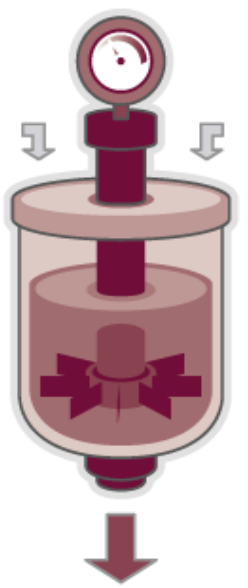
Evaluating a biocatalysts for given process:

1.Biocatalyst productivity

2.Reactor productivity

3.Product yield

4.Biocatalyst stability



1. Biocatalyst productivity: turnover number

,

In catalysis it refers to:

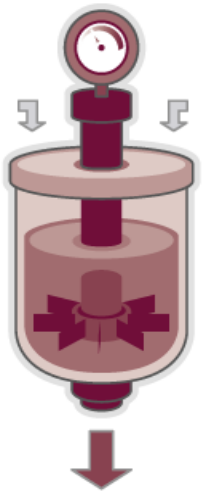
the number of moles of product formed per mole of catalyst over the reaction period.

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

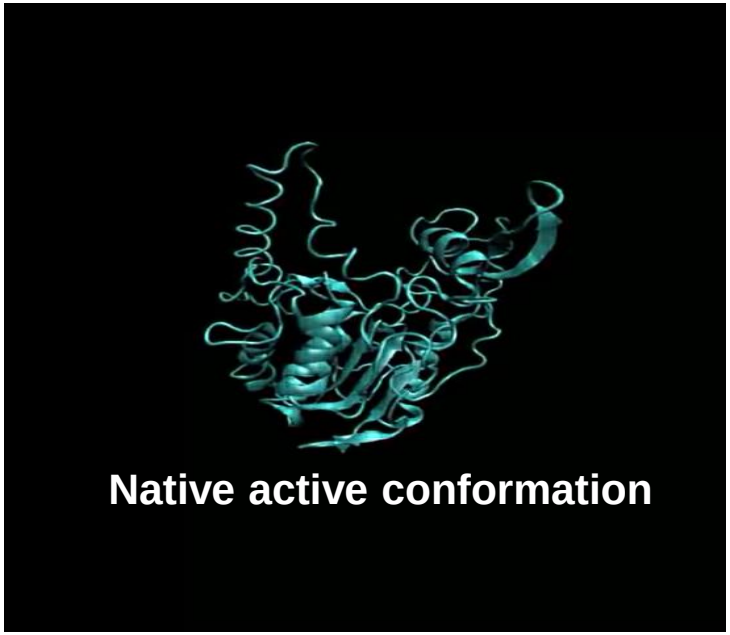
- Inactivation under operational conditions

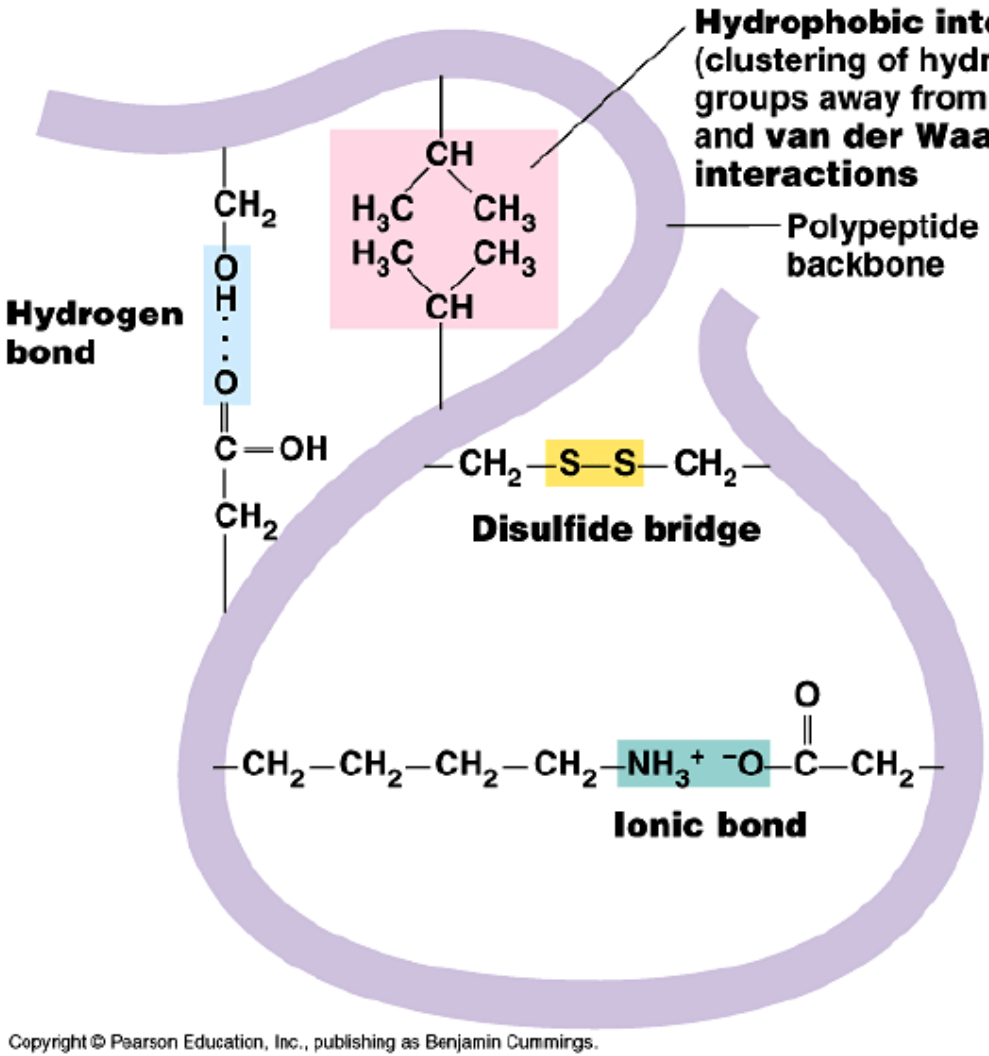
Possibly stability should be studied under conditions as closer as possible to the final operational system

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.



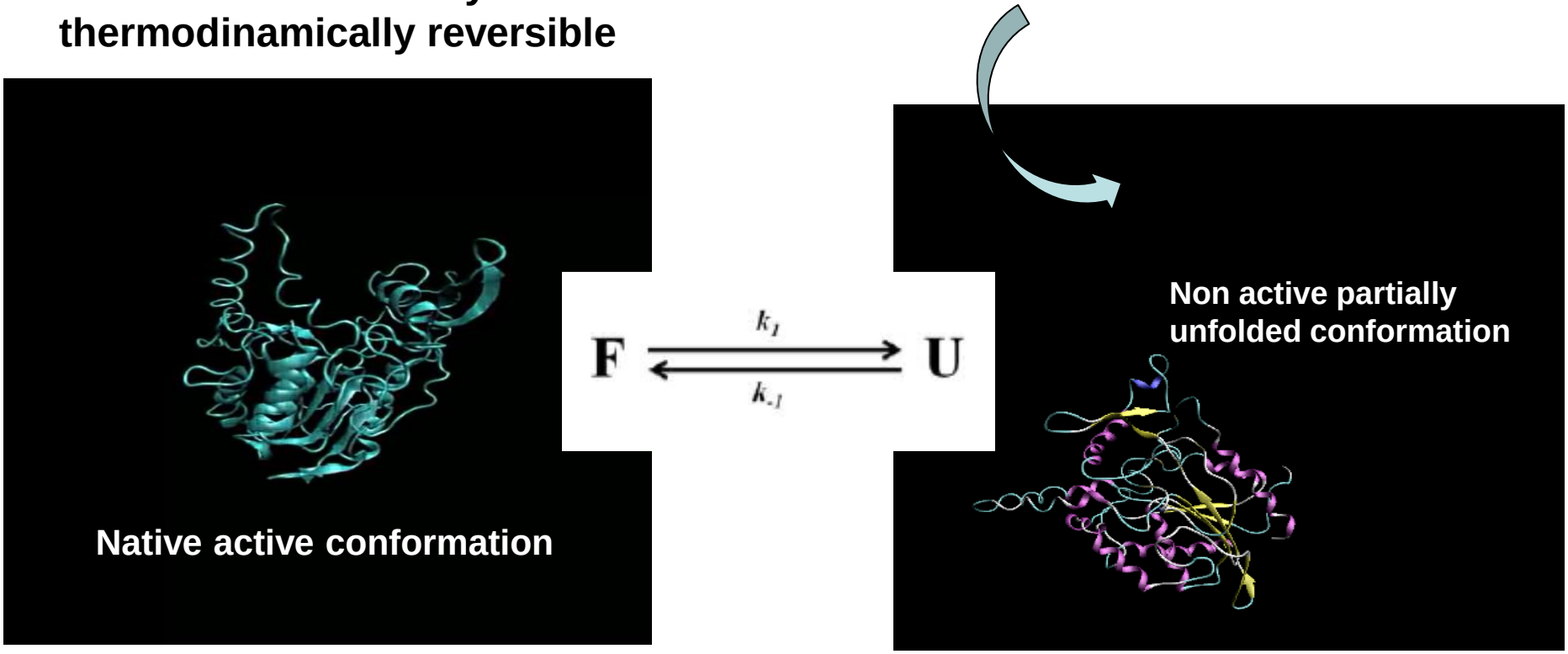


Such hydrophobic collapse is necessarily accompanied and guided by (secondary) structural hydrogen-bond formation between favorable peptide linkages in parallel with their desolvation .

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



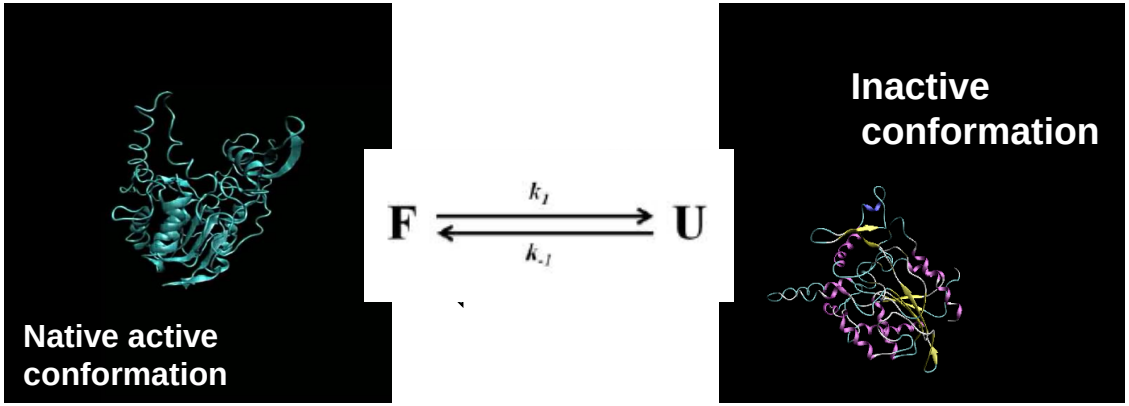
Thermodynamic stability

Thermodynamic free energy changes between the folded state and unfolded state using the free energy equation:

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

Difference between Gibbs free energy of the folded state and Gibbs free energy of the unfolded state.

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

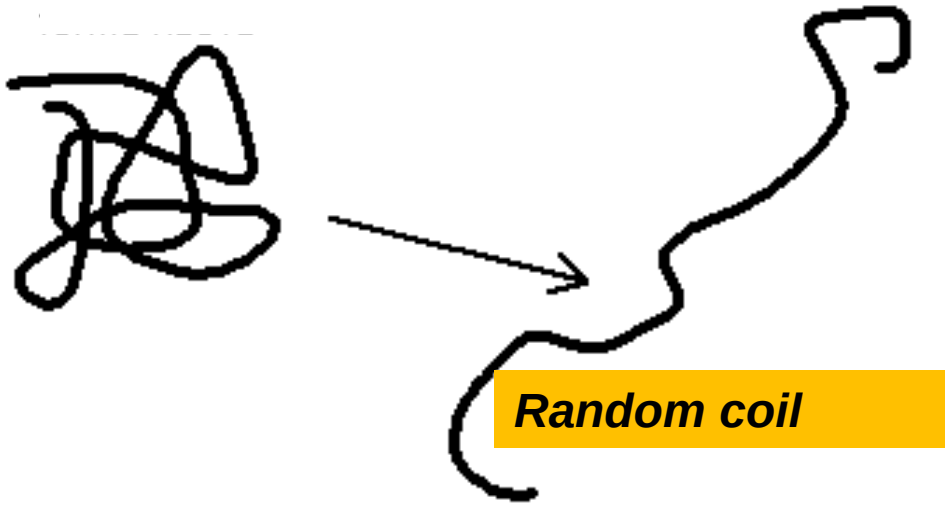


Thermodynamic stability

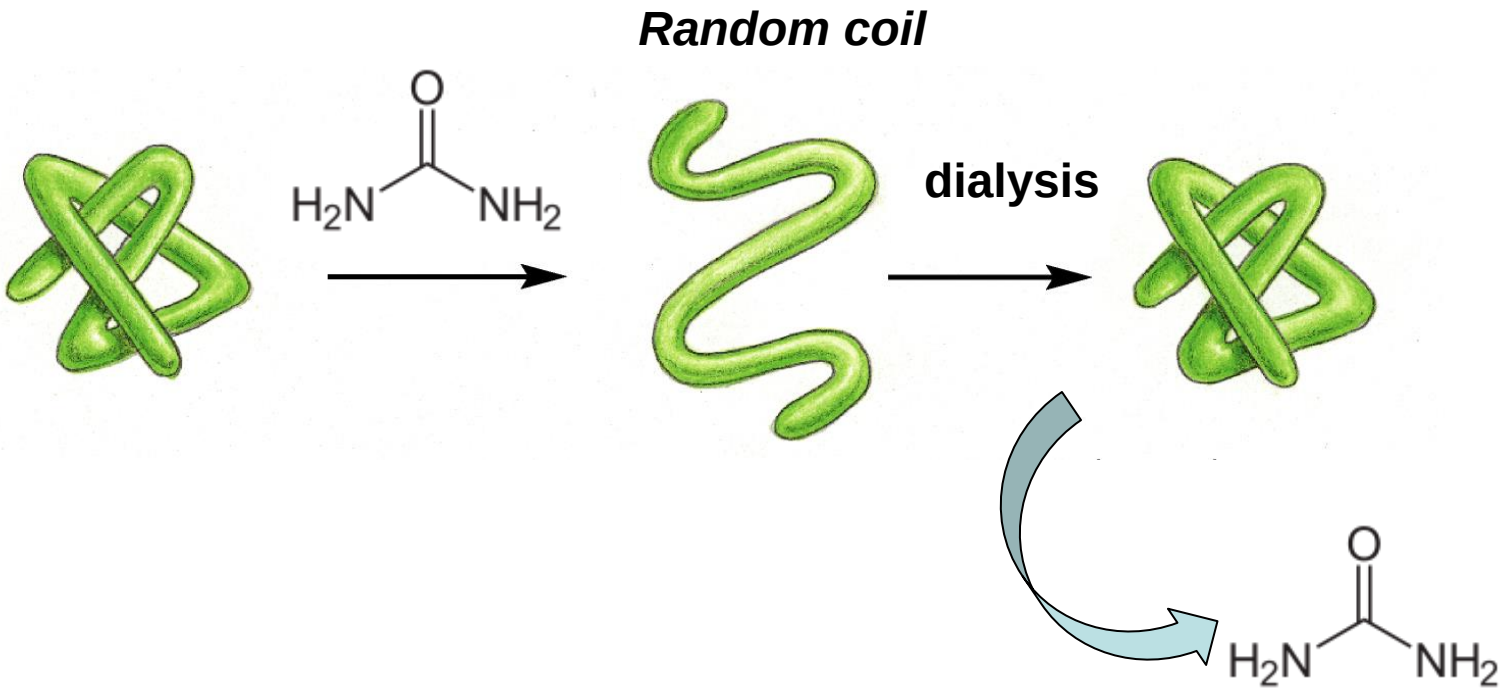
From the theoretical and experimental evidences, the folded state is only little more stable than the unfolded state and this means that small negative value, < 20 kcal/mol, can determine the stability of a protein.

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

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.



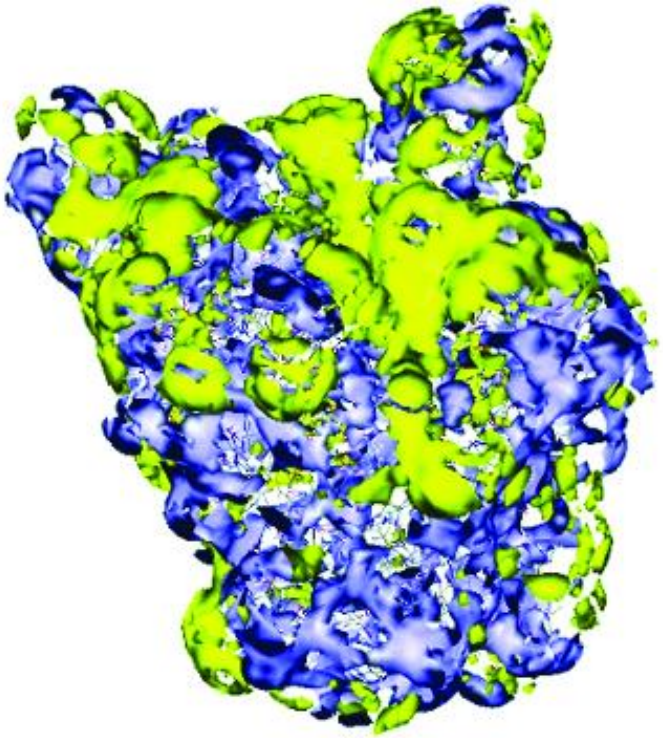
Example: reversible denaturation with urea



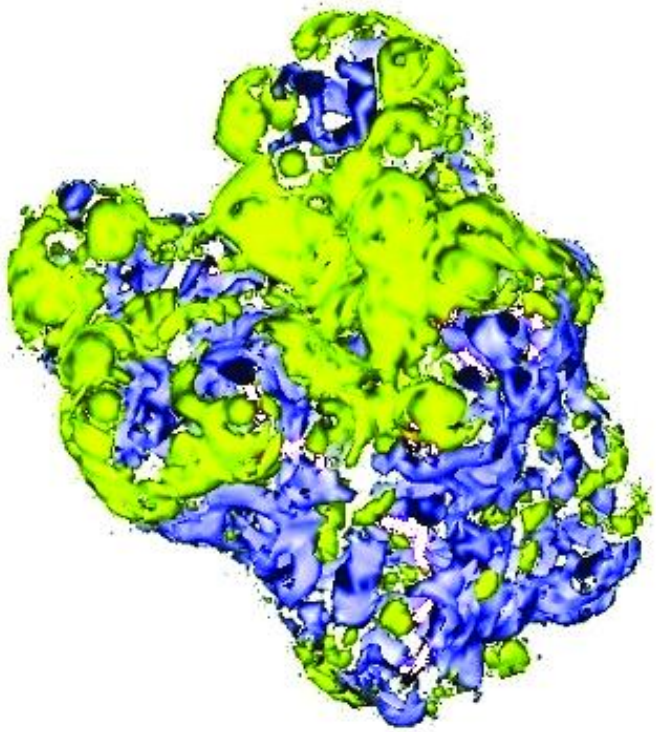
Conformational modifications sometimes are necessary for displaying enzyme activity

Lipases undergo activation at the water-lipid interface

Pseudomonas cepacia LIPASE



closed conformation in water

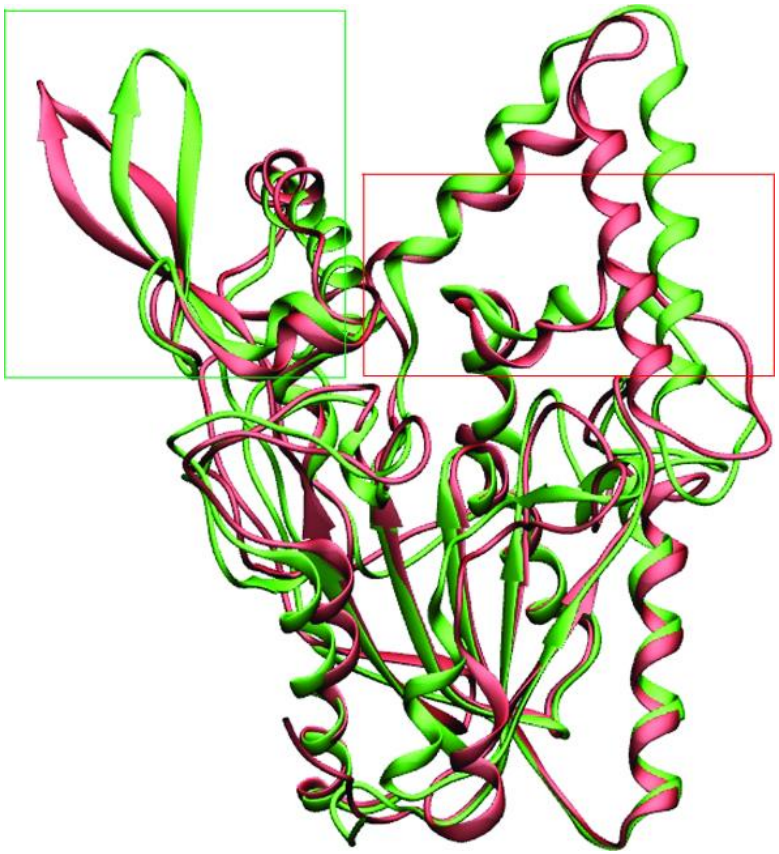


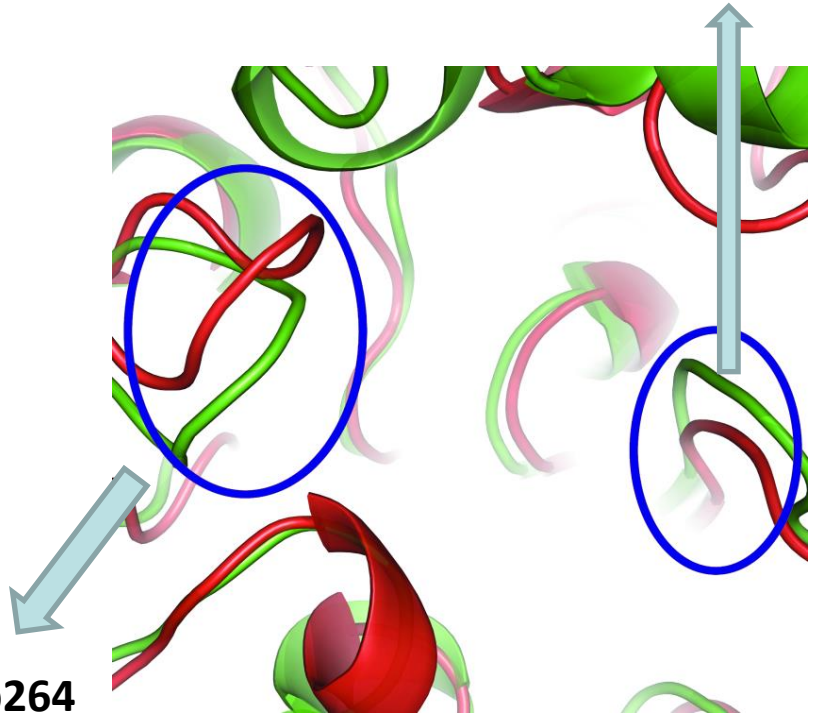
open conformation in hydrophobic media

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

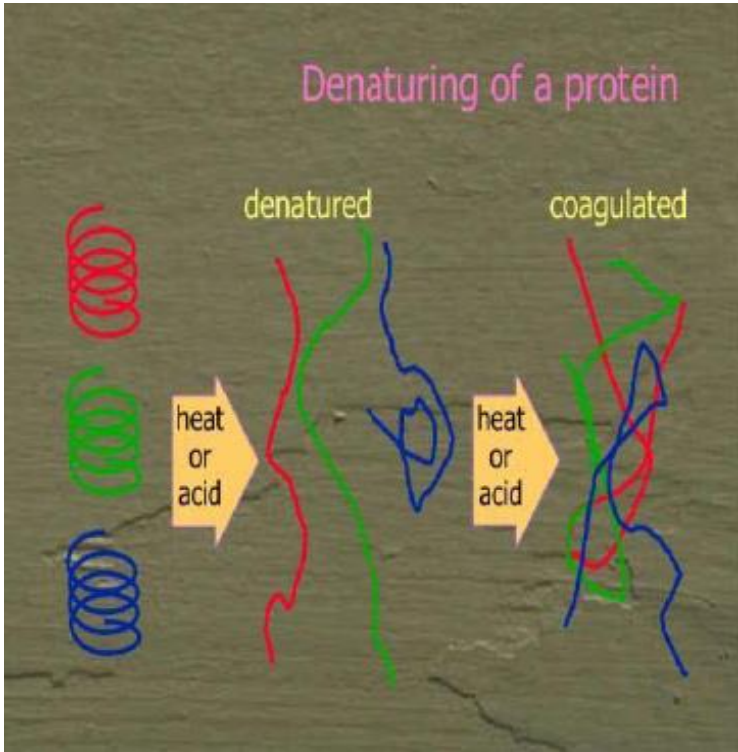
V. Ferrario & al., *Adv. Synth. Catal.,* 2011, 353, 2466.

Conformational modifications sometimes are necessary for displaying enzyme activity

Kinetic stability

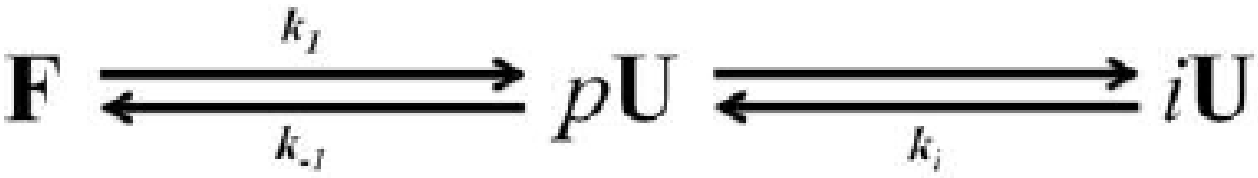
Most mesophilic proteins, however, unfold irreversibly. They unfold into inactive structures (scrambled structures), and they often form aggregates (intermolecular mechanism).

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**

Effect of temperature on enzymatic reactions

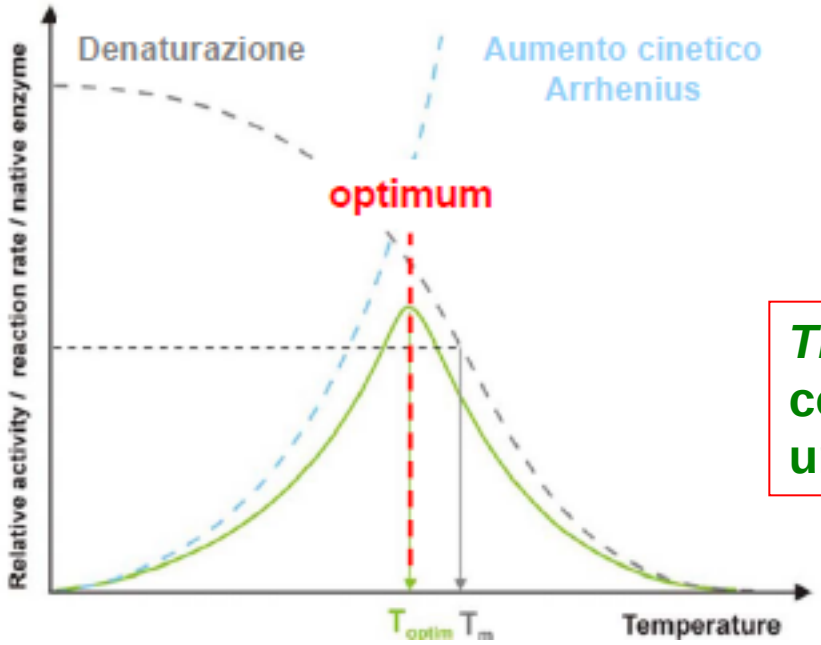
$$k = A \cdot e^{-E/RT}$$

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



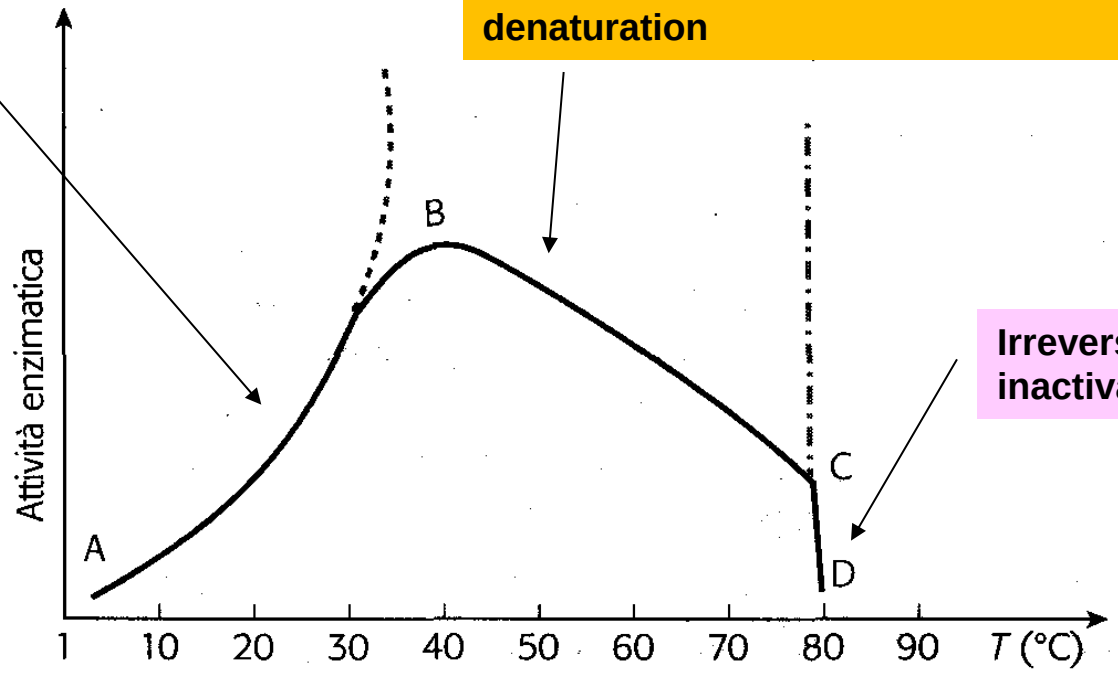
***Tm*, temperature corresponding to 50% of unfolded protein**

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



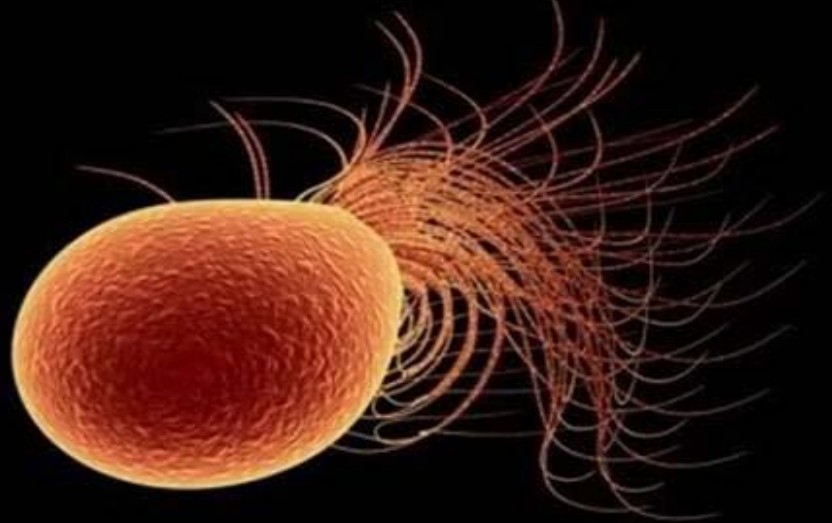
Irreversible inactivation.

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

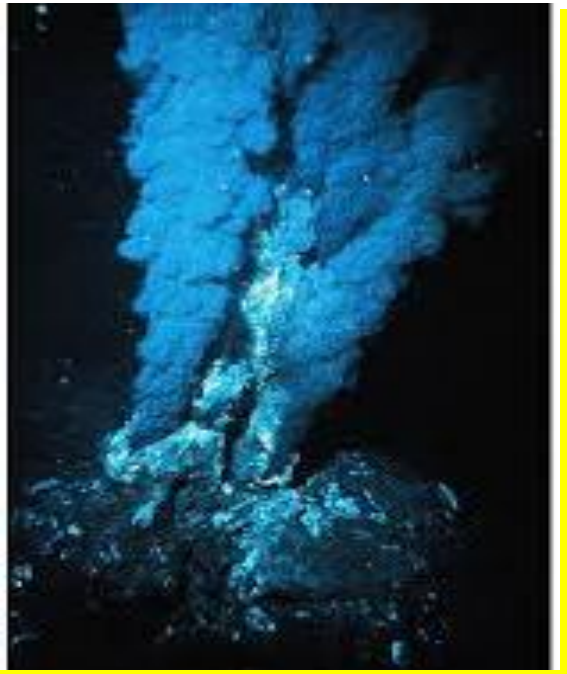
Hyperthermophiles microorganisms
have been isolated almost exclusively from environments with temperatures in the range of 80 to 115°C. Hot natural environments include continental solfataras, deep geothermally heated oil-containing stratifications, shallow marine and deep-sea hot sediments, and hydrothermal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

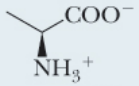
Hyperthermophilic and mesophilic enzymes are highly similar: (i) the sequences of homologous hyperthermophilic and mesophilic proteins are typically 40 to 85% similar; (ii) their three-dimensional structures are superposable ; and (iii) they have the same catalytic mechanisms

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

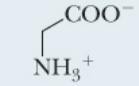
Residue(s)	Amino acid composition (%) of:		Variation of composition in hyperthermophilic relative to mesophilic proteins	
	Mesophilic proteins[b]	Hyperthermophilic proteins[c]		
A	8.09 ± 1.54	6.82 ± 1.42	−1.27	
C	1.10 ± 0.18	0.86 ± 0.27	−0.24	
D	5.06 ± 0.18	4.63 ± 0.54	−0.43	
E	6.45 ± 0.54	8.55 ± 0.95	+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	

Tabella 27.1 I 20 amminoacidi comuni che si trovano nelle proteine

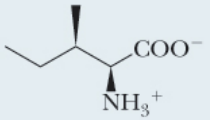
Catene laterali non polari



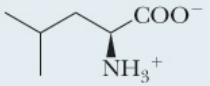
Alanina (Ala, A)



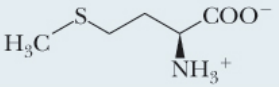
Glicina (Gly, G)



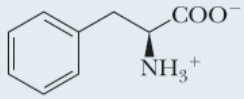
Isoleucina (Ile, I)



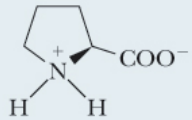
Leucina (Leu, L)



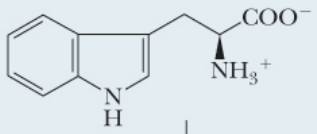
Metionina (Met, M)



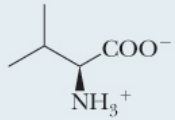
Fenilalanina (Phe, F)



Prolina (Pro, P)

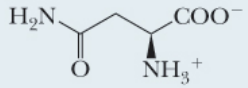


Triptofano (Trp, W)

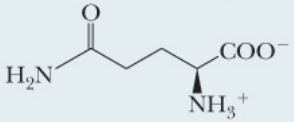


Valina (Val, V)

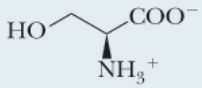
Catene laterali polari



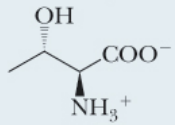
Asparagina (Asn, N)



Glutammina (Gln, Q)

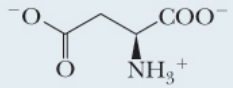


Serina (Ser, S)

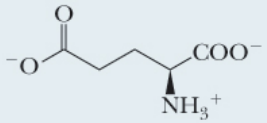


Treonina (Thr, T)

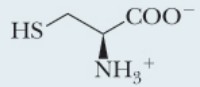
Catene laterali acide



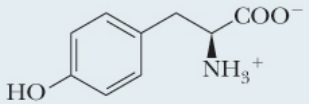
Acido aspartico (Asp, D)



Acido glutammico (Glu, E)

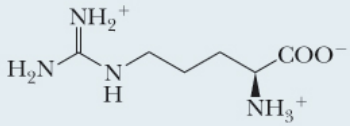


Cisteina (Cys, C)

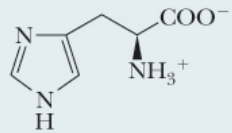


Tirosina (Tyr, Y)

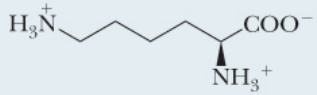
Catene laterali basiche



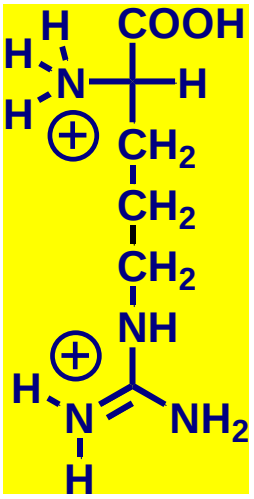
Arginina (Arg, R)



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

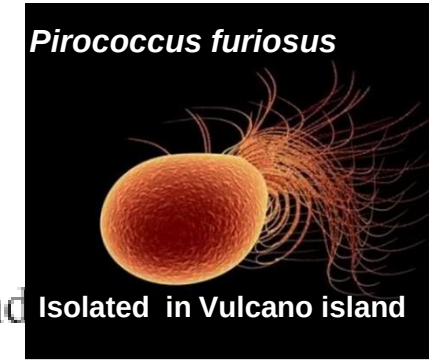


TABLE 6. Comparison of the ion pair contents of *P. furiosus* and *Clostridium symbiosum* GDHs[a]

(glutammato deidrogenasi)

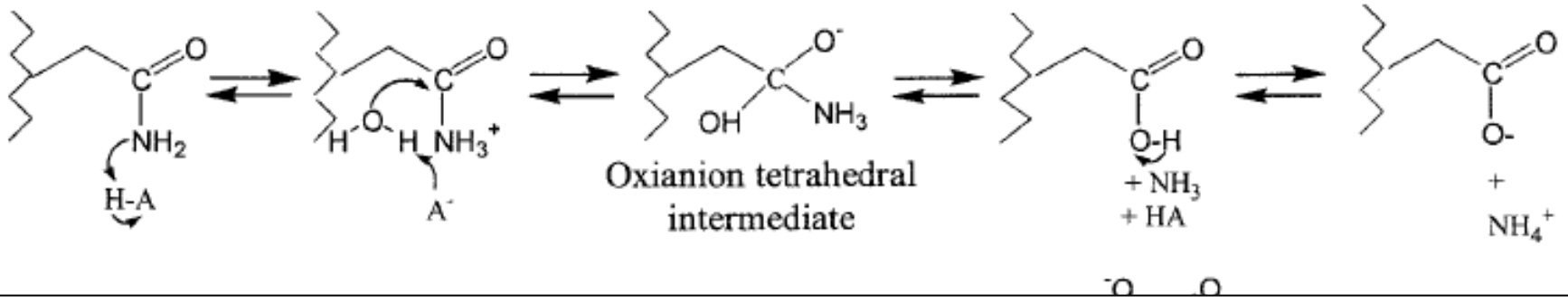
Characteristic	Value for:	
	C. symbiosum	*P. furiosus*
No. of IPs[b] per subunit	26	45
No. of IPs per residue	0.06	0.11
% of charged residues forming IPs	40	54
% of IPs formed by Arg/Lys/His	46/31/23	64/27/9
% of IPs formed by Asp/Glu	46/54	47/53
% of all Arg forming IPs	55	90
No. of residues forming 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 268 with permission of the publisher.

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 (ONH) 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.

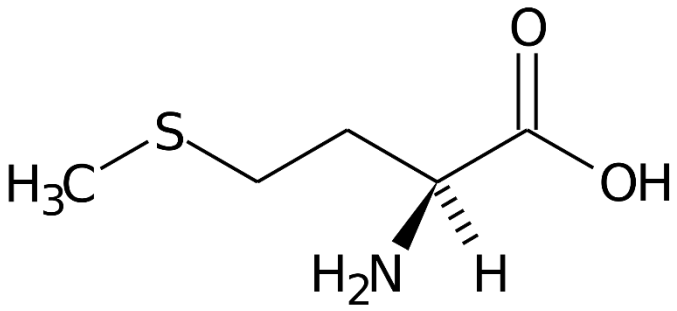
Protases must be stable against proteolitic attack!

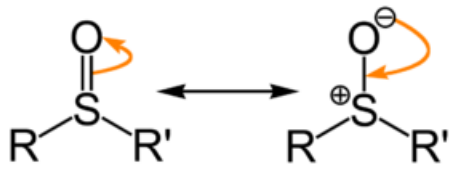
Cysteine oxidation.

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**
- **Pressure**
- **Mixing**
- **Interfaces (adsorption of proteins)**
- **Shear force**
- **Denaturing agents (urea)**

Practical and technical applications of thermostable enzymes

- **higher reaction rate at higher T**
- **lower resk of microbial contaminations**

Bioconversion reactions and applications of thermostable enzymes

Enzyme	Temperature range (°C)	Bioconversions	Applications
α-Amylase (bacterial)	90–100	Starch → dextrose syrups	Starch hydrolysis, brewing, baking, detergents
α-Amylase (fungal)	50–60	Starch → dextrose syrups	Production of maltose
Pullulanase	50–60	Starch → dextrose syrups	Production of glucose syrups
Xylanase	45–65, 105[a]	Craft pulp → xylan + lignin	Pulp and paper industry
Chitinase	65–75[b]	Chitin → chitobiose	Food, cosmetics, pharmaceuticals, agrochemicals
		Chitin → *N*-acetyl glucosamine (chitibiase)	
		N-acetyl glucosamine → glucosamine (deacetylation)	
		Chitin → chitosan (deacetylase)	
Cellulase	45–55, 95[c]	Cellulose → glucose	Cellulose hydrolysis, polymer degradation in detergents
Protease	65–85	Protein → amino acids and peptides	Baking, brewing, detergents, leather industry
Lipase	30–70	Fat removal, hydrolysis, interesterification, alcholysis, aminolysis	Dairy, oleo chemical, detergent, pulp, pharmaceuticals, cosmetics and leather industry
DNA polymerase	90–95	DNA amplification	Genetic engineering/PCR

DNA polymerase from *Thermus aquaticus*: Taq polymerase

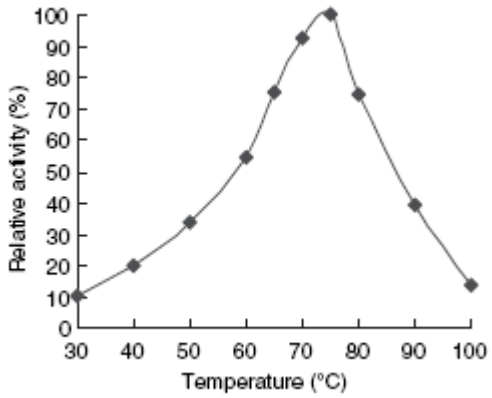
XYLANASE

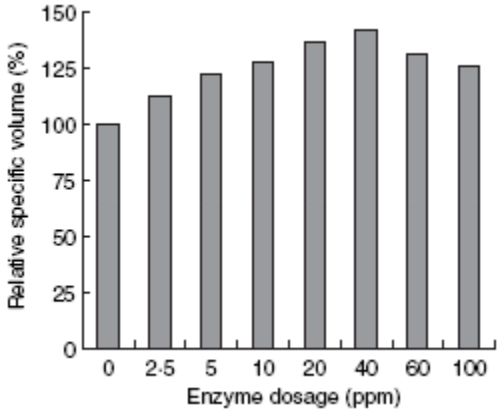
Pulp and paper industry

Bakery

Higher quality of bread

Enzyme active at 60-75°C , also during cooking





Proteases in detergents

Source microorganisms and properties of thermostable proteolytic enzymes

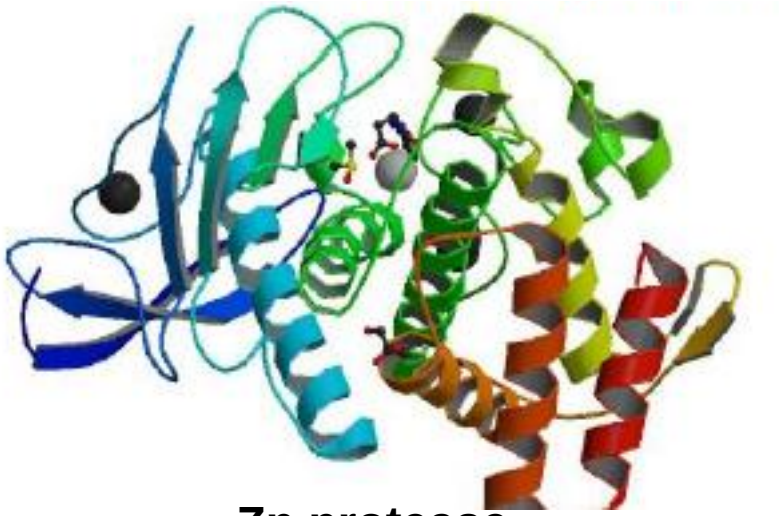
Organism	Enzyme properties	
	Optimal temperature (°C)	Optimal pH
Bacillus brevis	60	10.5
Bacillus licheniformis	70	9.0
Bacillus stearothermophilus	60	–
Bacillus stearothermophilus	85	–
Bacillus sp. JB-99	80	6–12
Bacillus stearothermophilus TP26	75	–
Bacillus sp. no. AH-101	80	12.0–13.0
Bacillus thermoruber	45	9
Pyrococcus sp. KODI	100	7
Staphylothermus marinus	–	9
Thermoacidophiles (archeal and bacterial origin)	60–70	7.0–8.5
Thermococcus aggreganes	90	7.0
Thermococcus celer	95	7.5
Thermococcus litoralis	85	8.5
Thermotoga maritema	95	9.5

Proteases in detergents

Bacillus thermoproteolyticus neutral proteinase

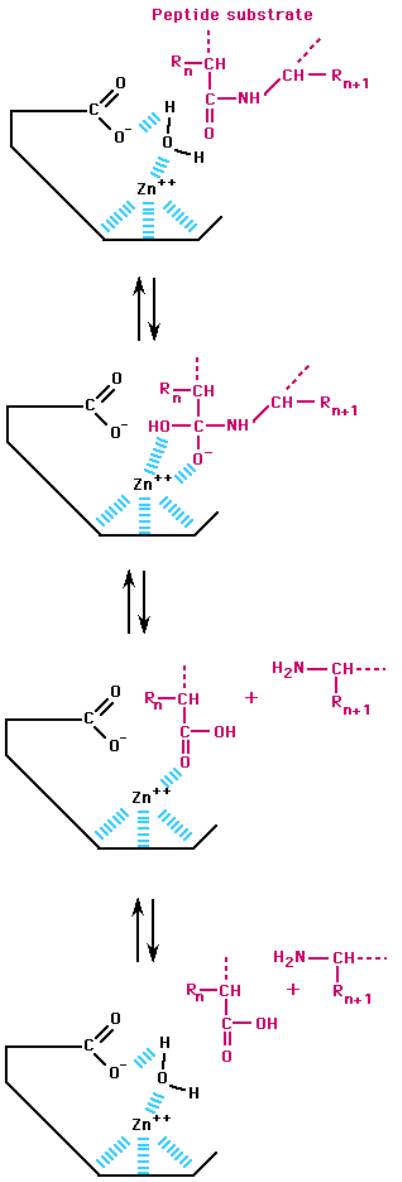
Thermolysine

Stable at 50-60°C



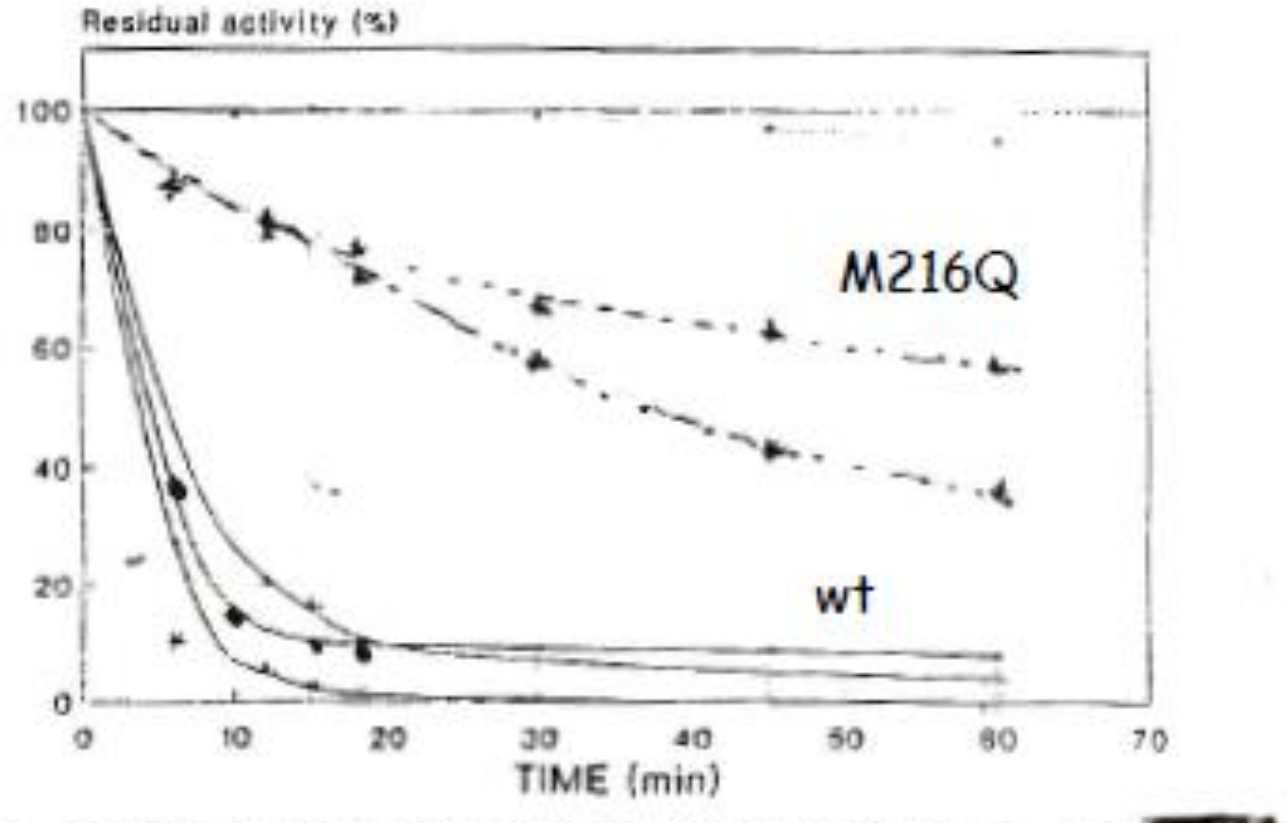
Zn protease

Catalytic mechanism of metallo proteinases



Stabilization of a protease towards oxidatve agents (peracids and H2O2): Genetic engineering

Mutation of Metionine into Glutamine



Thermostable DNA polymerase in molecular biology

DNA polymerase

Recombinant DNA

- DNA molecules formed by laboratory methods of genetic recombination to bring together genetic material from multiple sources
- Proteins resulting from the expression of recombinant DNA within living cells are termed *recombinant proteins*
- Expression of foreign proteins requires the use of specialized *expression vectors*

The first prerequisite is to obtain a sufficient DNA quantity (amplification)

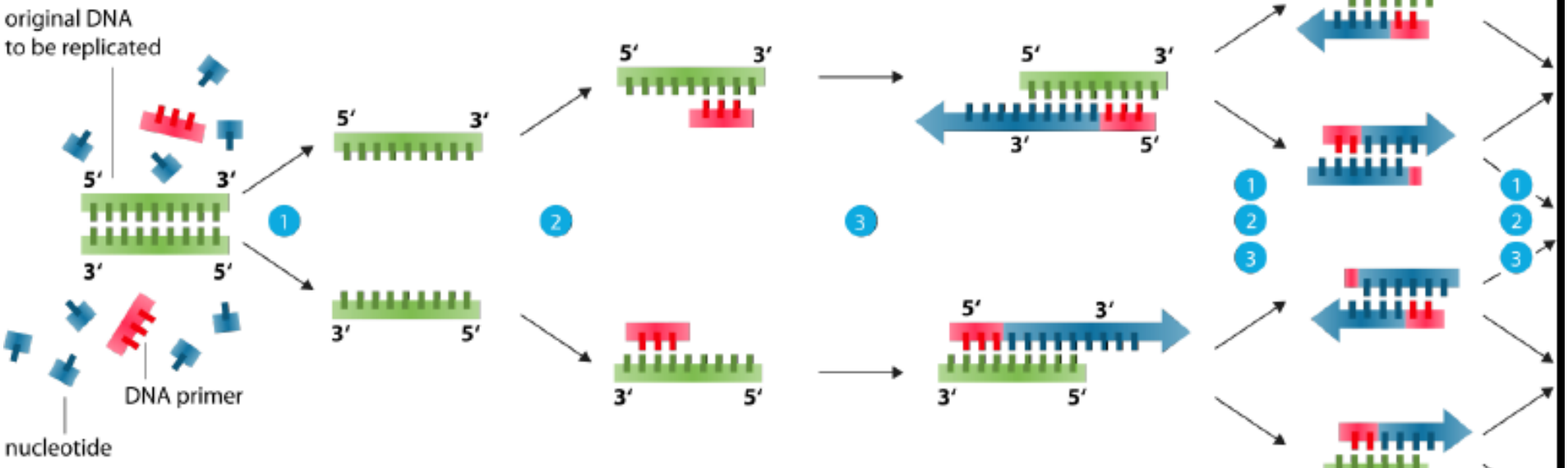
DNA amplification is obtained by *molecular cloning* or by PCR (Polymer Chain Reaction)

PCR - Polymerase Chain Reaction

- PCR is a technology used mainly for amplification and/or isolation of specific DNA sequences
- The method relies on thermal cycling, consisting of cycles of repeated heating and cooling of the reaction for DNA melting and enzymatic replication of the DNA
- PCR employ a thermostable DNA polymerase, such as Taq polymerase (DNA polymerase from *Thermus aquaticus*)

Taq polymerase enzymatically assembles a new DNA by using single-stranded DNA as a template and DNA oligonucleotides (DNA primers), which are required for initiation of DNA synthesis

PCR - Polymerase Chain Reaction



1. **Denaturation** at 94-96°C
2. **Annealing** at ~68°C
3. **Elongation** at ca. 72 °C

Molecular cloning

1. Choice of host organism and cloning vector
2. Preparation of cloning DNA vector
3. Introduction of cloning vector into host organism
4. Screening for clones with desired DNA inserts

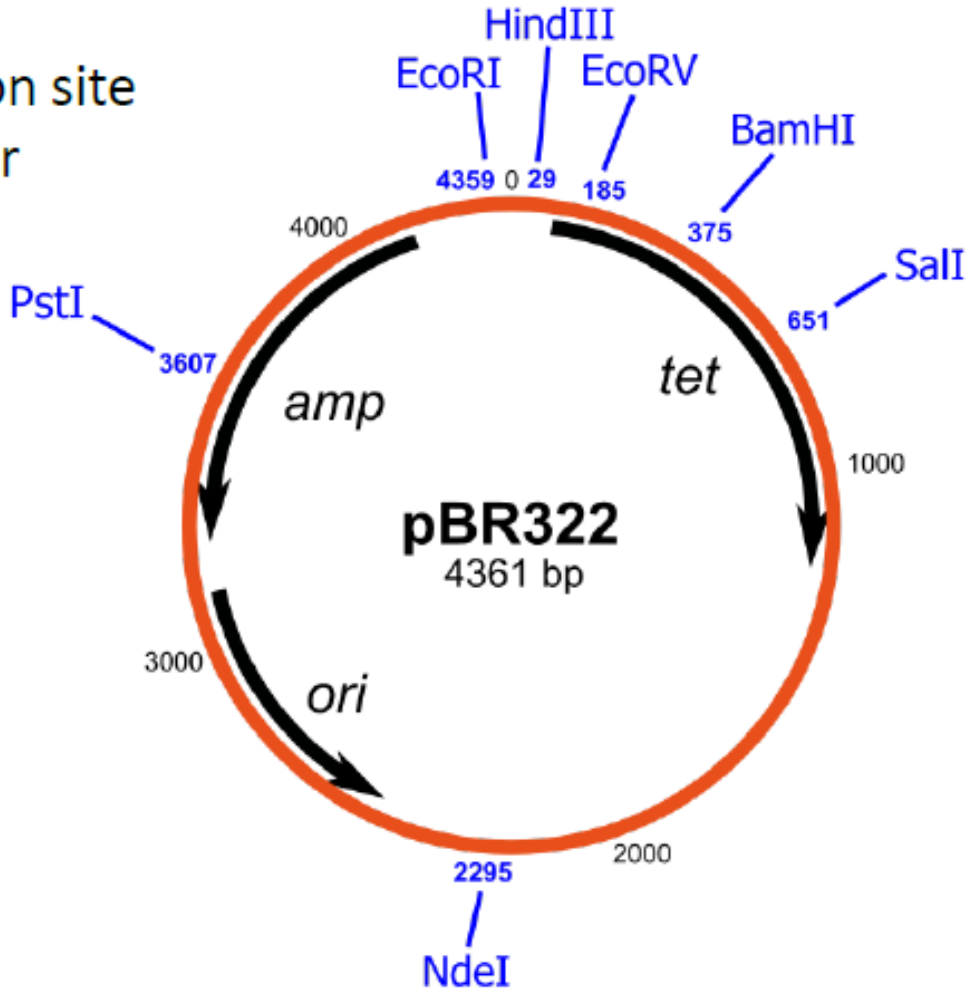
The great majority of molecular cloning experiments begin with a laboratory strain of the bacterium *Escherichia coli* and a *plasmid* cloning vector

A plasmid is a small DNA molecule physically separated from a chromosomal DNA that can replicate independently

Plasmid vector

Essential plasmid elements:

- Origin of replication
- At least one cloning/restriction site
- At least one selectable marker

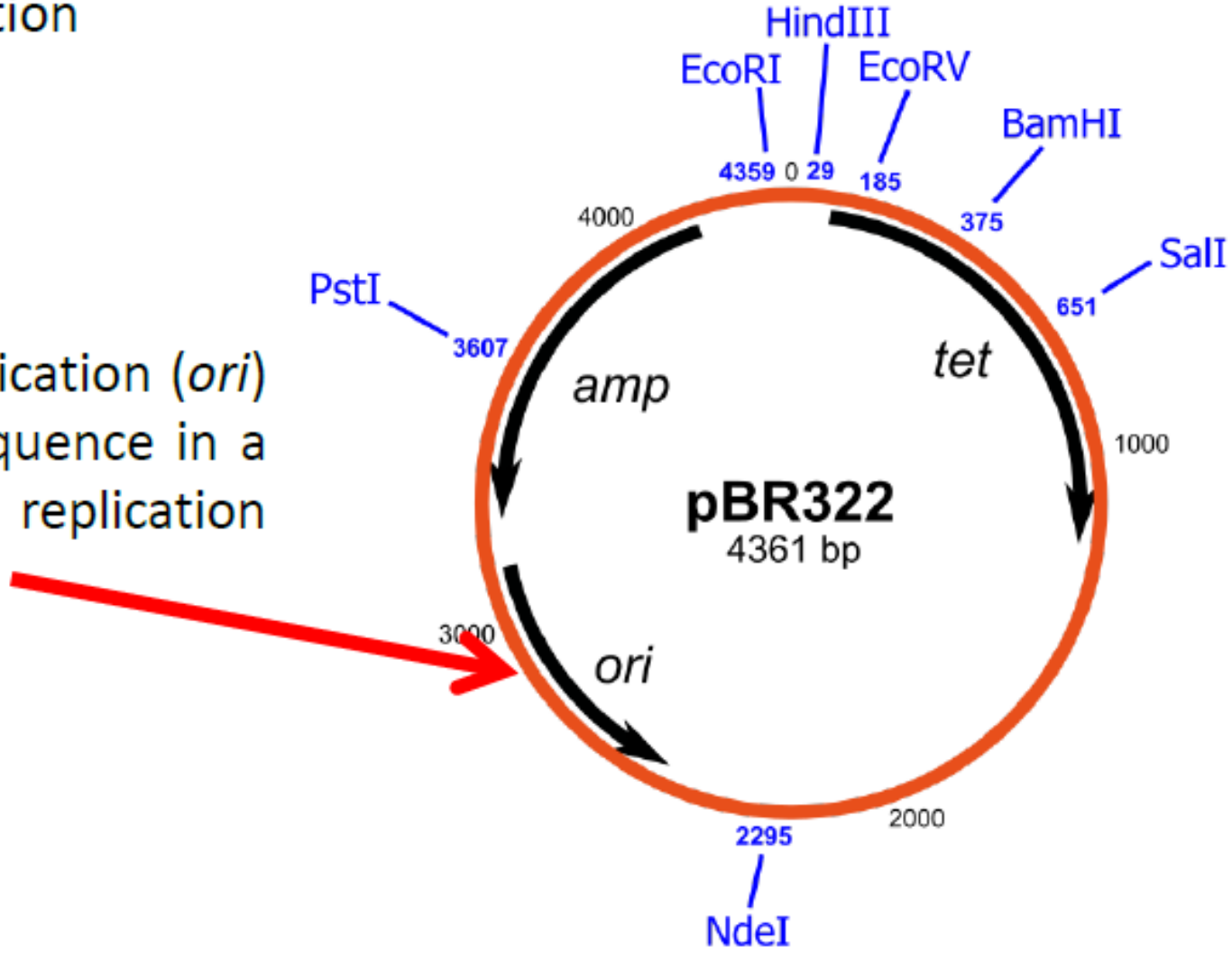


Plasmid vector

Essential plasmid elements:

- Origin of replication

The origin of replication (*ori*) is a particular sequence in a genome at which replication is initiated

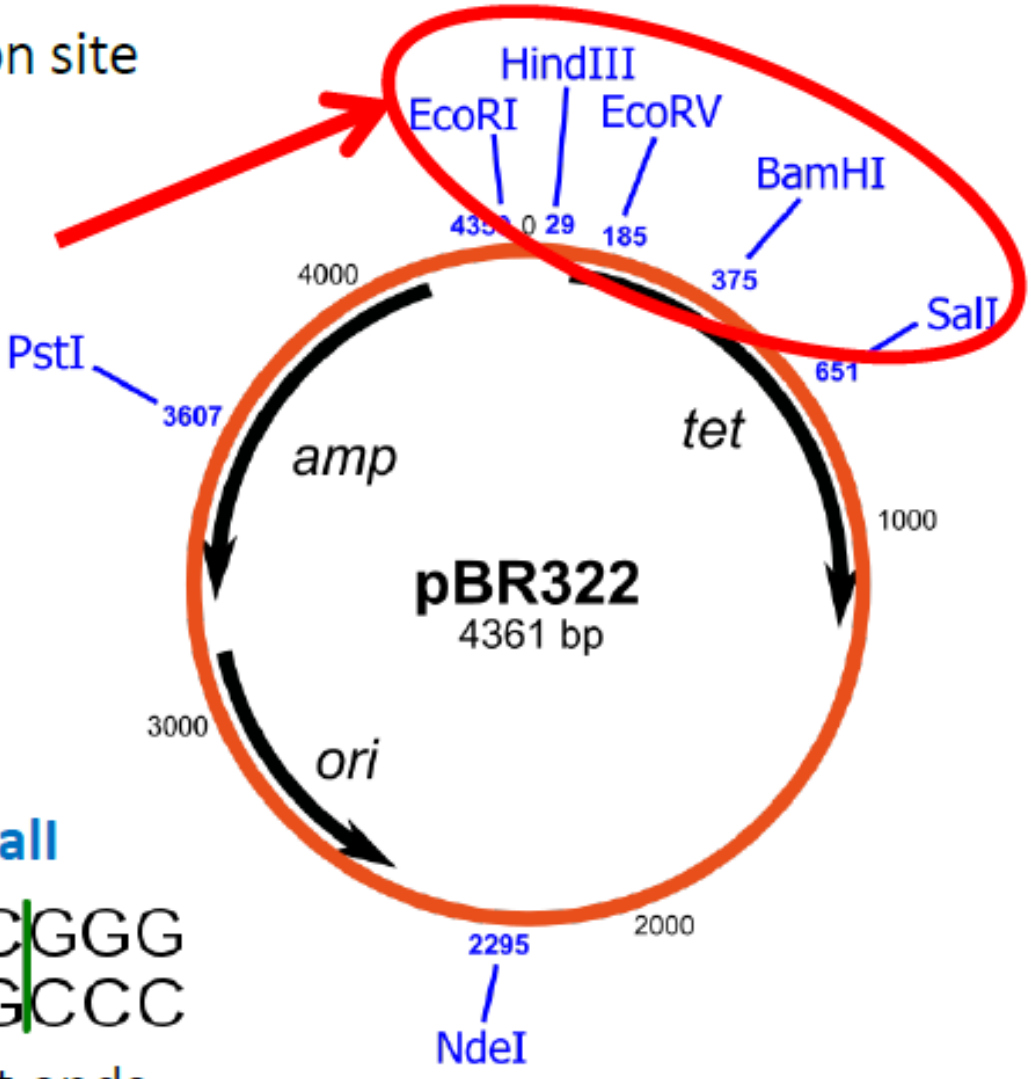


Plasmid vector

Essential plasmid elements:

- At least one cloning/restriction site

A restriction site is a nucleotide sequence specifically recognized by endonuclease enzymes (or restriction enzymes) that cuts DNA at or near the restriction sites



EcoRI

G A A T T C
C T T A A G

Sticky ends

SalI

C C C G G G
G G G C C C

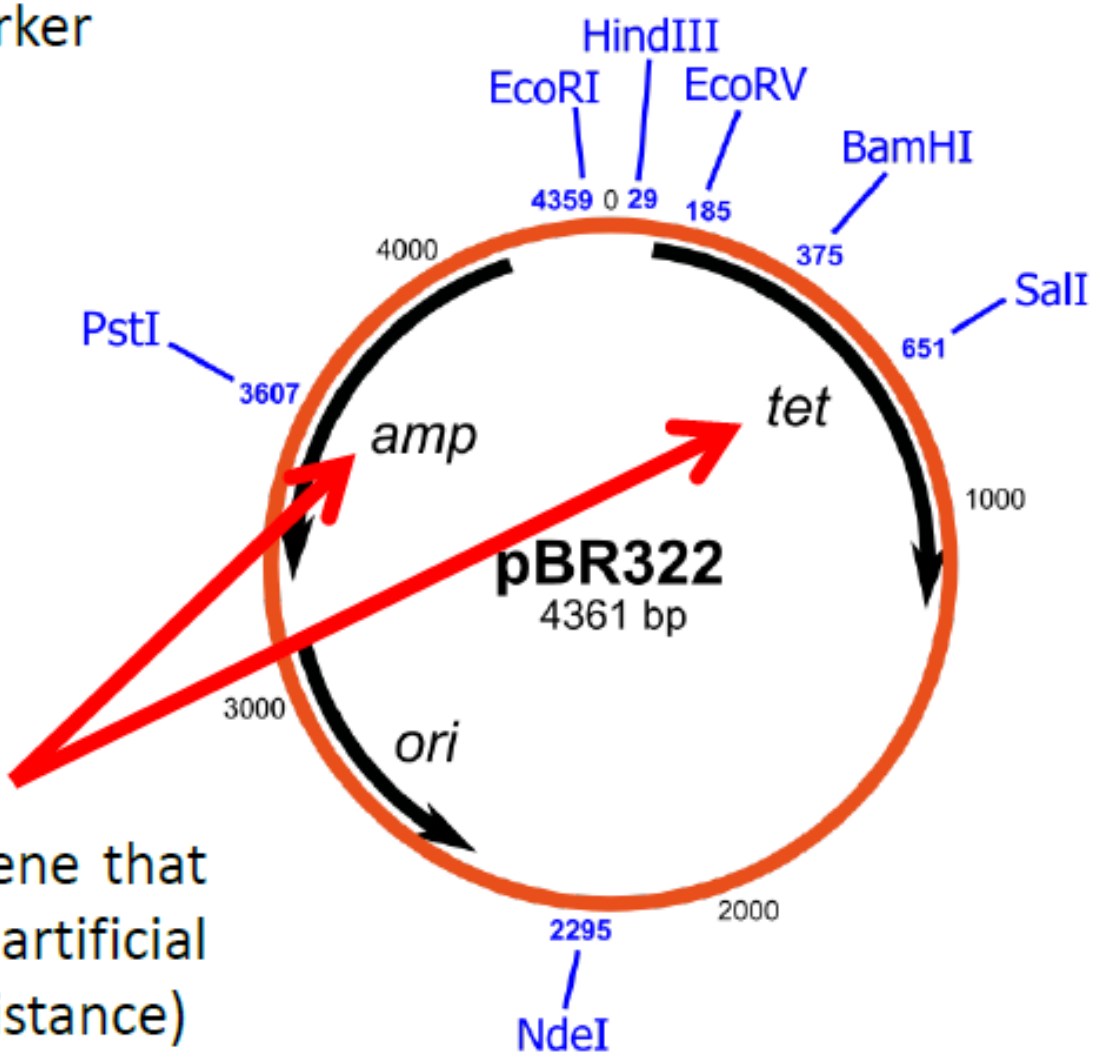
Blunt ends

Plasmid vector

Essential plasmid elements:

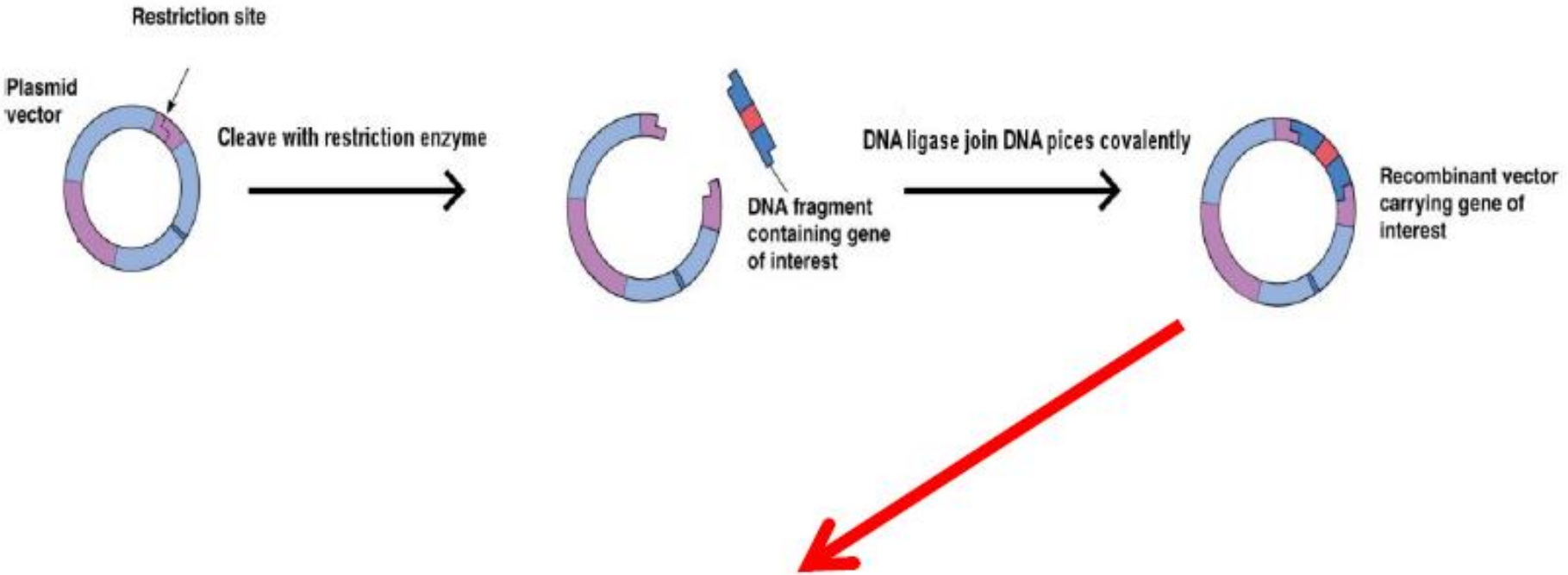
- At least one selectable marker

tet = tetracicline resistance
amp= ampicillin resistance

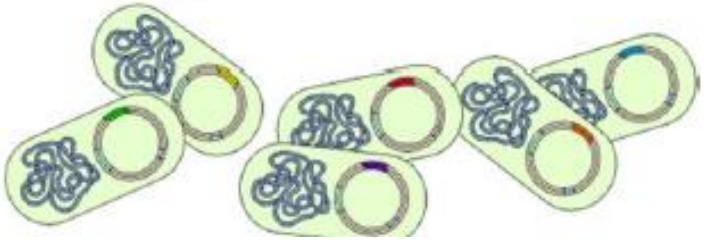


A selectable marker is a gene that confers a trait suitable for artificial selection (i.e. antibiotic resistance)

DNA insertion



Plasmid is inserted into the cloning host (i.e. *E. coli*)

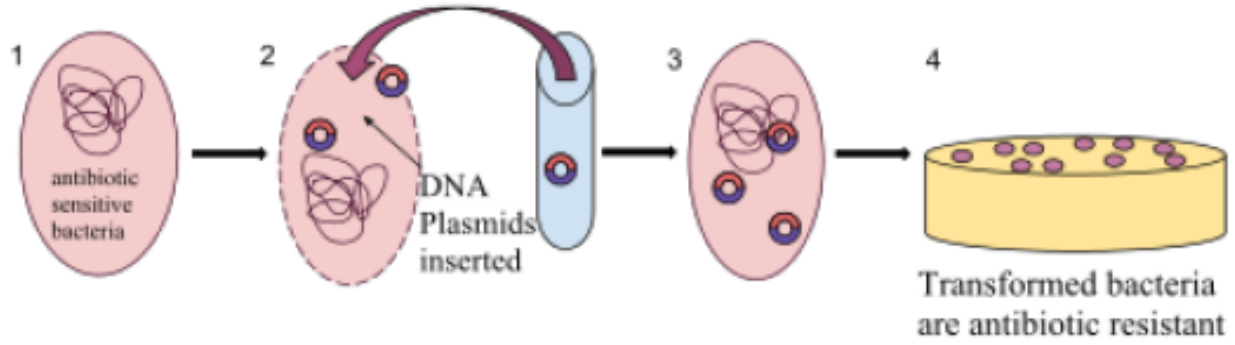


Cell transformation
Insert exogenous DNA in host microorganism

Main techniques:

- Heat shock
- Divalen cations ($CaCl_2$)
- Electroporation (electric field of 10-20 kV/cm)
- Enzyme treatment

Increase cell permeation



Glycosylation of proteins is a natural tool for increasing protein stability

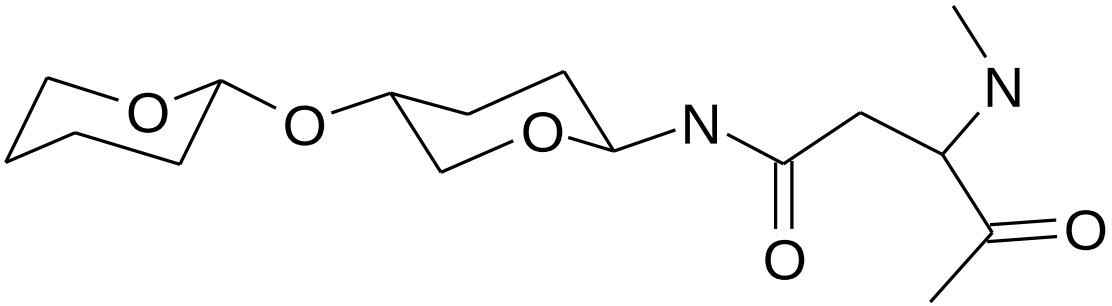
Several roles have been suggested for the carbohydrate moieties of glycoproteins among which

- **stabilization of protein conformation,**
- **protection from proteolysis**
- **Prevention of aggregation**

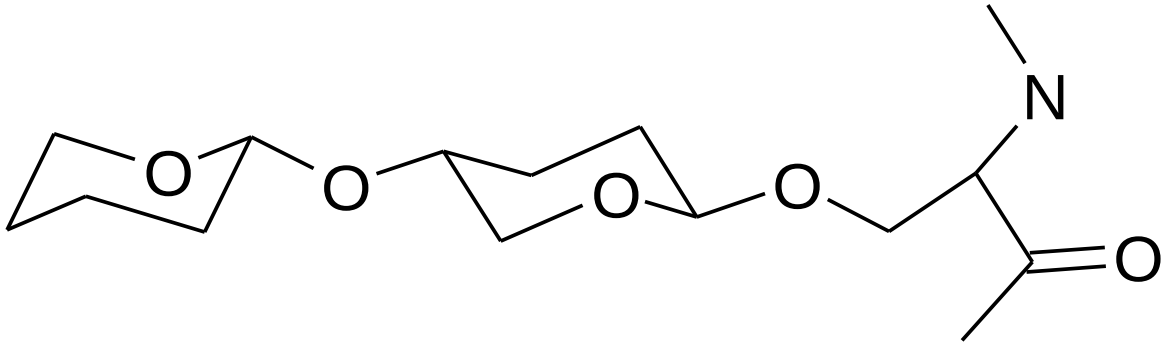
There are two main type of protein glycosylation:

- **N-glycosylation, in which the glycan (10-35 mannose residues) is attached to an Asn residue present in the tripeptide consensus sequon Asn-XSer/Thr (where X can be any amino acid except Pro),**

- **O-glycosylation, in which the glycan is attached to a Ser or Thr residue.**

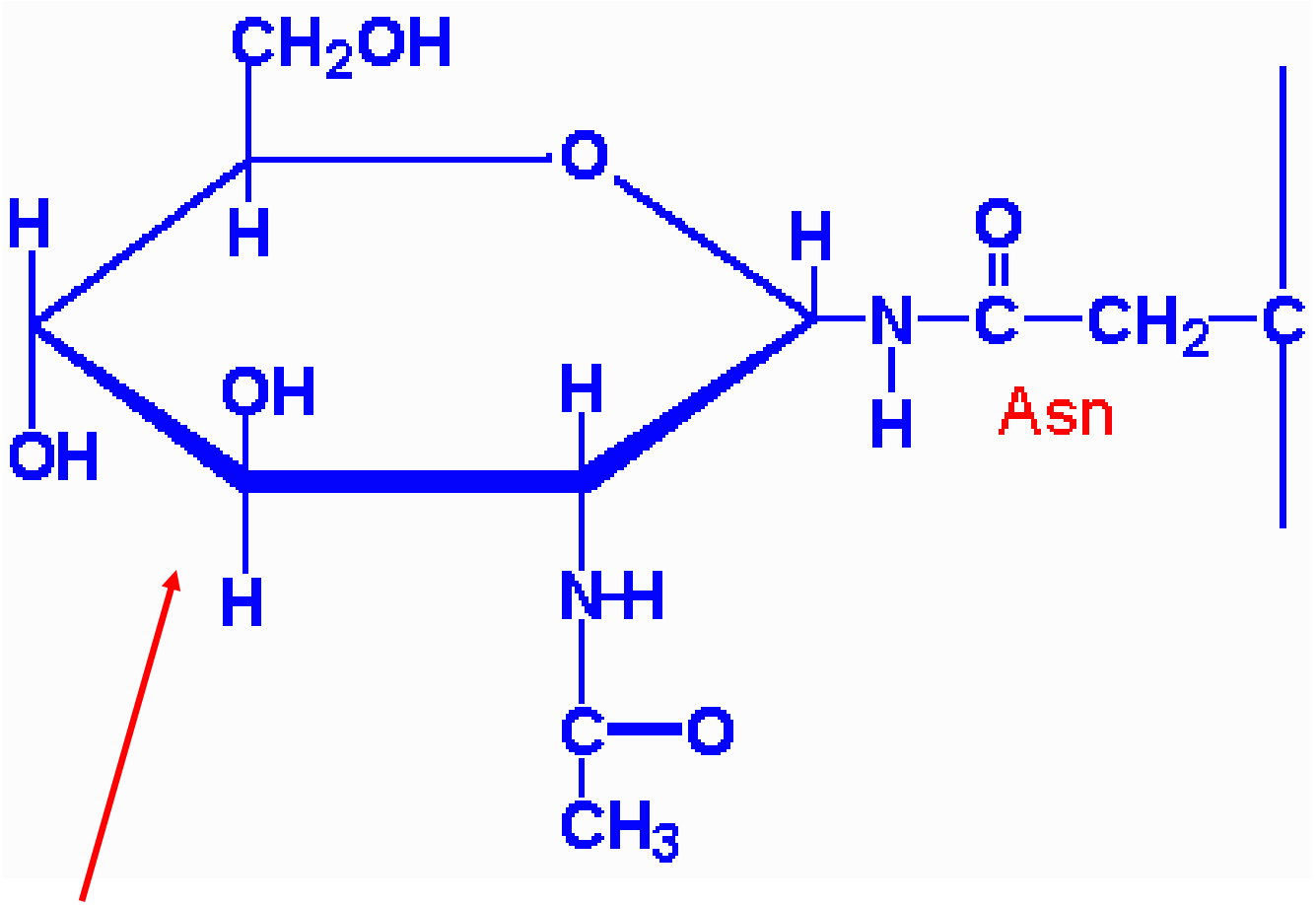


Asn



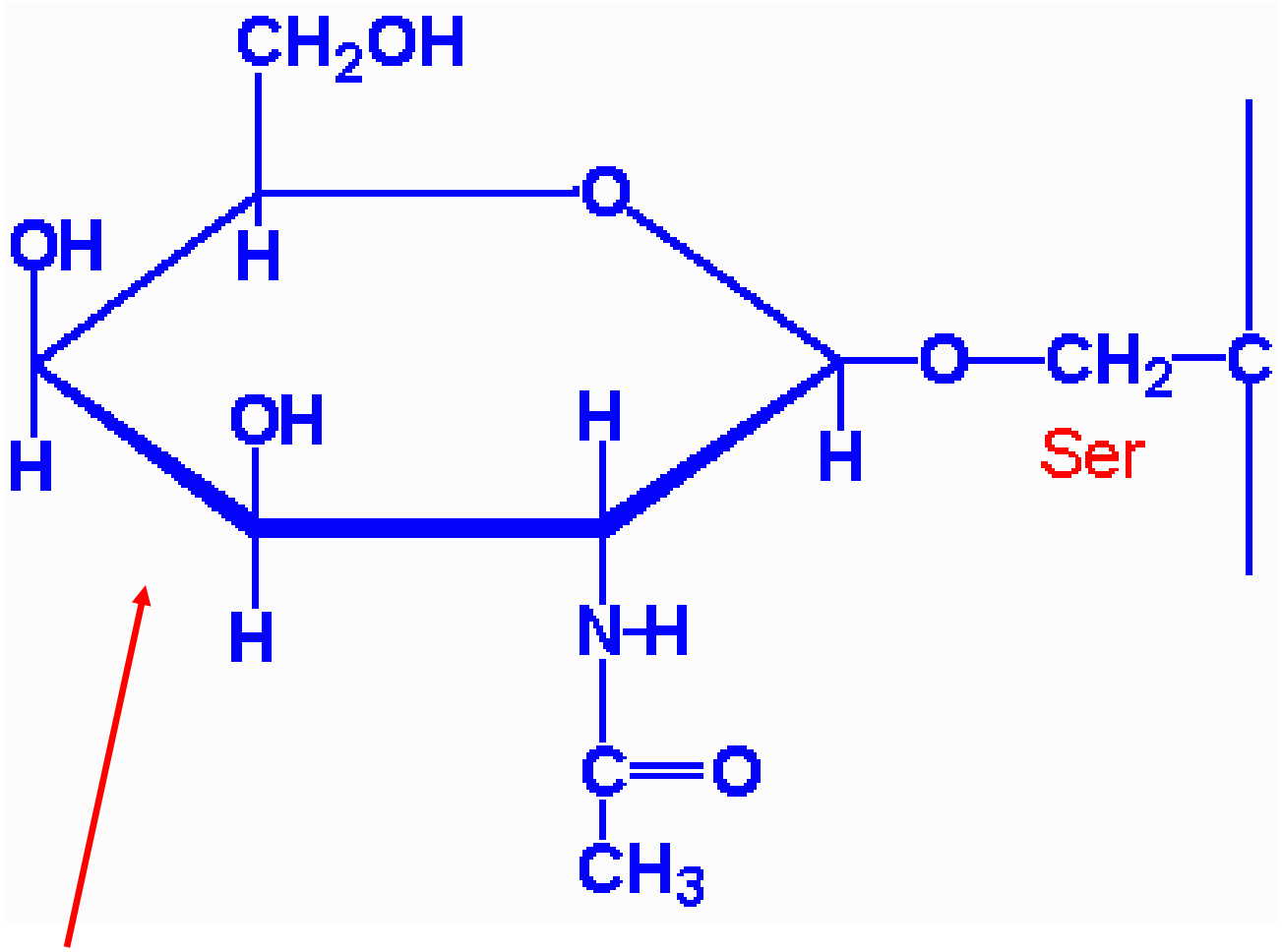
Ser

N-glycosidic bond



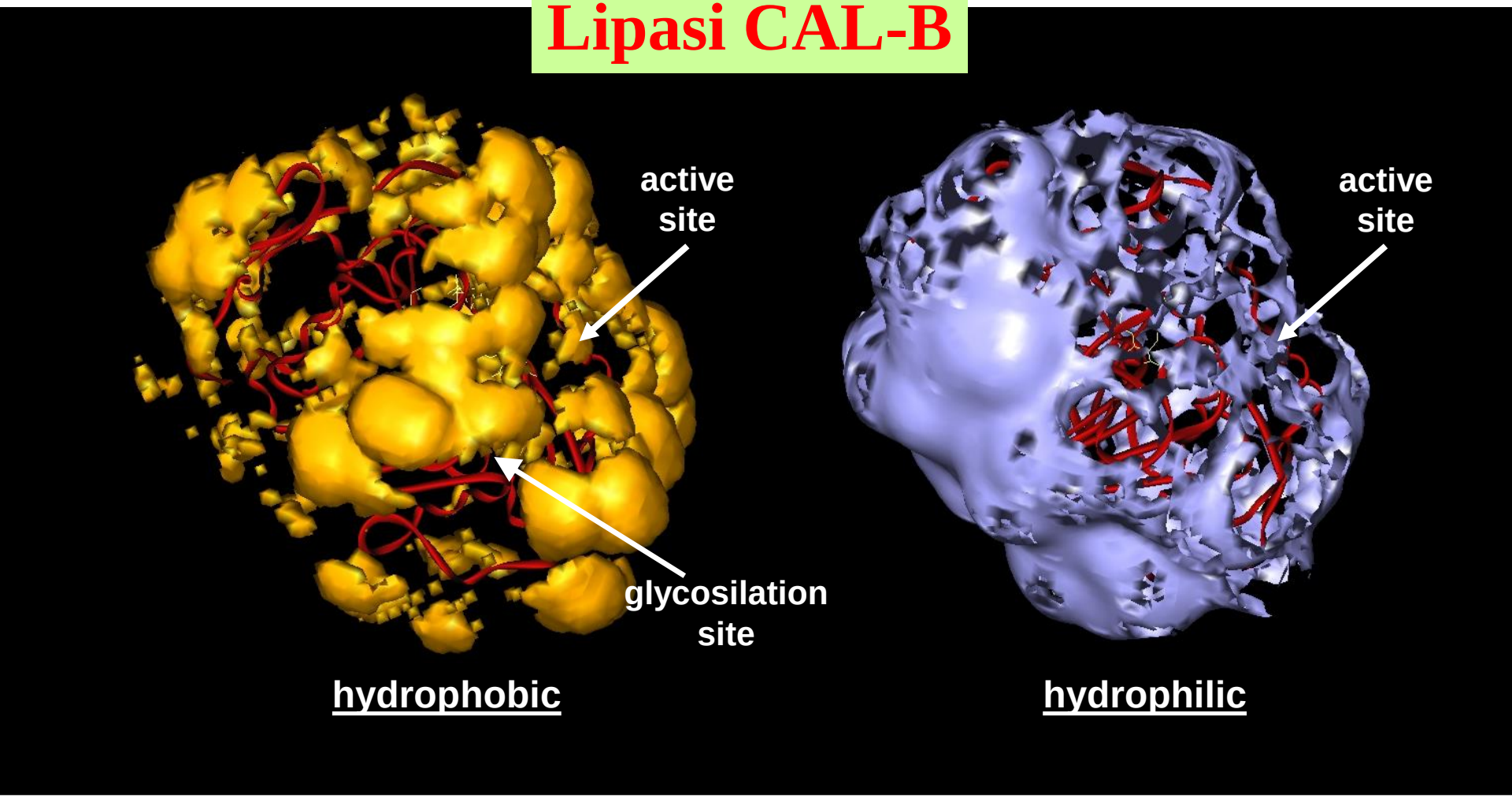
2-N-Acetylglucosammine

O-glycosidic bond



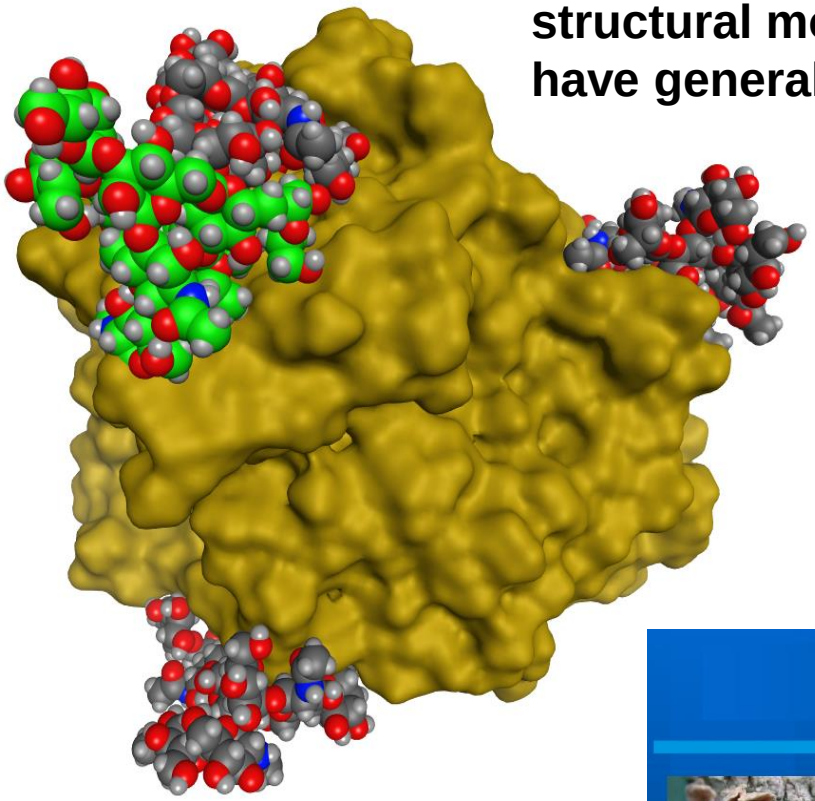
2-N-Acetylglucosammine

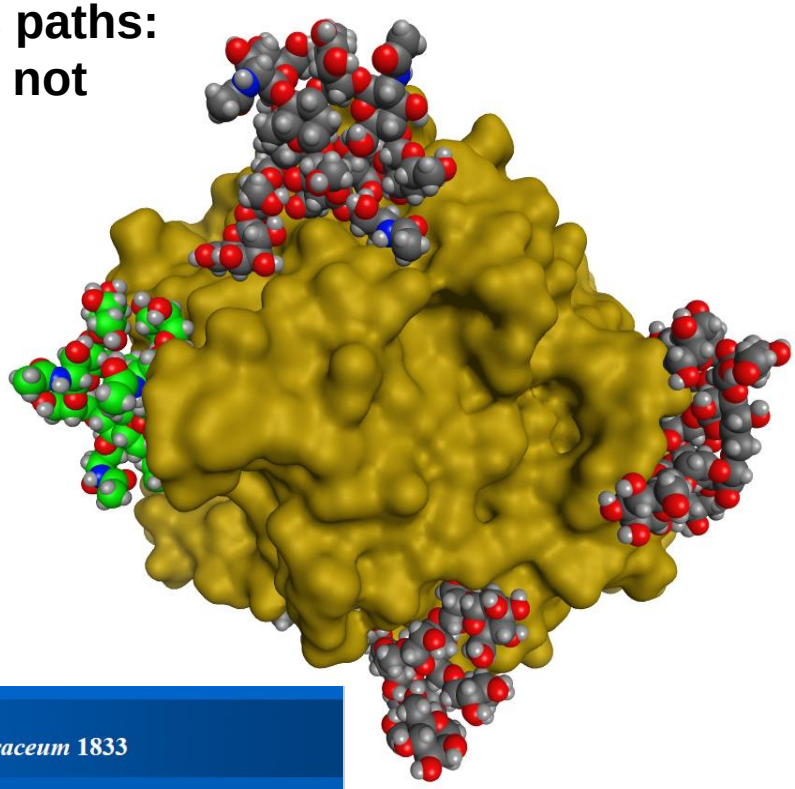
Lipasi CAL-B



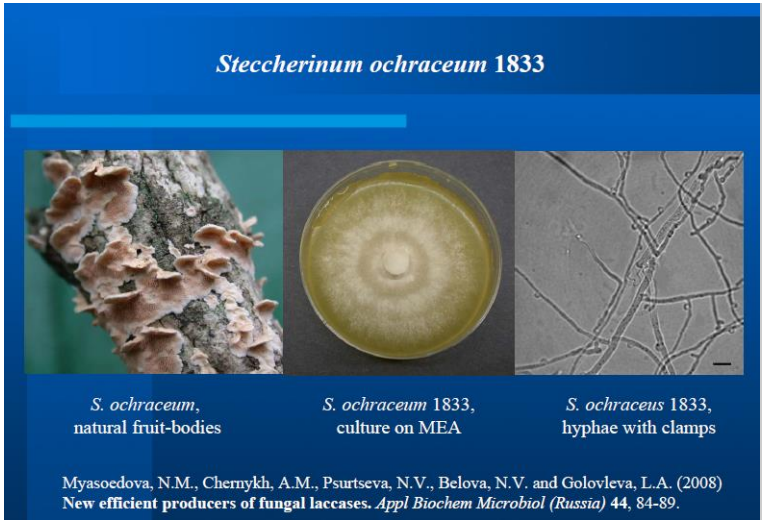
Glycans are generally insertedin the proximity of hydrophobic regions on the surface for stabilizing against hydration

Glycosylation occurs with broadly heterogeneous paths: structural models does not have general validity





Laccase from fungi: degrades lignin



Glycosylation improves thermal stability

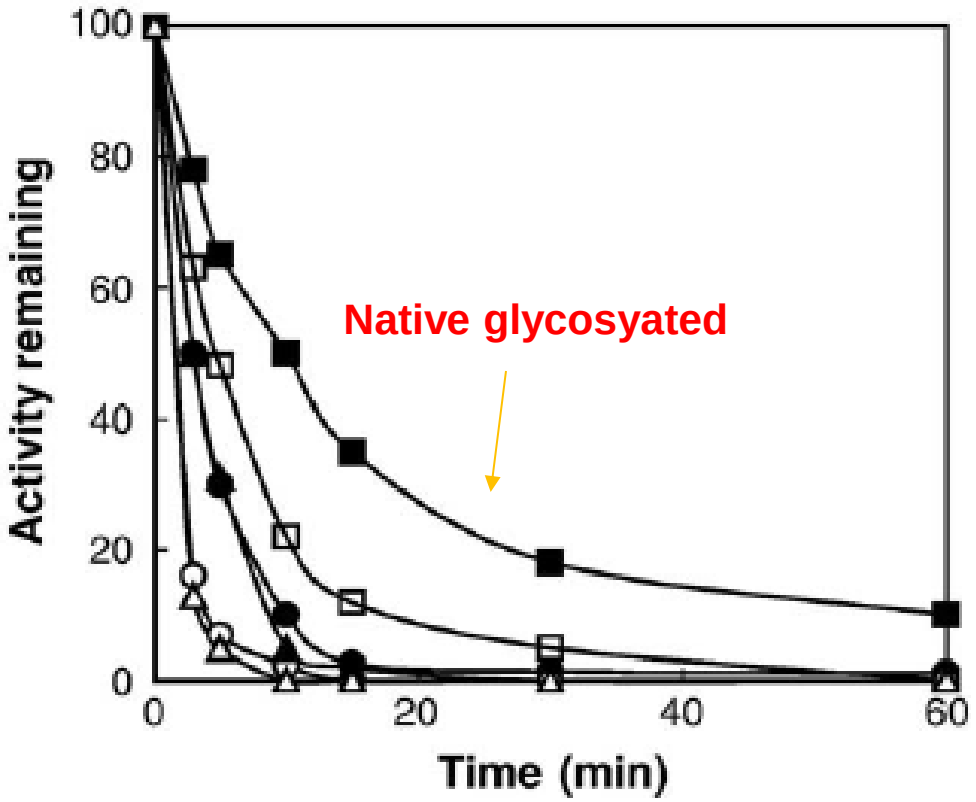


Fig. 4. Irreversible thermoinactivation of native and deglycosylated forms of glucoamylase at 70°C: Native (●), deglycosylated (○) at pH 3; native (■), deglycosylated (□) at pH 4.8; native (▲), deglycosylated (△) at pH 8. Further details are described under Materials and methods.

Glycosylation prevents aggregation

- **improves the solubility of unfolded or partially folded proteins**

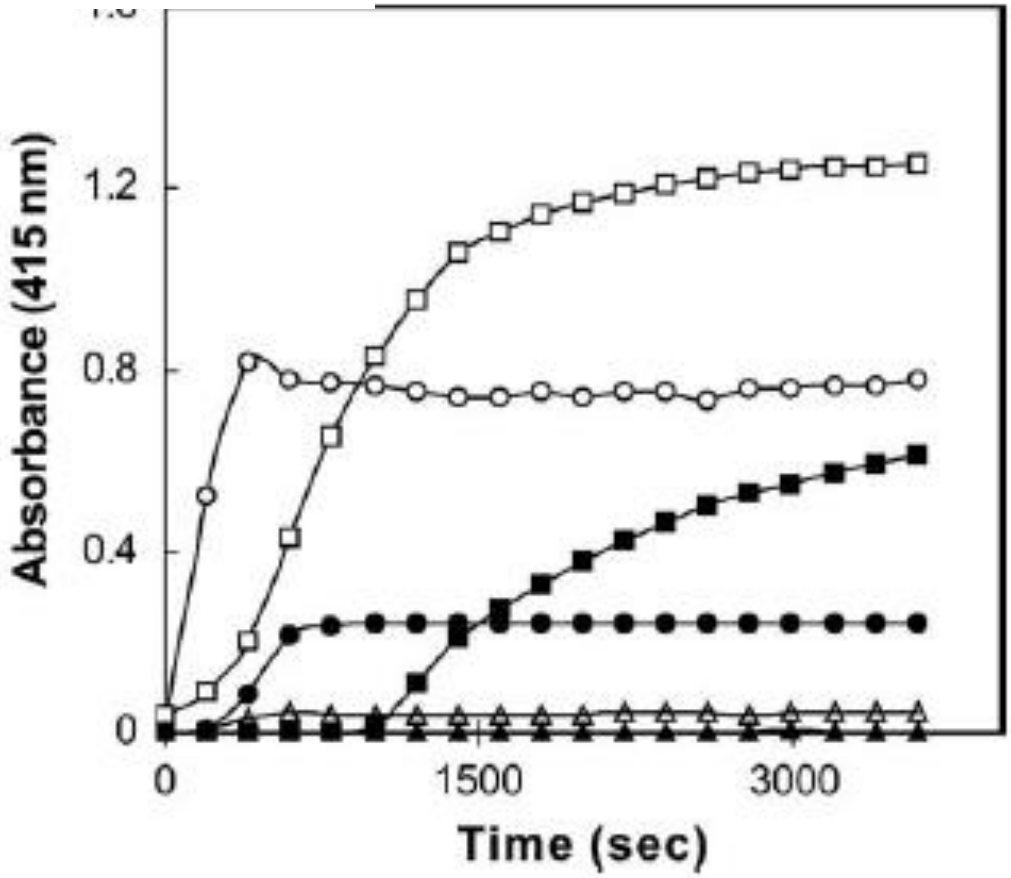


Fig. 5. Aggregation of native and deglycosylated forms of glucoamylase at 70°C: Native (●), deglycosylated (○) at pH 3; native (■), deglycosylated (□) at pH 4.8; native (▲), deglycosylated (△) at pH 8. For further details, please see Materials and methods.

Guidelines for Reporting of BIOCATALYTIC REACTIONS

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Opinion

Guidelines for reporting of biocatalytic reactions

Lucia Gardossi[1], Poul B. Poulsen[2], Antonio Ballesteros[3], Karl Hult[4], Vytas K. Švedas[5], Đurđa Vasić-Rački[6], Giacomo Carrea[7], Anders Magnusson[8], Andreas Schmid[8], Roland Wohlgemuth[9] and Peter J. Halling[10]

[1] Dipartimento di Scienze Farmaceutiche, Università di Trieste, Piazzale Europa 1, 34127 Trieste, Italy
[2] Novozymes A/S, Krogshoejvej 36, DK-2880 Bagsvaerd, Denmark
[3] Department of Biocatalysis, CSIC Institute of Catalysis, Madrid, Spain
[4] Department of Biochemistry, School of Biotechnology, Royal Institute of Technology (KTH), AlbaNova University Center, SE-106 91 Stockholm, Sweden
[5] Faculty of Bioengineering and Bioinformatics, Belozersky Institute of Physicochemical Biology, Lomonosov Moscow State University, Moscow 119991, Russia
[6] Faculty of Chemical Engineering and Technology, University of Zagreb, Savska c.16, 10000 Zagreb, Croatia
[7] Laboratory of Chemical Biotechnology, Istituto di Chimica del Riconoscimento Molecolare, CNR, via Mario Bianco 9, 20131 Milan, Italy
[8] Department of Biochemical & Chemical Engineering, TU Dortmund University, Emil Figge Str. 66, D-44227 Dortmund, Germany
[9] Sigma-Aldrich, Research Specialties, Industriestrasse 25, CH-9470 Buchs, Switzerland
[10] WestCHEM, Department of Pure & Applied Chemistry, University of Strathclyde, Glasgow G1 1XL, Scotland, UK

Focusing on differences:

Emphasis often just preparative

- Reproducibility may be more important that full characterisation
- Crude/impure enzymes acceptable
- Valuable to make clear comparison with alternative catalyst or condition

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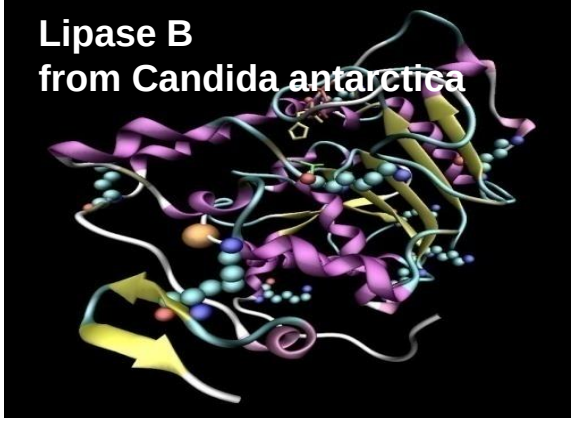
Section on Applied Biocatalysis

Identification of the enzyme

	Data required to allow the reproducibility of the results	***Explanations and additions from the viewpoint of applied biocatalysis***
Identity of the enzymes	Name of Reaction Catalyst EC-Number Sequence accession number (nucleotide sequence code) Organism/species & strain	•*Biocatalysts may often be* ***less well characterised*** *than usual in pure enzymology. The only requirement, which is equally important for biocatalysis, is that the catalysts used must be* ***specified unambiguously***
Additional information on the enzyme	Post-translational modifications	
Preparation	Description Artificial modifications Enzyme or protein purity	•*Information about* ***impurities*** *in the biocatalyst* •***Additives*** *included deliberately* •*Description of* ***immobilized*** *derivative preparation and characterization*

Commercial enzyme products:

- manufacturer with name and address
- complete product name
- code from manufacturer
- date of sample/batch n°



Product	Appearance	Protein content (from the manufacturer)	Specific hydrolytic activity
Lipozyme CALB L	Viscous brown-yellowish liquid Small proteic impurities ì	6% Declared content for 1g solution: 440 mg water 250 mg sorbitol 250 mg glycerol 60 mg protein 2 mg sodium benzoate 1 mg potassium sorbate	4800-5200 U/ml
CALB c-Lecta	White powder. Lyophilized	10-20%	30 U/mg_{prep}
Chyrazyme L-2	White powder. Lyophilized	43%	60-70 U/mg_{prep}
Novozym 435	Immobilized on polymeric beads	n.a. produced by submerged fermentation of a genetically modified Aspergillus microorganism and **adsorbed** on a macroporous resin	2000-2500 U/g_{prep}

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Assay conditions

Topics	Data required to allow the reproducibility of the results	*Explanations and additions from the viewpoint of applied biocatalysis*
Assay conditions	•Measured reaction •Assay temperature •Assay pressure •Assay pH •Buffer & concentrations •Metal salt(s) & concentrations •Other assay components •Substrates & concentration ranges •Enzyme/protein concentration •Variable components •Total assay mixture ionic strength	➢*If reaction mixture is, or may be multiphase:* ***agitation*** *conditions* ➢*If reaction mixture is non-aqueous (e.g. based on organic solvents, ionic liquids, gasses, supercritical fluids)* ***residual water content*** *or water activity must be specified* ➢*pH electrode* ***calibration*** *basis*

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Multiphase systems: agitation

Agitation may affect mass transfer rates that are limiting

Details:

- **stirring (magnetic? overhead?) or shaking (reciprocal horizontal? reciprocal vertical? rotary? inversion? other?)**
- **dimensions of the vessel**
- **fractional filling with liquid**
- ***others……***

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Activity assay: Reporting activity

	Data required to allow the reproducibility of the results	***Explanations***
Activity	•Initial rates of the reaction measured •Proportionality between initial velocity and enzyme concentration •Specific activity	➢*A statement of* ***conversion or product concentration at a given time*** *is acceptable only when describing a biocatalytic synthesis – but not for kinetic studies.*

Units for reporting biocatalyst activity

specific activity should be given in units like

mmol $min^{-1}mg^{-1}$

It is acceptable to specify

- **a rate of concentration change together with a biocatalyst concentration**,
- *or* **a rate in terms of moles per unit time together with a biocatalyst amount.**

Be careful to specify the basis on which a mass of biocatalyst is presented. This may be the mass of the entire preparation (for example including an immobilization support), the mass of protein present within it, or even the mass of an active enzyme within it. For some biocatalysts a wet mass may be given, but a dry mass is always more precisely defined.

Reporting activity assay

Topics	Data required to allow the reproducibility of the results	*Explanations and additions*
Methodology	•Assay method •Type of assay •Stopping procedure •Direction of the assay •Reactant determined •Reaction stoichiometry	➢*Description of **sampling** procedure to deal with multiple phase reaction mixture* ➢*Report observations on **phase separation***

Sampling multiphase reaction mixtures

A safe (but more laborious) option:

to terminate and extract the entire reaction mixture, using separate reaction vessels to explore different time points.

Obtaining meaningful samples for monitoring reaction progress:

- **the analytical data will account only for a small fraction of the total volume of this phase,**
- **the behaviour of the reaction mixture as a whole will be perturbed by their removal**

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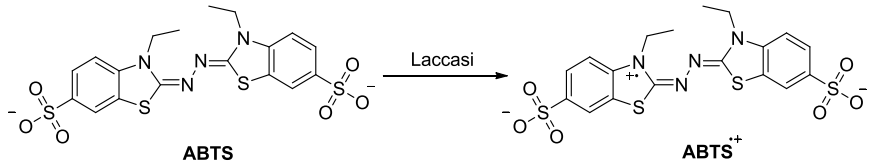
Section on Applied Biocatalysis

Test di attività delle laccasi: ossidasi



L'attività enzimatica delle laccasi è stata determinata tramite il test di ossidazione del 2,2'-azino-bis-(3-etilbenzotiazolino-6-acido solfonico) (ABTS). In una cuvetta è stato posto un volume noto di enzima compreso tra 1 e 200 µL di soluzione di enzima e la soluzione è stata portata al volume di 900 µL con tampone citrato 0,05 M a pH 3. Sono stati aggiunti 100 µL di soluzione 0,02 M di ABTS in tampone sodio citrato 0,05 M a pH 3 ed è stato monitorato l'incremento dell'assorbanza a 420 nm per 1 minuto.

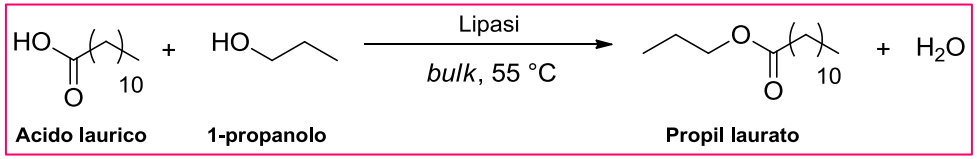
Un'unità di attività (espressa in U/mL) del biocatalizzatore viene definita come la quantità di enzima necessaria per catalizzare la conversione di una µmole di substrato per un minuto a 25 °C.

L'attività è stata calcolata con la seguente formula:

$$Attività = \frac{\Delta A/min}{36} * \frac{V_{totale}}{V_{enzima}}$$

Dove V_{totale} è il volume della soluzione nella cuvetta (1000 µL) e V_{enzima} è il volume della soluzione enzimatica a concentrazione proteica nota analizzato (µL). Il valore di attività enzimatica è stato calcolato su una media di tre misure ed è indicato con la deviazione standard.

Test di attività sintetica della lipasi



L'attività sintetica è stata testata attraverso l'esterificazione enzimatica dell'acido laurico e l'1-propanolo.
In una vial da 20 mL sono stati posti 1,2 g di acido laurico (6 mmol) e 0,45 mL di 1-propanolo (6 mmol). La soluzione è stata termostata a 55 °C e mantenuta sotto agitazione (250 rpm) all'*orbital shaker*. Al tempo t=0 sono stati prelevati 0.1 mL e pesati in un becher. Alla soluzione iniziale sono stati aggiunti circa 50 mg di biocatalizzatore e la miscela è stata lasciata sotto agitazione per 15 minuti. Ad intervalli regolari sono stati prelevati 0,1 mL della miscela, pesati in un becher e successivamente diluiti in becher con 7 mL di etanolo sotto agitazione magnetica continua. Sono state aggiunte 3 gocce di soluzione di fenolftaleina come indicatore. I campioni sono stati quindi titolati con una soluzione 0,1 M di KOH in etanolo fino viraggio persistente della soluzione a rosa chiaro. Le misure sono state effettuate in duplicato.

Un'unità di attività (U/$g_{biocatalizzatore}$) del biocatalizzatore viene definita come la quantità di enzima immobilizzato necessaria per produrre una µmole di propil laurato in un minuto a 55 °C senza solvente.

Il valore di acidità ad un determinato tempo è stato calcolato tramite la seguente formula:

$$AV = \frac{MM_{KOH} * M_{KOH} * V_{KOH}}{m_{campione}}$$

Dove MM_{KOH} è la massa molecolare del KOH (56,11 g/mol), M_{KOH} è la molarità della soluzione di KOH in etanolo (0,1 mol/L) e V_{KOH} è il volume di KOH (mL) usato per titolare il campione e $m_{campione}$ è la massa (g) del campione prelevato ad un determinato tempo. La resa percentuale di propil laurato è stata calcolata come segue:

$$\%_{propil\ laurato} = 100 * \frac{AV_0 - AV_t}{AV_0}$$

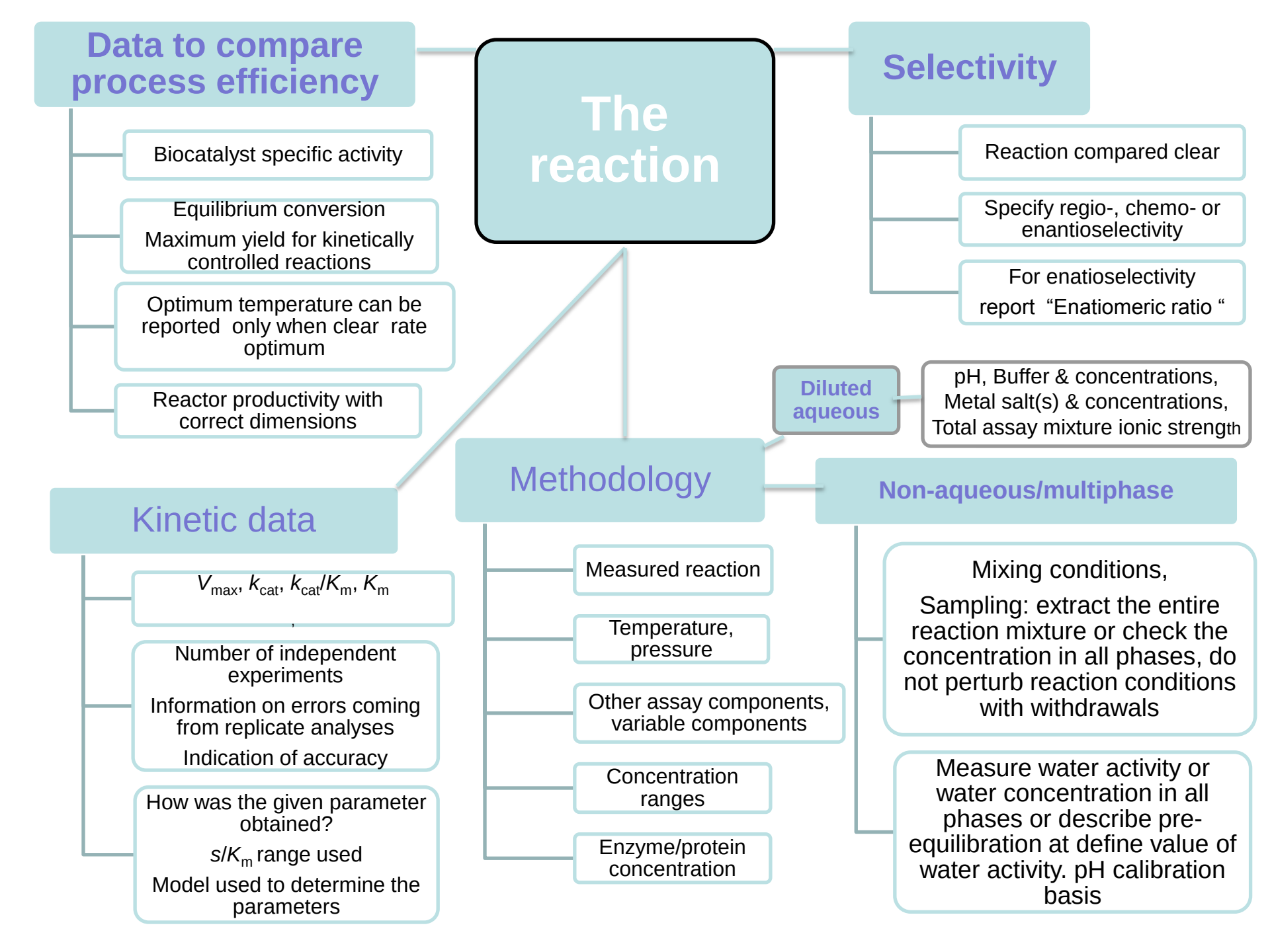
La quantità di propil laurato prodotta è stata calcolata come segue:

$$\mu mol_{propil\ laurato} = \frac{\%_{propil\ laurato} * 6000}{100}$$

Il valore di attività sintetica è stato calcolato con la seguente formula:

$$Attività = \frac{\mu mol_{propil\ laurato}}{m_{biocatalizzatore} * t}$$

Dove $m_{biocatalizzatore}$ è la massa (g) del biocatalizzatore usato nel saggio e t è il tempo (min) a cui è stato effettuato il prelievo. Il valore di attività enzimatica è stato calcolato su una media di due misure ed è stato indicato con la deviazione standard.


The reaction
Data to compare process efficiency
Biocatalyst specific activity
Equilibrium conversion
Maximum yield for kinetically controlled reactions
Optimum temperature can be reported only when clear rate optimum
Reactor productivity with correct dimensions
Selectivity
Reaction compared clear
Specify regio-, chemo- or enantioselectivity
For enatioselectivity
report "Enatiomeric ratio "
Kinetic data
V_{max}, k_{cat}, k_{cat}/K_m, K_m
Number of independent experiments
Information on errors coming from replicate analyses
Indication of accuracy
How was the given parameter obtained?
s/K_m range used
Model used to determine the parameters
Methodology
Measured reaction
Temperature, pressure
Other assay components, variable components
Concentration ranges
Enzyme/protein concentration
Diluted aqueous
pH, Buffer & concentrations,
Metal salt(s) & concentrations,
Total assay mixture ionic strength
Non-aqueous/multiphase
Mixing conditions,
Sampling: extract the entire reaction mixture or check the concentration in all phases, do not perturb reaction conditions with withdrawals
Measure water activity or water concentration in all phases or describe pre-equilibration at define value of water activity. pH calibration basis

- Rigorous experimental planning
- Detailed reporting of experimental conditions