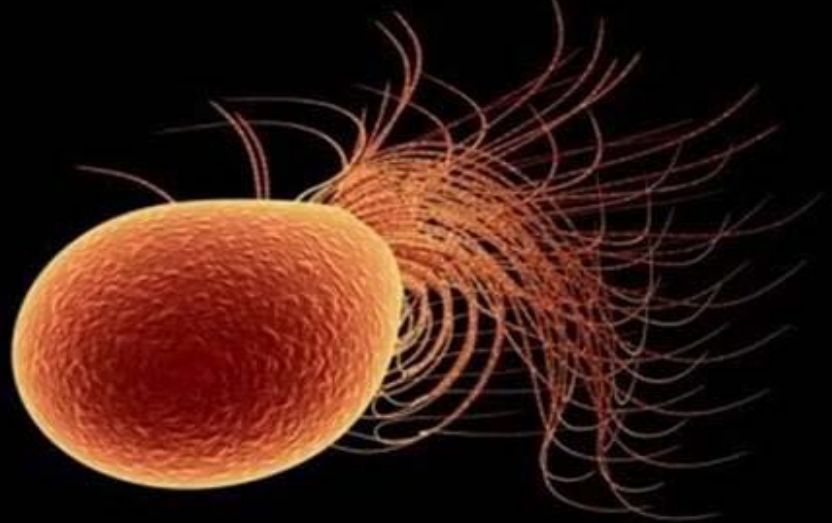


Thermostable enzymes from:

- Thermophilic organisms (60-80°C)

- Hyperthermophilic organisms (>80°C)

Pirococcus furiosus



Isolated on Vulcano island In 1986

(Fire ball)

- **Grows at T > 100°C**
- **Contains tungsten**
- **Duplicates very fast (35 min)**
- **Is active at pH 5 - 9**

Thermophilic enzymes:

Thermophilic organisms grow optimally between 50 and 80°C.
Their enzymes show thermostability properties which fall between those of hyperthermophilic and mesophilic enzymes.
Thermophilic enzymes typically do not function well below 40°C.

https://www.nps.gov/yell/learn/nature/thermobacteria.htm

Thermophilic Bacteria in Yellowstone National Park

Name	pH and Temperature	Description	Location
Cyanobacteria *Calothrix*	pH 6–9 30–45°C (86–113°F)	Color: dark brown mats Metabolism: photosynthesis by day; fermentation by night	•Mammoth Hot Springs •Upper, Midway, and Lower geyser basins
Phormidium	pH 6–8 35–57°C (95–135°F)	Color: orange mats Metabolism: photosynthesis	•Mammoth Hot Springs •Upper, Midway, and Lower geyser basins
Oscillatoria	pH 6–8 36–45°C (96–113°F)	Color: orange mats Metabolism: photosynthesis; oscillating moves it closer to light sources.	•Mammoth Hot Springs •Chocolate Pots
Synechococcus	pH 7–9 52–74°C (126–165°F)	Color: green mats Metabolism: photosynthesis by day; fermentation by night	•Mammoth Hot Springs •Upper, Midway, and Lower geyser basins
Green Sulfur *Chlorobium*	pH 6–9 32–52°C (90–126°F)	Color: dense, dark green mats Metabolism: anaerobic photosynthesis—produces sulfate and sulfur, not oxygen.	•Mammoth Hot springs •Calcite Springs
Green non-sulfur *Chloroflexus*	pH 7–9 35–85°C (95–185°F)	Color: green mats Metabolism: anaerobic photosynthesis	•Mammoth Hot Springs •Upper, Midway, and Lower geyser basins
Aquifex *Hydrogenobaculum*	pH 3–5.5 55–72°C (131–162°F)	Color: yellow and white streamers Metabolism: uses hydrogen, hydrogen sulfide and carbon dioxide as energy sources; can use arsenic in place of hydrogen sulfide.	•Norris Geyser Basin •Amphitheater Springs
Deinococcus-Thermus *Thermus*	pH 5–9 40–79°C (104–174°F)	Color: bright red or orange streamers; contains carotenoid pigments that act as sunscreen.	•Lower Geyser Basin

Hyperthermophilic enzymes:

-unique structure-function properties of high thermostability and optimal activity at temperatures above 70°C. Some of these enzymes are active at temperatures as high as 110°C and above.

Do not function well below 40°C.
Current theory suggests that hyperthermophiles were the first life-forms to have arisen on Earth.

Hyperthermophilic enzymes can serve as model systems for understanding enzyme evolution, molecular mechanisms for protein thermostability, and the upper temperature limit for enzyme function.

This knowledge can lead to the development of more efficient protein engineering strategies and a wide range of biotechnological applications.

Solfatara: Pozzuoli

Sulfolobus solfataricus **was first isolated in the Solfatara volcano. Other species can be found throughout the world in areas of volcanic or geothermal activity, such as geological formations called mud pots which are also known as *solfatare***

Consiglio Nazionale delle Ricerche

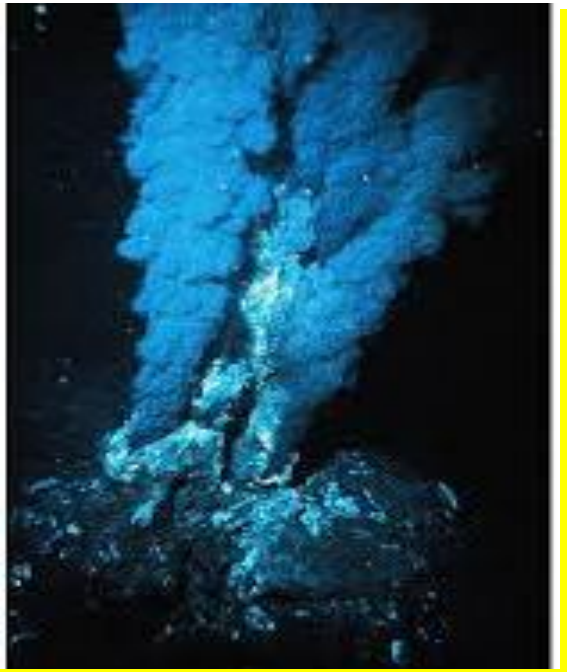
Hyperthermophiles microorganisms
have been isolated almost exclusively from environments with temperatures in the range of 80 to 115°C. Hot natural environments include continental solfataras, deep geothermally heated oil-containing stratifications, shallow marine and deep-sea hot sediments, and hydrothermal vents located as far as 4,000 m below sea level .

Hyperthermophiles have also been isolated from hot industrial environments (e.g., the outflow of geothermal power plants and sewage sludge systems). Deep-sea hyperthermophiles thrive in environments with hydrostatic pressures ranging from 200 to 360 atm.




Some of these species are barotolerant or even barophilic.

The most thermophilic organism known, ***Pyrolobus fumarii***, grows in the T range of 90 to 113°C.



Discovered in 1997 in a black smoker hydrothermal black smoker vent at the Mid Atlantic Ridge

T at which life is possible is probably not much above 113°C.

Above 110°C, amino acids and metabolites become highly unstable (ATP is spontaneously hydrolyzed in aqueous solution at T>140°C) and hydrophobic interactions weaken significantly.

Hyperthermophilic and mesophilic enzymes are highly similar: (i) the sequences of homologous hyperthermophilic and mesophilic proteins are typically 40 to 85% similar; (ii) their three-dimensional structures are superposable ; and (iii) they have the same catalytic mechanisms

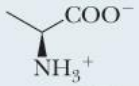
TABLE 4. Relative amino acid compositions of mesophilic and hyperthermophilic proteins[a]

Residue(s)	Amino acid composition (%) of:		Variation of composition in hyperthermophilic relative to mesophilic proteins	
	Mesophilic proteins[b]	Hyperthermophilic proteins[c]		
A	8.09 ± 1.54	6.82 ± 1.42	−1.27	
C	1.10 ± 0.18	0.86 ± 0.27	−0.24	
D	5.06 ± 0.18	4.63 ± 0.54	−0.43	
E	6.45 ± 0.54	8.55 ± 0.95	+2.10	← Glu
F	4.61 ± 0.78	4.40 ± 0.82	−0.21	
G	6.70 ± 0.96	7.16 ± 0.68	+0.46	
H	2.04 ± 0.21	1.57 ± 0.16	−0.47	
I	7.40 ± 1.69	7.82 ± 1.64	+0.42	
K	6.81 ± 2.00	7.61 ± 2.16	+0.80	
L	10.43 ± 0.55	10.21 ± 0.68	−0.22	
M	2.42 ± 0.28	2.29 ± 0.25	−0.13	
N	4.90 ± 1.20	3.52 ± 0.94	−1.38	
P	3.77 ± 0.77	4.36 ± 0.99	+0.59	
Q	3.99 ± 0.75	1.78 ± 0.22	−2.21	
R	4.33 ± 0.98	5.57 ± 1.16	+1.24	← Arg
S	6.08 ± 0.57	5.54 ± 1.01	−0.54	
T	5.09 ± 0.57	4.34 ± 0.23	−0.75	
V	6.35 ± 0.75	8.05 ± 0.68	+1.70	
W	1.02 ± 0.31	1.06 ± 0.20	+0.04	
Y	3.30 ± 0.43	3.82 ± 0.33	+0.52	
A, G	14.79	13.98	−0.81	
D, E	11.51	13.18	+1.67	
K, R, H	13.18	14.75	+1.57	
S, T	11.17	9.88	−1.29	
N, Q	8.99	5.3	−3.69	Asn, Gln
I, L, M, V	26.60	28.37	+1.77	
F, W, Y	8.93	9.28	+0.35	

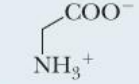
- **Glu and Arg promote the formation of salt bridges (increased stability)**
- **Asn and Gln are prone to covalent modification and degradation**

Tabella 27.1 I 20 amminoacidi comuni che si trovano nelle proteine

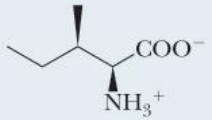
Catene laterali non polari



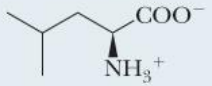
Alanina (Ala, A)



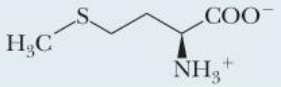
Glicina (Gly, G)



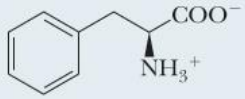
Isoleucina (Ile, I)



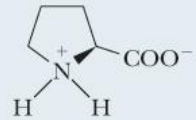
Leucina (Leu, L)



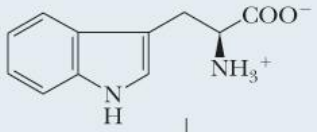
Metionina (Met, M)



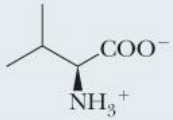
Fenilalanina (Phe, F)



Prolina (Pro, P)

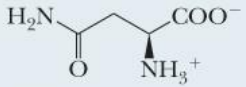


Triptofano (Trp, W)

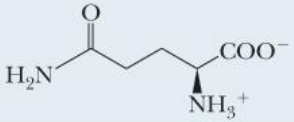


Valina (Val, V)

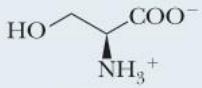
Catene laterali polari



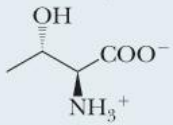
Asparagina (Asn, N)



Glutammina (Gln, Q)

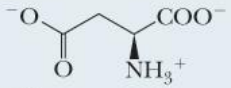


Serina (Ser, S)

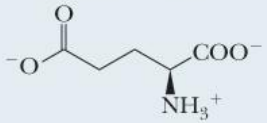


Treonina (Thr, T)

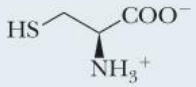
Catene laterali acide



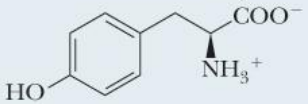
Acido aspartico (Asp, D)



Acido glutammico (Glu, E)

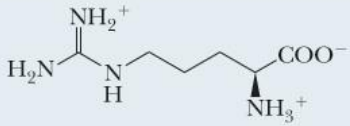


Cisteina (Cys, C)

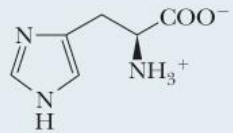


Tirosina (Tyr, Y)

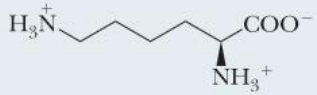
Catene laterali basiche



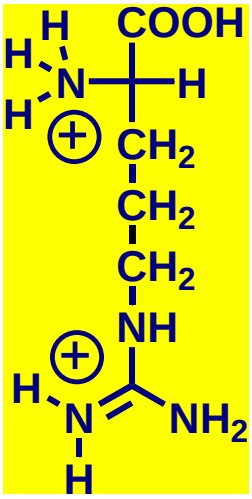
Arginina (Arg, R)



Istidina (His, H)



Lisina (Lys, K)



Role of Arg in stabilization of protein

Several properties of Arg residues suggest that they would be better adapted to high temperatures than Lys residues: the Arg -guanido moiety has a reduced chemical reactivity due to its high pKa and its resonance stabilization.

The guanido moiety provides more surface area for charged interactions than the Lys amino group does.

Last, because its side chain pKa (approximately 13) is more than 1 unit above that of Lys (11.1), Arg more easily maintains ion pairs and a net positive charge at elevated temperatures (pKa values drop as the temperature increases).

Occurence of of ionic bridges in hyperthermophilic enzymes

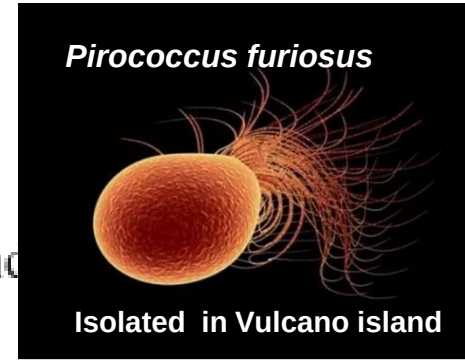


TABLE 6. Comparison of the ion pair contents of *P. furiosus* and *Clostridium symbiosum* GDHs[a]

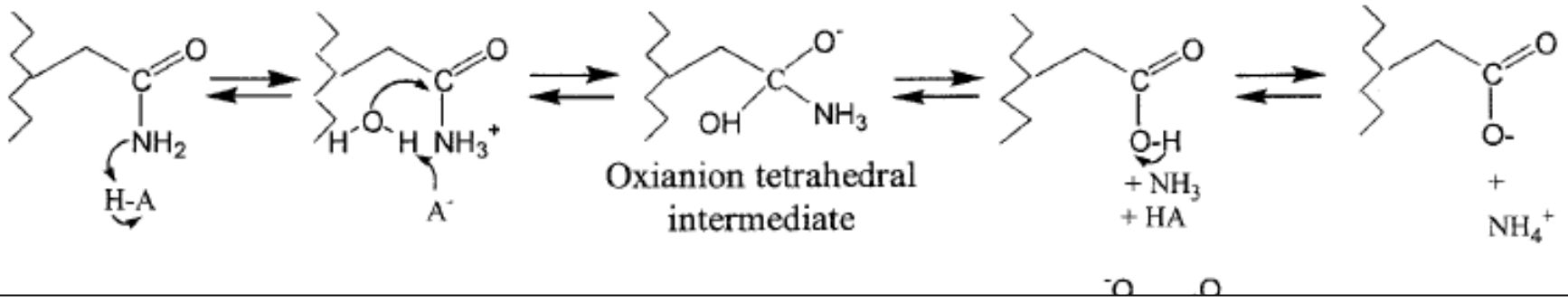
Characteristic (glutammato deidrogenasi)	Value for: *C. symbiosum*	*P. furiosus*
No. of IPs[b] per subunit	26	45
No. of IPs per residue	0.06	0.11
% of charged residues forming IPs	40	54
% of IPs formed by Arg/Lys/His	46/31/23	64/27/9
% of IPs formed by Asp/Glu	46/54	47/53
% of all Arg forming IPs	55	90
No. of residues forming 2 IPs	6	17
No. of residues forming 3 IPs	1	5
No. of 2/3/4-residue networks[c]	72/24/12	54/24/12
No. of 5/6/18-residue network[c]	0/0/0	12/6/3
% of IPs in networks of >3 residues[c]	23	62
No. of intersubunit IPs[c]	30	54
No. of interdomain IPs	1	7

[a] Adapted from reference 368 with permission of the publisher.
[b] IP, ion pair.

Irreversible covalent modifications causing enzyme inactivation

Deamidation of Asn and Gln: covalent irreversible inactivation

A. Deamidation by the general acid-base mechanism



In the general acid-base mechanism, a general acid (HA) protonates the Asn (or Gln) amido group. A general base (A2 or OH2) attacks the carbonyl carbon of the amido group or activates another nucleophile . The transition state is supposed to be an oxyanion tetrahedral intermediate.

Inactivation of enzymes by proteolitic attack and hydrolysis of peptide bond

A current working hypothesis is that hyperthermophilic enzymes are more rigid than their mesophilic homologues at mesophilic temperatures and that rigidity is a prerequisite for high protein thermostability.

Rigidity is also corrrelated to higher stability to proteolitic attack

Table 1
Specificity of proteases[a]

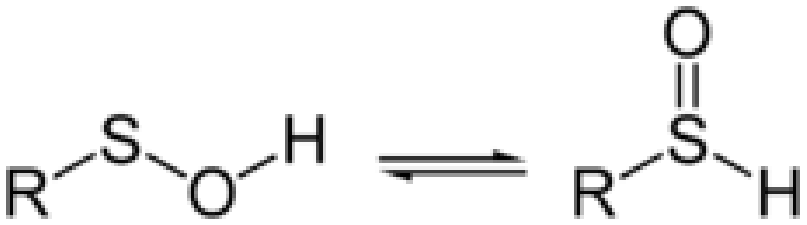
Enzyme	Preferred cleavage site[b] N-terminal	C-terminal
Serine proteases		
Trypsin	–Arg (or Lys)–	↓ Yaa–
Achromobacter protease	–Lys–	↓ Yaa–
Chymotrypsin, subtilisin	–Trp (or Tyr, Phe, Leu)–	↓ Yaa–
Elastase, α-lytic protease	–Ala (or Ser)–	↓ Yaa–
Proline-specific protease	–Pro–	↓ Yaa–
Staphylococcus V8 protease	–Asp (or Glu)–	↓ Yaa–
Carboxypeptidase Y	–Xaa–	↓ Yaa–
Thiol proteases		
Papain, *Streptococcus* protease	–Phe (or Val, Leu)-Xaa–	↓ Yaa–
Clostripain, cathepsin B	–Arg–	↓ Yaa–
Cathepsin C	H-X-Phe (or Tyr, Arg)–	↓ Yaa–
Metal proteases		
Thermolysin	–Xaa–	↓ Leu (or Phe)–
Myxobacter protease II	–Xaa–	↓ Lys–
Aspartic proteases		
Pepsin	–Phe (or Tyr, Leu)–	↓ Trp (or Phe, Tyr)–

[a]Data from Ref. 3.
[b]Xaa, various amino acid residues; Yaa, various amino acid residues, ester or amide.

Protases must be stable against proteolitic attack!

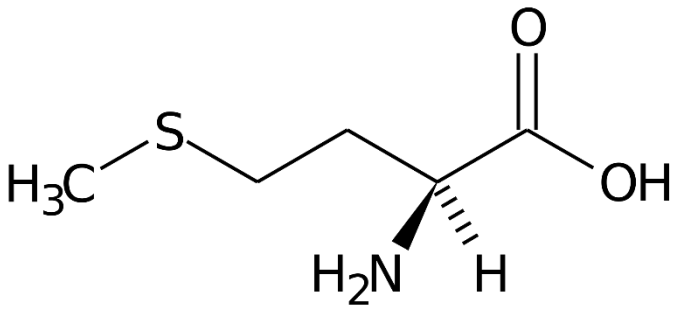
Cysteine oxidation.

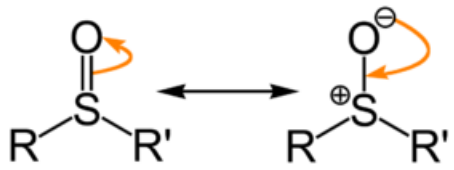
Cysteines are the most reactive amino acids in proteins. Their autooxidation, usually catalyzed by metal cations (especially copper), leads to the formation of intramolecular and intermolecular disulfide bridges or to the formation of sulfenic acid .



Sulfenic acids exhibit tautomerism, with the structure on the left predominating
Sulfenic acids are generally unstable

Methionine can be oxidized to sulfoxide





Sulfoxide

High ΔG values for inactivation of hyperthermophilic enzymes (above 100 kcal/mol) suggest that the limiting step in their inactivation is still unfolding.

These different observations suggest that chemical modifications (e.g., deamidation, cysteine oxidation, and peptide bond hydrolysis) take place only once the protein is unfolded.

chemical modifications make denaturation irreversible.

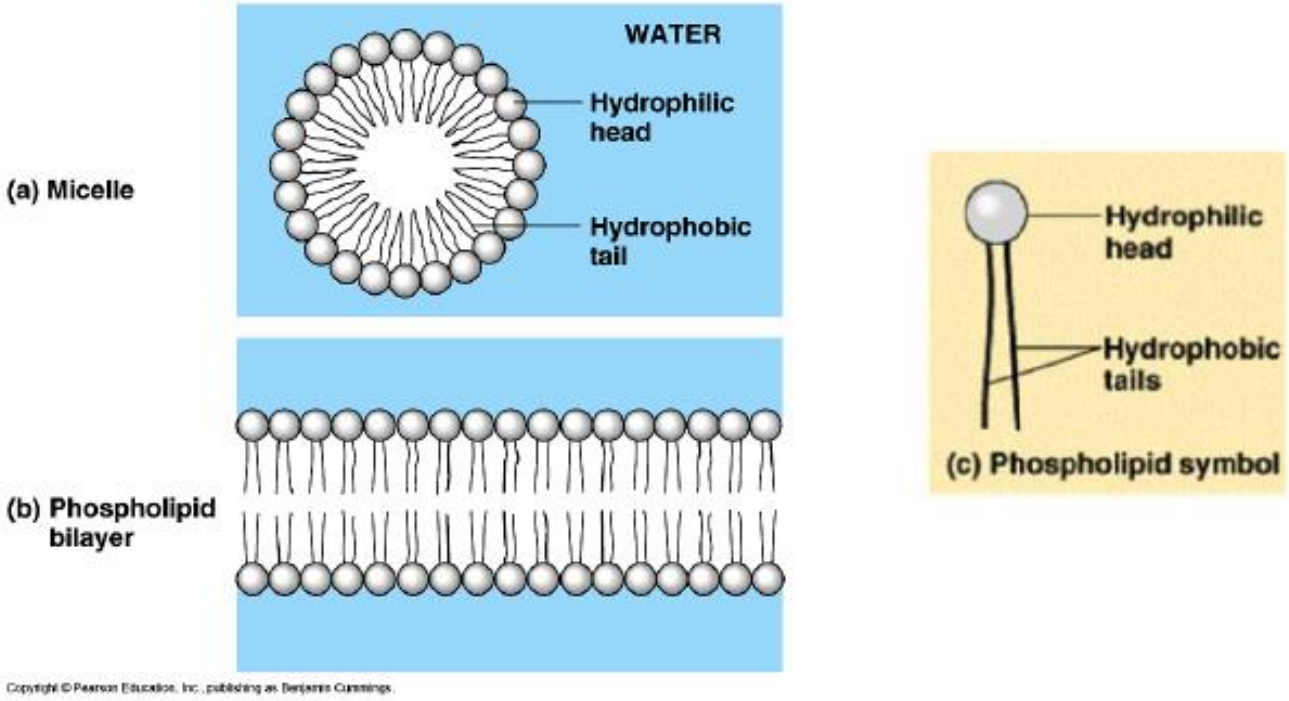
b) Irreversible folding process : kinetic stability

$$\mathbf{F} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} p\mathbf{U} \underset{k_i}{\rightleftharpoons} i\mathbf{U}$$

Other physical factors affecting protein stability

- T
- **pH**
- **Ionic species**
- **Oxidative metals**
- **Solvents**
- **Dehydration**
- **Surfactants**
- **Cold** (***e.g. freezing and thawing***)
- **Pressure** (Under higher pressure, proteins take up water into empty cavities)
- **Mixing**
- **Interfaces** (adsorption of proteins: whipped egg white)
- **Shear forces**
- **Denaturing agents (urea)**

Surfactants effect



Hydrophobic and hydrophilic portions of proteins are incorporated into micelles :
unfolding thermodynamically favoured

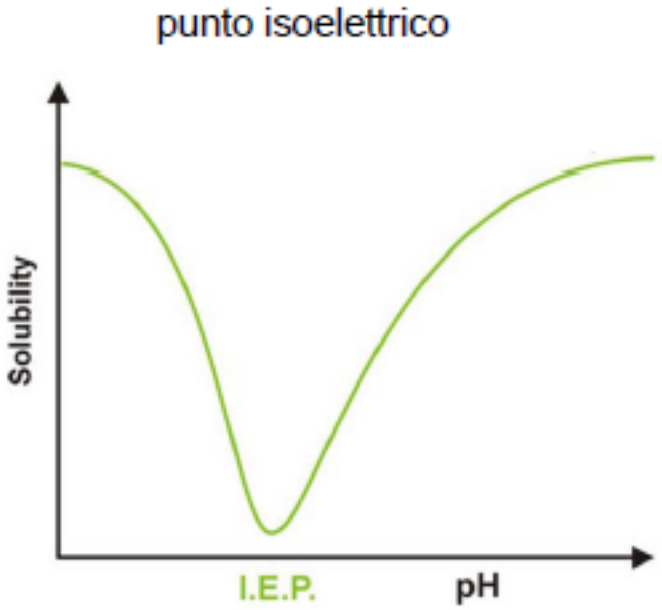
pH effect

On catalytic activity: protonation of side chains involved in catalysis

On conformation and stability (e.g. salt bridges determining quaternary structure of olygomers; denaturation, solubility-precipitation)

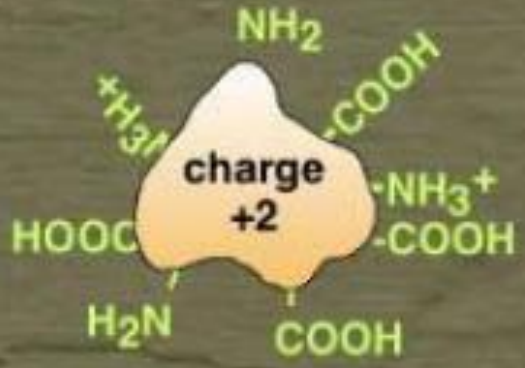
pK_a values of some amino acids

Amino acid	pK_a values (25°C) α-COOH group	α-NH_3^+ group	Side chain
Alanine	2.3	9.9	
Glycine	2.4	9.8	
Phenylalanine	1.8	9.1	
Serine	2.1	9.2	
Valine	2.3	9.6	
Aspartic acid	2.0	10.0	3.9
Glutamic acid	2.2	9.7	4.3
Histidine	1.8	9.2	6.0
Cysteine	1.8	10.8	8.3
Tyrosine	2.2	9.1	10.9
Lysine	2.2	9.2	10.8
Arginine	1.8	9.0	12.5

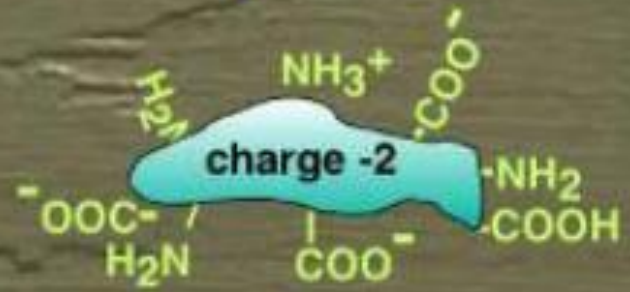


Effect of pH on proteins

lower pH

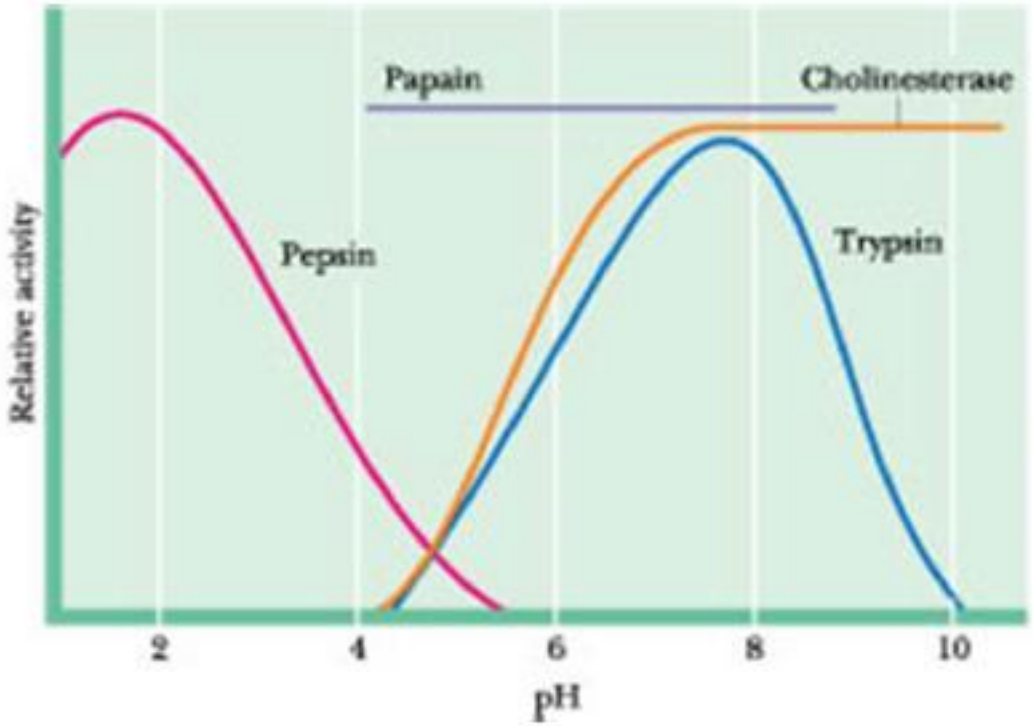


raise pH



Changing pH will alter charge on protein. This alters their solubility and may change their shape.

Different enzymes present different pH / activity profiles



Optimum pH of Some Enzymes	
Enzyme	Optimum pH
Pepsin	1.5
Catalase	7.6
Trypsin	7.7
Fumarase	7.8
Ribonuclease	7.8
Arginase	9.7