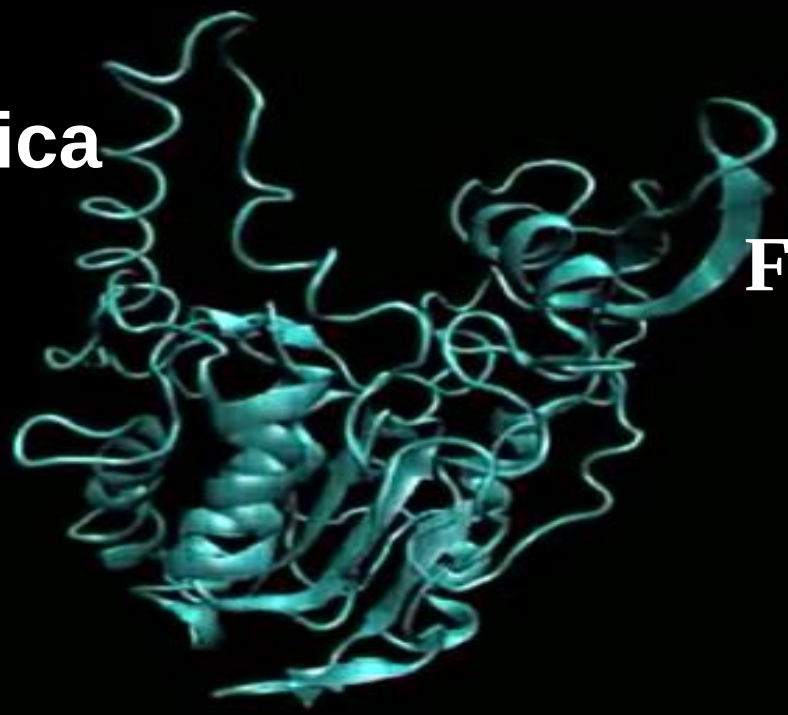
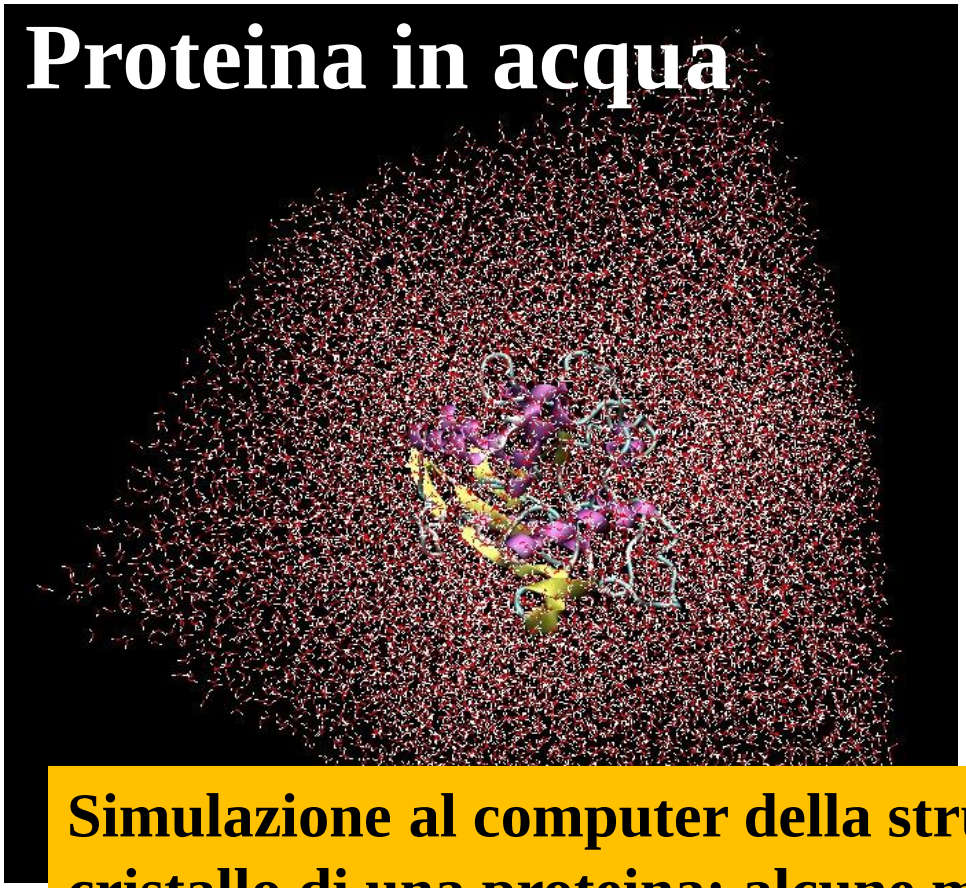
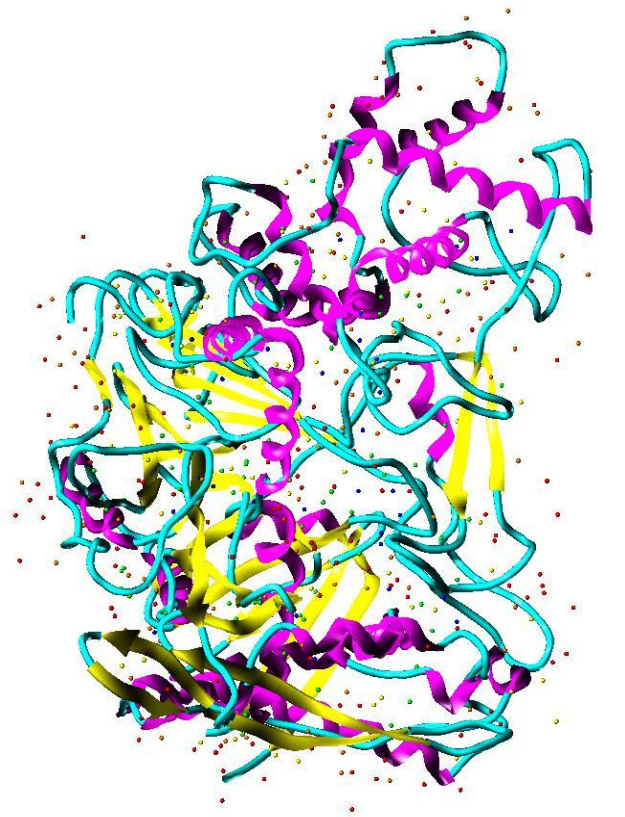
Struttura terziaria



Simulazione al computer della struttura tridimensionale di un cristallo di una proteina globulare (lipasi, un enzima)

La conformazione delle proteine dipende dall'ambiente esterno: la catena polipeptidica tende ad assumere la conformazione più stabile in funzione delle condizioni ambientali



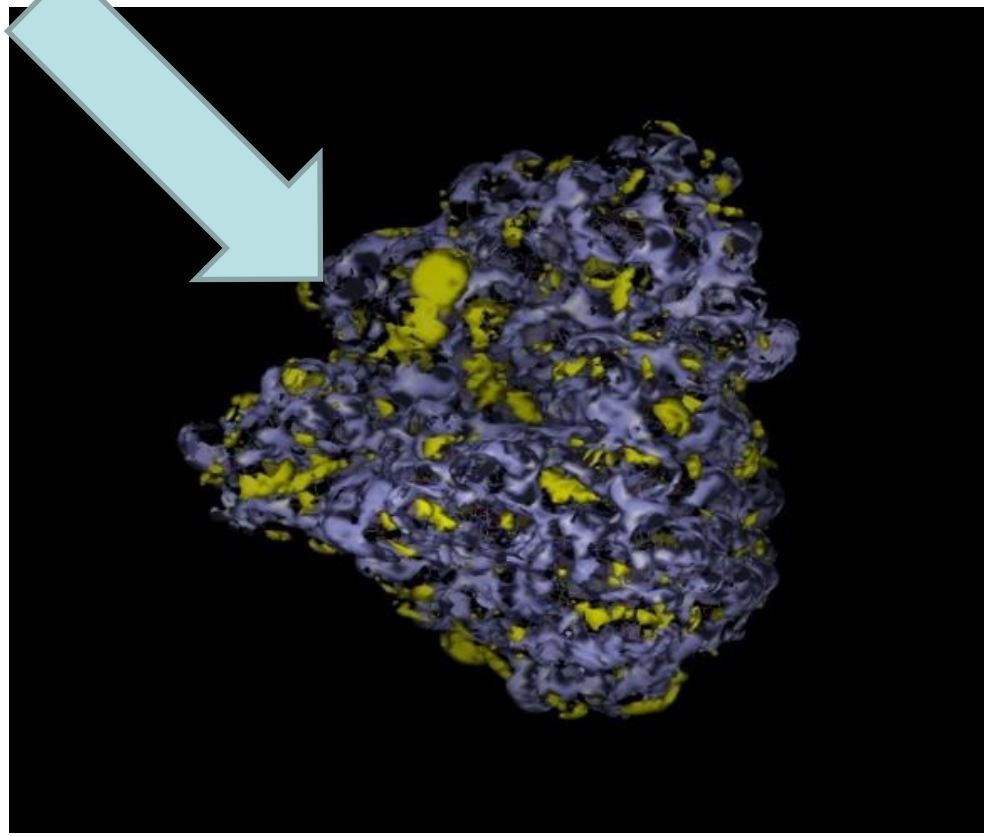


Simulazione al computer della struttura tridimensionale di un cristallo di una proteina: alcune molecole di acqua sono necessarie per il mantenimento della conformazione nativa

La superficie delle proteine globulari è idrofilica perché esposta all'ambiente acquoso mentre l'interno ed il sito attivo sono maggiormente idrofobici

Aree idrofiliche

Apertura del sito attivo

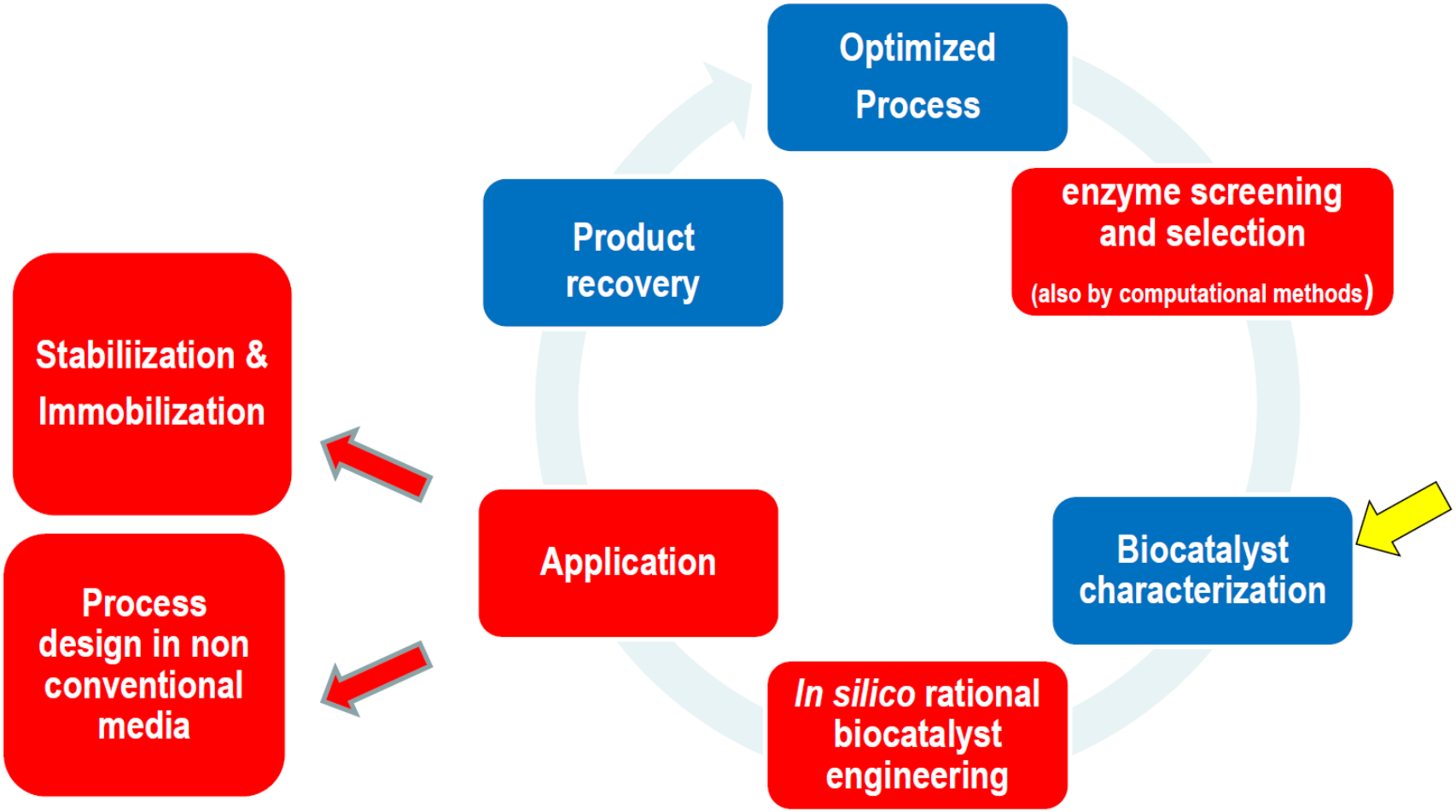


Penicillina G amidasi

Biocatalysts in chemical reactions and organic synthesis

Strategies for planning biocatalyzed reactions

Enzymes are characterized by enzymologists in diluted buffers, therefore in conditions quite distant from the operational conditions in industry



Most often biocatalysts are used under non phisiological conditions, the so called:

non conventional media

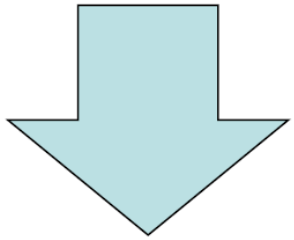
……anything different from a dilute aqueous solution.

*Most often a **multi-phase system**, some examples:*

Neat substrates + Native enzyme + Traces of solvent

Neat substrates + Immobilized enzyme

The point of view of kinetic studies in fundamental enzymology: enzyme solubilized in buffer



Most often models developed by enzymologists cannot be used in applied biocatalysis

Mass transfer and diffusion limitations are most often rate determining

Parameters and equation refer to simplified ideal conditions

Equazione di Michaelis – Menten (1913)

Leonor Michaelis
(1875–1949)

Maud Leonora Menten
(1879–1960)

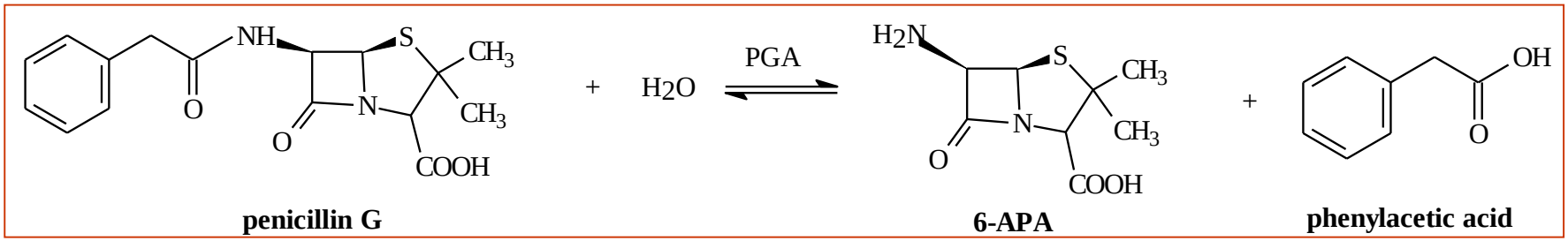
$$V = k_{cat}[E]_T \frac{[S]}{K_S + [S]}$$

oppure

$$v = V_{max} \frac{[S]}{K_M + [S]}$$

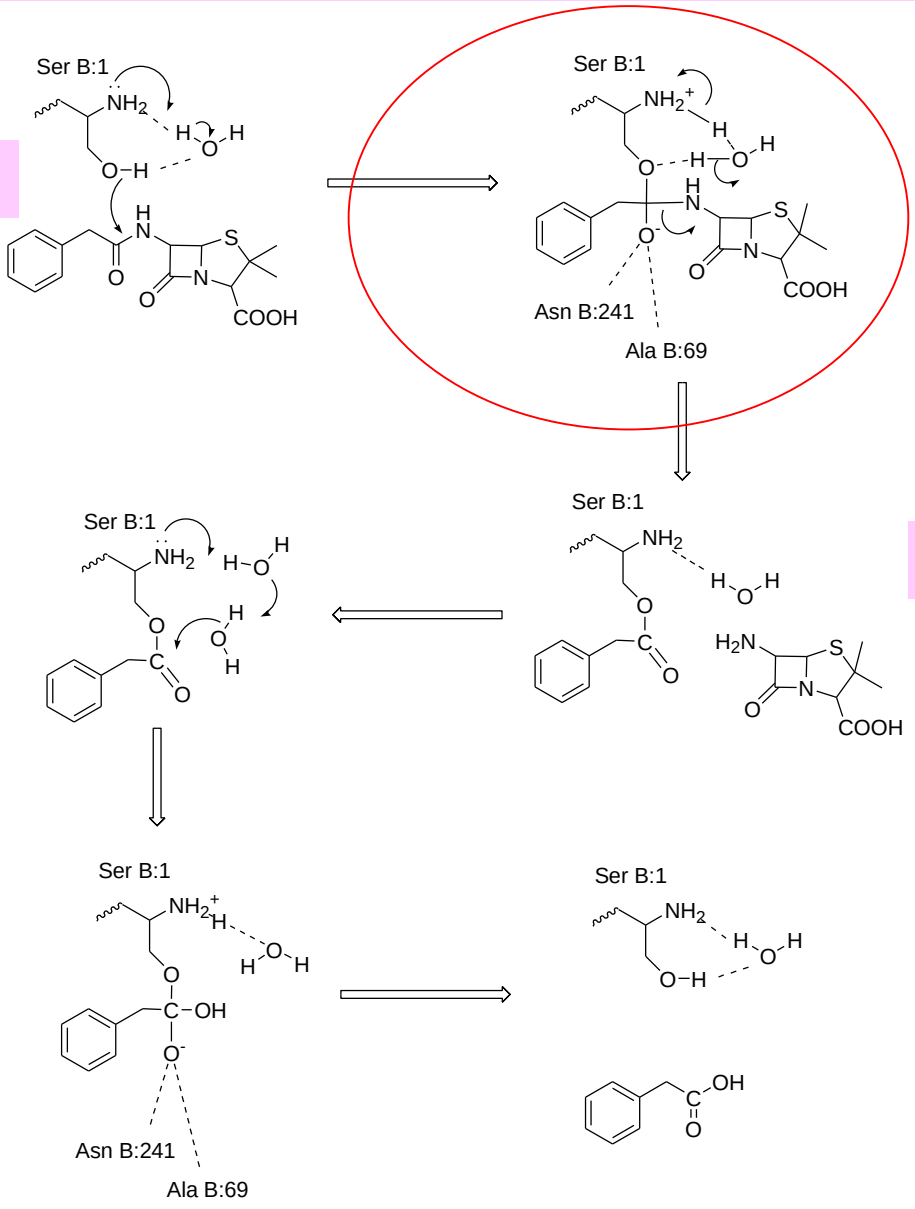
In most cases enzymes in biocatalysis are used because of their specificity and selectivity

Case study: hydrolysis of penicillin G to 6-amminopenicillanic acid, precursor of semisynthetic penicillins



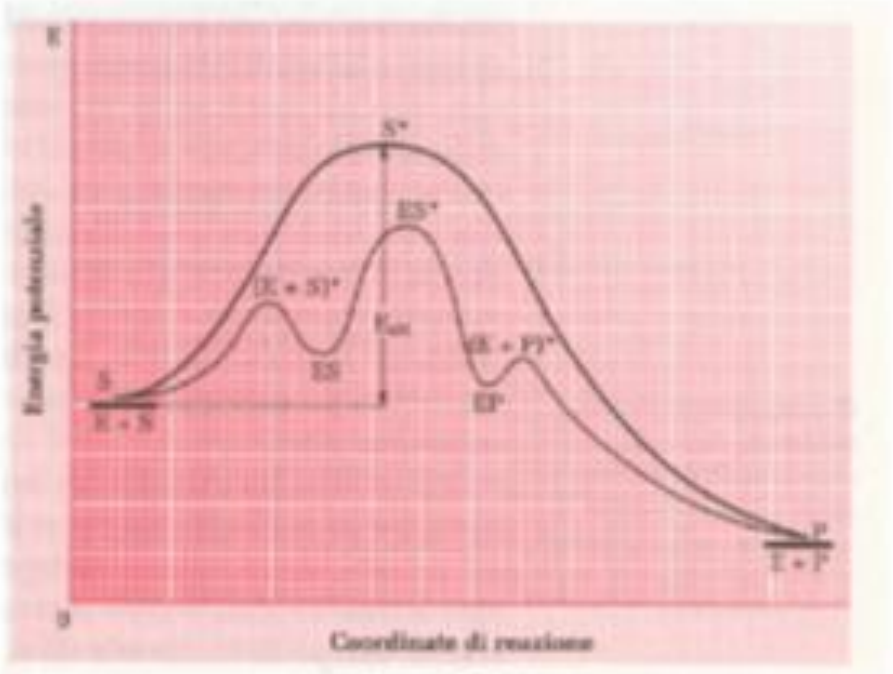
Mechanism of the hydrolysis of an amide bond catalyzed by peniccilin G amidase (PGA)

Serine hydrolase



Tegrahedral Intermediate

Acyl-enzyme



$E + S \rightleftharpoons ES \longrightarrow ES^* \longrightarrow EP \rightleftharpoons E + P$

Specific activity: k_{cat}

k_{cat} (time^{-1})

First order rate constant for the conversion of ES, ES*, EP

All species involving E

Specificity

k_{cat}/k_m specificity constant

Specific activity

affinity

K_{cat}/K_M must be calculated at low concentration of Substrate, when the enzyme is not saturated

Penicillin G amidase from different microorganisms display different specificity towards pen G

Enzima	Substrato	T(°C)	pH	k_{cat}/K_M ($M^{-1}s^{-1}$)
Penicillina amidasi				
E. coli	Penicillina G	25.0	7.8	4 800 000
A. faecalis	Penicillina G	25.0	7.8	10 000 000
K. citrofila	Penicillina G	25.0	7.8	3 000 000

Selectivity

k_{cat}/k_m specificity constant

$$\text{selectivity} \quad \frac{(k_{cat}/k_m)_A}{(k_{cat}/k_m)_B}$$

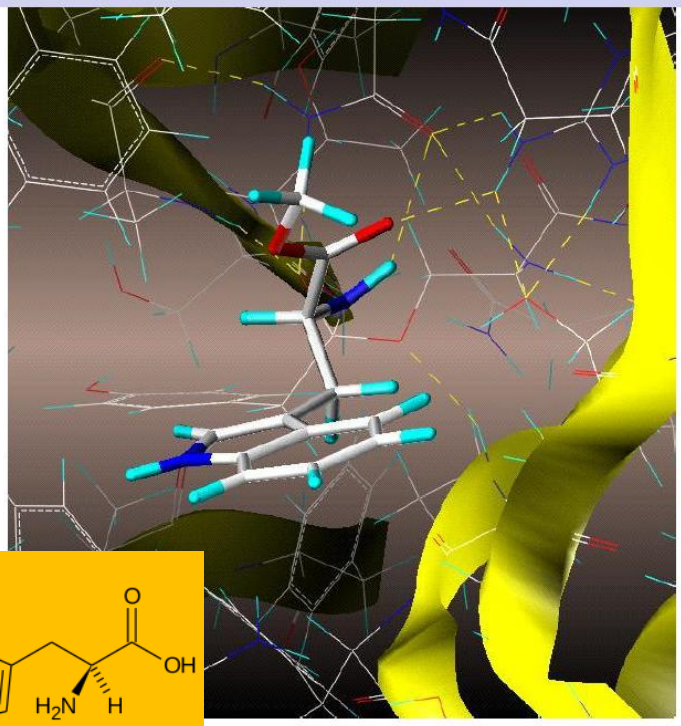
Ratio between the specificity constants

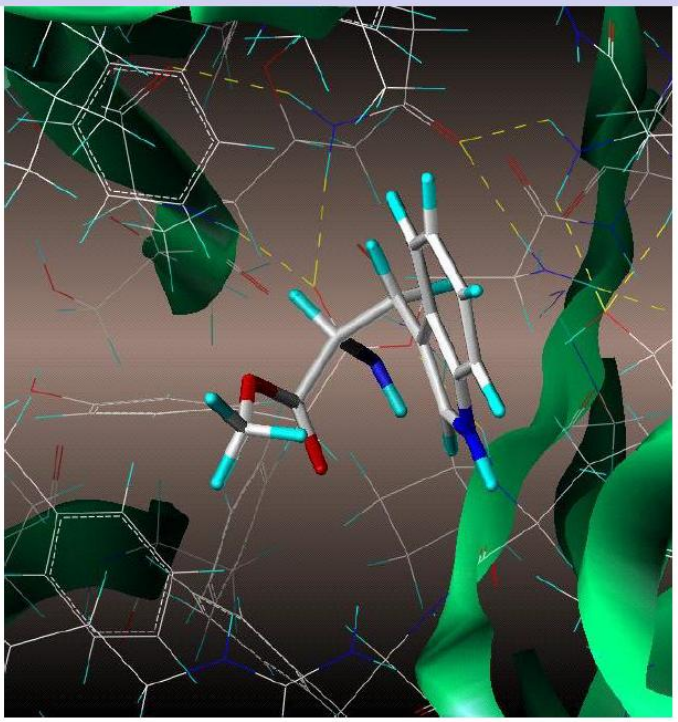
How the enzyme is able to discriminate between A and B

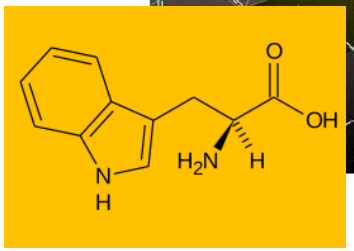
(chemo-regio-enantio-selectivity)

Enantio-selettività degli enzimi

I due enantiomeri vengono riconosciuti in maniera diversa: le interazioni con gli amminoacidi chirali del sito attivo determina diverse energie di legame







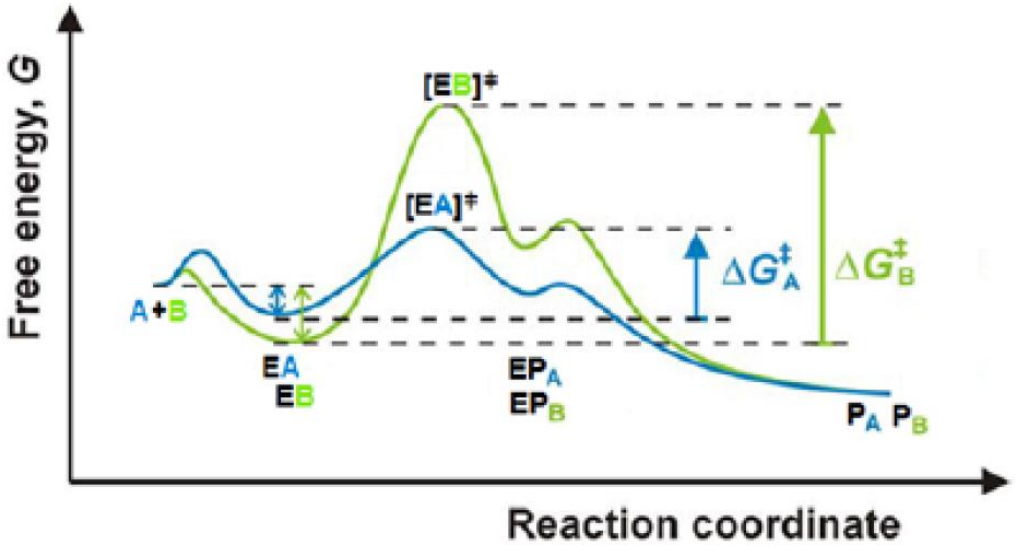
S-triptofano

R-triptofano

L'enantiomero S stabilisce interazioni stabilizzanti (legami H)

L'enzima catalizza la trasformazione solo dell'enantiomero S

Enzyme enantioselectivity: enantiomer S *vs* R



$\Delta\Delta G^{\neq}$ [kcal/mol]	V_S/V_R	e.e. [%]
0.118	1.2	10
0.651	3	50
1.74	19	90
2.17	39	95
3.14	199	99
4.50	1,999	99.9

What is really relevant in biocatalysis? Turnover number: biocatalyst productivity

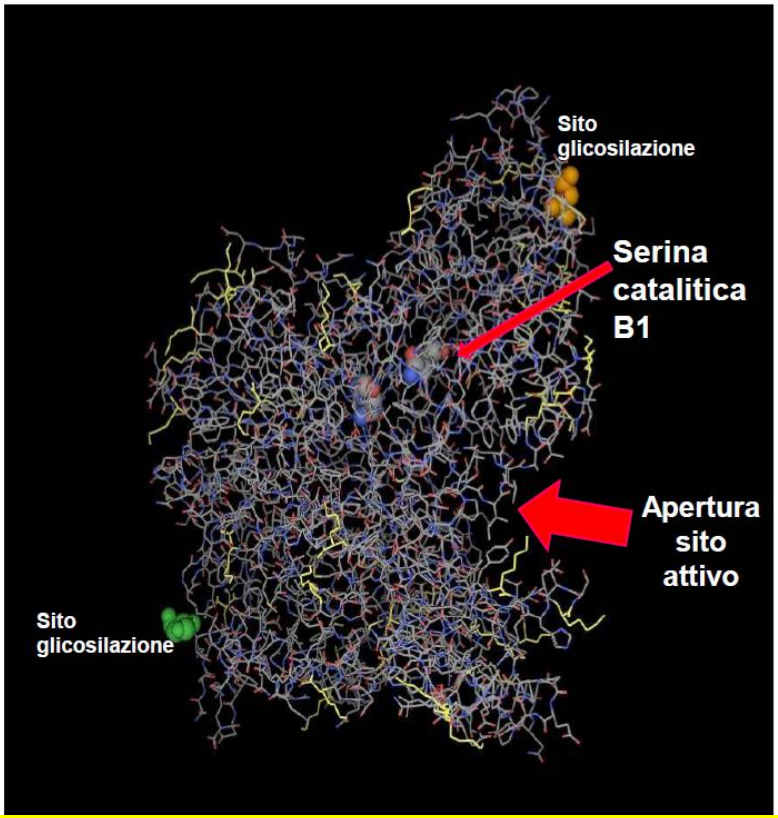
In catalysis it refers to:

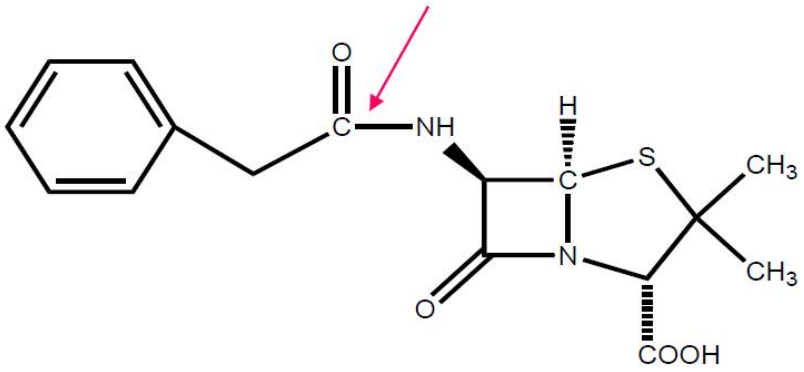
the number of moles of product formed per mole of catalyst over the reaction period.

dimensionless ratio:

(mol product) / (mol enzyme)

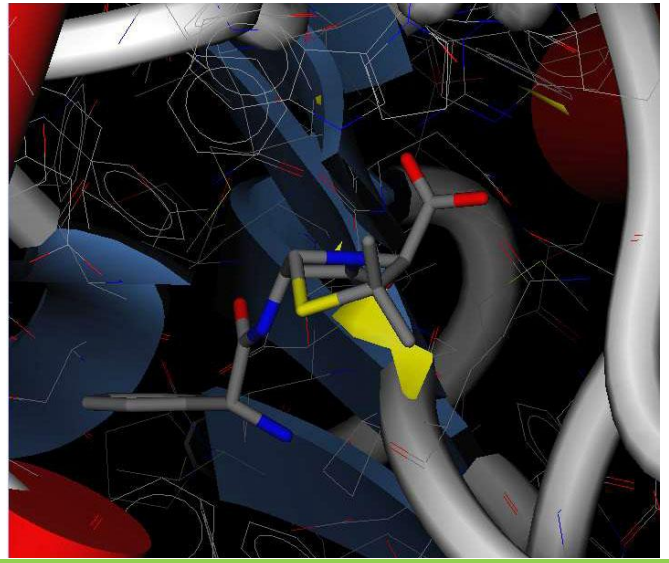
Molecular simulation for the study of enzyme-substrate recognition: penicillin G inside penicillin G amidase (PGA), a serine hydrolase (dimer)

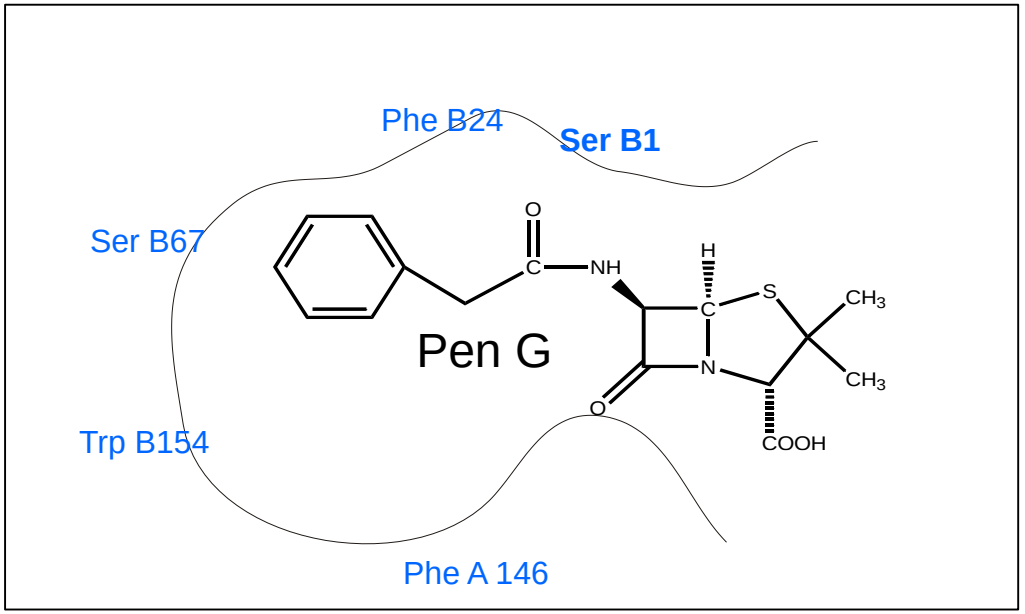




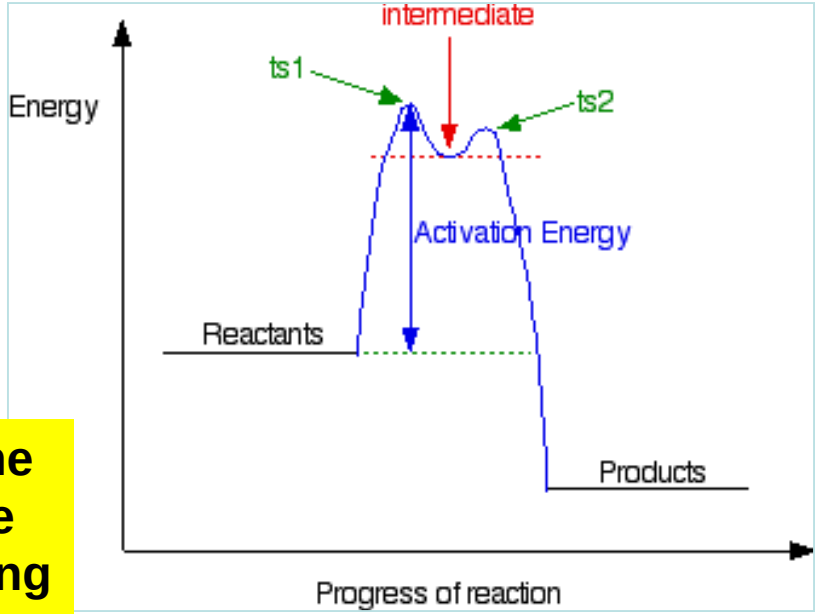
Penicillin G

Molecular models: a computational simulation of tridimentional structures



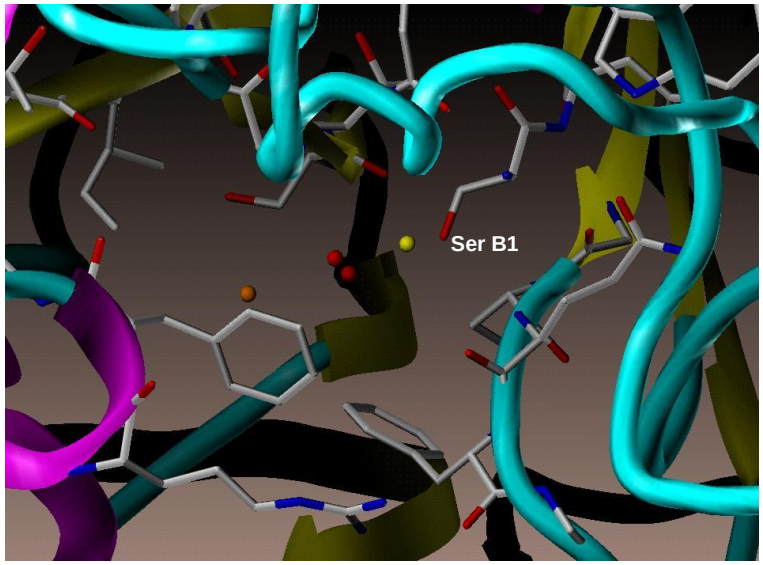


Docking of the ground state gives information only on enzyme-substrate recognition: affinity, Km



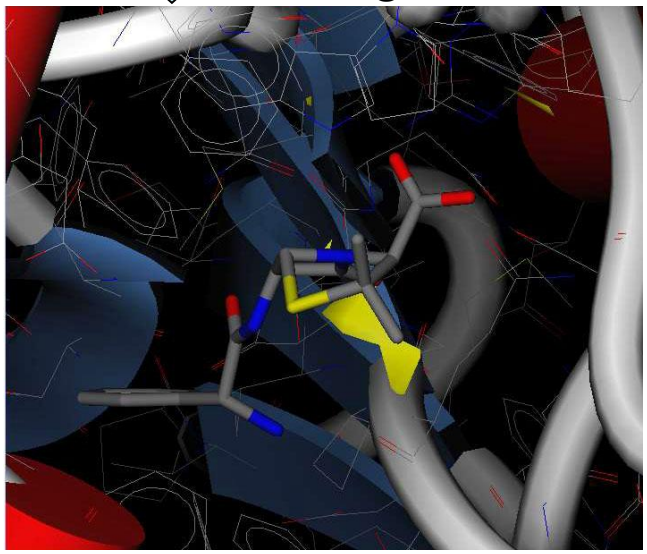
Information on Kcat derives from the study of energies associated to the transition state of the rate determining step of the reaction

Molecular modeling and docking can simulate enzyme-substrate interactions: computation of energies of interaction (enthalpy), affinity.

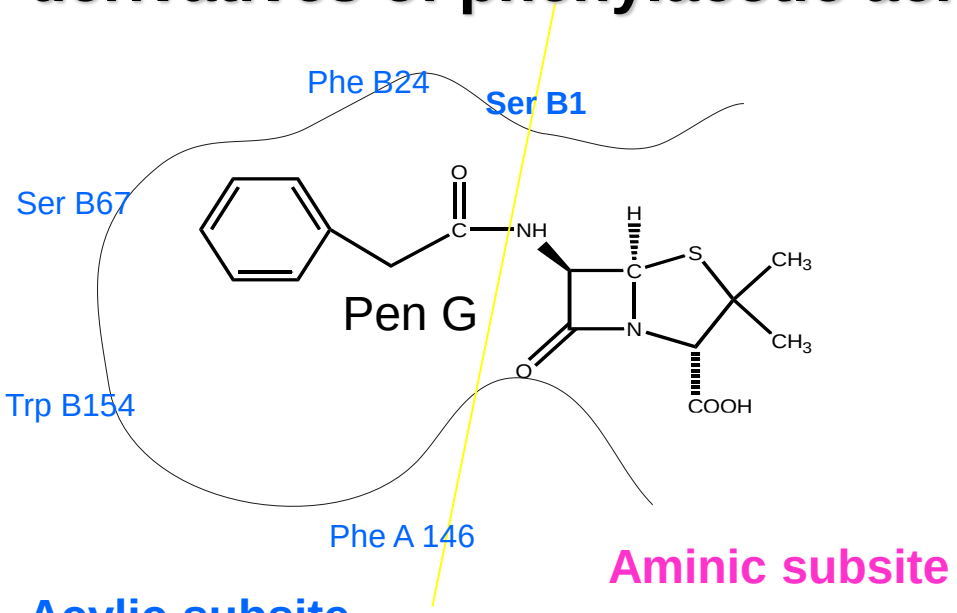


active site 3D structure with water molecules

Docking the substrate



Penicillin G Amidase (PGA) recognizes substrates that are derivatives of phenylacetic acid

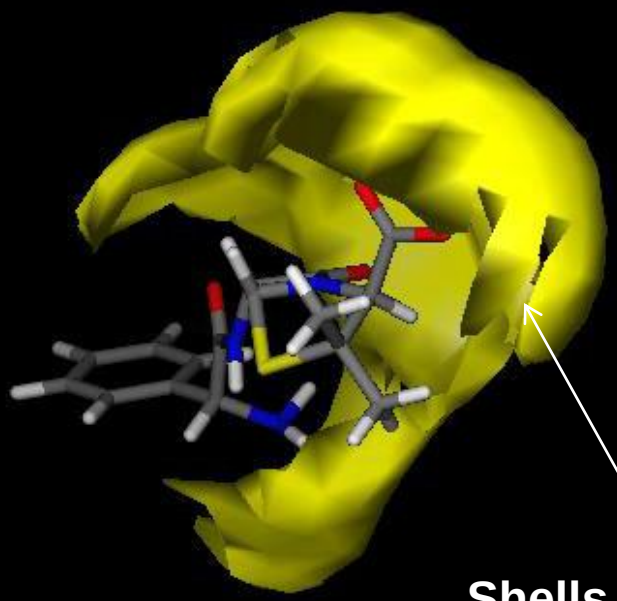


Docking simulates enzyme-substrate recognition: affinity, Km

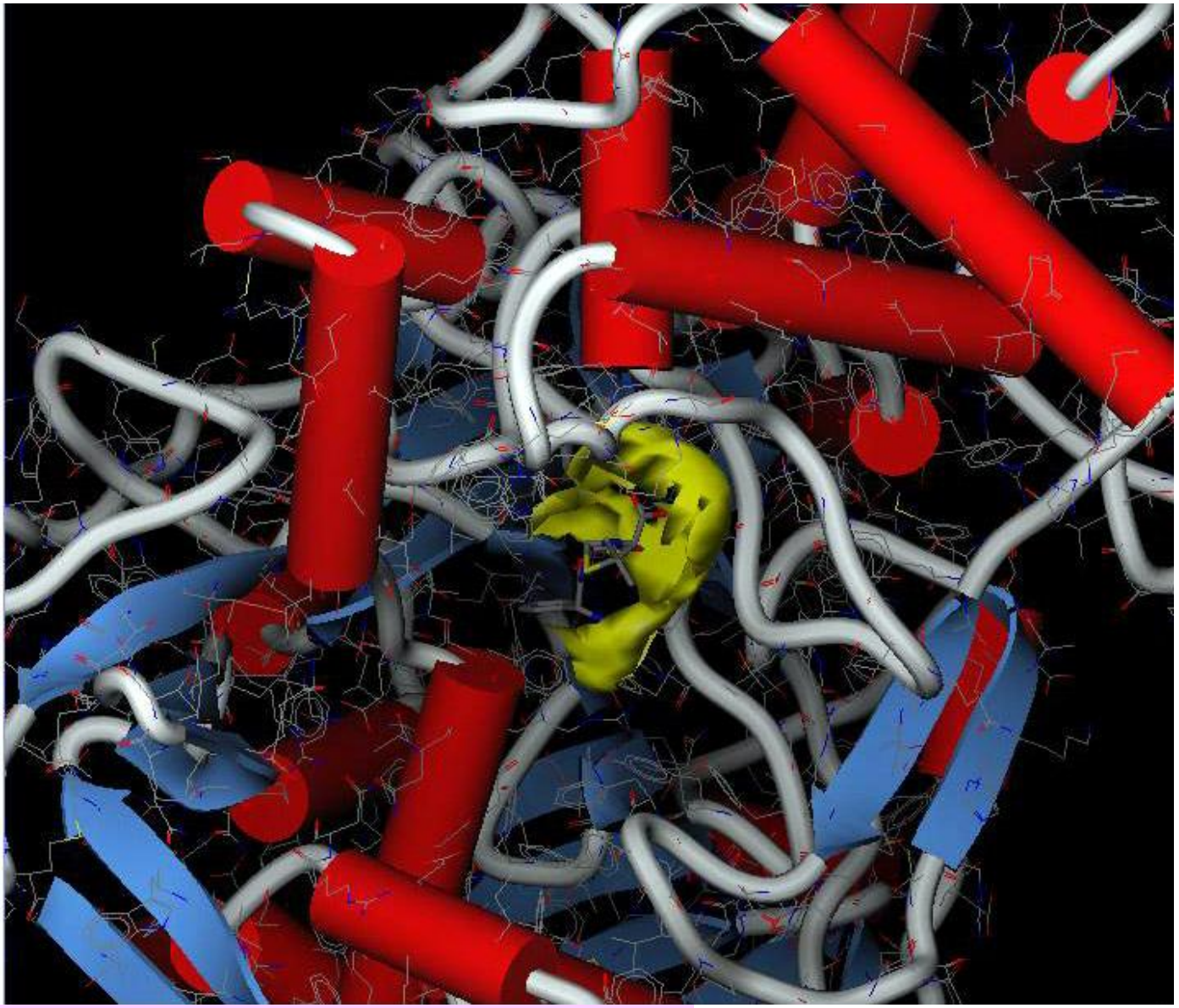
What is not accounted by docking? Solvation and desolvation

1.

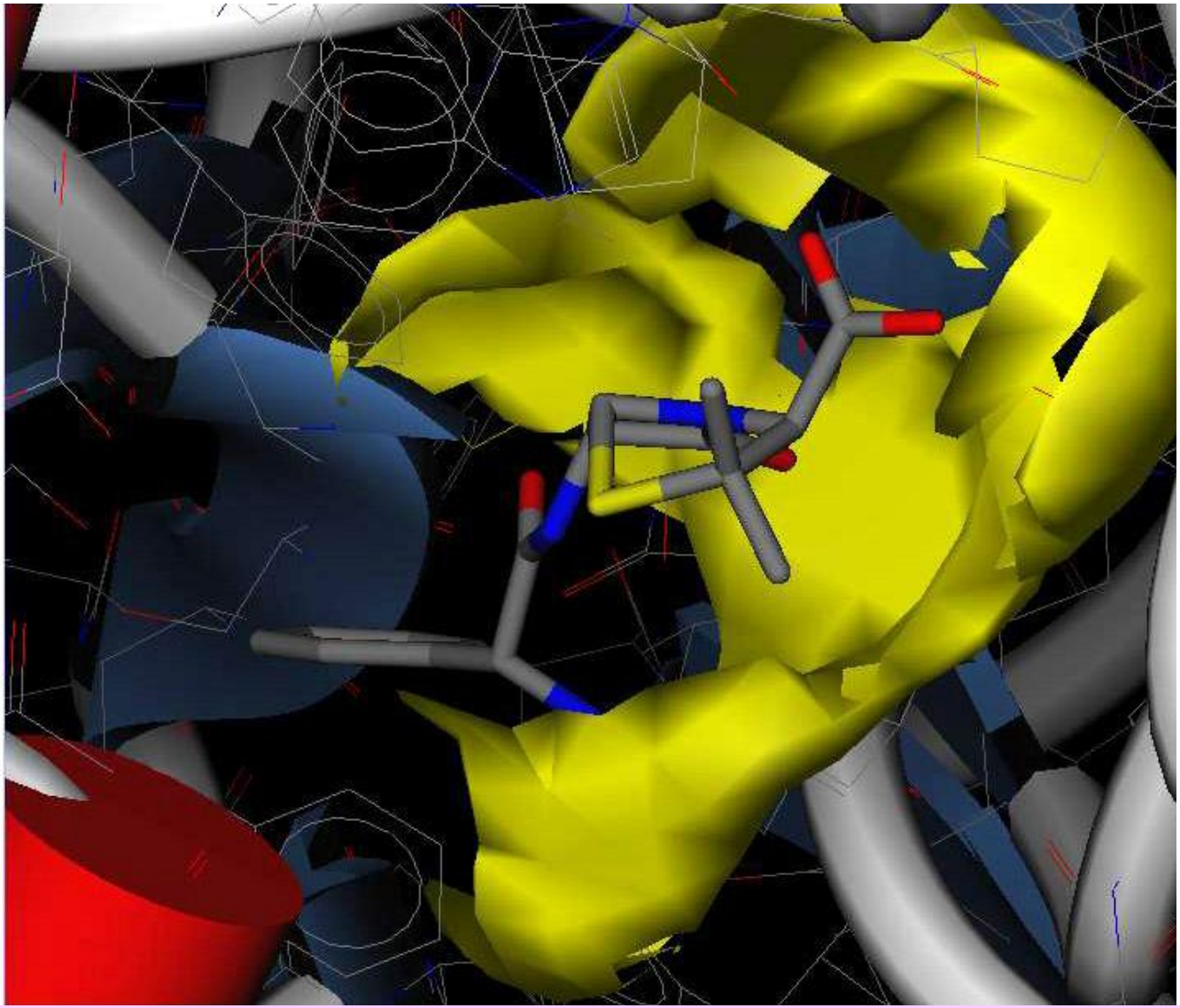
Penicillin before enzyme-substrate recognition



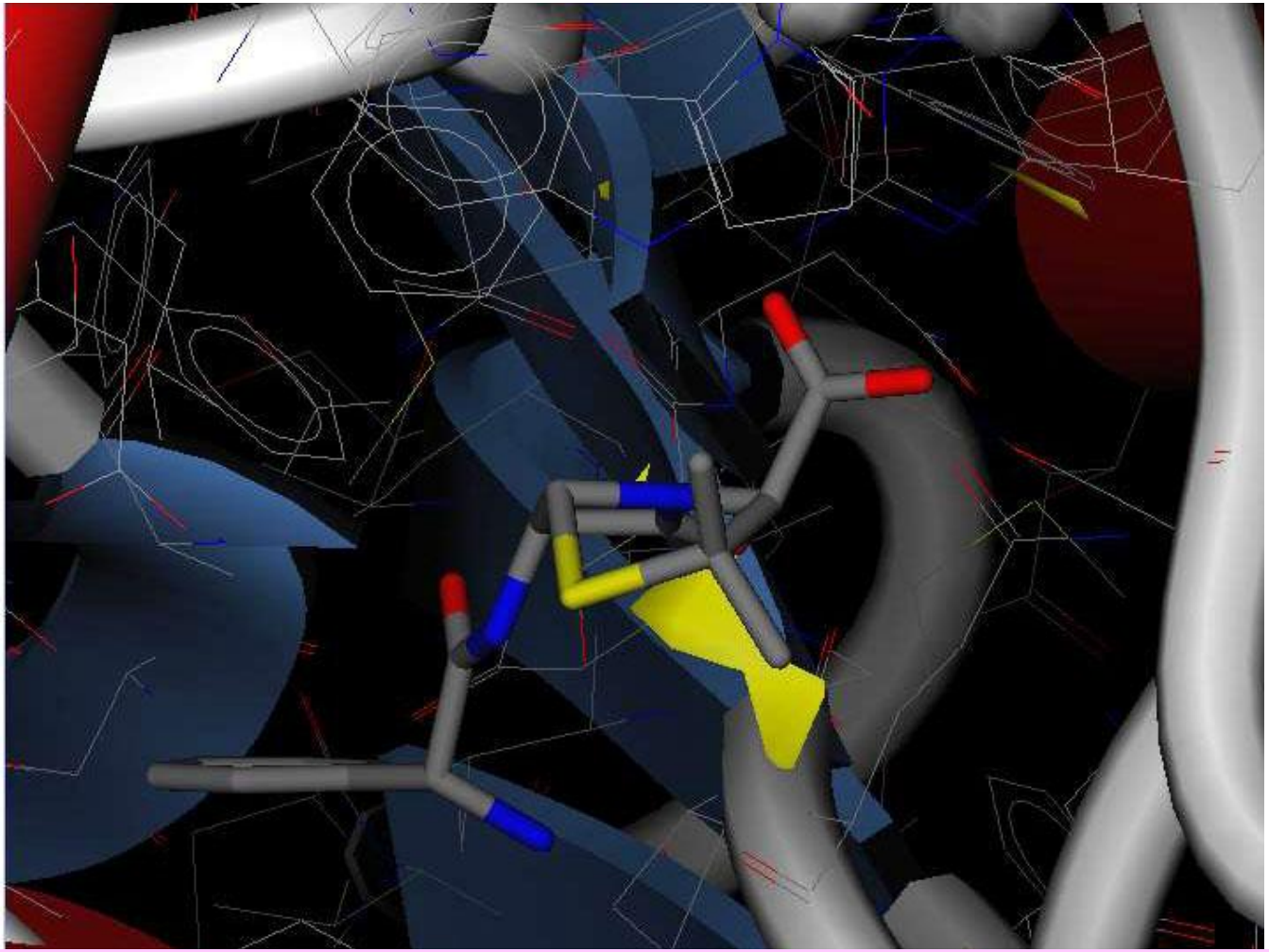
Shells of water solvating the substrate in the aqueous bulk medium



2. The substrate enters the active site



3. To enable enzyme substrate interactions/recognition, part of the water molecules must be displaced (desolvation)



4. After displacement of water molecules, the substrate establish new electrostatic interactions with residues in the active site

The process:
Biocatalysis in non conventional media

How biocatalysts work under non-physiological conditions

Neat substrates + Native enzyme + Traces of solvent

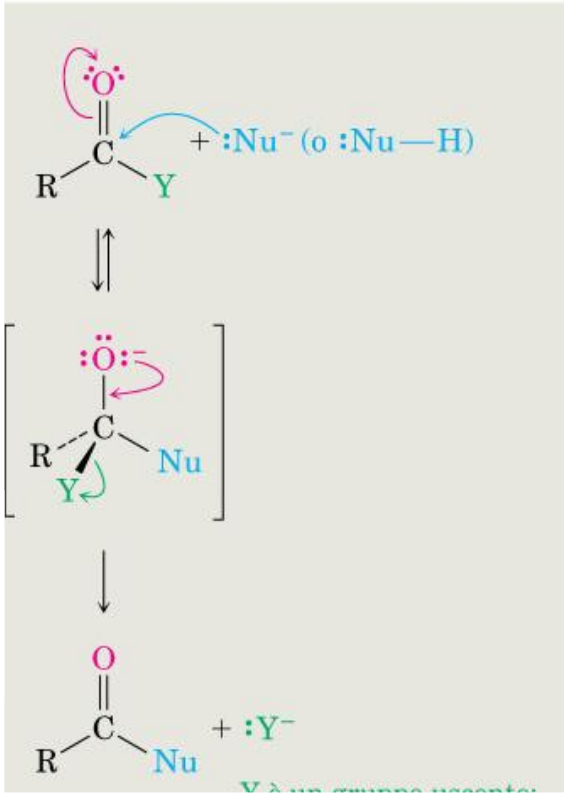
Neat substrates + Immobilized enzyme

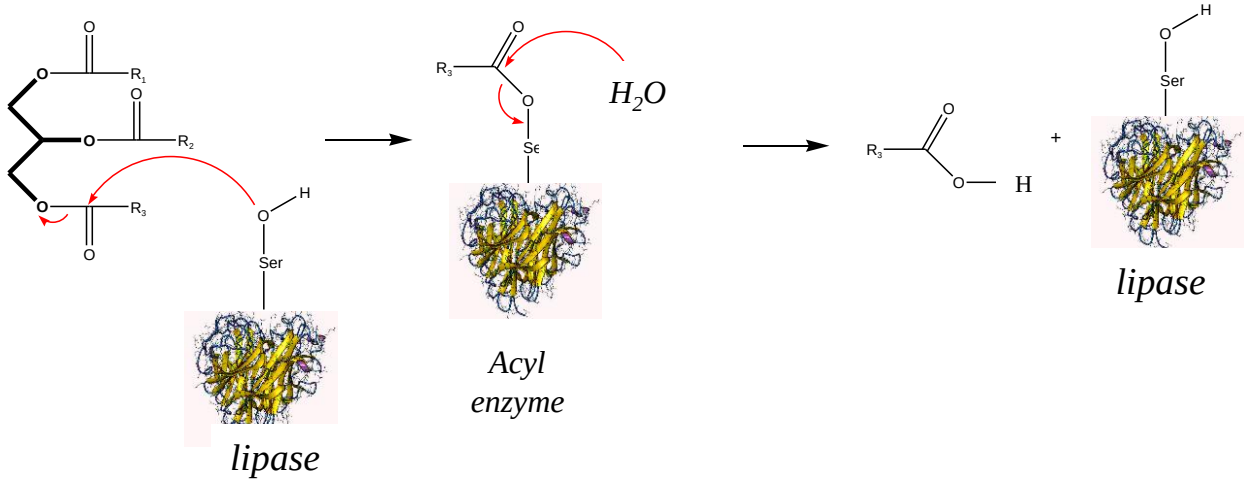
Low -water media: advantages

- Shift of thermodynamic equilibrium
- Recovery of products
- Solubility of hydrophobic substrates
- Microbial contamination negligeble
- Side-reactions reduced
- Simple recycling of the catalyst

Shifting the equilibrium towards the synthesis of acyl bond

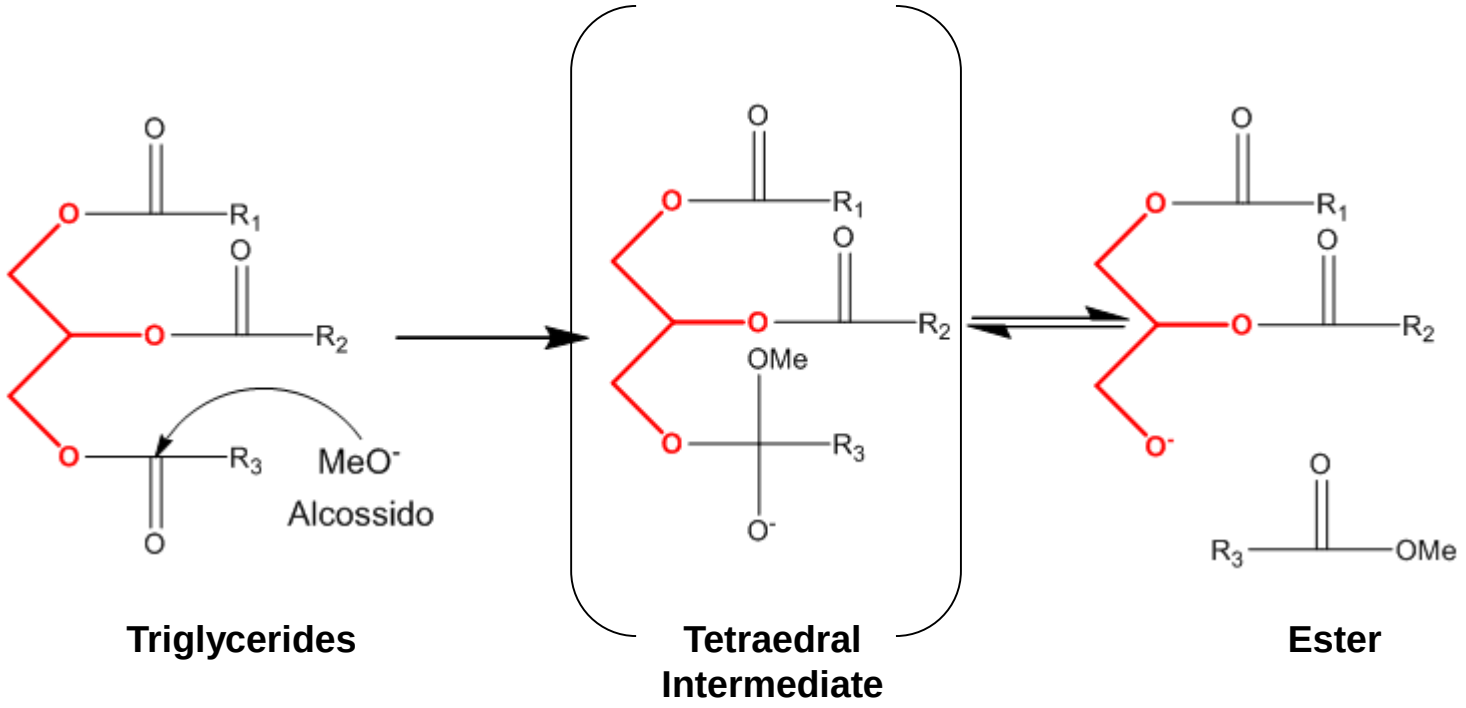
Esterases
Amidases
Lipases
Peptidases





Chemical synthesis of biodesel

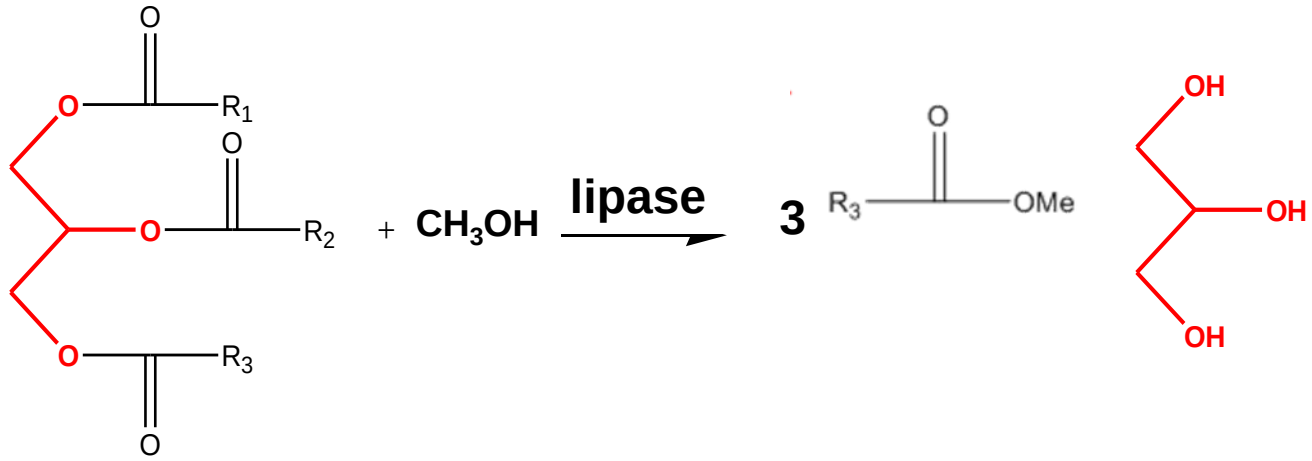
Alkaline or acid conditions



Triglycerides **Tetraedral Intermediate** **Ester**

High temperatures
Separation of by product at the end of the processes (distillation)
Catalysts disposal
Low quality of glicerol

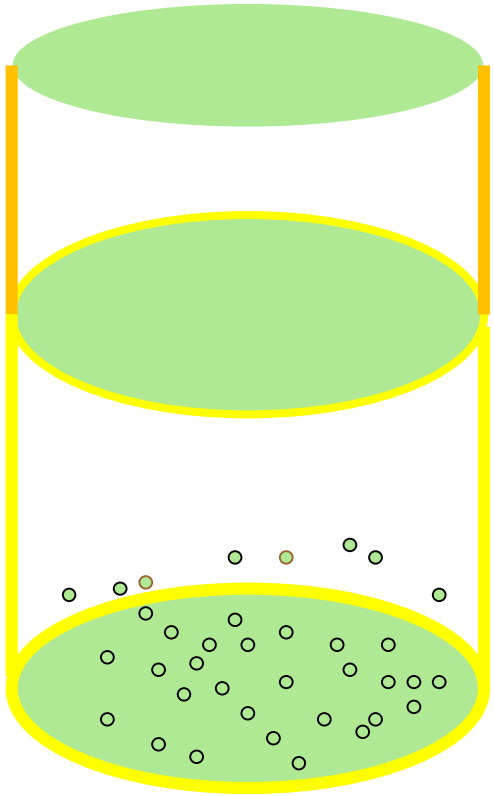
Industrial examples: enzymatic esterification/transesterification of fats and oils



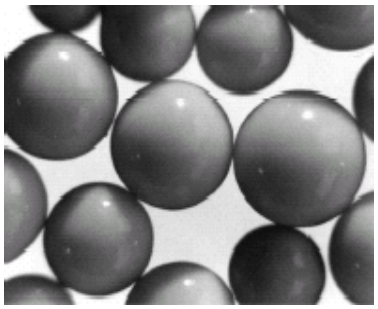
Trigliceride (TG) Alcol Esteri Glicerolo

Biodiesel

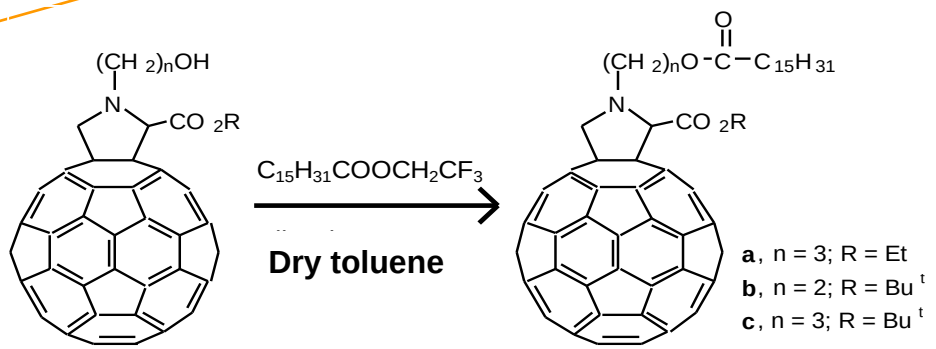
Low-water media: different systems



Immobilized



Enzymes suspended in organic solvent



Tetrahedron Lett., 1998, 39, 7791-7794.

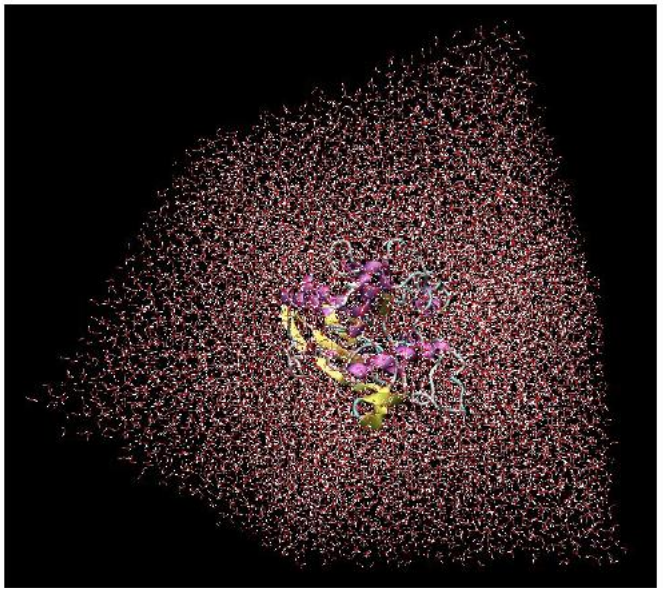
The contribution of water to protein structure

Protein hydration is very important for their three-dimensional structure and activity. Indeed, **proteins lack activity in the absence of hydrating water.**

The aqueous structuring around proteins is affected out to at least **1 nanometer** from its surface.

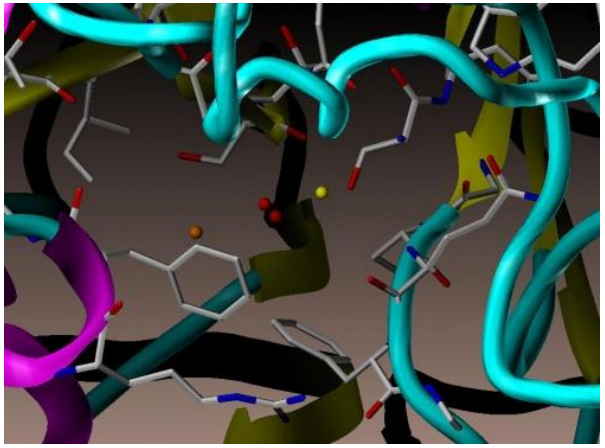
In solution they possess a **conformational flexibility with** a wide range of **hydration states**,

Equilibrium between these states will depend on the **activity of the water** (a_w); that is, the freedom that the water has to hydrate the protein.

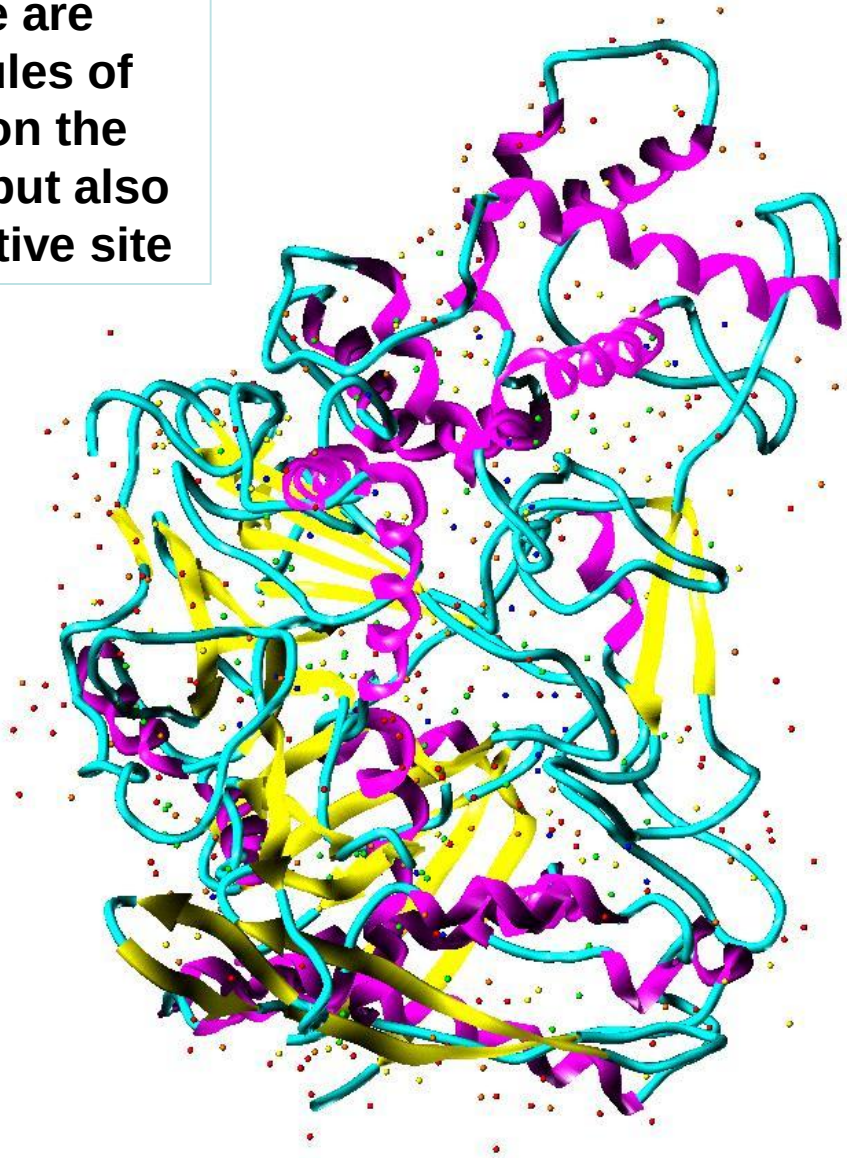


Biocatalysts in organic solvents: residual water in PGA

Active site



There are molecules of water on the surface but also in the active site

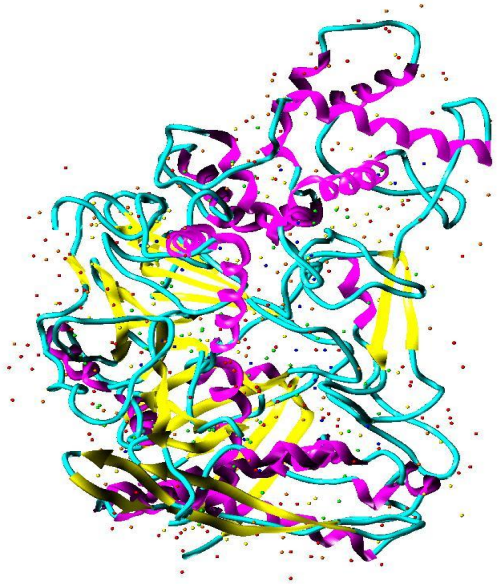


β

Violet
Blue
Green
Yellow
Orange
Red

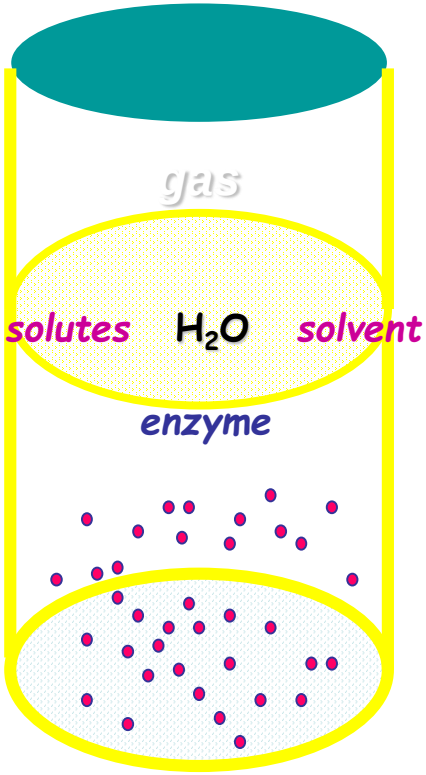
Strenght of bond

Low water media: residual water and water activity (a_w)



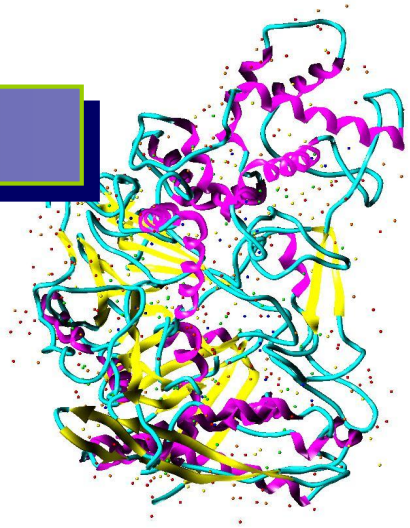
- It is not sufficient to state the amount of added water

The concept of water activity can be assimilated to the "free" water present in the system, which is available to react or hydrate other molecules. When a system reaches the equilibrium, the water activity (or the "free water") will be the same in all phases. Therefore, the reaction and the enzyme activity will be affected by the a_w rather than by the water concentration in the solvent.



a_w

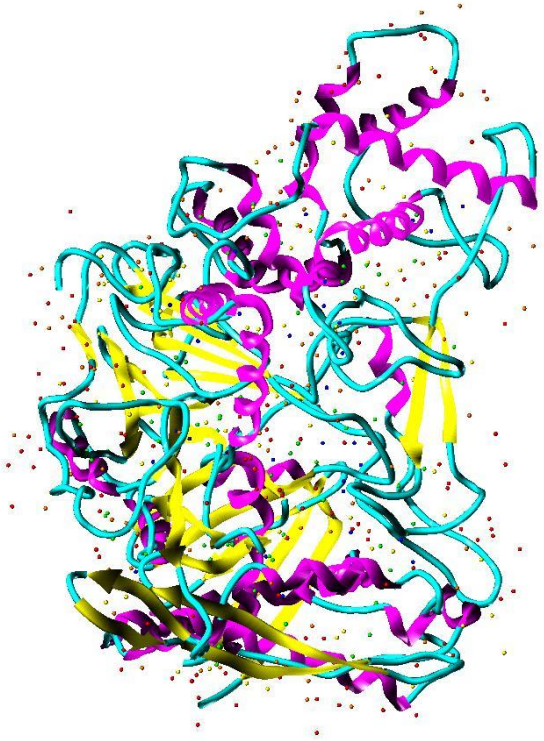
Log P of organic solvents and effect on enzymatic activity

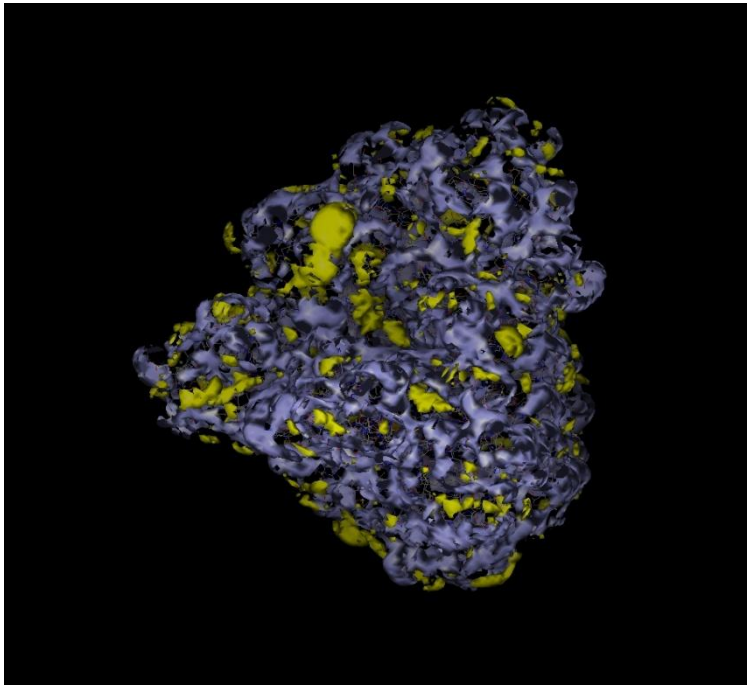


Reaction media
-can compete for water molecules on the surface thus inducing denaturation
-can remove water molecules essential for the mechanism of action: enzyme retains its conformation but looses its activity

Log P	Water-Miscibility	Effects on enzyme activity
-2.5 to 0	Completely miscible	Used to solubilise lipophilic substrates in concentrations of 20-50% v/v without deactivating the enzyme
0 to 2	Partially miscible	Limited use due to rapid enzyme deactivation
2 to 4	Low miscibility	May be used with caution
> 4	Immiscible	**Ensures high retention of activity**

Penicillin G amidase in organic solvent: active when sufficiently hydrated

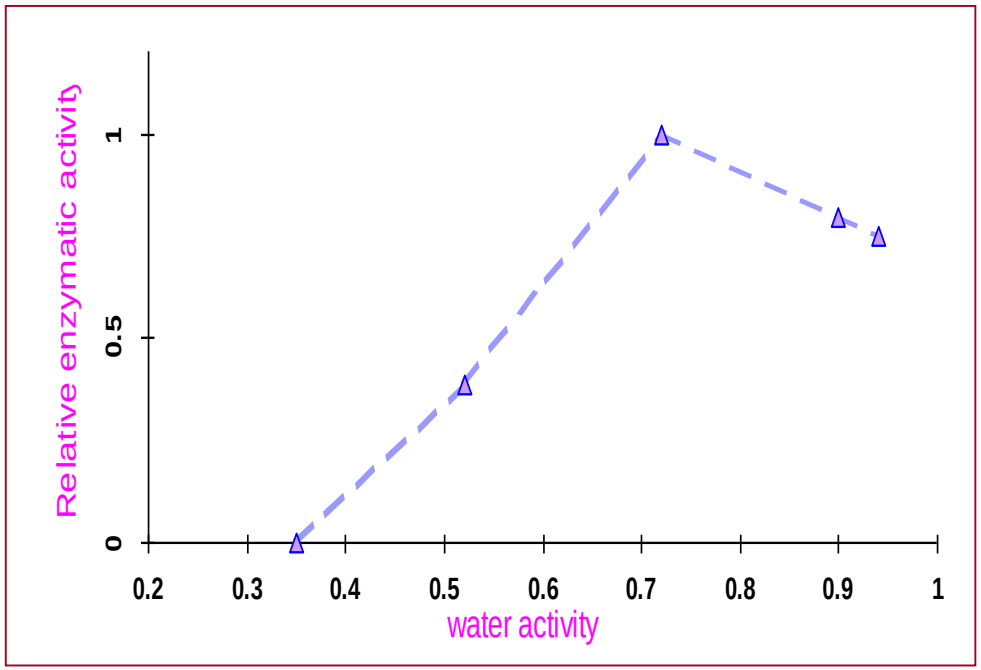


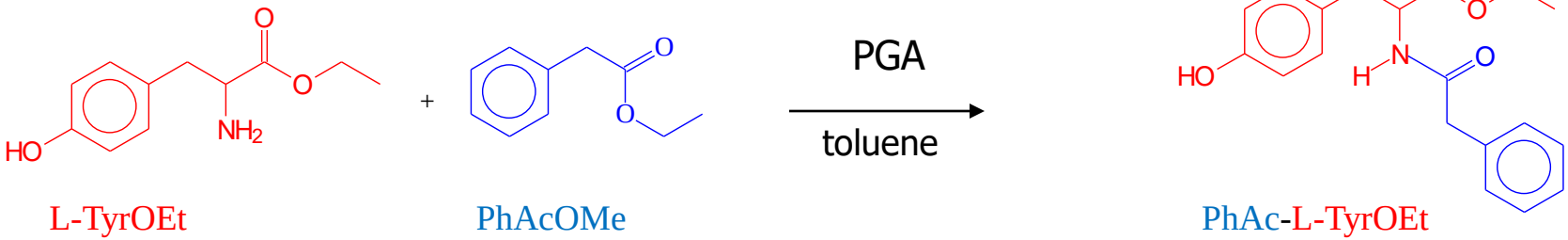


The blue areas indicate hydrophylic residues: the surface of most globular proteins and enzymes is hydrophylic

The hydration of the biocatalyst will depend on the amount of "free water" (i.e. water activity) rather then on the amount of total water present in the system.

a_w effect on synthetic activity of PGA in organic solvent



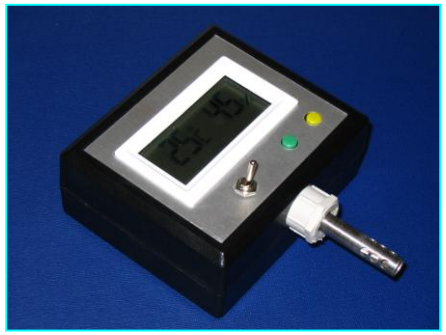


L-TyrOEt PhAcOMe PhAc-L-TyrOEt

Ebert, C.; Gardossi, L.; Linda, P., *Tetrahedron Lett.*, 1996, *37*, 9377-9380

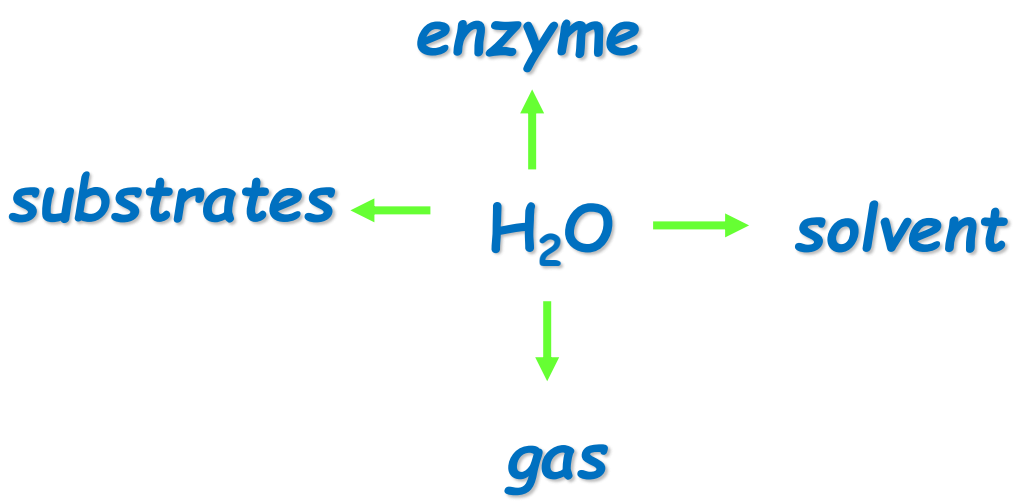
Ebert, C.; Gardossi, L.; Linda, P, *J. Mol. Catal. B*, 1998, *5*, 241-244.

Basso A., De Martin L., Ebert C., Gardossi L., Linda P., Zlatev, V., *J. Mol. Catal. B*, 2001, *11*, 851-855.



How measuring or controlling the water activity

Since at the equilibrium the "free water" will be the same in all phases, it can be measured in the most accessible one, generally the gas phase *via* the measurement of vapour pressure of water.



a) Evaluating water activity by measuring water pressure in the gas phase of the close system at the equilibrium

b) adjust to a defined water activity all ingredients/phases by using pairs of hydrated salts "buffering" the a_w

Water activity values for different coupples of hydrated salts

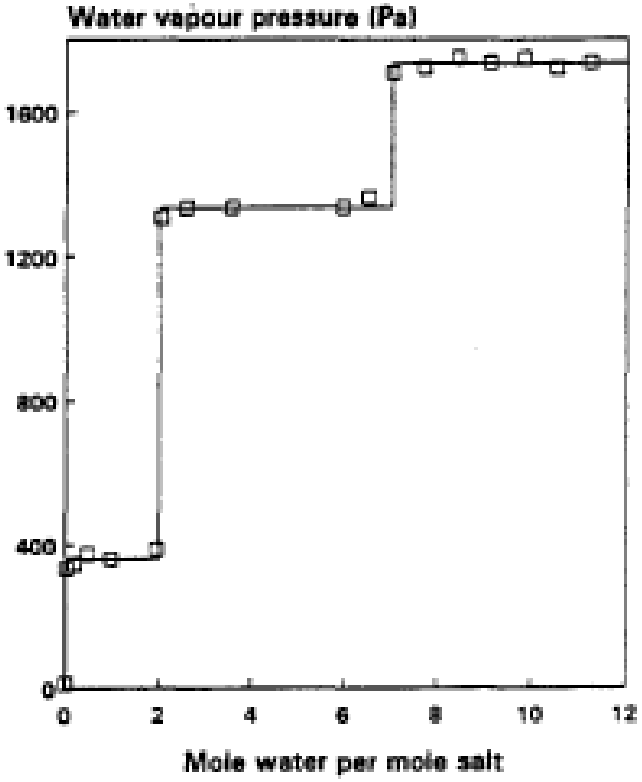


Figure 1. Water binding isotherm for Na_2HPO_4. Data for 20 °C taken from Menzel and Sieg (1932).

Salt pair	Temperature (°C) 20	25	30	35	40	50	60	70	80
Na-tartr.2/?	*0.85*	*0.86*	-	-	-	-	X	X	X
Na_2SO_4.10/0	**0.76**	**0.80**	**0.83**	X	X	X	X	X	X
Na_2HPO_4.12/7	**0.74**	**0.80**	**0.85**	0.90	X	X	X	X	X
Na_2CO_3.10/7	**0.72**	**0.75**	**0.79**	X	X	X	X	X	X
Na_2CO_3.7/1	0.66	0.70	0.74	0.78	X	X	X	X	X
$ZnSO_4$.7/6	0.58	0.63	0.68	0.73	X	X	X	X	X
$Na_2B_4O_7$.10/5?	0.58	0.61	0.65	0.69	*0.72*	*0.80*	-	X	X
Na_2HPO_4.7/2	**0.57**	**0.61**	0.65	0.69	0.73	X	X	X	X
$ZnSO_4$.6/1	**0.55**	**0.59**	**0.62**	**0.66**	**0.69**	X	X	X	X
$Sr(OH)_2$.8/1	*0.52*	0.56	0.61	0.65	0.70	*0.80*	-	-	-
$KCr(SO_4)_2$.12/6	*0.48*	0.51	0.55	0.58	0.62	0.69	0.77	X	X
$Na_4P_2O_7$.10/0	0.46	0.49	0.52	**0.56**	**0.59**	**0.67**	**0.75**	**0.83**	0.92
$K_4Fe(CN)_6$.3/0?	*0.42*	*0.45*	*0.48*	*0.52*	*0.55*	*0.62*	*0.69*	*0.76*	*0.84*
Na_2HAsO_4.7/5	0.39	0.43	0.46	*0.50*	*0.53*	-	X	X	X
$Na_2S_2O_3$.5/2	**0.34**	**0.37**	**0.40**	**0.43**	0.45	X	X	X	X
NaBr.2/0	**0.33**	**0.35**	**0.38**	**0.41**	**0.43**	**0.49**	X	X	X
$SrCl_2$.6/2	**0.33**	**0.35**	**0.37**	**0.40**	**0.43**	**0.49**	**0.55**	X	X
$CuSO_4$.5/3	0.30	**0.32**	**0.35**	**0.38**	**0.41**	**0.48**	**0.55**	**0.63**	0.72
$Ba(OH)_2$.8/1	0.28	0.31	0.34	0.37	0.40	*0.47*	*0.54*	-	-
$CoCl_2$.6/4	*0.28*	0.31	0.33	0.36	0.39	X	X	X	X
Na_2HAsO_4.5/1	0.28	0.30	0.33	0.35	*0.37*	*0.42*	-	X	X
NaAc.3/0	*0.25*	*0.28*	*0.30*	*0.32*	*0.35*	*0.41*	X	X	X
Na_2CO_3.1/0	0.22	0.24	0.27	0.29	0.32	0.38	0.44	0.51	0.59
$NiCl_2$.4/2	-	*0.23*	*0.25*	*0.27*	*0.29*	*0.33*	*0.37*	X	X
$BaCl_2$.2/1	*0.20*	*0.23*	*0.25*	0.28	0.30	0.36	0.43	0.50	0.58
$CuSO_4$.3/1	0.196	0.22	**0.24**	**0.26**	**0.28**	**0.33**	**0.39**	**0.45**	**0.52**
$MgHPO_4$.3/1	-	-	-	-	*0.26*	*0.25*	*0.25*	*0.25*	*0.24*
$Zn(NO_3)_2$.6/4	0.166	0.175	0.184	X	X	X	X	X	X
Na_2HPO_4.2/0	0.150	0.163	0.177	*0.191*	*0.21*	-	-	-	-
$BaBr_2$.2/1	0.139	0.156	0.174	0.193	0.21	0.26	0.31	0.37	0.44
$NH_4Al(SO_4)_2$.12/3?	-	-	-	-	-	*0.24*	*0.30*	*0.38*	*0.46*
NaI.2/0	*0.111*	*0.121*	*0.130*	*0.141*	*0.151*	*0.174*	*0.198*	X	X
$Ca(NO_3)_2$.2/0	0.085	0.094	0.105	0.116	0.128	0.154	X	X	X
$SrBr_2$.6/1	*0.084*	0.092	0.100	0.109	0.119	0.139	0.162	0.186	0.21
Li_2SO_4.1/0	-	*0.095*	*0.101*	*0.108*	*0.114*	*0.128*	*0.143*	*0.159*	*0.175*
$Cd(NO_3)_2$.4/2	*0.079*	*0.087*	*0.096*	*0.106*	*0.117*	X	X	X	X
$SrCl_2$.2/1	0.071	0.079	**0.088**	**0.098**	**0.109**	**0.132**	**0.159**	**0.189**	**0.22**
$Cd(NO_3)_2$.2/0	-	-	*0.044*	*0.048*	0.053	0.063	X	X	X
$CaCl_2$.2/1	0.037	0.040	0.043	0.046	0.049	0.055	0.062	0.069	0.076
$MgCl_2$.6/4	0.036	0.039	0.041	0.043	0.046	0.050	0.056	0.061	0.066
LiBr.2/1	*0.021*	*0.025*	*0.030*	X	X	X	X	X	X
LiCl.1/0	*0.017*	*0.020*	*0.023*	*0.026*	*0.029*	0.037	0.047	0.058	*0.071*
$MgCl_2$.4/2	*0.01 to 0.02 throughout*								
$Zn(NO_3)_2$.4/2	0.007	0.011	0.019	0.032	X	X	X	X	X
LiI.3/2	-	-	-	-	-	*0.018*	0.025	0.035	X
$BaCl_2$.1/0	-	-	*0.006*	*0.008*	*0.009*	0.014	0.021	0.030	0.042
LiI.2/1	-	-	-	-	-	-	*0.018*	0.020	X
$BaBr_2$.1/0	-	-	*0.006*	*0.006*	*0.007*	0.008	0.010	0.012	0.013

Thermodynamically controlled synthesis with substrate suspension in toluene at controlled a_w

toluene (1mL), a_w=0.73

Z-L-Phe-COOH + L-PheOEt → **Z-L-Phe-L-PheOEt** ↓

80 μmol *80 μmol*

Solid *liquid*

H_2O

>93% isolated yield (48h)

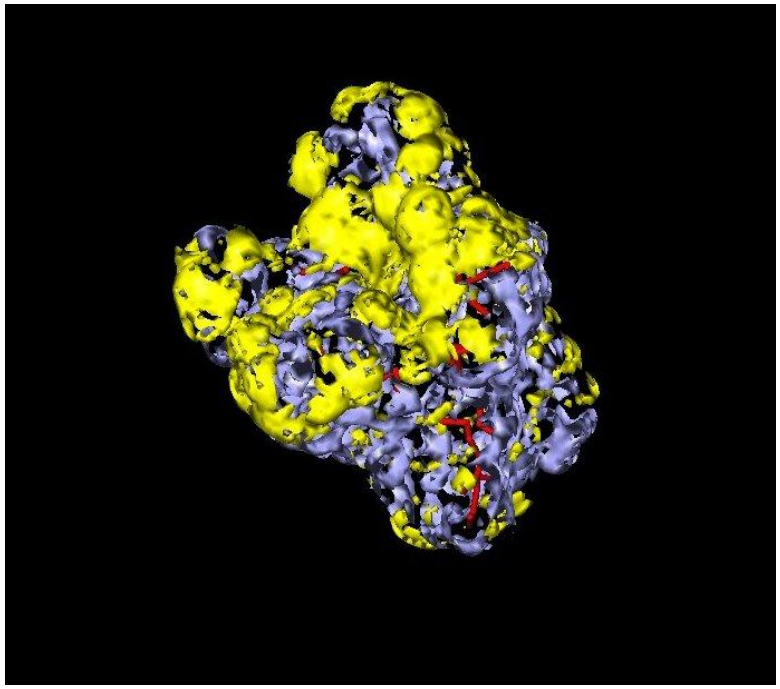
Enzyme	Acyl donor	Nucleophile	Conv. (%)	Time (h)
Thermolysin	Z-L-Phe-COOH	L-Phe-OEt (s)	**98**↓	48
Thermolysin	Z-L-Phe-COOH	L-Tyr-OEt (s)	**97**↓	144
Thermolysin	Z-L-Phe-COOH	L-Leu-NH_2 (s)	**95**↓	96

Basso A., De Martin L., Ebert C., Gardossi L., Linda P., *Chem. Comm.*, **2000**, 467-468.

Not all enzymes need the same amount of «free water»

hydrophobic

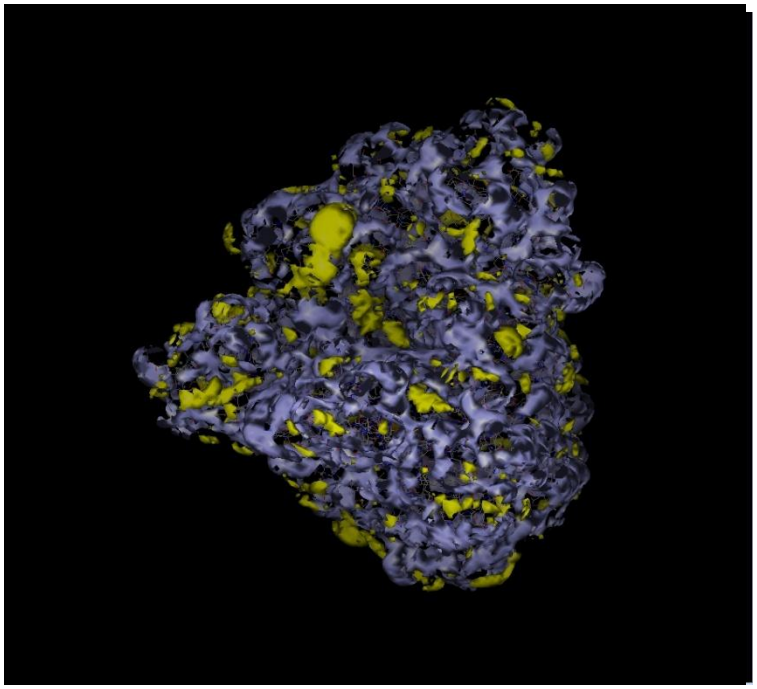
hydrophilic



Lipase from *P. cepacia*

↓

Very active even at low water activity (<0.2)



(PGA) Amidase from *E. coli*

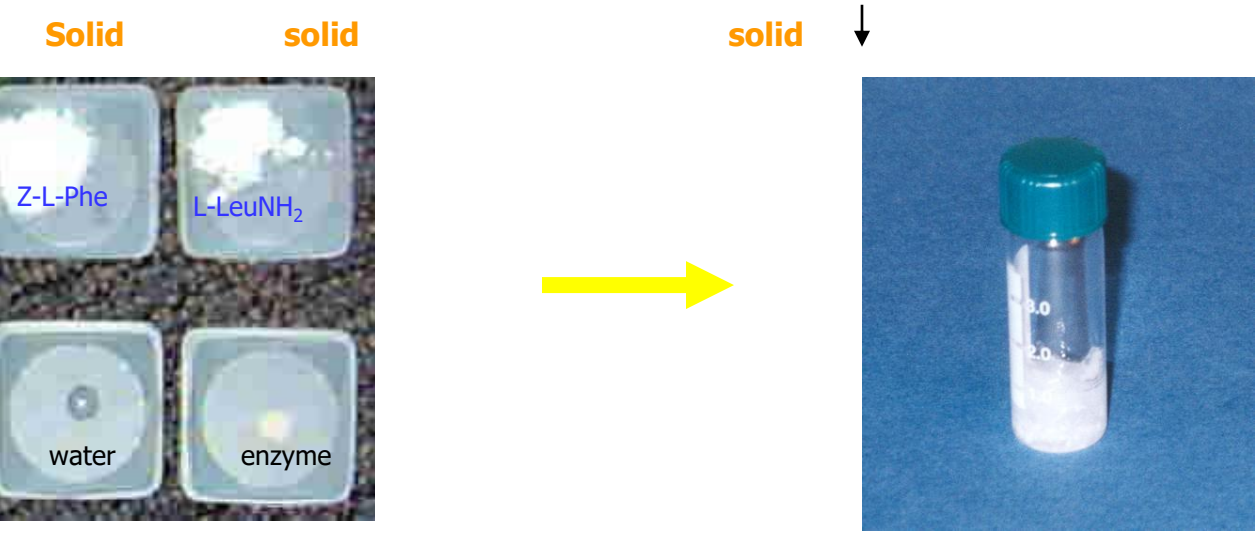
↓

Active only at water activity > 0.4

Adv. Synth. Catal, 2007, 349, 877-886.

Some examples of even “more desperate “ experimental conditions

Precipitation driven "solid to solid" peptide synthesis: product solubility must be lower than substarte solubility



Ulijn R. V., De Martin L., Halling P. J., Janssen A.E.M., Gardossi L., Moore B. D., Biotech. Bioeng., **2002**, *80*, 509-515.

Ulijn R. V., De Martin L., Gardossi L., Halling P.J. , Current Org. Chem, **2003**, *7*, 1333-1346.

When is precipitation driven synthesis feasible?
It depends on the thermodynamics of the reaction

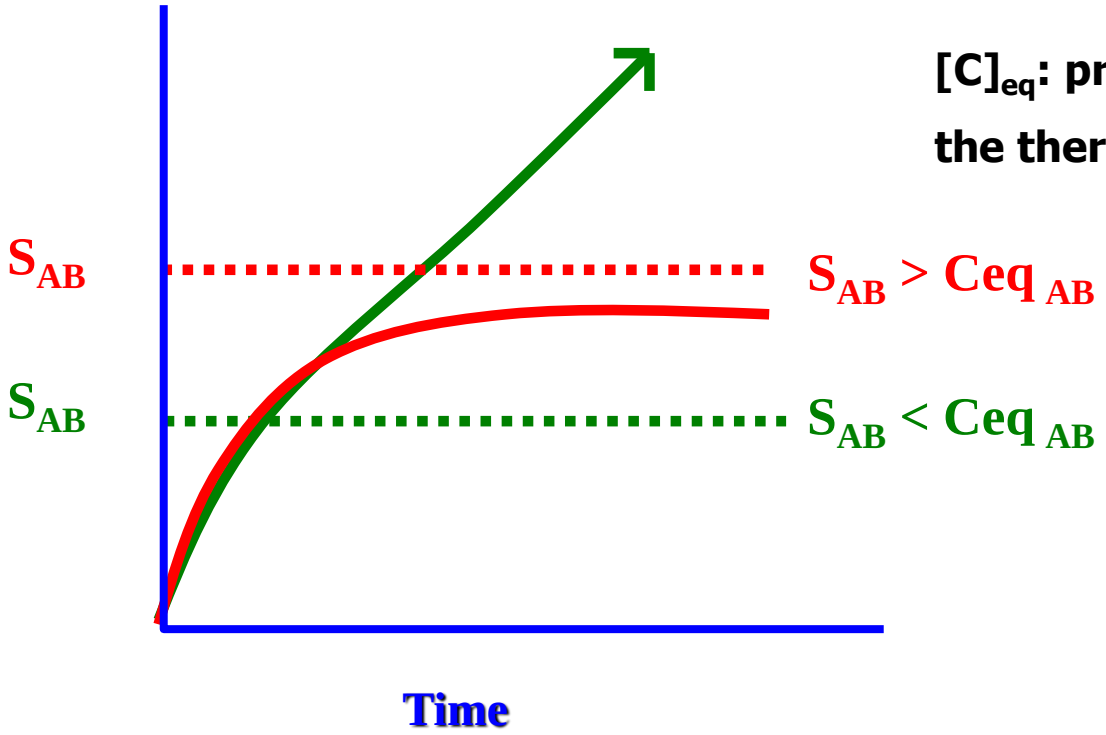
$$A + B \rightleftharpoons AB + H_2O$$

When:

- $S_{AB} < [C]_{eq}$ the product precipitates and the thermodynamic equilibrium is reached only when the substrate in excess is completely consumed.

S_{AB}: product solubility in the solvent;

$[C]_{eq}$: product concentration in solution at the thermodynamic equilibrium



Rules for Solvent Selection

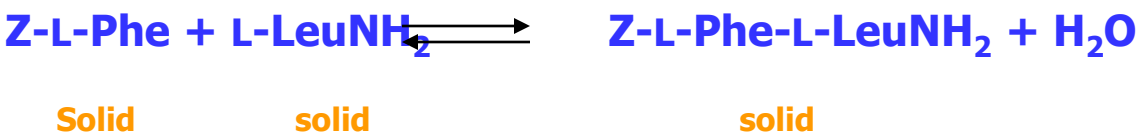
- **The yield of crystalline product can be maximised by choosing a solvent where product solubility is lowest**

- **for hydrophobic targets water is generally a good choice**

- **in the synthesis of hydrophilic targets good yields are expected in hydrophobic solvents**

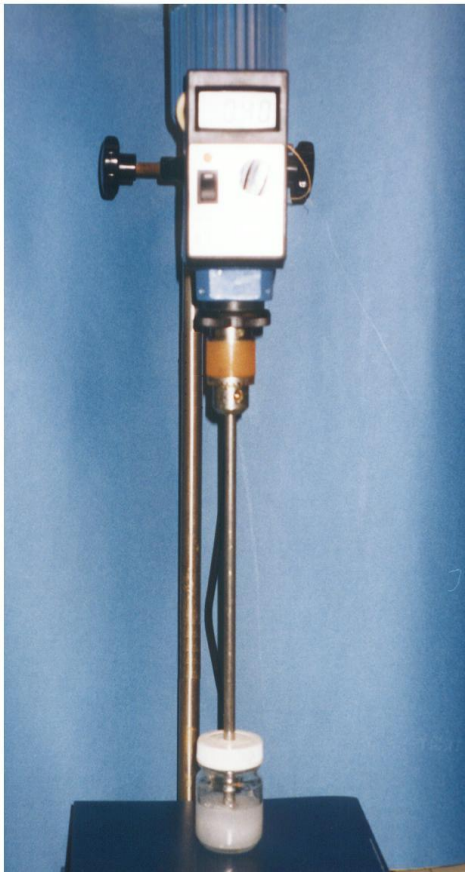
Always use an excess of the most soluble compound

Thermodynamically controlled synthesis of Z-L-Phe-L-LeuNH$_2$ catalysed by immobilized Thermolysin in toluene



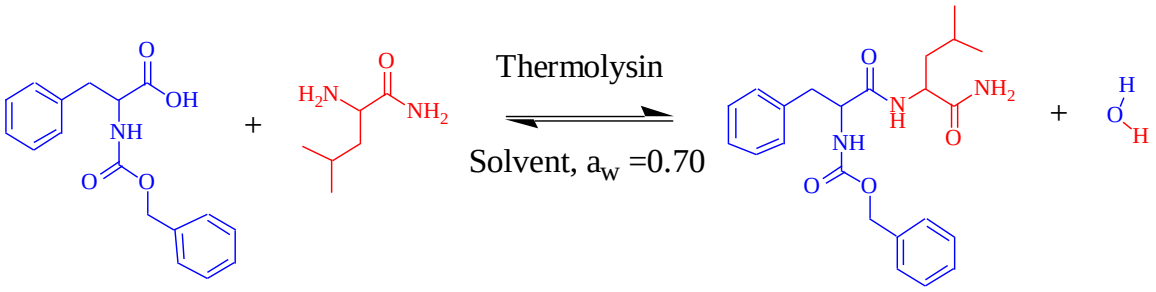
- 2 millimoles of Z-L-Phe and L-LeuNH$_2$ in 20mL toluene
- Conv. after 8h: > 99%
- 96% (1.92 millimoles) of pure solid product recovered by rinsing the enzymel
- Enzyme recycled 4 times

Mechanic stirrer

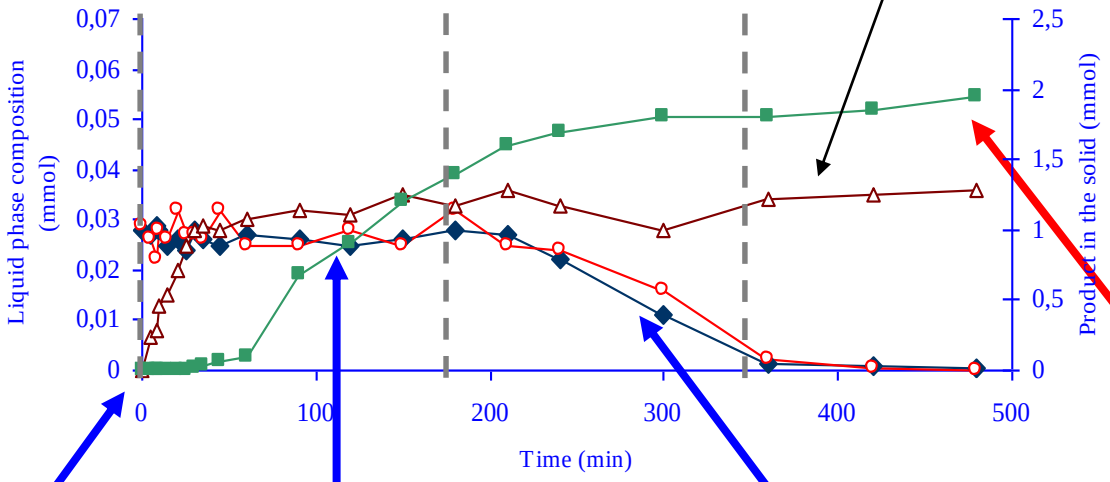


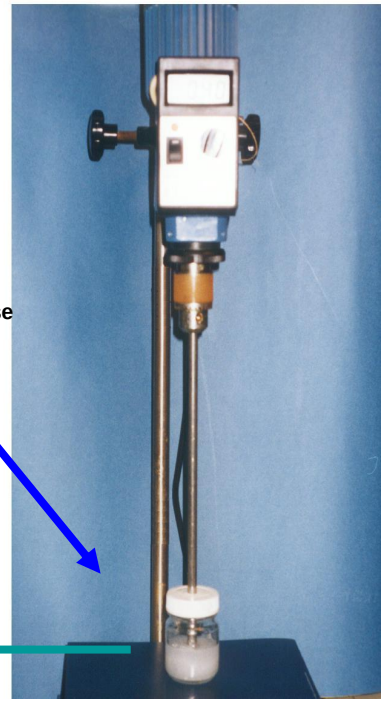
L. De Martin, PhD Thesis, University of Trieste, 2001.

How does it work?



Z-L-Phe (2 mmol) **L-LeuNH$_2$ (2 mmol)**





Product precipitation occurs when [ZPheLeuNH2] equals $S_{ZPheLeuNH2}$

The solution phase reaches a steady state situation

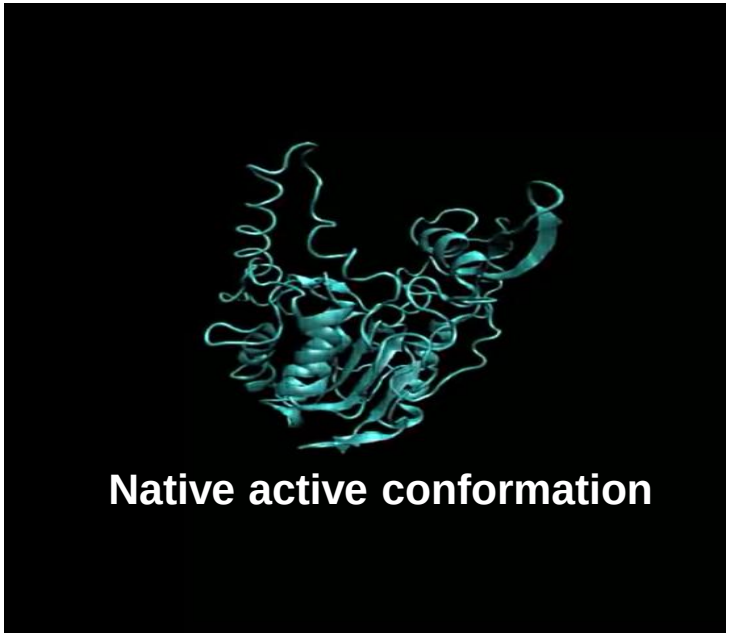
-Substrates in solution reach the equilibrium concentration
-No more excess solid substrate is present

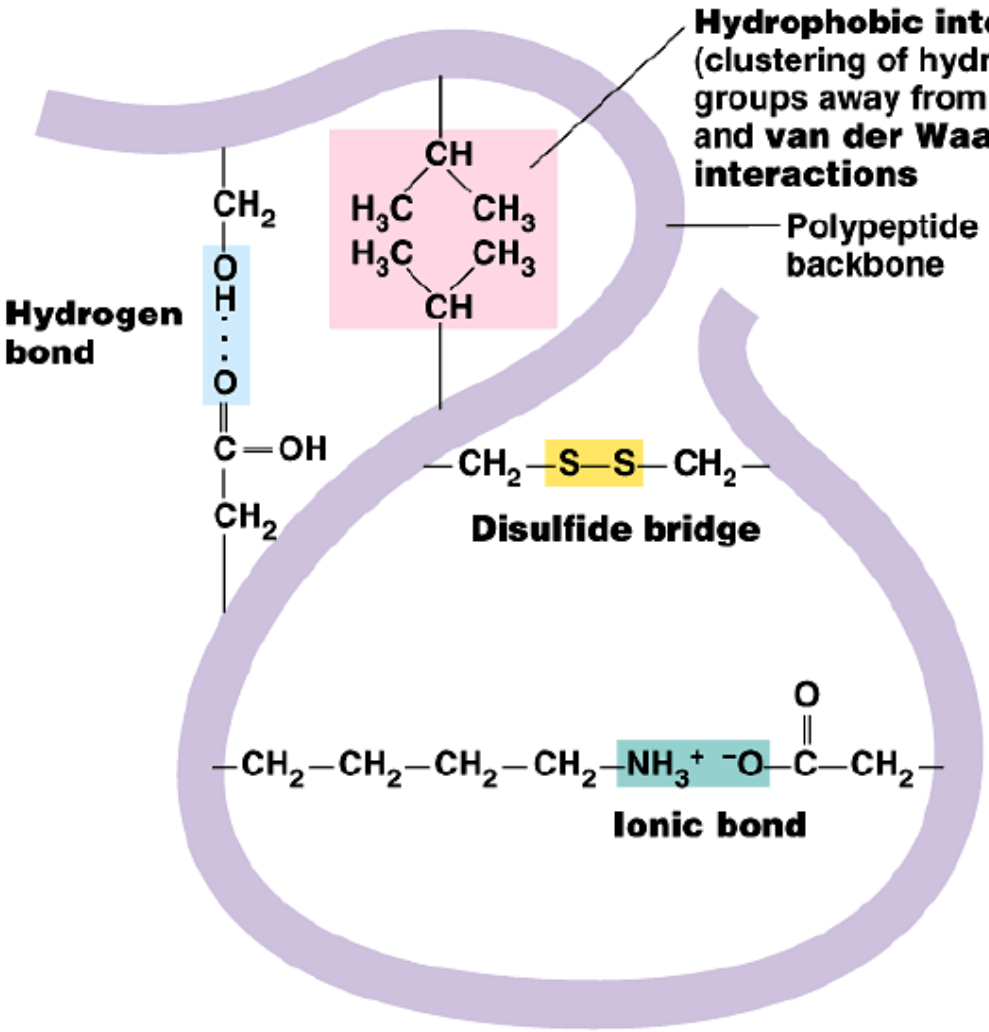
- Conv. after 8 hours > 99%
- 96% (1.92 millimol) of pure solid product at the equilibrium

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.



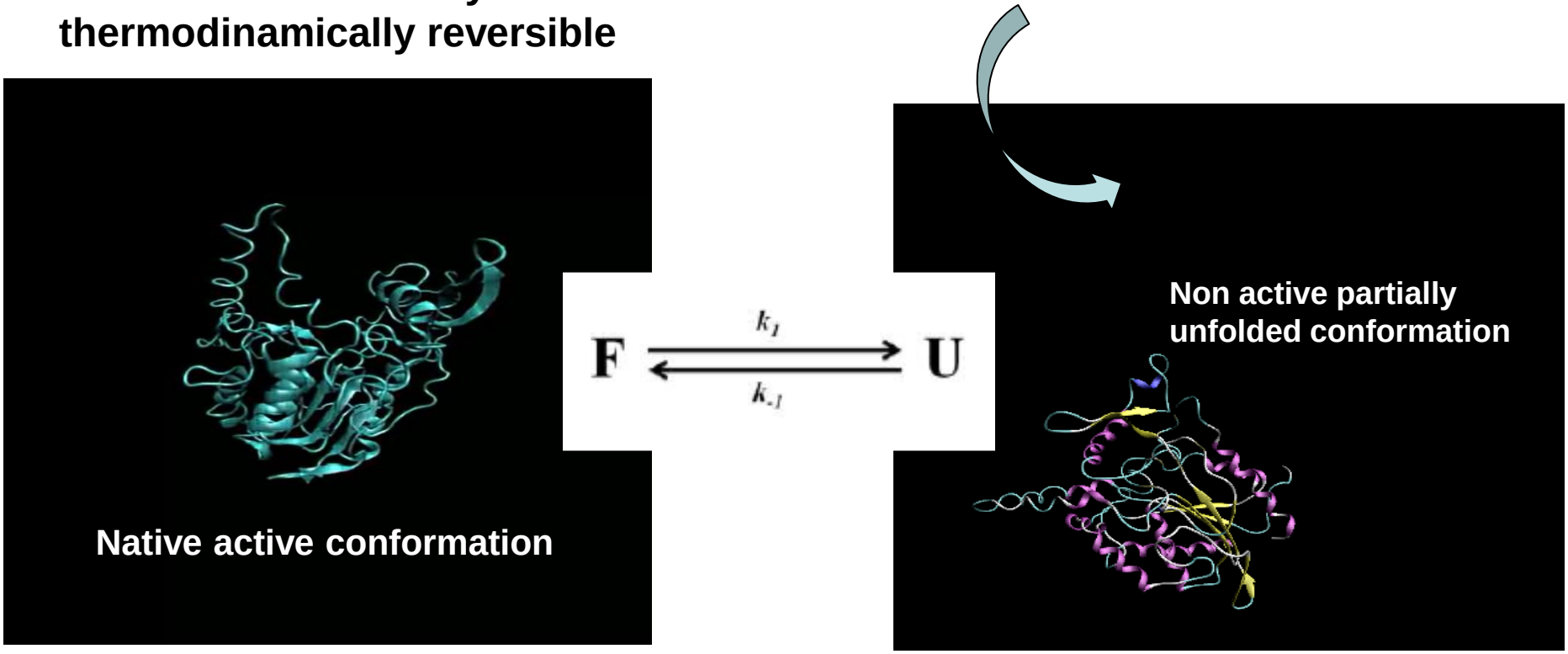


Such hydrophobic collapse is necessarily accompanied and guided by (secondary) structural hydrogen-bond formation between favorable peptide linkages in parallel with their desolvation .

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

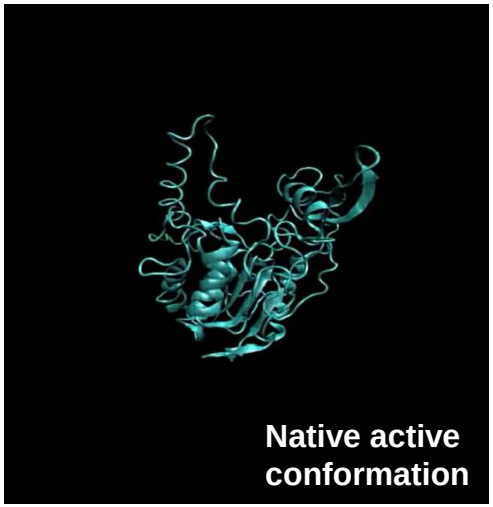


Thermodynamic stability

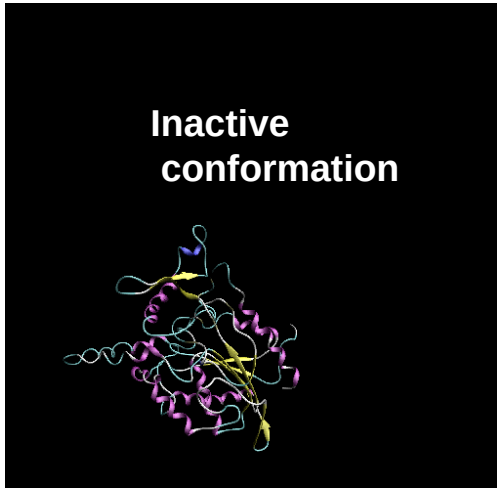
The Gibbs free energy changes between the folded state and unfolded state is described using the free energy equation:

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

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



$$F \rightleftharpoons U$$

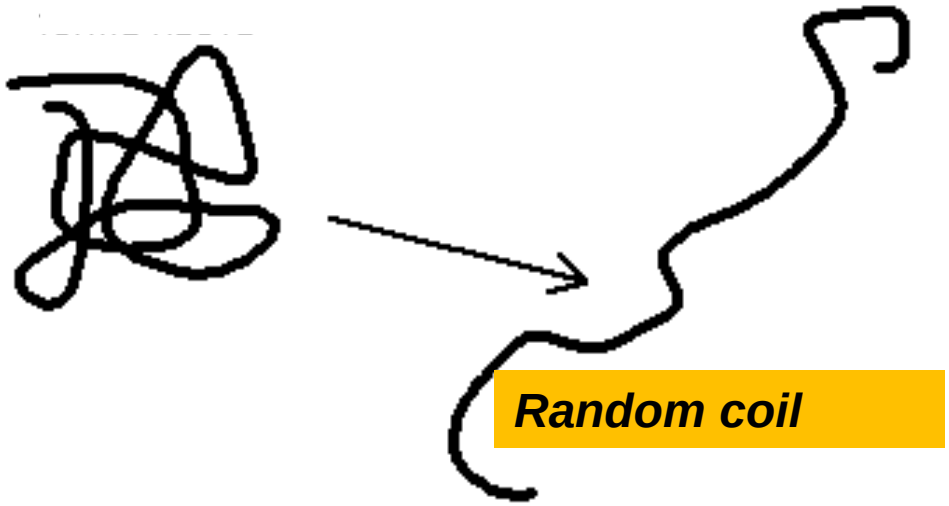


Thermodynamic stability

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.

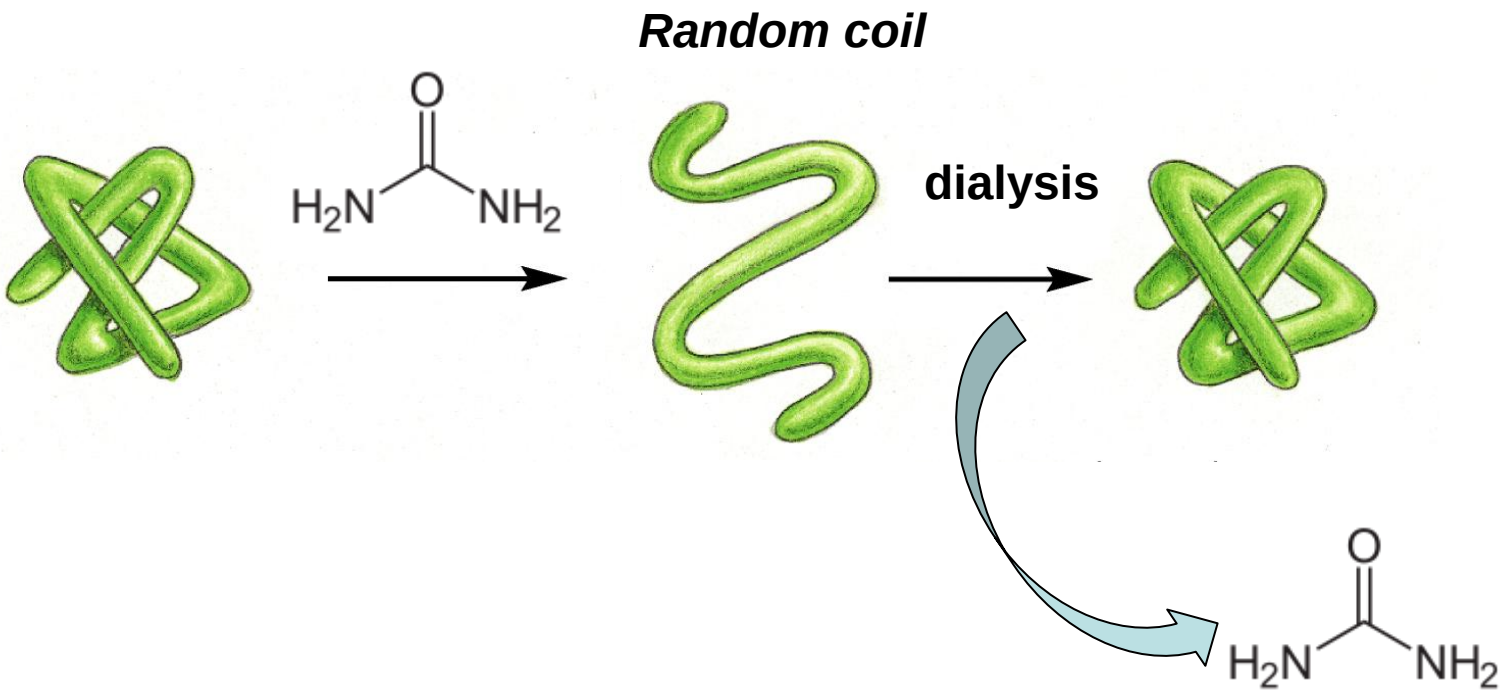
$$\Delta G_f = G_f - G_u = \Delta H - T\Delta S$$

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

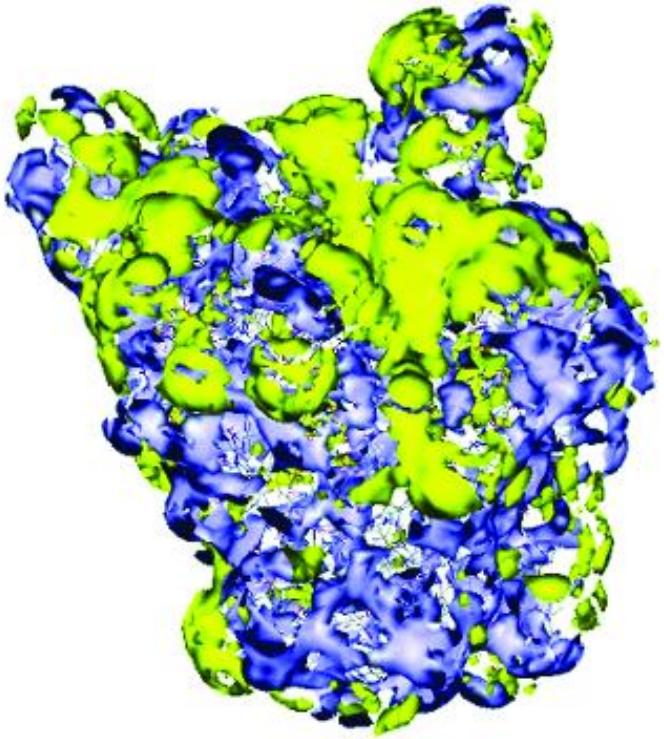
Example: reversible denaturation with urea



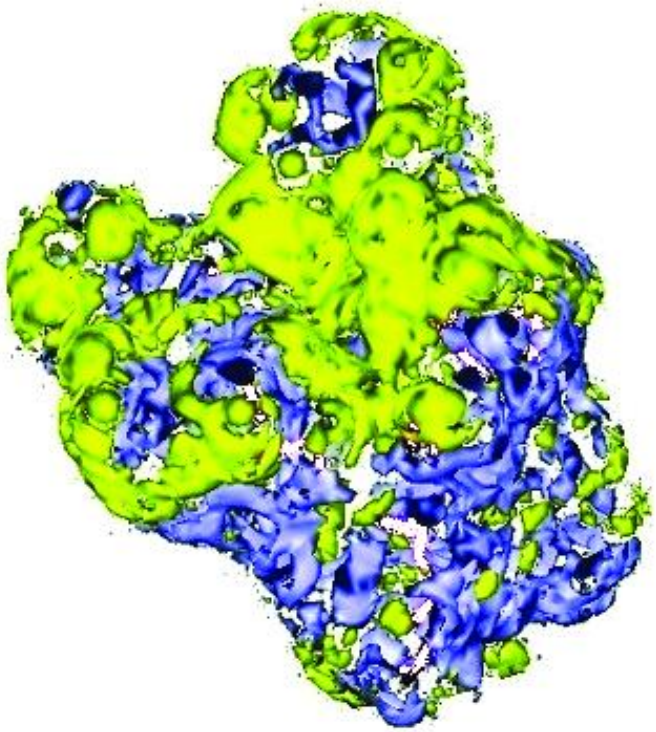
Conformational modifications sometimes are necessary for displaying enzyme activity

Lipases undergo activation at the water-lipid interface

Pseudomonas cepacia LIPASE



closed conformation in water

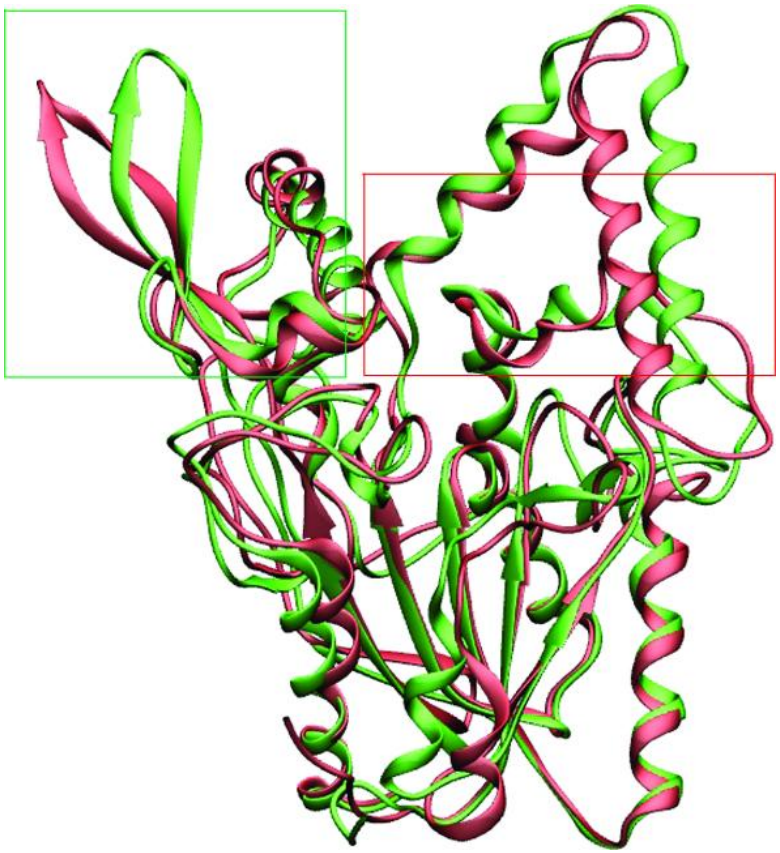


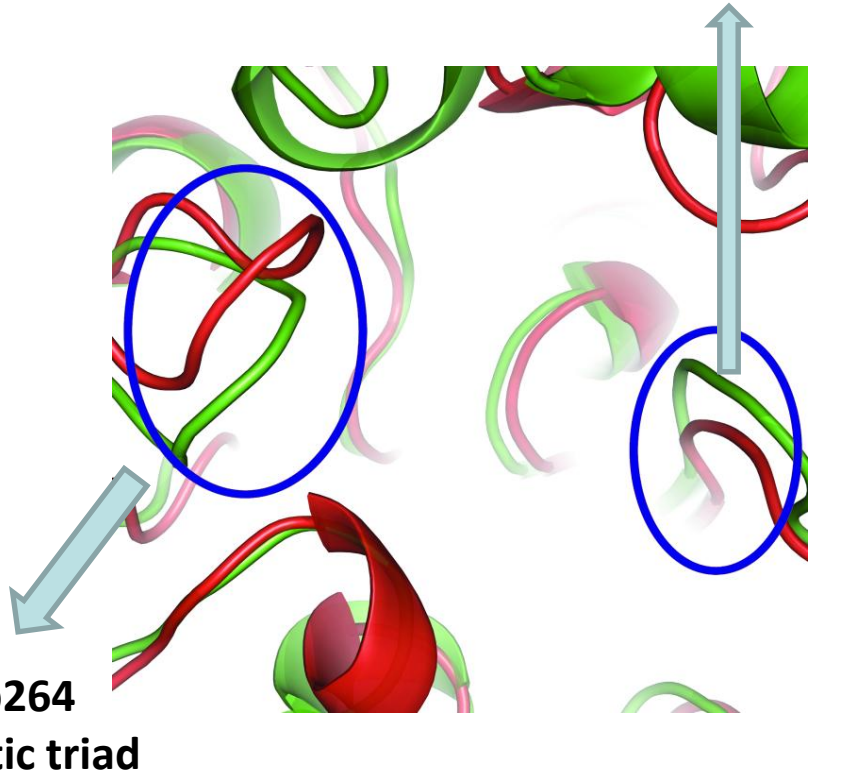
open conformation in hydrophobic media

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.

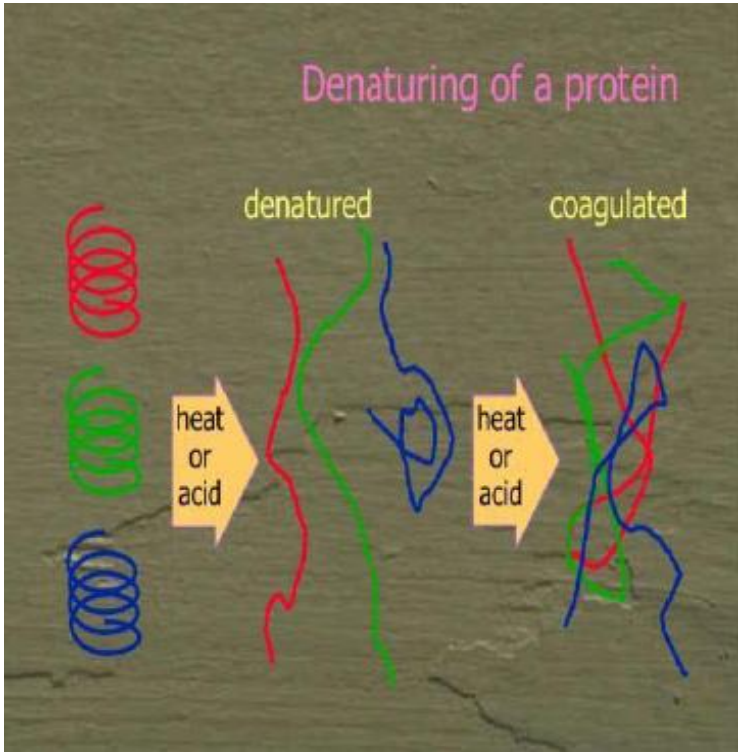
Conformational modifications of a lipase enable the access of hydrophobic substrates to the active site

Open in hydrophobic media; close in water

Kinetic stability

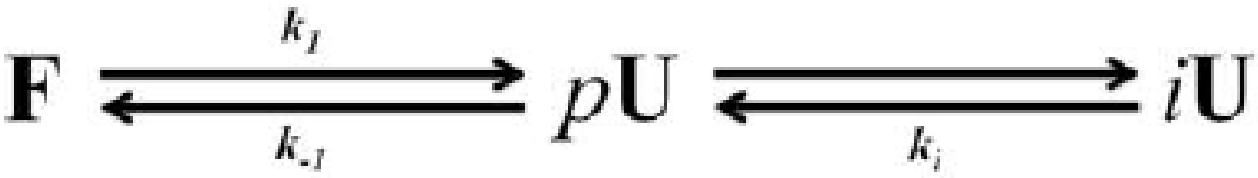
Most mesophilic proteins undergo irreversible unfolding upon extreme changes of the environment. They unfold into inactive structures (scrambled structures), and they often form aggregates (intermolecular mechanism).

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

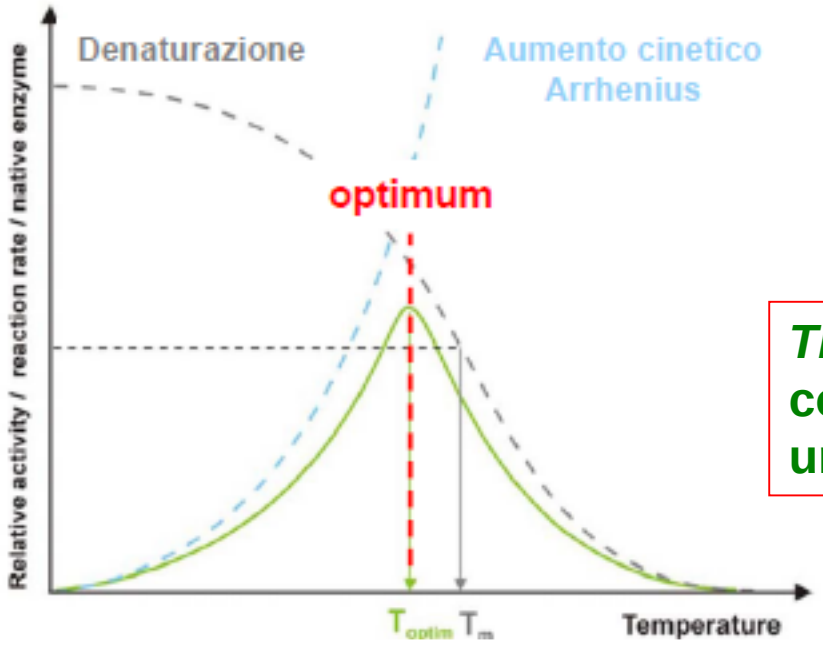
$$k = A \cdot e^{-E/RT}$$

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**

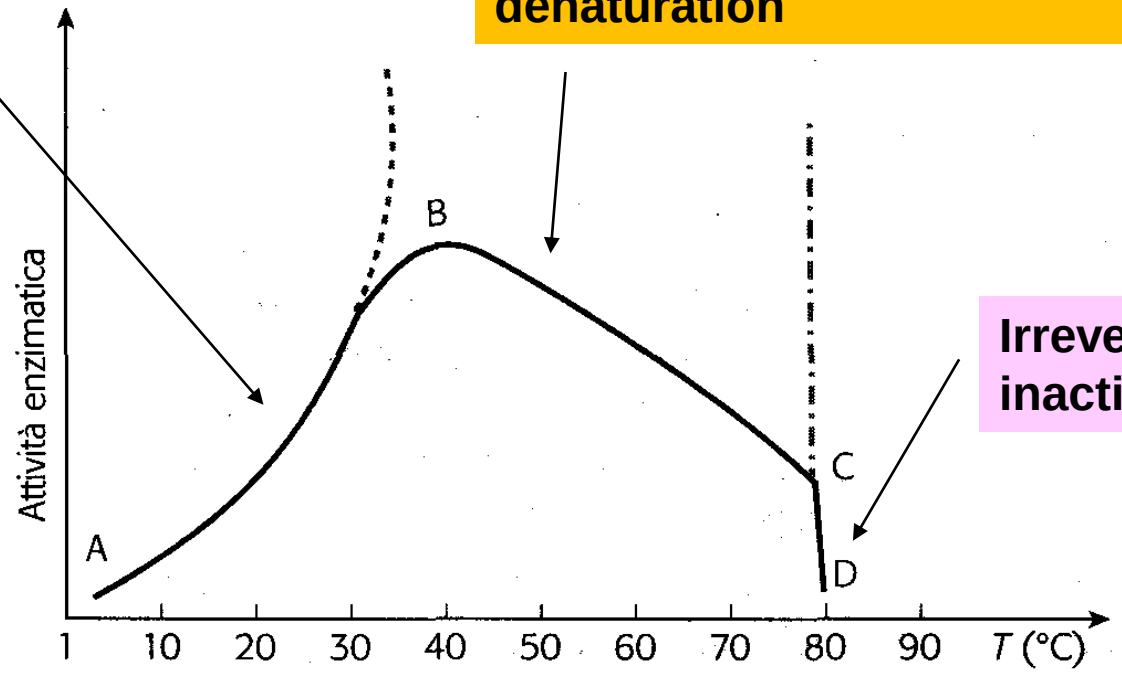
Different enzymatic reactions carried out at different temperatures. Reaction rates are used to build up the profile

Effect of temperature on an enzymatic reaction

Increase due to Arrhenius effect

combined effect of Arrhenius and denaturation

Irreversible inactivation.

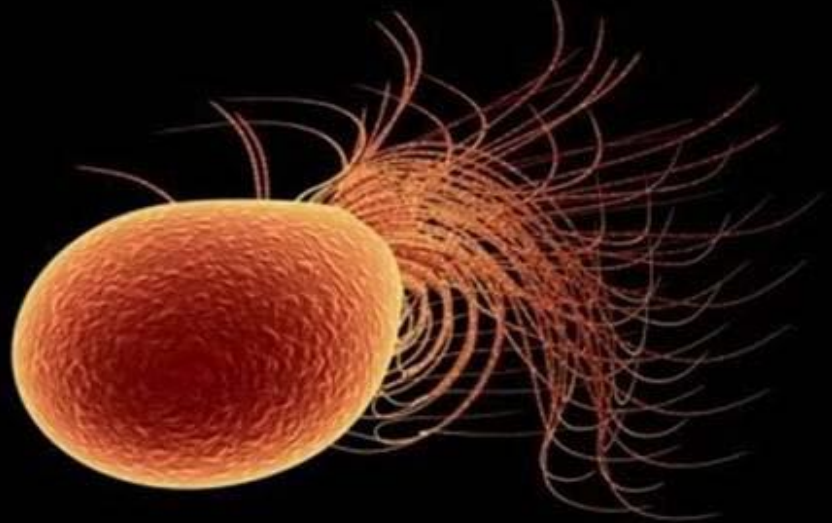


Thermostable enzymes from:

- 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

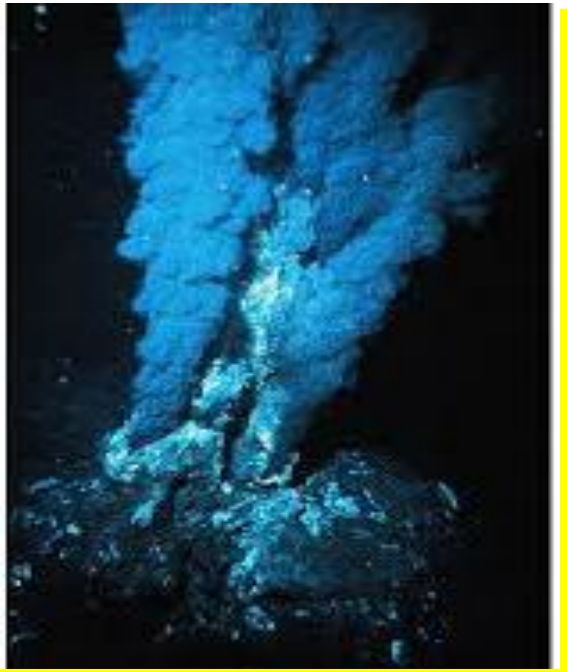
Hyperthermophiles microorganisms
have been isolated almost exclusively from environments with temperatures in the range of 80 to 115°C. Hot natural environments include continental solfataras, deep geothermally heated oil-containing stratifications, shallow marine and deep-sea hot sediments, and hydrothermal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

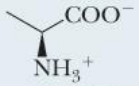
TABLE 4. Relative amino acid compositions of mesophilic and hyperthermophilic proteins[a]

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	

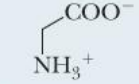
- **Glu and Arg promote the formation of salt bridges (increased stability)**
- **Asn and Gln are prone to covalent modification and degradation**

Tabella 27.1 I 20 amminoacidi comuni che si trovano nelle proteine

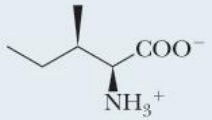
Catene laterali non polari



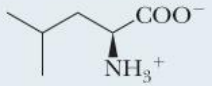
Alanina (Ala, A)



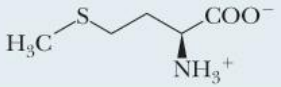
Glicina (Gly, G)



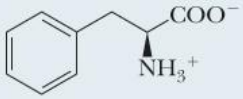
Isoleucina (Ile, I)



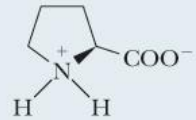
Leucina (Leu, L)



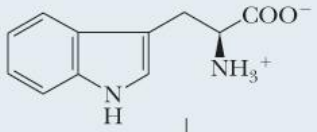
Metionina (Met, M)



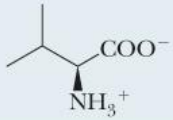
Fenilalanina (Phe, F)



Prolina (Pro, P)

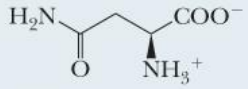


Triptofano (Trp, W)

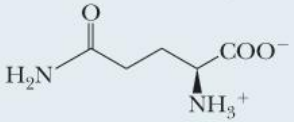


Valina (Val, V)

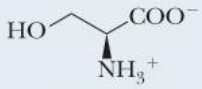
Catene laterali polari



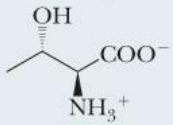
Asparagina (Asn, N)



Glutammina (Gln, Q)

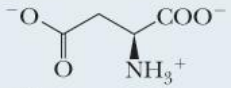


Serina (Ser, S)

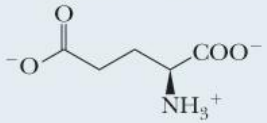


Treonina (Thr, T)

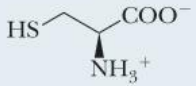
Catene laterali acide



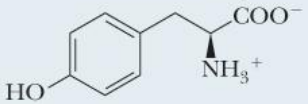
Acido aspartico (Asp, D)



Acido glutammico (Glu, E)

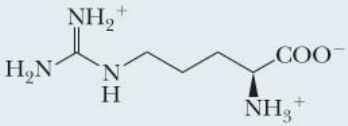


Cisteina (Cys, C)

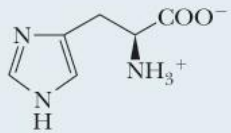


Tirosina (Tyr, Y)

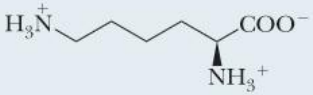
Catene laterali basiche



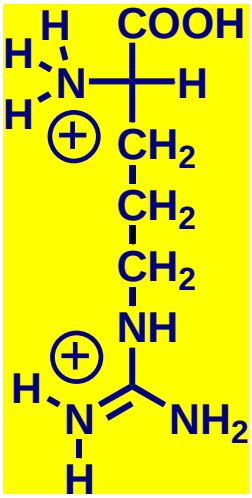
Arginina (Arg, R)



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 ionic bridges in hyperthermophilic enzymes

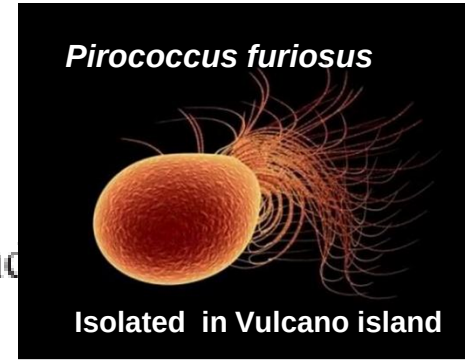


TABLE 6. Comparison of the ion pair contents of *P. furiosus* and *Clostridium symbiosum* GDHs[a]

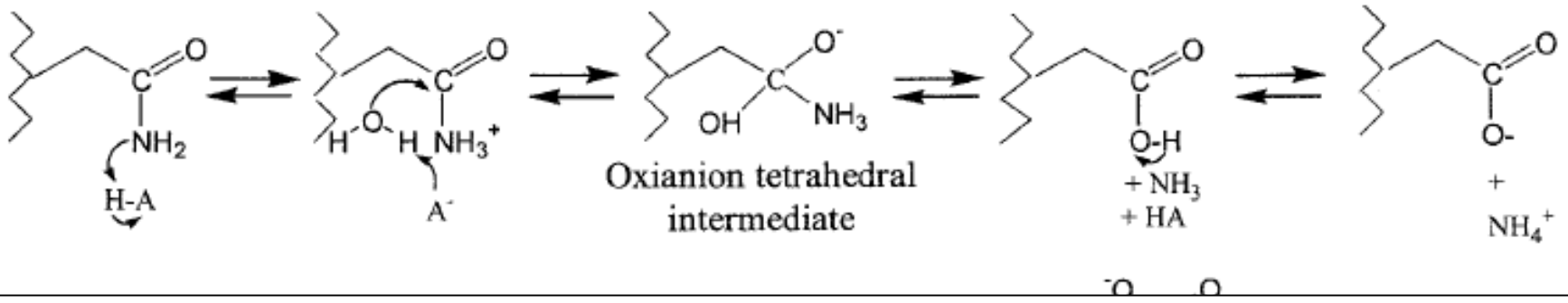
Characteristic (glutammato deidrogenasi)	Value for: *C. symbiosum*	*P. furiosus*
No. of IPs[b] per subunit	26	45
No. of IPs per residue	0.06	0.11
% of charged residues forming IPs	40	54
% of IPs formed by Arg/Lys/His	46/31/23	64/27/9
% of IPs formed by Asp/Glu	46/54	47/53
% of all Arg forming IPs	55	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]

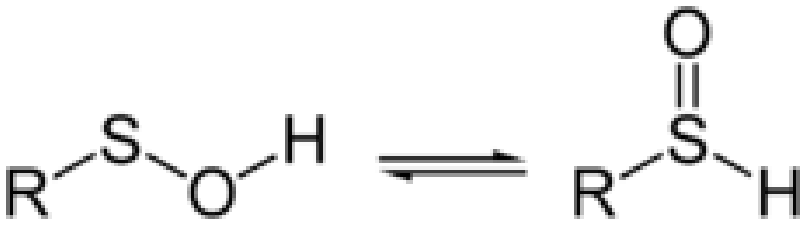
Enzyme	Preferred cleavage site[b] N-terminal	C-terminal
Serine proteases		
Trypsin	–Arg (or Lys)–	–Yaa–
Achromobacter protease	–Lys–	–Yaa–
Chymotrypsin, subtilisin	–Trp (or Tyr, Phe, Leu)–	–Yaa–
Elastase, α-lytic protease	–Ala (or Ser)–	–Yaa–
Proline-specific protease	–Pro–	–Yaa–
Staphylococcus V8 protease	–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!

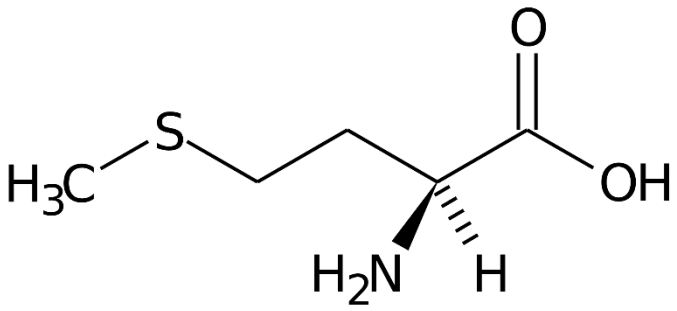
Cysteine oxidation.

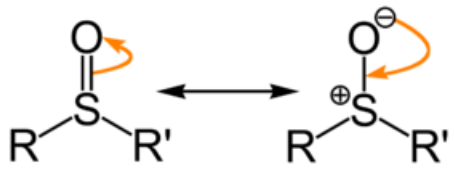
Cysteines are the most reactive amino acids in proteins. Their autooxidation, usually catalyzed by metal cations (especially copper), leads to the formation of intramolecular and intermolecular disulfide bridges or to the formation of sulfenic acid .



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.

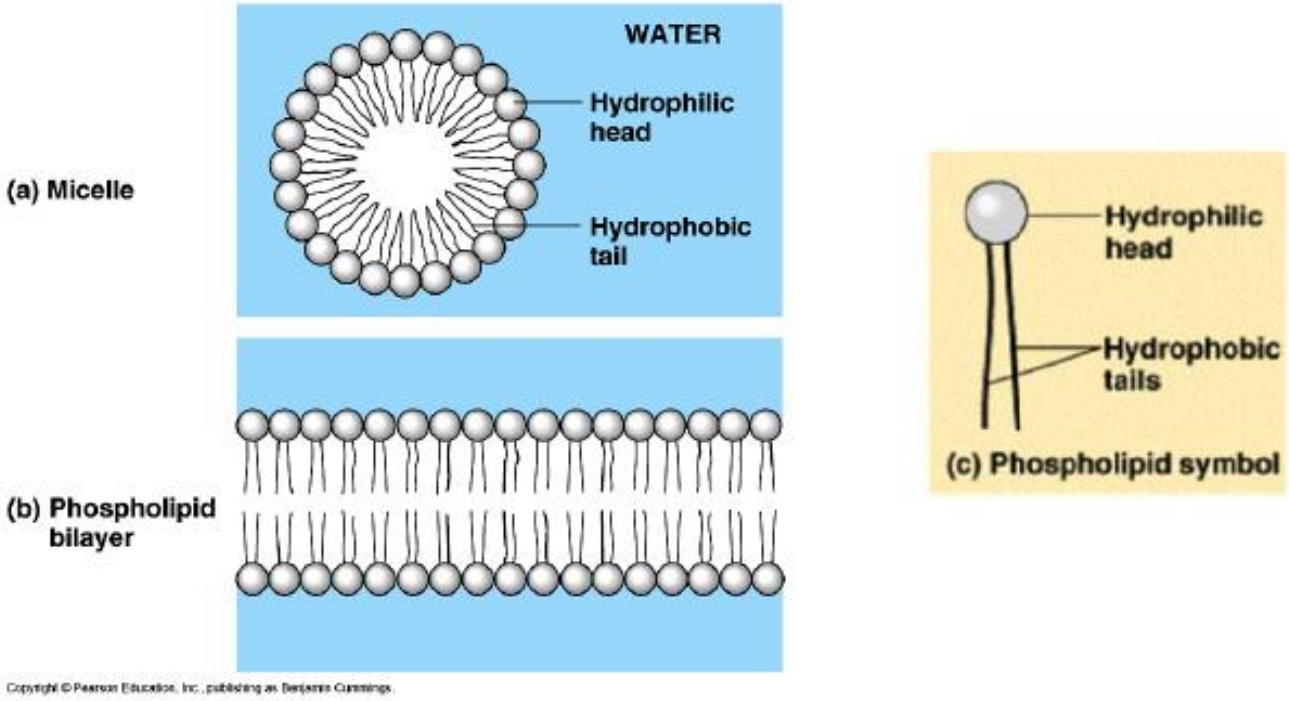
b) Irreversible folding process : kinetic stability

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

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

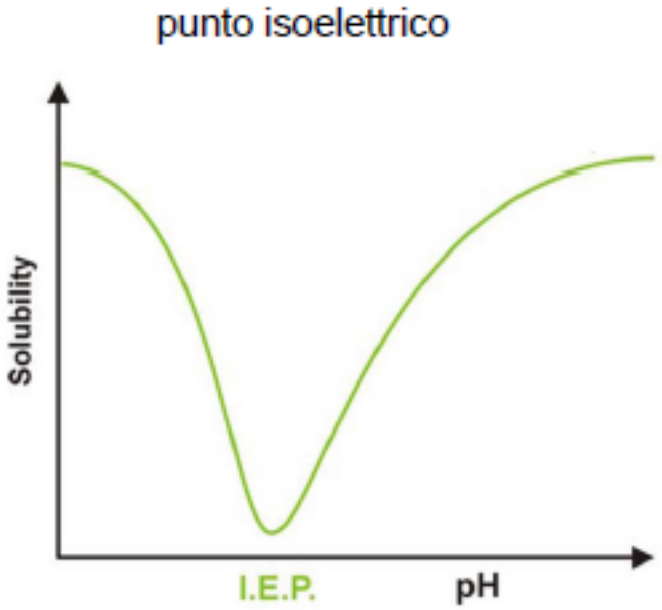
pH effect

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)

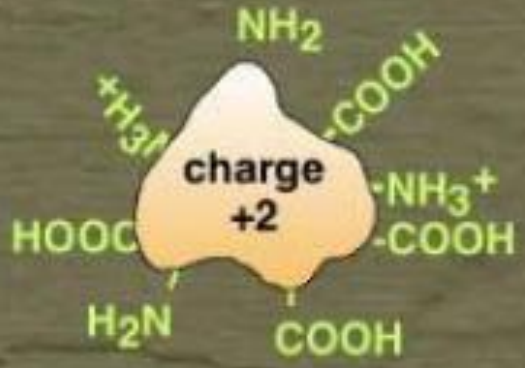
pK_a values of some amino acids

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

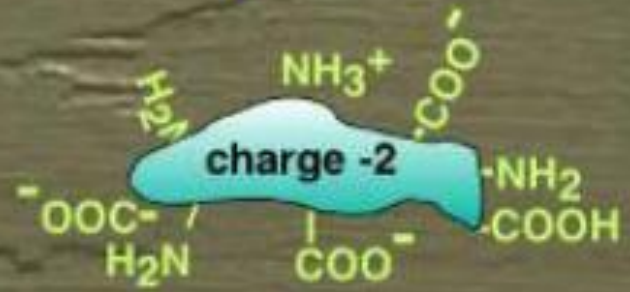


Effect of pH on proteins

lower
pH

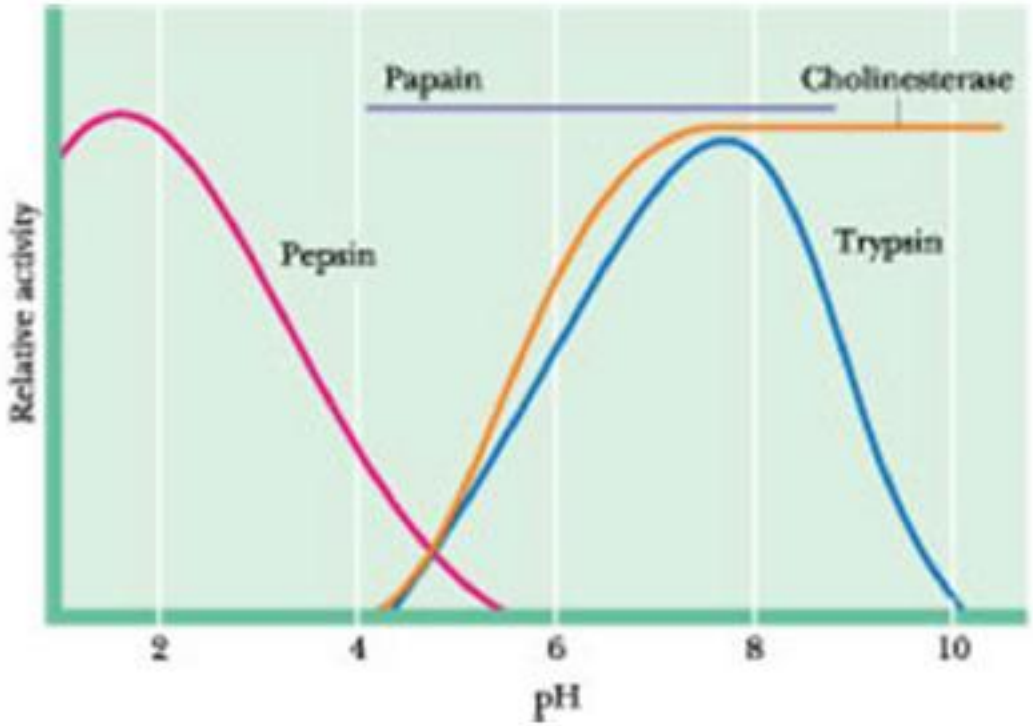


raise
pH



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