Selected Problems of Molecular Biophysics

(1100-5BM15)

Jan M. Antosiewicz

Division of Biophysics
Institute of Experimental Physics
Faculty of Physics

LECTURE 19

December 6, 2023

Problem 4: Biomolecular processes dependent on pH (part 1): peptide synthesis and enzeme catalysis

http://www.fuw.edu.pl/~jantosi/

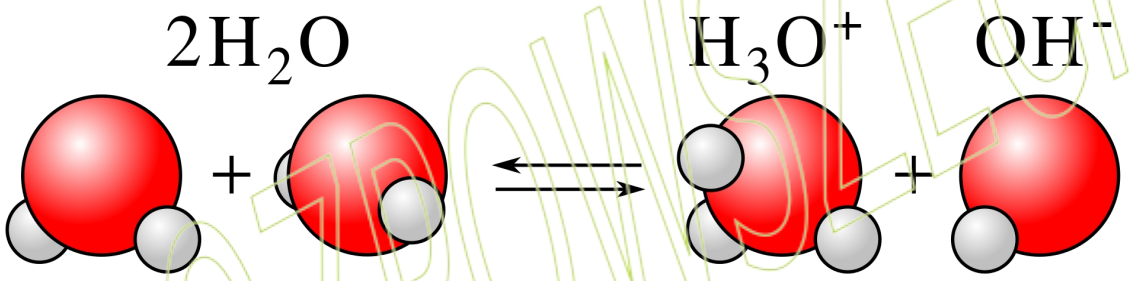
jantosi@fuw.edu.pl

Among all possible chemical reactions that occur in water, the most fundamental is the water dissociation reaction. In aqueous solution, water will self-ionize and form hydroxide (OH^-) and hydronium (H_3O^+) ions.

NIE ROZPOWSZECHNIAJ

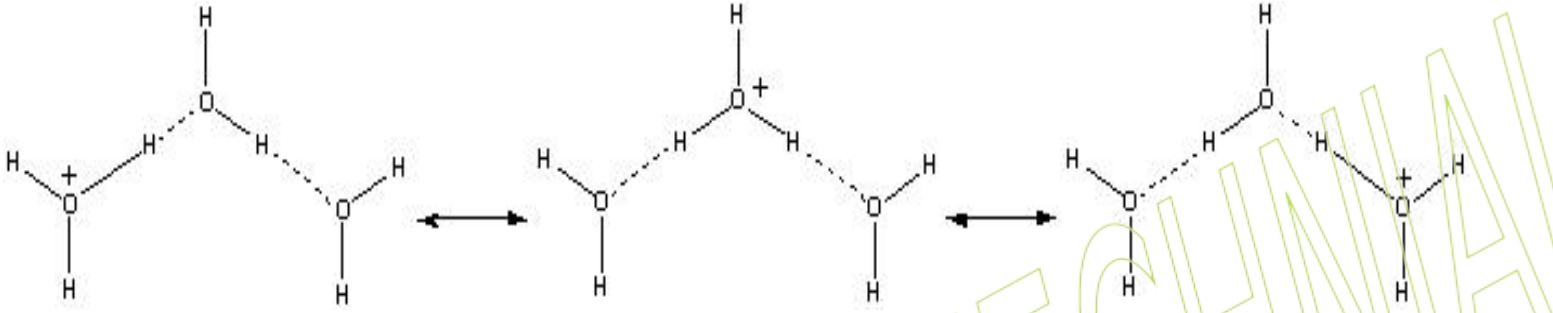
Mahmoud Moqadam and coworkers, *Local initiation conditions for water autoionization*, PNAS 115:E4569-E4576 (2018)

The dissociation of a water molecule in liquid water is the fundamental event in acid-base chemistry, determining the pH of water. Because of the short time scales and microscopic length scales involved, the dynamics of this autoionization have not been directly probed by experiment. Here, the autoionization mechanism is revealed by sampling and analyzing *ab initio* molecular dynamics trajectories. We identify the rare fluctuations in solvation energies that destabilize an oxygen-hydrogen bond. [P. L. Geissler, C. Dellago, David Chandler, J. Hutter, M. Parrinello, *Autoionization in Liquid Water*, SCIENCE 291:2121-2124 (2001)]



The following sequence of events has been proposed on the basis of electric field fluctuations in liquid water. Random fluctuations in molecular motions occasionally (about once every 10 hours per water molecule) produce an electric field strong enough to break an oxygen–hydrogen bond, resulting in a hydroxide (OH^-) and hydronium ion (H_3O^+); the hydrogen nucleus of the hydronium ion travels along water molecules by the Grotthuss mechanism and a change in the hydrogen bond network in the solvent isolates the two ions, which are stabilized by solvation. Within 1 picosecond, however, a second reorganization of the hydrogen bond network allows rapid proton transfer down the electric potential difference and subsequent recombination of the ions. This timescale is consistent with the time it takes for hydrogen bonds to reorientate themselves in water. [wikipedia, citations Science 2001 and older]

Mechanizm Grotthusa



Średnio co 0.5 ms cząsteczka wody „chwyta” proton, „trzyma go” przez 1 ps i podaje dalej (mechanizm Grotthusa, 1806)

W publikacji z 1806 roku, “Teoria dekompozycji cieczy przez prąd elektryczny”, Theodor Grotthus wyjaśnił zjawisko elektrolizy wody zakładając, że atomy tlenu sąsiadujących ze sobą cząsteczek wody przekazują sobie atom wodoru. Wówczas sądzono, że cząsteczka wody to HO.

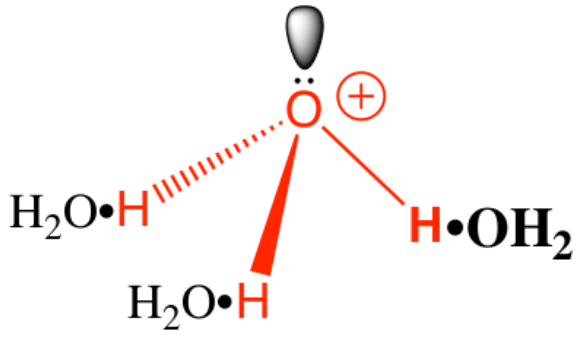
Elektroliza wody do jej dekompozycja na tlen i wodór pod wpływem przepuszczania przez nią prądu elektrycznego. W 1800 roku Alessandro Volta wynalazł baterię elektryczną, a kilka tygodni poźniej William Nicholson i Anthony Carlisle wykorzystali ją do przeprowadzenia elektrolizy wody. Wyjaśnienie tego zjawiska stało się wyzwaniem dla najtęższych umysłów epoki.



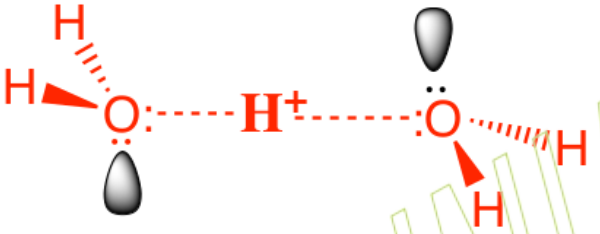
Dyfuzja w wodzie, w polu elektrycznym

Kation	Mobilność [$cm^2V^{-1}s^{-1}$]
Na^+	$0.519 \cdot 10^{-3}$
K^+	$0.762 \cdot 10^{-3}$
H^+	$3.620 \cdot 10^{-3}$

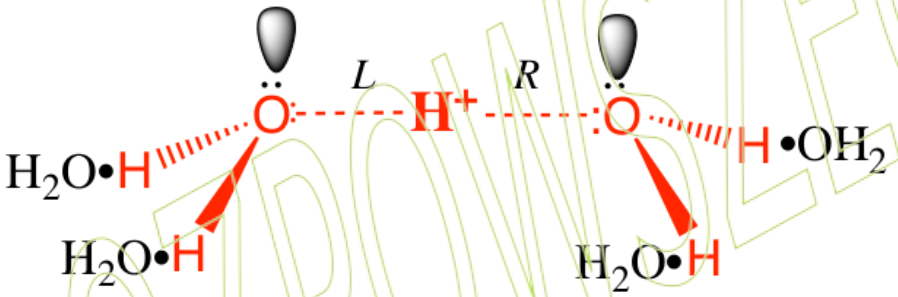
NIE ROZDAWISZECHNA!



A. Eigen cation: $H_3O^+ \cdot (H_2O)_3$



B. Zundel cation: $H^+ \cdot (H_2O)_2$



C. Reed/Stoyanov cation: $H^+ \cdot (H_2O)_2 \cdot (H_2O)_4$

(expanded Zundel)

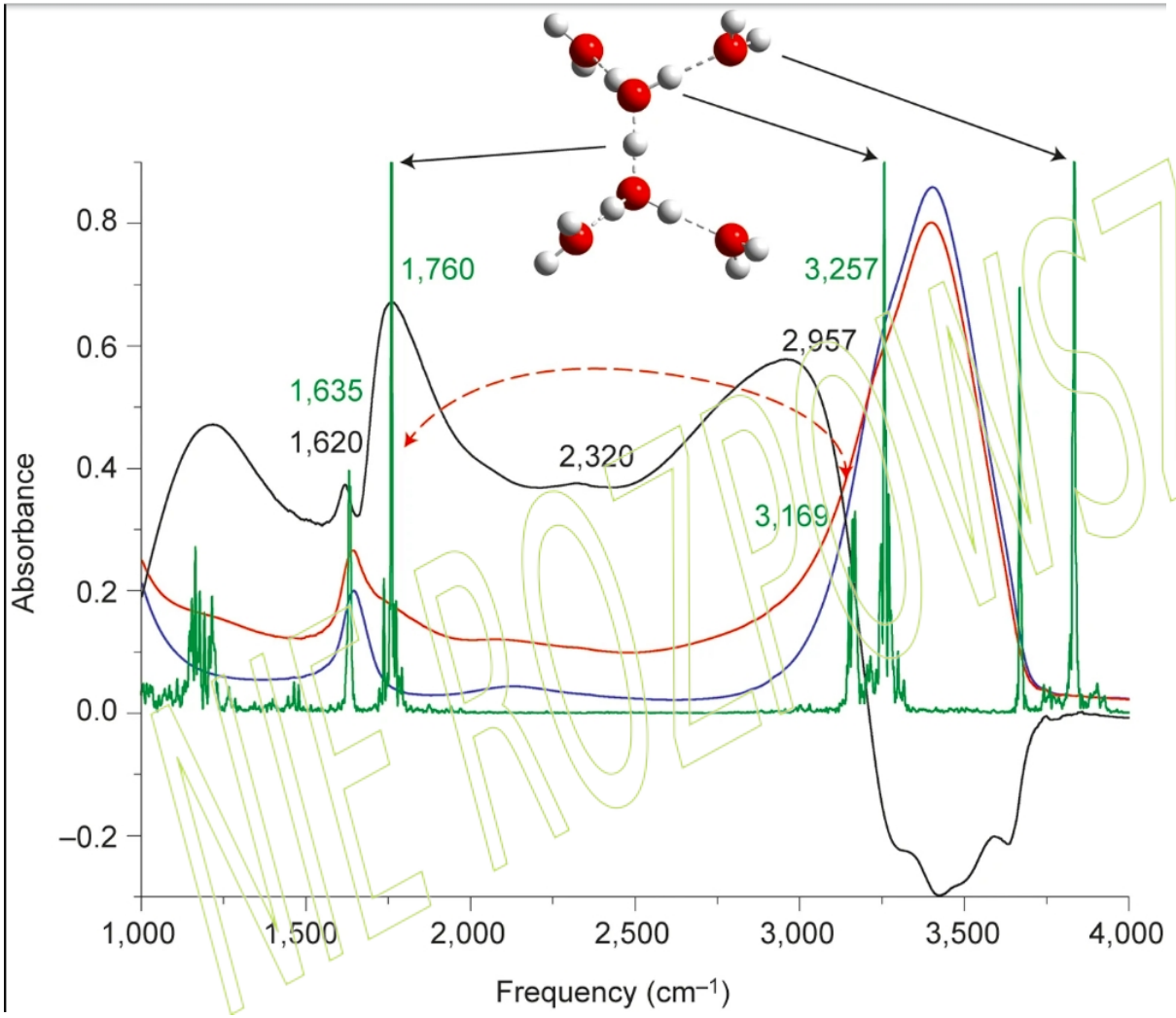
NIE DO ZPOWSZECHNEJ

The two most widely accepted $H^+(aq)$ structures were posed in the 1960s by Eigen and Zundel, and both structures feature more than a single water of hydration. The Eigen cation (Figure A) is an $H_9O_4^+$ tetrahydrate in which an H_3O^+ entity is hydrogen-bonded to three waters in a second hydration shell. These H-bonds are the "normal" type seen in bulk water and ice. The Zundel cation (Figure B) is an $H_5O_2^+$ dihydrate where H^+ is shared equally between two inner shell water molecules. The two central hydrogen bonds are symmetrical, and they are unusually short and strong. Stoyanov and Reed have proposed a hexahydrate $H_{13}O_6^+$ ion (Figure C) with a Zundel-like inner core (but with longer, symmetrical H-bonds) and four H-bonded water molecules in the second hydration shell. The proton's +1 charge is delocalized over this entire hexahydrate complex.

Written as H_3O^+, the hydronium ion is a useful fiction but where feasible, should be replaced with the simple notation $H^+_{(aq)}$ as a signifier of the hexahydrate complex.

Todd Silverstein, Frontiers in Molecular Sciences, 2021, 8, Art. 764009

While there is some qualitative understanding of how excess protons behave in acidic aqueous solutions, there is still much to learn about the structures they form with water molecules. Now, 2D infrared spectroscopy has enabled the definite identification of the 'Zundel cation' in protonated liquid water and an approximate determination of its lifetime.



Measured infrared spectrum of liquid water (blue), of 4 M aqueous HCl (red), and their difference (black). Superimposed (green line) is the simulated spectrum of the $H^+(H_2O)_6$ Zundel-like isomer, scaled by a factor of 1.01 to match the 1,760 cm^{-1} band. Compared with the liquid-phase difference spectrum (black), its low frequency bands are in good agreement, but the doublet near 3,200 cm^{-1} occurs on the blue shoulder of the 2,957 cm^{-1} band, where no absorption feature is easily discernible. The three peak frequencies marked in black are best attributed to the Eigen cation, which therefore also plays an important role in aqueous acid solutions. The free-OH stretching modes above 3,600 cm^{-1} are irrelevant to the liquid phase. Dashed red arrow indicates the coupling revealed by the 2DIR experiment.

Recently, it was shown that the gas-phase $H^+(H_2O)_6$ cluster exists as both Zundel and Eigen cations, and both IR spectra were accurately measured [Heine, N. et al. J. Am. Chem. Soc. 135, 8266–8273 (2013)].

Noam Agmon, *The acid test for water structure*, Nature Chemistry 8:206-207 (2016)

INTERNATIONAL UNION OF PURE AND APPLIED CHEMISTRY

MEASUREMENT OF pH.
DEFINITION, STANDARDS, AND PROCEDURES
(IUPAC Recommendations 2002)

Pure Appl. Chem., Vol. 74, No. 11, pp. 2169–2200, 2002.

Working Party on pH
R. P. BUCK (CHAIRMAN), S. RONDININI (SECRETARY), A. K. COVINGTON (EDITOR), F. G. K. BAUCKE, C. M. A. BRETT, M. F. CAMÕES, M. J. T. MILTON, T. MUSSINI, R. NAUMANN, K. W. PRATT, P. SPITZER, AND G. S. WILSON

1.1 pH, a single ion quantity

The concept of pH is unique among the commonly encountered physicochemical quantities listed in the IUPAC Green Book in that, in terms of its definition,

$$pH = -\log_{10} a_{H^+}$$

it involves a single ion quantity, the activity of the hydrogen ion, which is immeasurable by any thermodynamically valid method and requires a convention for its evaluation.

2.1 Hydrogen ion activity

pH was originally defined by Sørensen in 1909 in terms of the concentration of hydrogen ions (in modern nomenclature) as pH = $-\log_{10}(c_{H^+}/c^\circ)$ where c_{H^+} is the hydrogen ion concentration in mol dm^{-3}, and c° = 1 mol dm^{-3} is the standard amount concentration. Subsequently, it has been accepted that it is more satisfactory to define pH in terms of the relative activity of hydrogen ions in solution

$$pH = -\log_{10} a_{H^+} = -\log_{10}\left(\frac{\gamma_{H^+} m_{H^+}}{m^o}\right) \qquad (1)$$

where a_{H^+} is the relative (molality basis) activity and γ_{H^+} is the molal activity coefficient of the hydrogen ion H^+ at the molality m_{H^+}, and m° is the standard molality. The quantity pH is intended to be a measure of the activity of hydrogen ions in solution. However, since it is defined in terms of a quantity that cannot be measured by a thermodynamically valid method, eq. 1 can be only a notional definition of pH.

chemical potential of hydrogen ion ($H^+_{(aq)}$) diluted in pure water

$$\mu_{H^+}(T,p,x_{H^+}) = \mu^{\ominus}_{H^+}(T,p) + \mu^{(m)}_{H^+}(T,p,x_{H^+})$$

$$a_{H^+}(T,p,x_{H^+}) \equiv \exp\left(\frac{\mu^{(m)}_{H^+}(T,p,x_{H^+})}{RT}\right) \quad \textbf{(Lewis)}$$

$$\gamma_{H^+}(T,p,x_{H^+}) \equiv \frac{a_{H^+}(T,p,x_{H^+})}{x_{H^+}}$$

$$\mu_{H^+}(T,p,x_{H^+}) = \mu^{\ominus}_{H^+}(T,p) + RT\ln a_{H^+}(T,p,x_{H^+}) = \mu^{\ominus}_{H^+}(T,p) + RT\ln x_{H^+} + RT\ln \gamma_{H^+}(T,p,x_{H^+})$$

$$pH = -\log_{10} a_{H^+} = -\log_{10}(\gamma_{H^+} x_{H^+}) = -\log_{10}\left(\frac{\gamma_{H^+} N_{H^+}}{N_{H^+} + N_{H_2O}}\right) = -\log_{10}\left(\frac{\gamma_{H^+} N_{H^+}}{N_{H_2O}} \cdot \frac{V}{V}\right) = -\log_{10}\left(\frac{\gamma_{H^+} c_{H^+}}{c_{H_2O}}\right)$$

$$pH = -\log_{10} a_{H^+} = -\log_{10}\left(\frac{\gamma_{H^+} c_{H^+}}{c^o}\right) \approx -\log_{10} c_{H^+}; \qquad c^o = 1\,M$$

Because γ ≈ 1 for concentrations below 0.01 M, the approximation in the above Equation, pH ≈ −log c_{H+}, holds true above pH 2. c^o the standard state concentration (Todd Silverstein, Frontires in Molecular Biosciences, 2021)

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Ionization of an acid

$$AH + H_2O \Leftrightarrow A^-_{(aq)} + H_3O^+_{(aq)} \qquad AH \Leftrightarrow A^-_{(aq)} + H^+_{(aq)}$$

$$K_a = \frac{a_{A^-} \cdot a_{H_3O^+}}{a_{AH}\, a_{H_2O}} \approx \frac{[A^-]\cdot[H^+]}{[AH][H_2O]} \qquad K_a = \frac{a_{A^-} \cdot a_{H^+}}{a_{AH}} \approx \frac{[A^-]\cdot[H^+]}{[AH]} = \exp\left(-\frac{\Delta G^o}{RT}\right)$$

$$a_\alpha = \gamma_\alpha c_\alpha \quad vs. \quad a_\alpha = \gamma_\alpha x_\alpha$$

$$pH = -\log_{10}[H^+] \qquad pK_a = -\log_{10}[K_a]$$

$$pH = pK_a + \log_{10}\frac{[A^-]}{[AH]} \equiv pK_a + \log_{10}\left(\exp\left(\frac{\Delta G^o}{RT}\right)\right)$$

NIE ROZPOWSZECHNIAJ

Henderson-Hasselbach equation

The problem of pH for small volumes

Debbie C. Crans and Nancy E. Levinger, The Conundrum of pH in Water Nanodroplets: Sensing pH in Reverse Micelle Water Pools, Accounts of Chemical Research, 45:1637–1645 (2012);

Wojciech Bal, Ewa Kurowska, Wolfgang Maret, The Final Frontier of pH and the Undiscovered Country Beyond, PLoS ONE 7:e45832/1-4 (2012);

Alan L. Rockwood, Meaning and Measurability of Single-Ion Activities, the Thermodynamic Foundations of pH, and the Gibbs Free Energy for the Transfer of Ions between Dissimilar Materials, ChemPhysChem 16:1978-1991 (2015);

Todd Silverstein, The proton in biochemistry: Impacts on bioenergetics, biophysical chemistry, and bioorganic chemistry, Frontiers in Molecular Biosciences, 2021, in press

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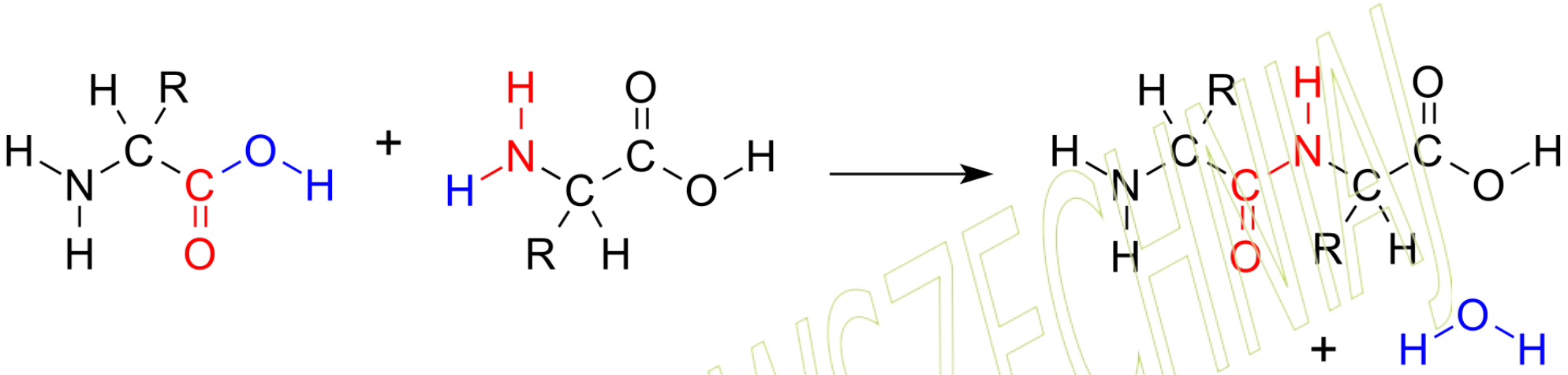
Alan L. Rockwood, *Meaning and Measurability of Single-Ion Activities, the Thermodynamic Foundations of pH, and the Gibbs Free Energy for the Transfer of Ions between Dissimilar Materials*, ChemPhysChem 2015, 16, 1978-1991.

A proper thermodynamic definition of single-ion activities is a very old and vexing unsolved problem in physical chemistry, as is the thermodynamic measurement of these same quantities. Single-ion activities are widely considered to be either unmeasurable or physically meaningless, but chemists, biologists, other scientists, and engineers simply cannot give up the idea of single-ion activities. For example, it would be hard to imagine a concept more widely used by chemists and biologists than that of pH, a concept that conceptually depends on the negative of the logarithm of hydrogen-ion activity, but the IUPAC Gold Book says: "pH cannot be measured independently because calculation of the activity involves the activity coefficient of single ion. Thus it can be regarded only as a notional definition." Similarly, National Bureau of Standards Special Publication 260-53 says this about the thermodynamic foundations of pH: "The use of pH in the expression $pH = -\log a_H$ is purely a formalism because a_H, a single (hydrogen) ion activity, is indeterminate." Consequently, the pH scale is based on standardized measurement schemes that are not based on a true thermodynamic method.

This paper takes a fresh approach to the problem, one that does not separate the problematical surface potential from the other contributions to the free energy. This approach rests entirely on classical equilibrium thermodynamics, which results in a single-ion activity that is thermodynamically well defined and physically meaningful.

The experimental method proposed in this work … places pH on a rigorous and defensible thermodynamic foundation.

Peptide bond formation



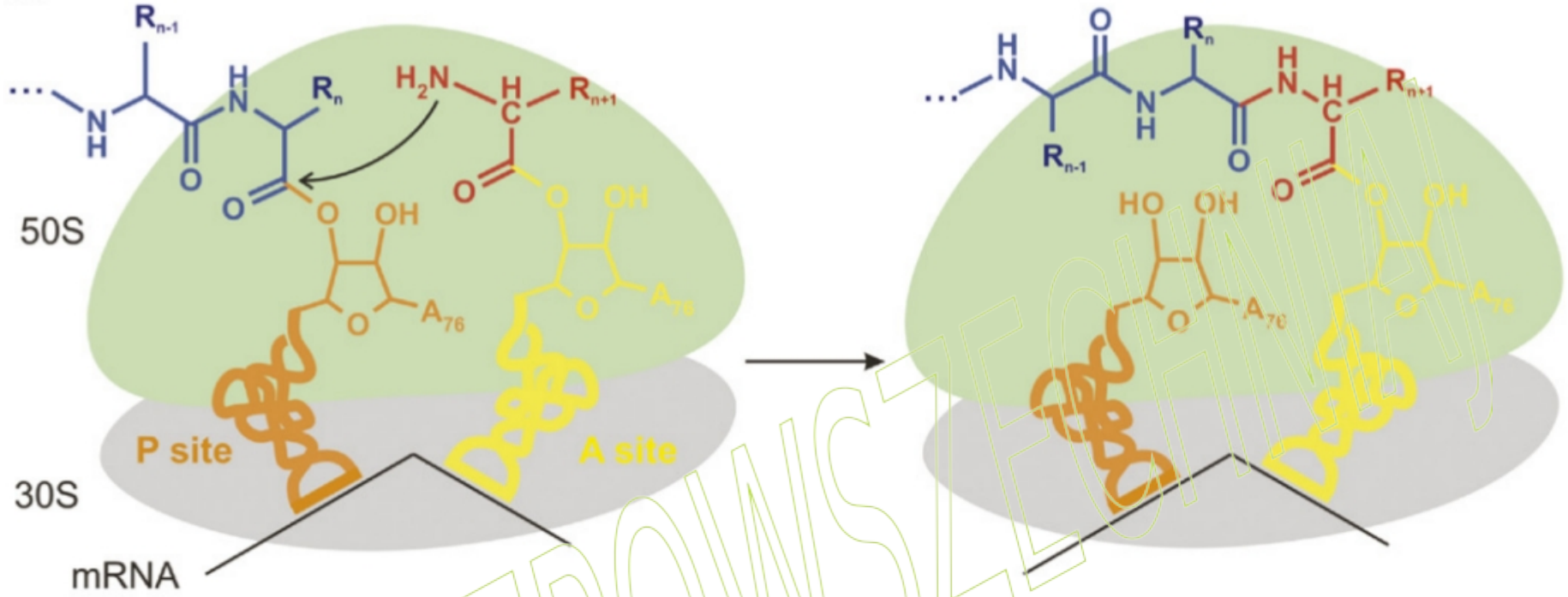
When two amino acids form a dipeptide through a peptide bond it is a type of condensation reaction. In this kind of condensation, two amino acids approach each other, with the non-side chain (C1) carboxylic acid moiety of one coming near the non-side chain (N2) amino moiety of the other. One loses a hydrogen and oxygen from its carboxyl group (COOH) and the other loses a hydrogen from its amino group (NH_2). This reaction produces a molecule of water (H_2O) and two amino acids joined by a peptide bond (-CO-NH-). The two joined amino acids are called a dipeptide.

The formation of the peptide bond consumes energy, which, in organisms, is derived from ATP. Organisms use enzymes to produce nonribosomal peptides, and ribosomes to produce proteins via reactions that differ in details from dehydration synthesis.

Peptide bond formation in living cells occurs at the peptidyl transferase center (PTC) of the large ribosomal subunit and involves the transfer of the peptidyl group from peptidyl-tRNA to aminoacyl-tRNA. Despite numerous kinetic and theoretical studies, many details of this reaction remain unclear [Chem. Pharm. Bull. 69:734-740 (2021)].

wikipedia

How ribosomes make peptide bonds



Ribosomes are molecular machines that synthesize proteins in the cell. Recent biochemical analyses and high-resolution crystal structures of the bacterial ribosome have shown that the active site for the formation of peptide bonds – the peptidyl-transferase center – is composed solely of rRNA.

The ribosome binds two tRNA substrates, one with the growing peptide chain attached by a high-energy ester linkage to its 3' hydroxyl (the peptidyl-tRNA in the P site, orange), and the other with a single amino acid esterified to its 3' hydroxyl (the aminoacyl-tRNA in the A site, yellow). During peptide-bond formation, the α-amino group of the A-site aminoacyl-tRNA attacks the carbonyl carbon of the P-site peptidyl-tRNA to produce a new peptidyl-tRNA that is one-amino-acid longer in the A site and a deacylated tRNA in the P site. The peptidyl-transferase center is on the 50S subunit (green). On the 30S subunit (gray), aminoacyl-tRNA are recognized according to the match between their anticodons and the codon of mRNA in the A site.

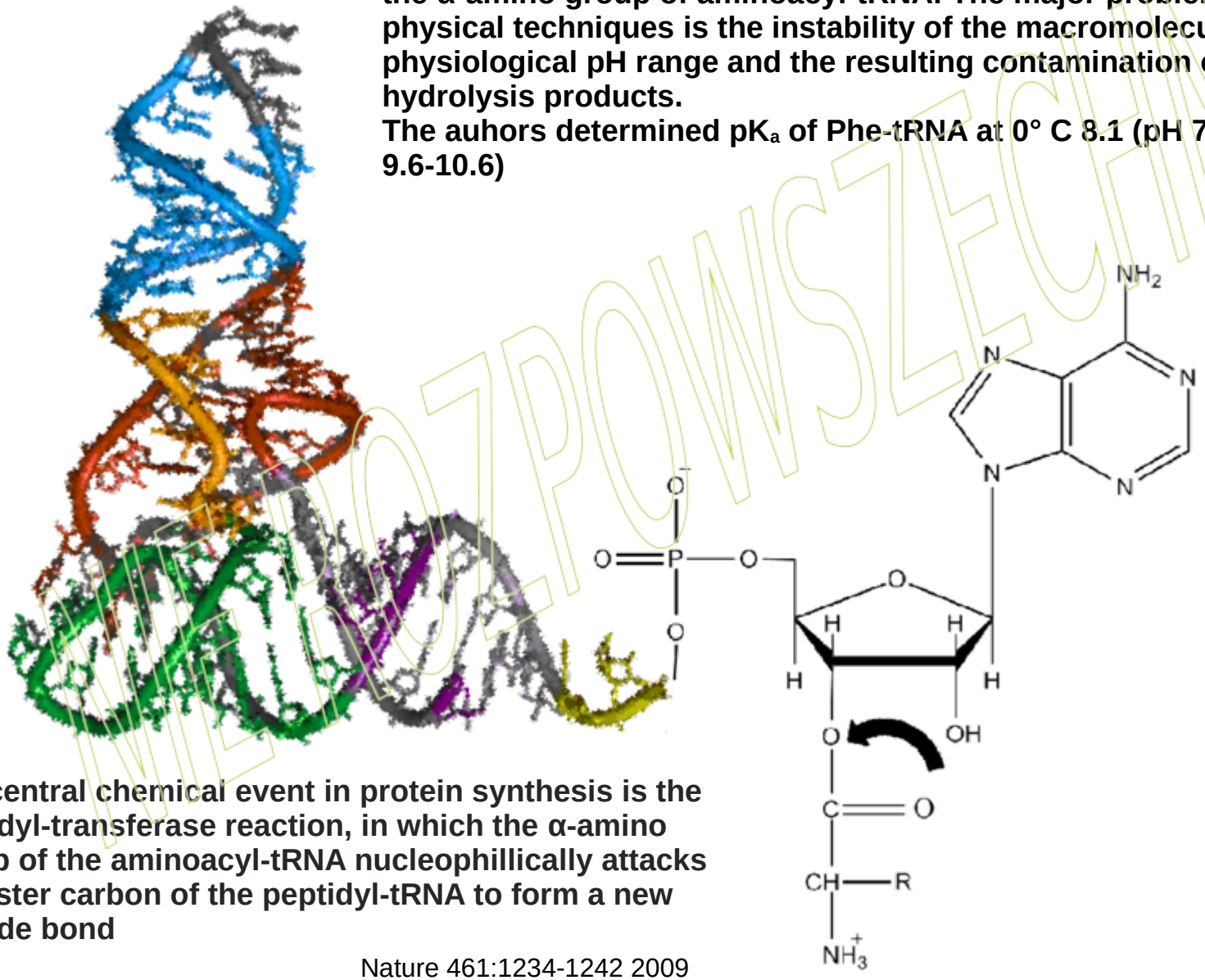
The second enzymatic activity associated with the peptidyl-transferase center is the hydrolytic cleavage of the ester bond in peptidyl-tRNA during termination of protein synthesis. In contrast to peptide-bond formation, which is an intrinsic activity of the ribosome and proceeds without auxiliary factors, peptide release requires specialized release factors that recognize termination codons and promote the hydrolysis of the P-site peptidyl-tRNA.

WEBOZPOWSZECHNY

TRENDS in Biochemical Sciences 32:20-26, 2007

Aminoacyl tRNA

Barend Kraal and Guido W. M. Swart, FEBS Lett. 121:86-90 (1980): Peptide bond formation is a condensation reaction in which the α-amino group of an aminoacyl-tRNA is brought into contact with the active ester linkage between the preceding tRNA and the nascent peptide. The reaction takes place close to the 3'- and 5'-ends of the tRNA, which have a special role in it. Until now no data have been available concerning the pK_a, and reactivity of the α-amino group of aminoacyl-tRNA. The major problem for conventional physical techniques is the instability of the macromolecule in the physiological pH range and the resulting contamination of the sample with hydrolysis products.
The auhors determined pK_a of Phe-tRNA at 0° C 8.1 (pH 7.0-9.4) and 9.7 (pH 9.6-10.6)



The central chemical event in protein synthesis is the peptidyl-transferase reaction, in which the α-amino group of the aminoacyl-tRNA nucleophillically attacks the ester carbon of the peptidyl-tRNA to form a new peptide bond

Nature 461:1234-1242 2009

wikipedia

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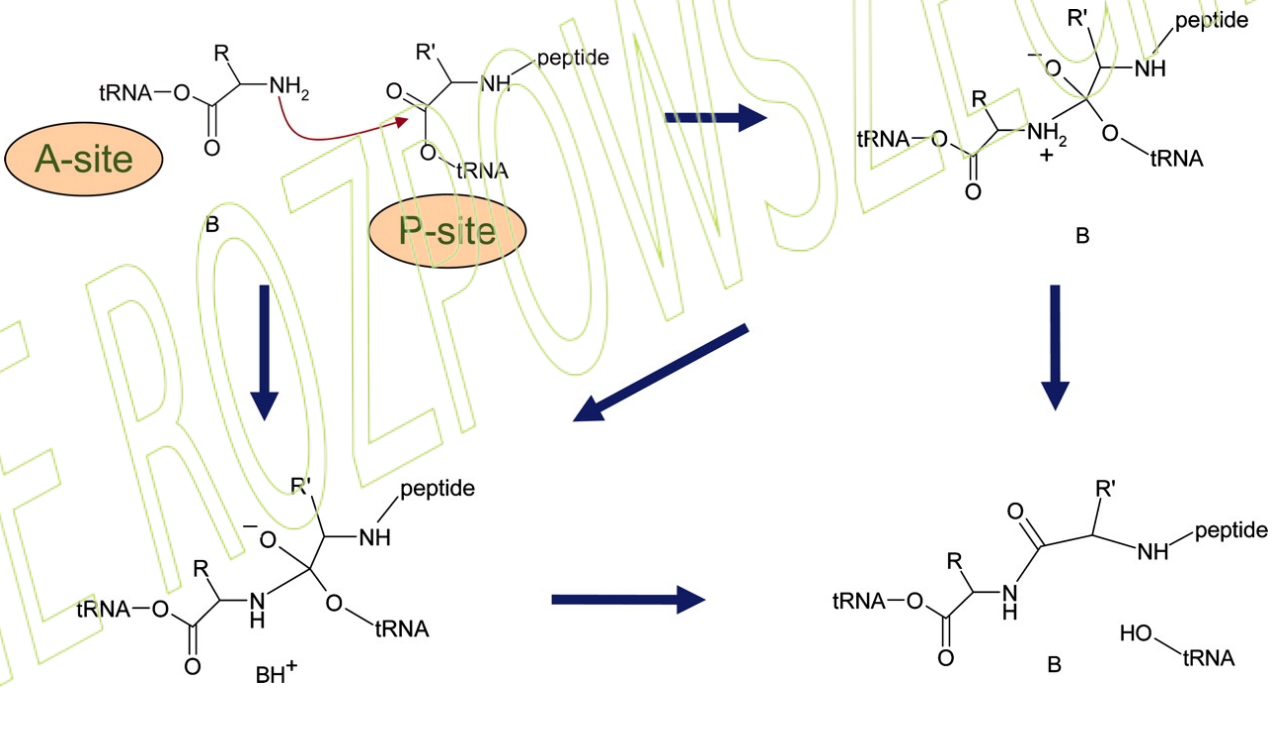
Mechanism of peptide bond synthesis on the ribosome

Stefan Trobro and Johan Åqvist

The ribosome translates genetic messages and catalyzes the synthesis of new proteins. The determination of high-resolution x-ray structures of ribosomal subunits has enabled considerable progress toward understanding the structural basis of the translation process.

…

Still, the actual mechanism of the peptidyl transfer reaction remains unclear. Although it is evident that the unprotonated form of the A-site α-amino nitrogen attacks the ester carbon of the donor peptidyl substrate, leading to a transient tetrahedral "intermediate," a major question is whether or not this process is subject to general acid–base catalysis by ribosomal groups.

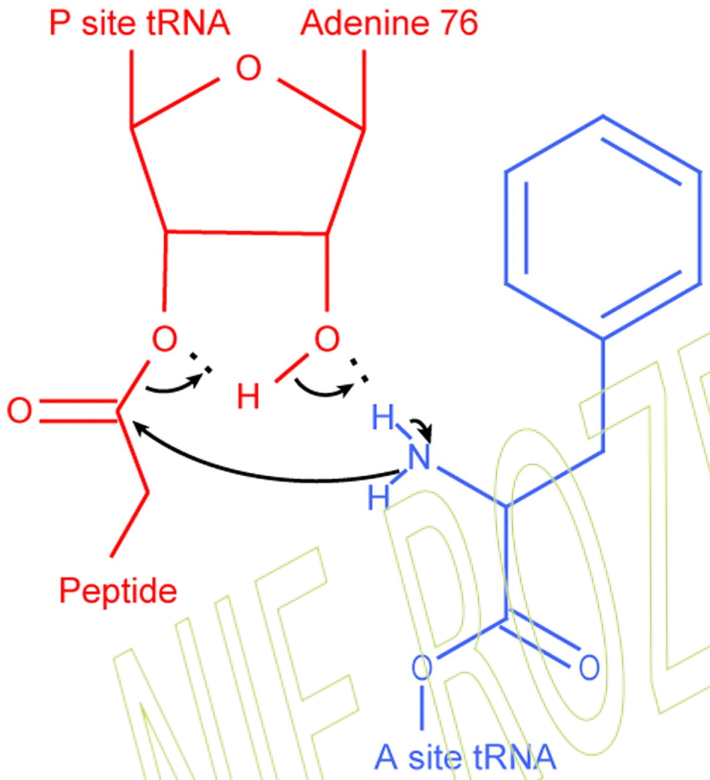


Possible mechanistic pathways for aminolysis of the ribosomal P-site peptidyl-tRNA ester bond by the A-site amino acid where the role of a putative rRNA base (B) is indicated.

PNAS 102:12395-12400 (2005)

NIE ROZPOWSZECHNIAĆ

Peptide bond formation (peptidyl transfer) is initiated by a nucleophilic attack of the α-amino group of the amino acid, ester linked to the A-site tRNA, on the ester carbonyl carbon of the peptide chain linked to the 3′-oxygen of the P-site tRNA. Peptidyl transfer requires that the α-amino group of the amino acid on the A-site tRNA is in charge neutral rather than in protonated NH_3^+ form.



The proposed mechanism of peptidyl transfer. A nucleophilic attack of the α-amino group of the A-site aa-tRNA (blue, here Phe-tRNA Phe) on the ester carbonyl carbon of the P-site peptidyl-tRNA (red) results in a six-membered transition state.

Aminoacyl-tRNA	pK_a^{aq} *	pK_a^{obs}
Asn-tRNA^{Asn}	6.8	5.9 ± 0.2
Phe-tRNA^{Phe}	7.2	6.1 ± 0.1
Ile-tRNA^{Ile}	7.8	6.1 ± 0.2
Ala-tRNA^{Ala}	7.9	6.3 ± 0.1
Gly-tRNA^{Gly}	7.8	7.36 ± 0.04
Pro-tRNA^{Pro}	8.6	7.8 ± 0.2

***pK_a of the α-NH_2 group of aa-tRNA in bulk water.**

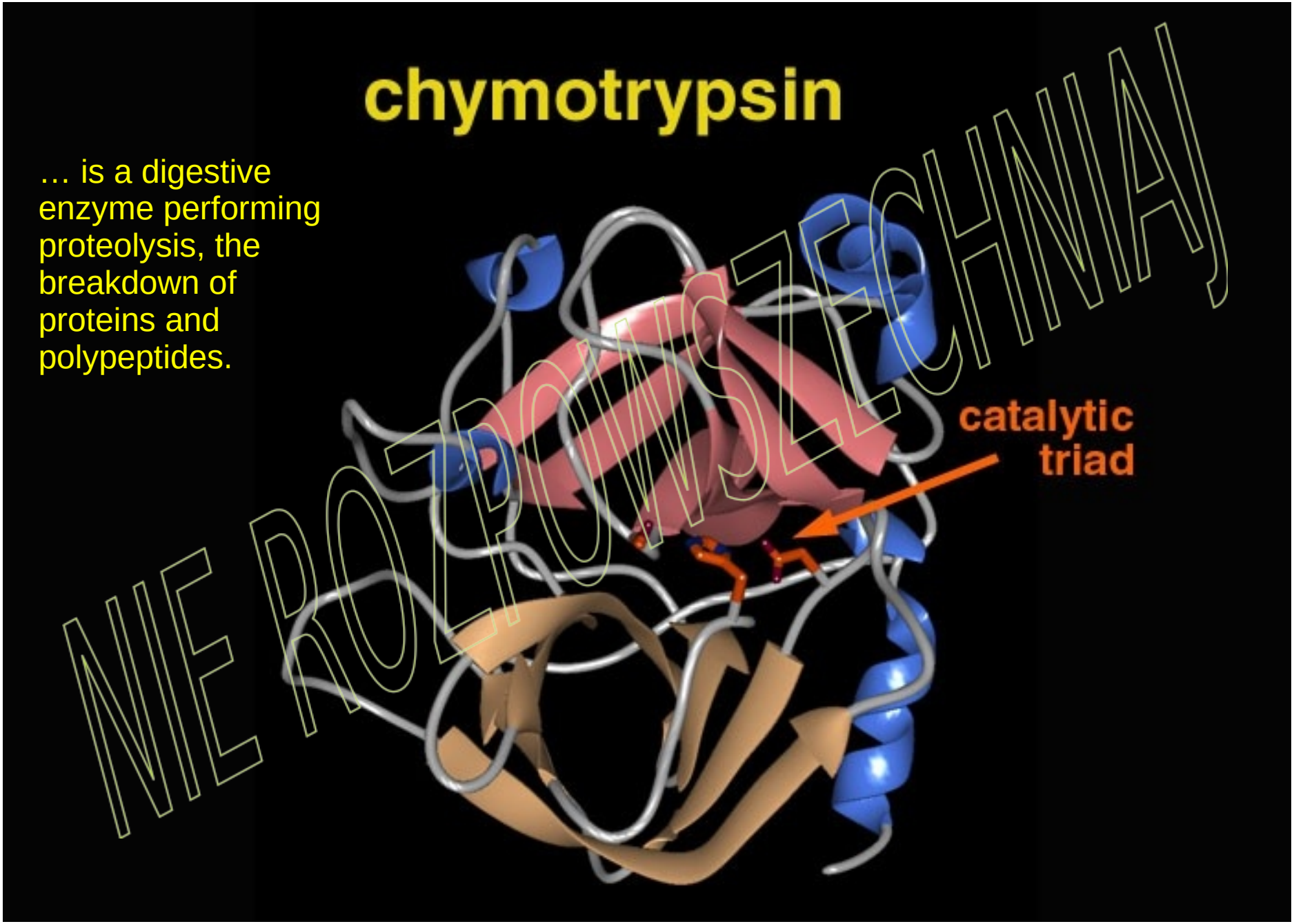
The pK_a-values of the α-amino group of different aa-tRNAs in solution at 20 °C were extrapolated from measured values of the pK_a of free amino acids and amino acid esters.

We studied the pH-dependence of ribosome catalyzed peptidyl transfer from fMet-tRNA^{fMet} to the aa-tRNAs Phe-tRNA^{Phe}, Ala-tRNA^{Ala}, Gly-tRNA^{Gly}, Pro-tRNA^{Pro}, Asn-tRNA^{Asn}, and Ile-tRNA^{Ile}, selected to cover a large range of intrinsic pK_a-values for the α-amino group of their amino acids. The peptidyl transfer rates were different at pH 7.5 and displayed different pH-dependence, quantified as the pH-value, pK_a^{obs}, at which the rate was half maximal. The pK_a^{obs}-values were downshifted relative to the intrinsic pK_a-value of aa-tRNAs in bulk solution.

PNAS 108:79-84 (2011)

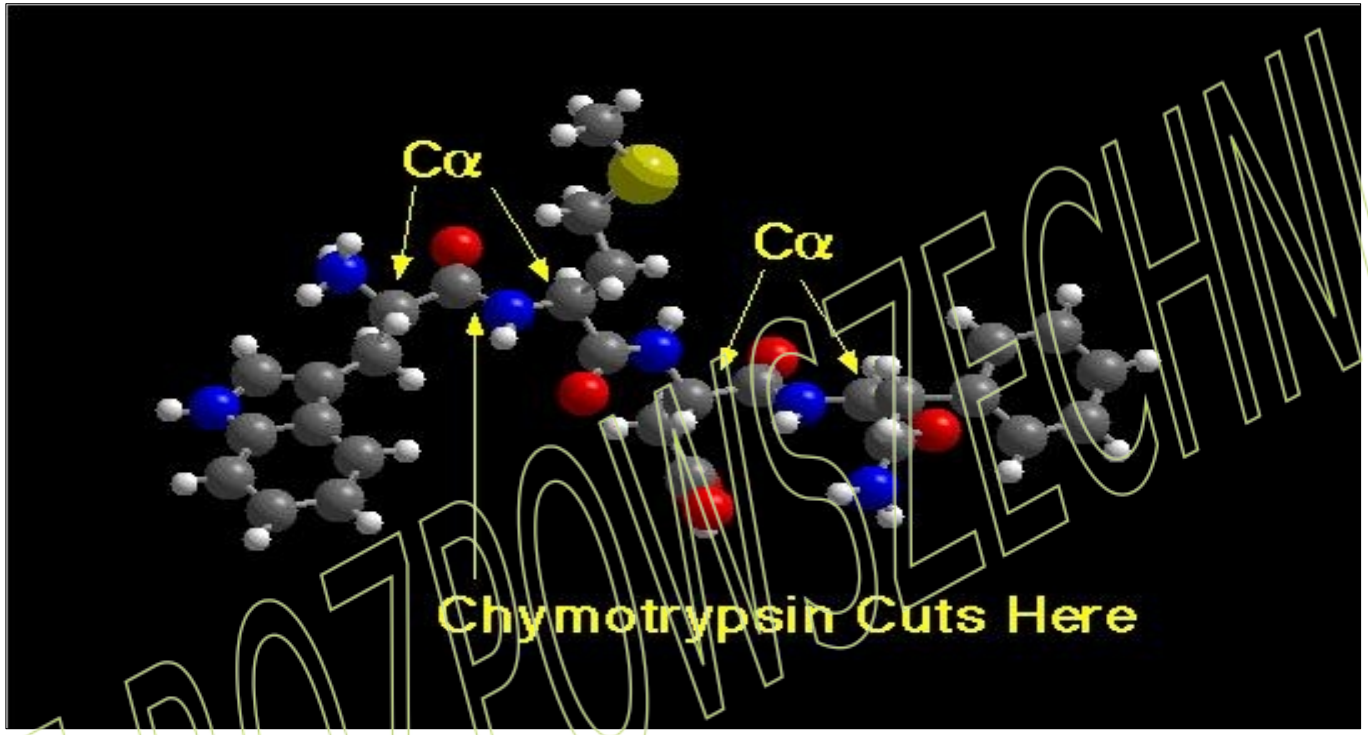
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Impact of H^+ on biochemical kinetics:



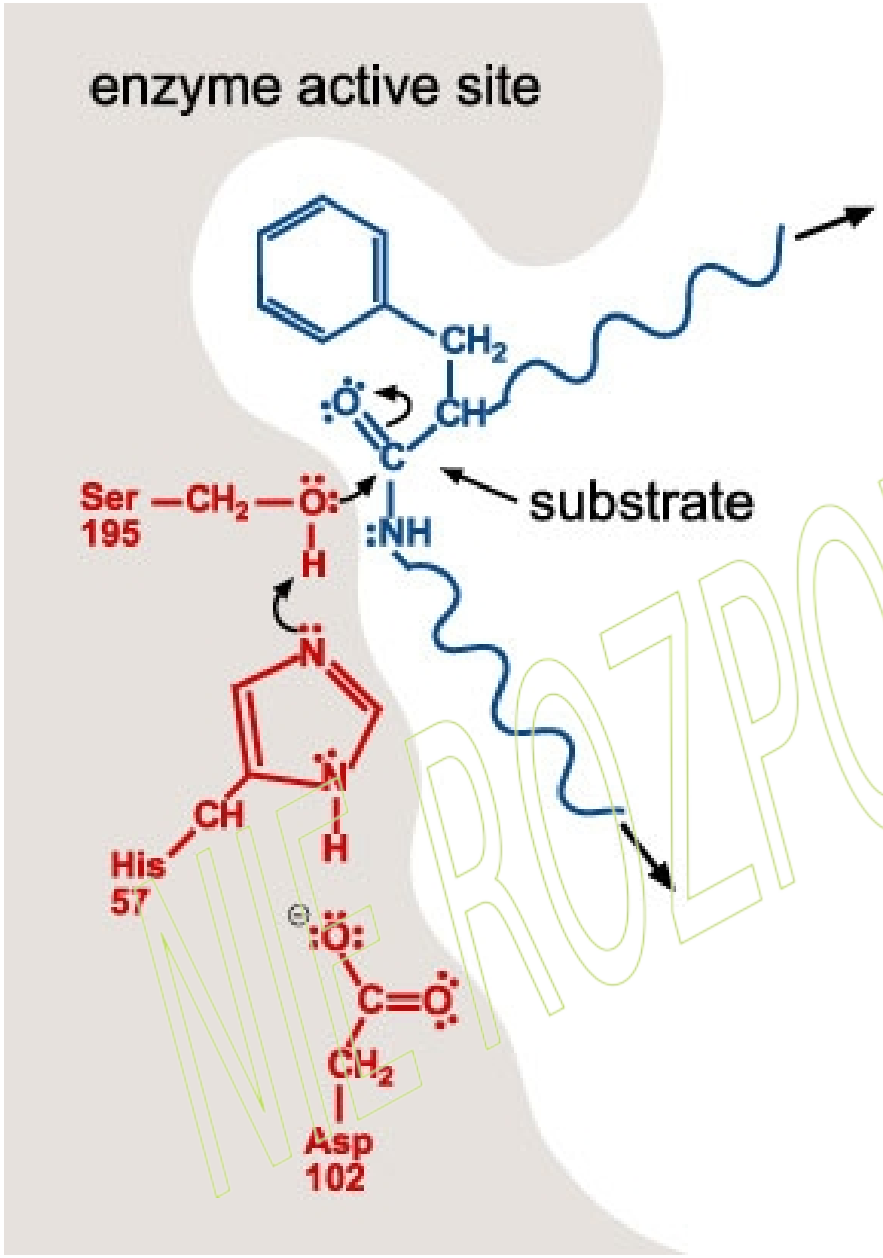
http://www.personal.psu.edu/sxt30/gallery_proteins.html

Protein hydrolysis by α-chymotrypsin



NIE ROZPOWSZECHNIAJ

Chymotrypsin is a mammalian digestive enzyme. Hydrolyzes peptide bonds. It has a selective effect on bonds on the carboxyl side of aromatic amino acids and those with large hydrophobic moieties (e.g. methionine). He's a serine protease, her the active center is composed of histidine, aspartic acid and serine. Next to cheese there is a non-polar "pocket" binding the hydrophobic side group of the amino acid, followed by cleavage of the peptide bond by the enzyme.



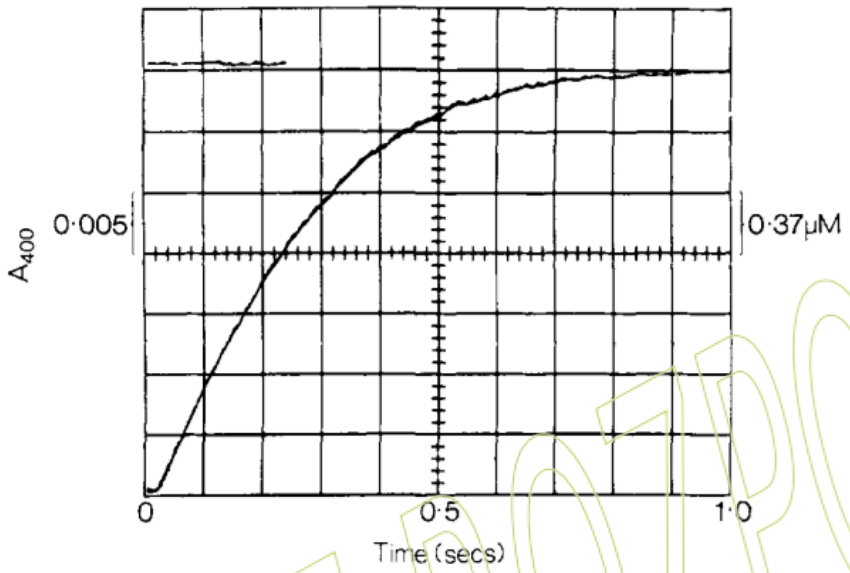
Stage #1: A polypeptide substrate moves into the active site of the enzyme. The shape, size, and amino acid sequence of chymotrypsins active site allow that part of the enzyme to bind a portion of a polypeptide that has nonpolar side chains, like those found in phenylalanine. Once the polypeptide is in the active site, an H^+ ion moves from the serine amino acid at position 195 (Ser-195) of the enzymes amino acid sequence to the histidine amino acid at position 57 (His-57). The oxygen atom in serines hydroxyl group then forms a covalent bond to the carbon of one of the substrates peptide bonds shifting the two electrons from one of the double bonds up to form a lone pair.

http://www.mpcfaculty.net/mark_bishop/chymotrypsin.htm

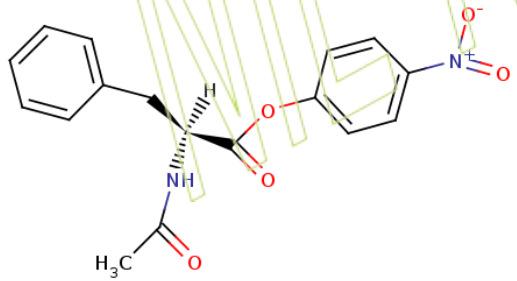
Anomalous pH Dependence of k_{cat}/K_M in Enzyme Reactions. Rate Constants for the Association of Chymotrypsin with Substrates

M. Renard and Alan R. Fersht
Biochemistry 12:4113-4718 (1973)

The hydrolysis of acetyl-L-phenylalanine p-nitrophenyl ester was monitored by the increase in absorbance of p-nitrophenyl at 340 nm using stopped-flow spectrophotometry of dead-time 4 msec and path length 1 cm.

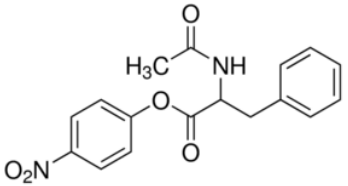


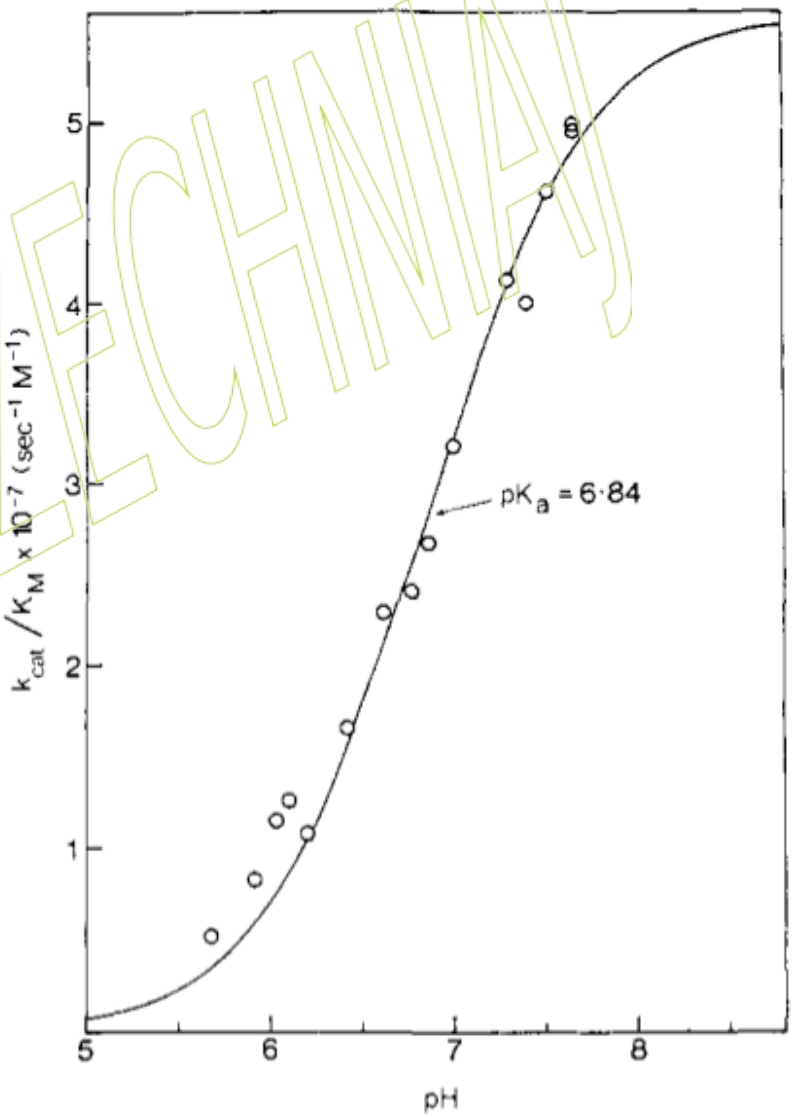
Typical trace of the release of p-nitrophenolate against time used for the determination of k_{cat}/K_M. Acetylphenylalanine p-nitrophenyl ester (10 μM) was mixed with 0.32 μM enzyme in pH 7.66 buffer in the stopped-flow spectrophotometer.



N-acetyl-L-phenylalanine-p-nitrophenyl ester

https://www.brenda-enzymes.org/structure.php?n=12813





k_{cat}/K_M against pH for the hydrolysis of acetyl phenylalanine p-nitrophenyl ester by δ-chymotrypsin at 25°.

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Michaelis-Menten Equation

$$E + S \underset{k_{-1}}{\overset{k_1}{\leftrightarrow}} ES \xrightarrow{k_{cat}} E + P$$

$$v = \frac{d[P]}{dt} = k_{cat}[ES]$$

For steady-state conditions, i.e. for [ES] = const., we have:

$$\frac{d[ES]}{dt} = k_1[E][S] - (k_{-1} + k_{cat})[ES] = 0$$

$$[ES] = \frac{k_1[E][S]}{k_{-1} + k_{cat}}$$

$$[ES] = \frac{k_1([E]_o - [ES])[S]}{k_{-1} + k_{cat}}$$

$$v = \frac{k_{cat}}{K_m}[E][S]$$

$$v = k_{cat} \cdot \frac{[E]_o[S]}{K_m + [S]}$$

$$K_m = \frac{k_{-1} + k_{cat}}{k_1}$$ **Michaelis constant**

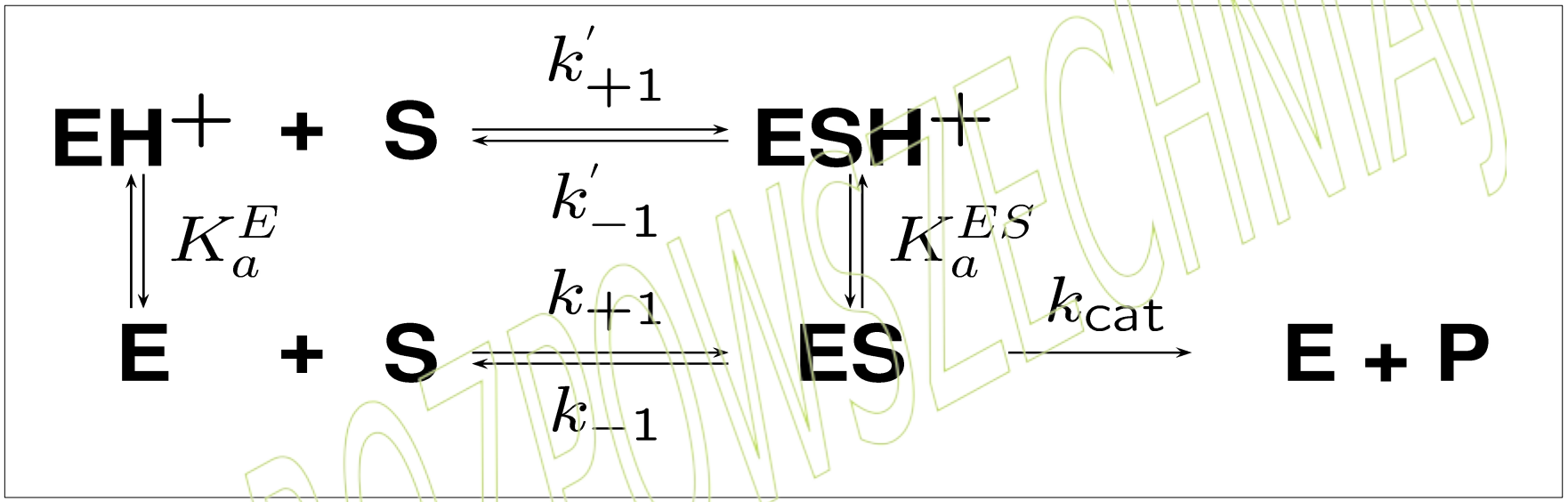
$$v_{max} = k_{cat}[E]_o$$ **Maximal velocity**

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Zależność działania enzymu od pH

(jedna grupa protonowalna w centrum aktywnym)

W przypadku proteaz serynowych, His znajdująca się w miejscu aktywnym musi być zdeprotonowana, aby mogła zajść reakcja hydrolizy wiązania peptydowego w substracie.



$$K_a^E = \frac{[E][H^+]}{[EH^+]} \qquad K_a^{ES} = \frac{[ES][H^+]}{[ESH^+]} \qquad [E]_o = [E] + [EH^+] + [ES] + [ESH^+]$$

$$[EH^+] = \frac{[E][H^+]}{K_a^E} \qquad [ESH^+] = \frac{[ES][H^+]}{K_a^{ES}}$$

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$$v=\frac{d[P]}{dt}=k_{cat}[ES] \qquad \frac{d[ES]}{dt}=-(k_{cat}+k_{-1})[ES]+k_{+1}[E][S]=0 \quad \Rightarrow \quad [ES]=\frac{[E][S]}{K_M}$$

$$[E]_o=[E]\cdot\left(1+\frac{[H^+]}{K_a^E}\right)+[ES]\cdot\left(1+\frac{[H^+]}{K_a^{ES}}\right)\equiv[E]\cdot\alpha+[ES]\cdot\beta$$

$$\Downarrow$$

$$[E]=\frac{[E]_o-[ES]\cdot\beta}{\alpha}$$

$$[ES]=\frac{[E][S]}{K_M}=\frac{\left(\frac{[E]_o-[ES]\cdot\beta}{\alpha}\right)\cdot[S]}{K_M} \quad \Rightarrow \quad [ES]=\frac{[E]_o\cdot[S]}{K_M\cdot\alpha+[S]\cdot\beta}$$

$$v=\frac{d[P]}{dt}=k_{cat}[ES]=k_{cat}\cdot\frac{[E]_o\cdot[S]}{K_M\cdot\alpha+[S]\cdot\beta}$$

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K_M i k_{cat} odnoszą się do enzymu ze zdeprotonowana jonizowalną grupą reaktywną. W przypadku enzymu, którego grupa reaktywna musi być uprotonowana aby zaszła reakcja, mielibyśmy K_M i k_{cat} odnoszace się do formy uprotonowanej grupy reaktywnej.

$$v = k_{cat} \cdot \frac{[E]_o \cdot [S]}{K_M \cdot \alpha + [S] \cdot \beta} = \frac{\frac{k_{cat} \cdot [E]_o}{\beta} \cdot [S]}{\frac{K_M \cdot \alpha}{\beta} + [S]} \equiv \frac{\frac{V_{max}}{\beta} \cdot [S]}{\frac{K_M \cdot \alpha}{\beta} + [S]} \equiv \frac{\tilde{V}_{max} \cdot [S]}{[S] + \tilde{K}_M}$$

$$\tilde{V}_{max} = \frac{k_{cat}[E]_o}{1 + [H^+]/K_a^{ES}}$$

$$\tilde{K}_M = \frac{K_M \cdot \left(1 + [H^+]/K_a^E\right)}{1 + [H^+]/K_a^{ES}}$$

$$v = \frac{V_{max}[S]}{[S] + K_M} \approx \frac{k_{cat}}{K_M}[E]_o[S]_o \quad (dla \ \ [S]_o \ll K_M)$$

$$v(pH) = \frac{\frac{V_{max}}{\beta} \cdot [S]}{[S] + \frac{K_M \cdot \alpha}{\beta}} \approx \frac{V_{max} \cdot [S]_o}{K_M \cdot \alpha} = \frac{k_{cat} \cdot [E]_o \cdot [S]_o}{K_M \cdot \left(1 + \frac{[H^+]}{K_a^E}\right)}$$

$$\frac{k_{cat}}{K_M \cdot \left(1 + \frac{[H^+]}{K_a^E}\right)}$$

pełni rolę stałej szybkości reakcji drugiego rzędu (dwucząsteczkowej) dla małych stężeń substratu w warunkach początkowych zachodzenia reakcji wyznaczona zależność v od pH daje nam zależne od pH k_{cat}/K_M, które zawiera w sobie stałą kwasowej dysocjacji grupy reaktywnej w apo-enzymie.

NIE ROZMNAŻAĆ

$$\tilde{k}_{cat} = \frac{k_{cat}}{1 + [H^+]/K_a^{ES}}$$

$$\tilde{K}_M = \frac{K_M \cdot \left(1 + [H^+]/K_a^{E}\right)}{1 + [H^+]/K_a^{ES}}$$

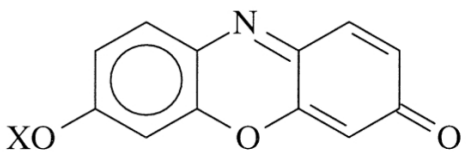
$$\left(\frac{\tilde{k}_{cat}}{K_M}\right) = \frac{k_{cat}}{K_M \cdot \left(1 + \frac{[H^+]}{K_a^{E}}\right)}$$

NIE ROZPOWSZECHNIAĆ

Studies on the α-chymotrypsin-catalysed hydrolysis of resorufin acetate and resorufin bromoacetate

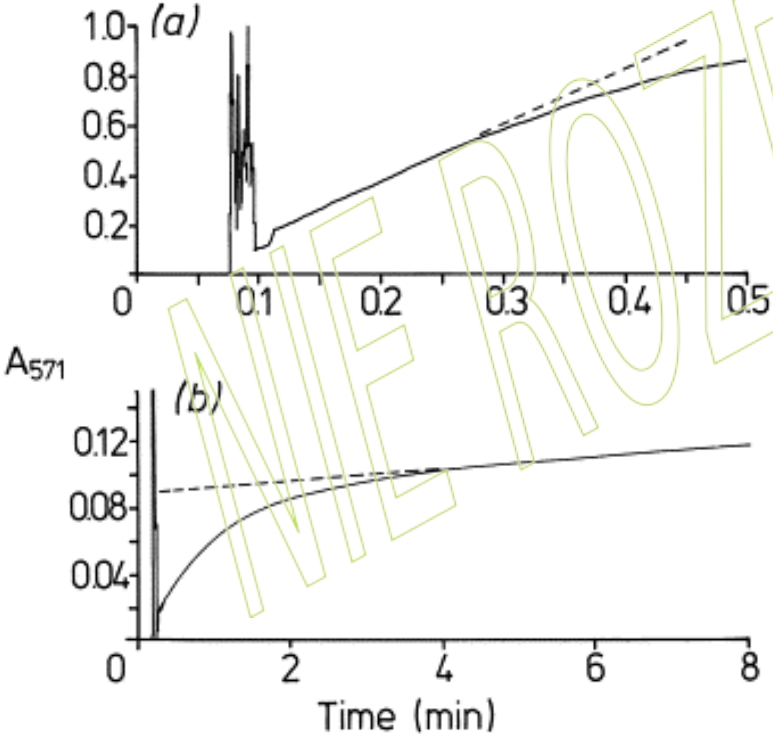
Trevor M. Kitson, BBA 1385:43-52 (1998)

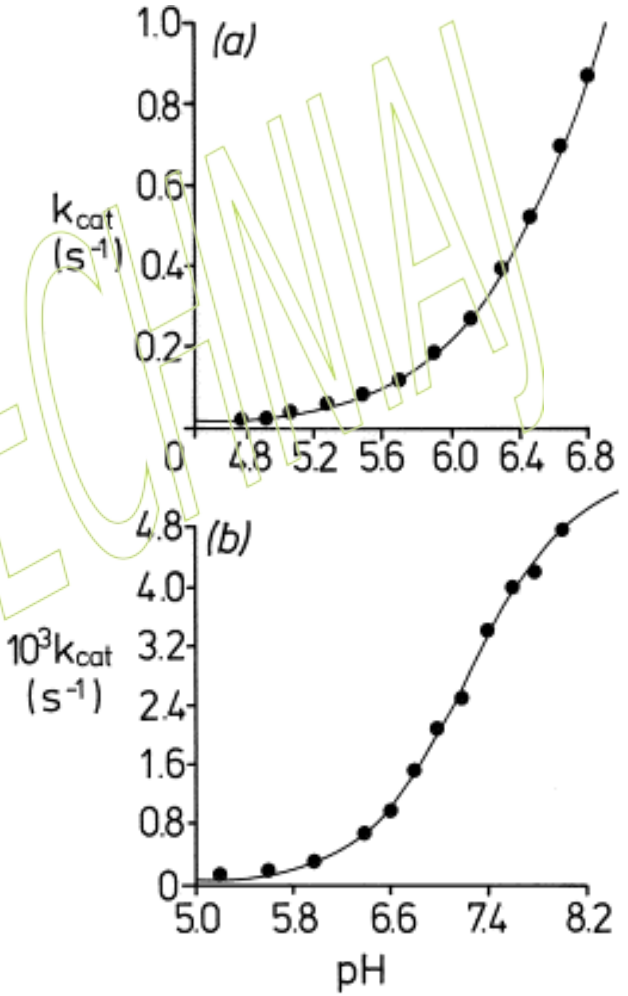
Methods: conventional spectrophotometry



Structure of the compounds: resorufin, X=H; resorufin acetate, X=CH_3CO; resorufin bromoacetate, X=$BrCH_2CO$.

Chymotrypsin-catalysed hydrolysis of resorufin esters.
(a) is a spectrophotometer trace showing the hydrolysis of resorufin bromoacetate (25 μM) catalysed by chymotrypsin (2.0 μM) in 50 mM sodium phosphate buffer, pH 6.0, at 25°C. Substrate was added at the point marked by the irregularity in the trace. The dashed line is a continuation of the linear part of the assay showing how the experimental trace tails off as substrate is used up.
(b) is a spectrophotometer trace showing the hydrolysis of resorufin acetate (25 μM) catalysed by chymotrypsin (2.0 μM) under the same conditions. The dashed line is a back-projection of the linear steady-state rate, from which the half-life of the burst phase was estimated graphically.





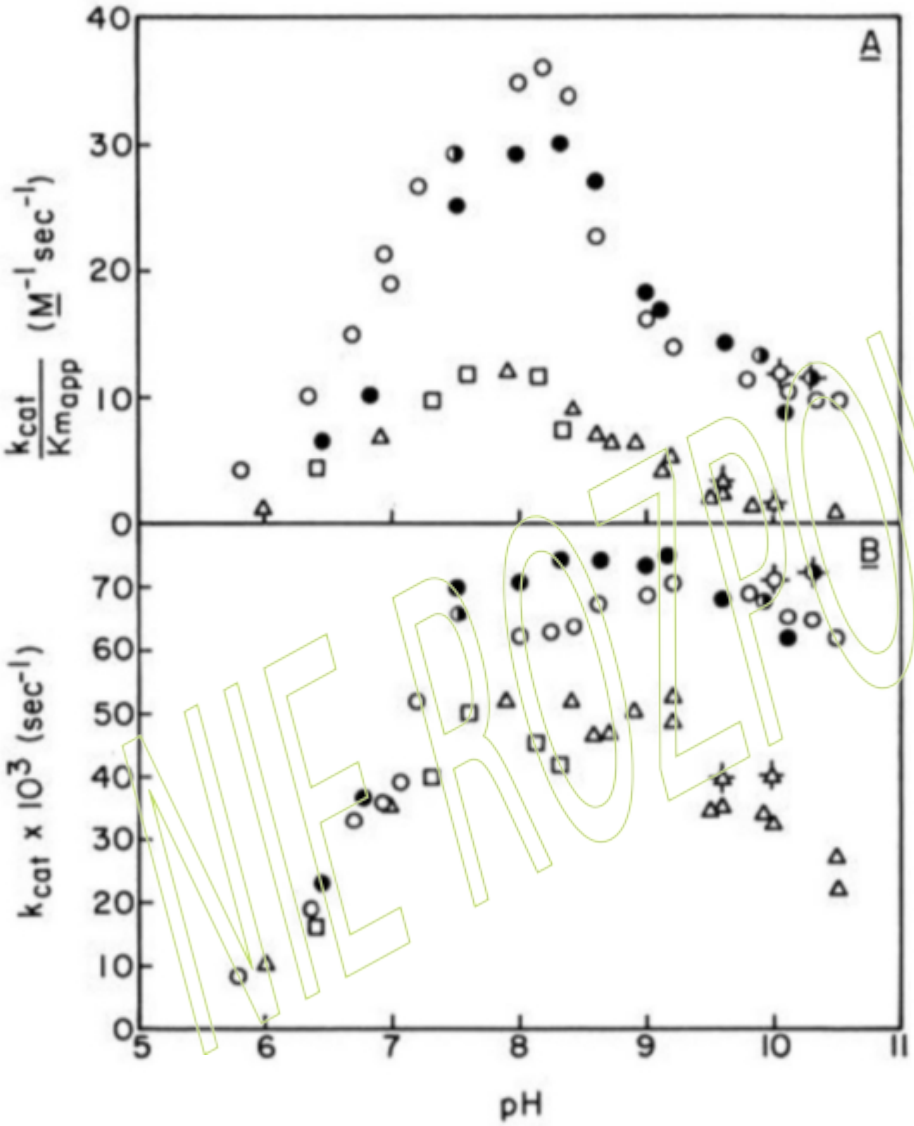
pH-Dependence of chymotrypsin-catalysed hydrolysis of resorufin esters. The steady-state rate of ester hydrolysis (after correction for spontaneous hydrolysis) was converted into values of k_{cat} over a range of pH using citrate/phosphate buffers. Each point is the average of two closely agreeing determinations. (a) substrate = resorufin bromoacetate (0.1 mM), [enzyme]=4.0 μM. The curve is the best-fit theoretical ionisa-tion curve and has pK_a=6.92. (b) substrate = resorufin acetate (0.2 mM) , [enzyme]=10 μM. The curve is the best-fit theoretical ionisation curve and has pK_a=7.25.

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Chymotrypsin-catalyzed hydrolysis of N-acetyl-L-tryptophanamide (ATA)

Albert Himoe, Paul C. Parks and George P. Hess
J. Biol. Chem. 242:919-929 (1967)

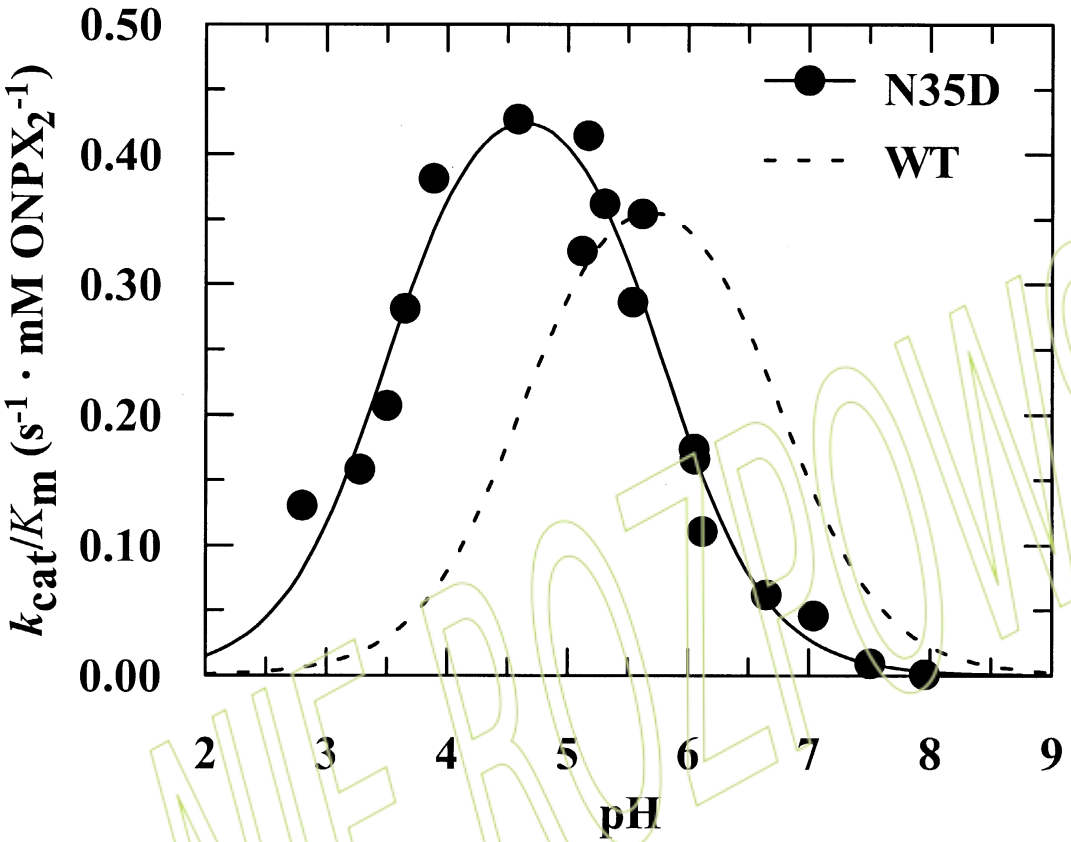
Methods: absorption 570 nm



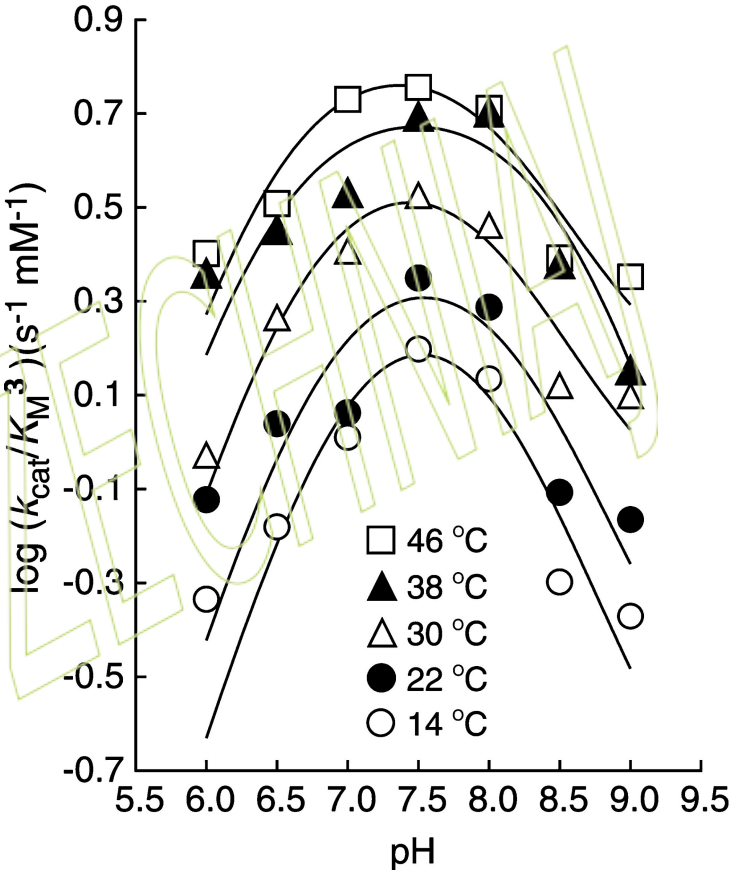
The activity pH profile of chymotrypsin is depicted in the Figure lead to distinct pK_a values for the enzyme: pK_{a1}=6.7 and pK_{a2}=9.6. It was later determined that pK_{a1} was due solely to the increase of k_{cat} with pH, whereas pK_{a2}= was due to both an increase of K_M and a decrease of k_{cat} with pH [Kahana and Shalitin, Isr. J. Chem. 12, 573-589 (1974)]. Based on knowledge of the structure and catalytic mechanism of chymotrypsin, we can hypothesize that the active site His-57 titrates with pK_{a1}=6.7, while its N-terminal Ile-16 titrates with pK_{a2}=9.6: His-57 must be deprotonated in order to base-catalyze the Ser-195 -OH nucleophile, while N-terminal Ile-16 must be protonated in order to form the key salt bridge that keeps the substrate binding cleft unblocked and stabilizes the optimal position of Asp-194 , Ser- 195, and other important nearby residues at the active site.

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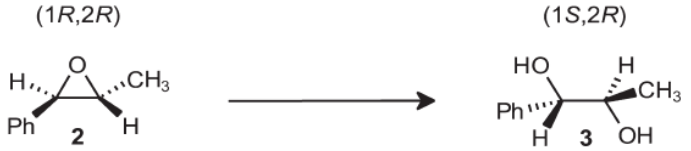
Enzymes with two catalytic groups at the active site. Dependence of enzyme kinetics on pH: bell−like shape pH dependences



pH-dependence of k_{cat}/K_M for Asn35Asp (N35D) mutant of Bacillus circulans xylanase (BCX) (•) at 25 C towards the synthetic substrate orthonitro-phenyl β-xylobioside ($ONPX_2$). Substitution of an Asp residue at position 35 shifts the pH optimum from 5.7 for the WT protein to 4.6 for the N35D mutant. The activity profile follows ionizations with pK_a values of 3.5 and 5.8 in the N35D enzyme (—) and 4.6 and 6.7 in the WT enzyme (- - -). M.D. Joshi et al., J. Mol. Biol., 299:255-279 (2000) (ksylany = rodzaj polisacharydów)



Effect of pH on k_{cat}/K_M for formation of compound 3 from compound 2, catalyzed by epoxide hydrolase from *Solanum tuberosum* (StEH1), at different temperatures. Fitted lines correspond to titrations involving two ionization events (source: Biochemistry, 49:2297-2304, 2010).



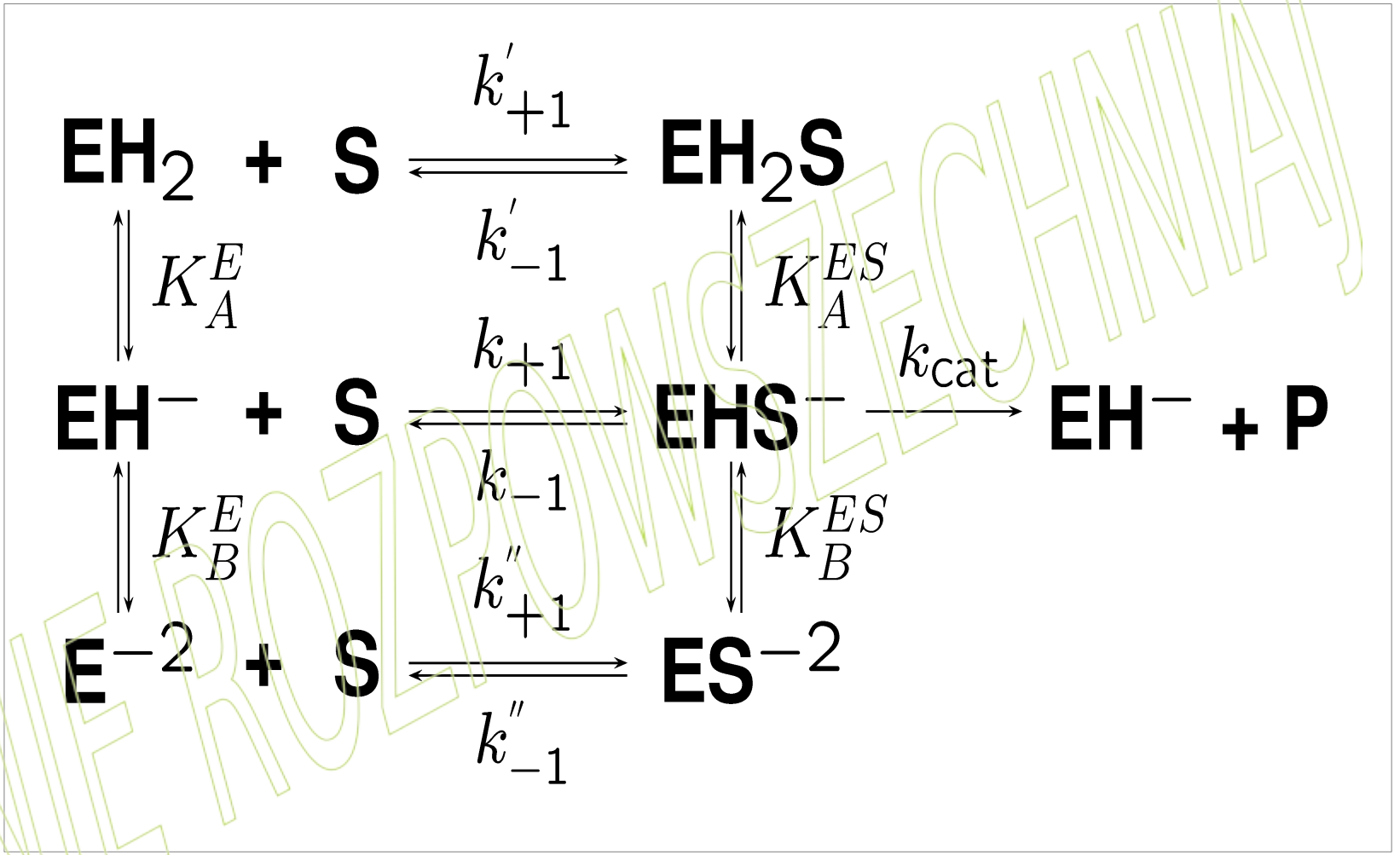
trans-2-methylstyrene oxide (2-MeSO), (1R,2R)-enantiomer

(1S,2R) diol

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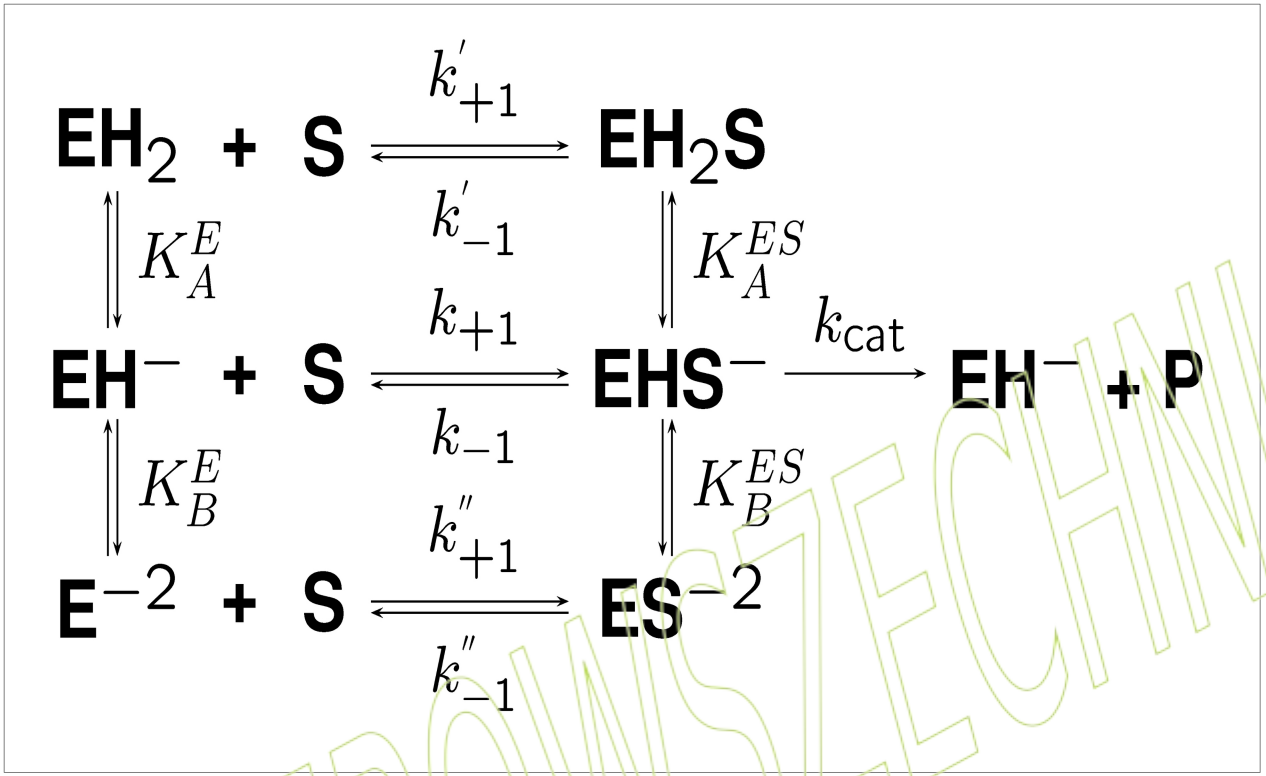
Zależność działania enzymu od pH

(dwie grupy protonowalne w centrum aktywnym)



$$v = \left(\frac{d[P]}{dt}\right) = k_{cat}[EHS^-]$$

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$$K_A^E=\frac{[EH^-][H^+]}{[EH_2]} \qquad K_A^{ES}=\frac{[EHS^-][H^+]}{[EH_2S]}$$

$$K_B^E=\frac{[E^{-2}][H^+]}{[EH^-]} \qquad K_B^{ES}=\frac{[ES^{-2}][H^+]}{[EHS^-]}$$

$$[E]_o=[EH_2]+[EH^-]+[E^{-2}]+[EH_2S]+[EHS^-]+[ES^{-2}]$$

$$[EH_2]=\frac{[EH^-][H^+]}{K_A^E} \qquad [EH_2S]=\frac{[EHS^-][H^+]}{K_A^{ES}}$$

$$[E^{-2}]=\frac{[EH^-]K_B^E}{[H^+]} \qquad [ES^{-2}]=\frac{[EHS^-]K_B^{ES}}{[H^+]}$$

$$[E]_o=\frac{[EH^-][H^+]}{K_A^E}+[EH^-]+\frac{[EH^-]K_B^E}{[H^+]}+\frac{[EHS^-][H^+]}{K_A^{ES}}+[EHS^-]+\frac{[EHS^-]K_B^{ES}}{[H^+]}$$

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$$[E]_o = \frac{[EH^-][H^+]}{K_A^E} + [EH^-] + \frac{[EH^-]K_B^E}{[H^+]} + \frac{[EHS^-][H^+]}{K_A^{ES}} + [EHS^-] + \frac{[EHS^-]K_B^{ES}}{[H^+]}$$

$$\Downarrow$$

$$[E]_o = [EH^-]\left(1 + \frac{[H^+]}{K_A^E} + \frac{K_B^E}{[H^+]}\right) + [EHS^-]\left(1 + \frac{[H^+]}{K_A^{ES}} + \frac{K_B^{ES}}{[H^+]}\right) = [EH^-]\alpha + [EHS^-]\beta$$

$$\frac{d[EHS^-]}{dt} = -(k_{cat} + k_{-1})[EHS^-] + k_{+1}[EH^-][S] = 0 \quad \Rightarrow \quad [EHS^-] = \frac{[EH^-][S]}{K_M}$$

$$[EH^-] = \frac{[E]_o - [EHS^-]\beta}{\alpha}$$

$$[EHS^-] = \frac{\frac{[E]_o - [EHS^-]\beta}{\alpha}[S]}{K_M} \quad \Rightarrow \quad [EHS^-] = \frac{[E]_o[S]}{K_M\alpha + [S]\beta}$$

$$v = \left(\frac{d[P]}{dt}\right) = k_{cat}[EHS^-] = k_{cat} \cdot \frac{[E]_o[S]}{K_M\alpha + [S]\beta}$$

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$$v = \left(\frac{d[P]}{dt} \right) = k_{cat}[EHS^-] = k_{cat} \cdot \frac{[E]_o[S]}{K_M \alpha + [S]\beta}$$

$$\alpha = \left(1 + \frac{[H^+]}{K_A^E} + \frac{K_B^E}{[H^+]} \right) \qquad \beta = \left(1 + \frac{[H^+]}{K_A^{ES}} + \frac{K_B^{ES}}{[H^+]} \right)$$

$$v = k_{cat} \cdot \frac{[E]_o \cdot [S]}{K_M \cdot \alpha + [S] \cdot \beta} = \frac{\frac{k_{cat} \cdot [E]_o}{\beta} \cdot [S]}{\frac{K_M \cdot \alpha}{\beta} + [S]} \equiv \frac{\frac{V_{max}}{\beta} \cdot [S]}{\frac{K_M \cdot \alpha}{\beta} + [S]} \equiv \frac{\tilde{V}_{max} \cdot [S]}{[S] + \tilde{K}_M}$$

$$\tilde{V}_{max} = \frac{V_{max}}{\beta} = \frac{V_{max}}{\left(1 + \frac{[H^+]}{K_A^{ES}} + \frac{K_B^{ES}}{[H^+]} \right)} \qquad \tilde{K}_M = \frac{K_M \cdot \alpha}{\beta} = \frac{K_M \cdot \left(1 + \frac{[H^+]}{K_A^E} + \frac{K_B^E}{[H^+]} \right)}{\left(1 + \frac{[H^+]}{K_A^{ES}} + \frac{K_B^{ES}}{[H^+]} \right)}$$

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