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DYNAMICS OF PROTON TRANSFER AND ENZYMATIC ACTIVITY

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The rate-determining elementary reaction step, i.e. proton transfer from the chymotrypsin active centre to the scissile substrate bond has been studied in the present work. On the basis of our theoretical results a hypothesis was formulated to explain chymotrypsin enzymatic efficiency. After ES complex formation excited vibrational states are populated in the enzyme molecule. In the rate-determining elementary reaction step, the proton transfer takes place from the first excited vibrational state of the N-H bond in the imidazole group of His57. This proton transfer is realised by quantum mechanical tunneling mechanism.

1. Introduction

For the process of hydrolysis of amide and ester substrates catalysed by chymotrypsin, the reaction mechanism has been proposed by Blow et al. [1–3] – eq. (1).

$$E + S \rightleftharpoons ES \rightleftharpoons TI_1 \rightleftharpoons AE + P_1 \overset{H_2O}{\rightleftharpoons} TI_2 \rightleftharpoons E + P_2. \quad (1)$$

An acylenzyme (AE) formation is rate-determining for amide substrate hydrolysis. On the other hand deacylation is rate-determining for ester substrates hydrolysis [4,5]. Experimental studies [5–8] support the existence of a tetrahedral intermediate (TI_1) in the acylation step of chymotrypsin action. For amide substrates, there is evidence that the formation of AE involves the breakdown of TI_1 [8,9]. Moreover, the measurement of the deuterium isotope effect $k_{AE}(H_2O)/k_{AE}(D_2O) \approx 2.2$ [5,10–13] in the AE formation indicates that proton transfer is the rate-limiting elementary reaction step in the breakdown of TI_1.

The catalytic mechanism of serine proteases, especially of chymotrypsin has been extensively studied by experimental methods [5,14] as well as by theoretical methods [15–24]. The results of theoretical works support the Blow's reaction mechanism-eq. (1) and also the proposed protomeric structure of chymotrypsin active centre [1]. The chymotrypsin active centre – charge relay system (fig. 1) – is a H-bonded chain connecting Asp102, His57 and Ser195. As suggested by Blow et al. [1], the reactive structure of active centre is structure II., fig. 1., where the negative charge is located on O^{γ}Ser195 and imidazole of His57 as well as carbonyl of Asp102 are neutral. The results of theoretical calculations confirm that Asp 102 acts as ultimate proton acceptor in the charge relay system. These results are in accordance with the experimental studies on pH dependence of the reaction rate of that catalytic process [25,26] and with ^{13}C NMR results [27], which state that at pH 7 (maximum catalytic activity of chymotrypsin), the residues of Asp102 and His57 are neutral. Zundel [38] by IR study of the model of H-bonds in charge-relay system has shown, that proton transfer equilibrium I-II (fig. 1) is controlled by the polarity of the environment. In the substrate bound state the water molecules are removed from that side of active centre and structure II with $^{\ominus}O^{\gamma}$Ser195 and neutral His57 and Asp102 is stabilised.

As indicated above, the rate limiting elementary reaction step of amide substrates hydrolysis is the proton transfer from imidazole of His57 to N atom of hydrolysed peptide bond – fig. 2. The rate constant of this step for specific substrates is $k_{AE} \approx 1.5\ s^{-1}$ (pH 7,

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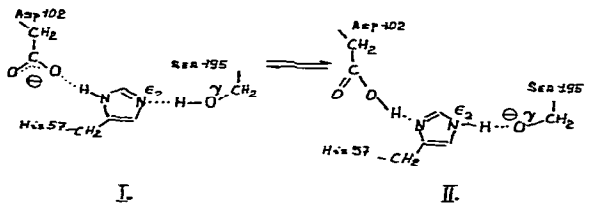

Fig. 1. The equilibrium structure of chymotrypsin active centre (pH ≈ 7–8).

T = 15 C) [14]. For specific ester substrates $k_{AE} \approx 100\ s^{-1}$ (pH 7, T = 25 C) and activation enthalpy and reaction enthalpy, respectively are $\Delta H^{\ddagger}_{AE} \approx 42$ kJ mole^{-1} and $\Delta H_{AE} \approx -8.5$ kJ mole^{-1} [28]. In comparison with uncatalysed hydrolysis, the rate of the enzymatic hydrolytic process is about 10^5 times higher [14]. Such a rate enhancement is surprising, because in accordance with Blow's reaction mechanism and reactive structure II of the active centre, the rate-determining reaction step is acid-catalysed by a very weak acid with $pK_a \sim 7$, i.e. neutral imidazole molecule.

In an effort to understand the physico-chemical nature of chymotrypsin catalytic efficiency, we have studied in the present work the dynamics of proton transfer from imidazole molecule to a substrate.

2. The dynamics of proton transfer

The theoretical study of chymotrypsin reaction path shows that in the stage of breakdown of TI_1, the linear

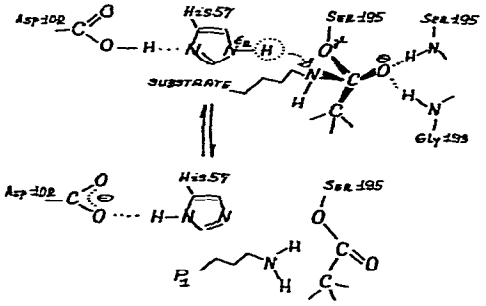

Fig. 2. The breakdown of tetrahedral intermediate (TI_1) and formation of acylenzyme (AE). The cycled hydrogen is transferred as proton to cleaved substrate bond.

H-bond is formed between $N^{\epsilon 2}$ atom of imidazole His57 and N atom of substrate leaving group [22]. The distance between the $N^{\epsilon 2}$ and N is 3 Å.

In the case of linear H-bonds, proton transfer process is satisfactorily described by the potential function of proton transfer along the stretching vibration of a particular bond [36].

Let us assume that during the proton transfer process, the internal coordinates of the other atoms of the H-bond network do not change. The system can be described by one-dimensional Schrödinger's equation:

$$\mathcal{H}(x)\Psi(x, t) = i\hbar(\partial/\partial t)\Psi(x, t) \qquad (2)$$

The hamiltonian of the system is

$$\mathcal{H}(x) = -\frac{1}{2\mu}\frac{d^2}{dt^2} + V(x) = T(x) + V(x),$$

where μ is the reduced mass of the transferred proton and $V(x)$ is the potential function, which for H^+ transfer is in general an asymmetric double well potential (ADWP) – fig. 3. For ADWP of proton transfer a very convenient analytical form was suggested [29].

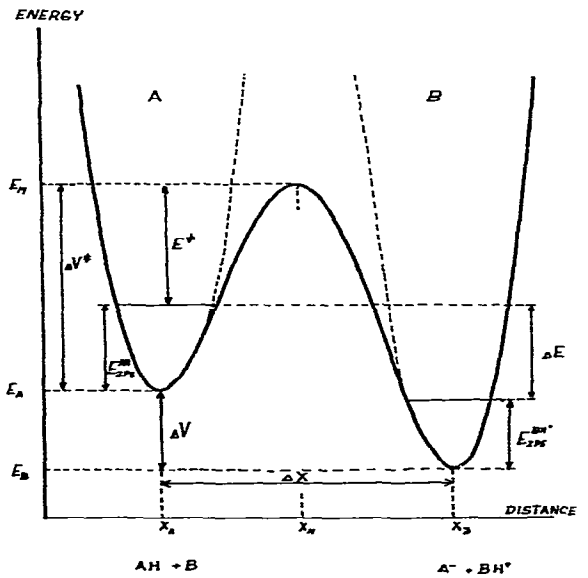

Fig. 3. The assymetric double well potential (ADWP) of proton transfer in H-bond; A-H ··· B.

$$V(x) = a_2x^2 + a_3 \exp(-a_4(x - a_5)^2). \quad (3)$$

The time-dependent wave function $\Psi(x, t)$ which describes the dynamical property of the system can be obtained by the following procedure:

We begin with the time-independent process;

$$\mathcal{H}(x)\phi_i(x) = E_i\phi_i(x) \quad (4)$$

with the same hamiltonian as in eq. (2),

$$\mathcal{H}(x) = \mathcal{H}_1(x) + \mathcal{H}_2(x);$$

$$\mathcal{H}_1(x) = T(x) + a_2x^2; \quad \mathcal{H}_2(x) = a_3 \exp[-a_4(x - a_5)^2].$$

The eq. (4) can be solved by variational method, using the eigenfunctions of the linear harmonic oscillator χ_n as the basis set.

$$\mathcal{H}_1(x)\chi_k(x) = \mathcal{E}_k\chi_k(x). \quad (5)$$

For the eigenfunctions $\phi_i(x)$ of the proton in H-bonded system described by ADWP– $V(x)$ we can write:

$$\phi_i(x) = \sum_k b_{ik}\chi_k(x) \quad (6)$$

Corresponding eigenvalues E_i are the vibrational energy levels of the proton in particular H-bonded system.

Because the hamiltonian of the system does not explicitly depend on the time, we can obtain the time-dependent wave function $\Psi(x, t)$ by unitary transformation:

$$\Psi(x, t) = \hat{u}(t, t_0)\Psi(x, t_0) \quad (7)$$

where $\hat{u}(t, t_0)$ is unitary operator of the time translation;

$$\hat{u}(t_1t_0) = \exp[(-i/\hbar)\mathcal{H}(x)(t - t_0)] \quad (8)$$

and $\Psi(x_1t_0)$ is a wave function of the proton initial state.

In the present work, the study of the dynamics of proton motion is based on the calculation of "tunneling time" derived from the probability density of the particle [37]. The following relation was used for calculation of the tunneling time;

$$\tau = \frac{\pi}{\omega_0} D^{-1/2}\left[1 + \left(\frac{\pi\Delta V}{\hbar\omega_0}\right)^2 D^{-1}\right]^{-1/2} \quad (9)$$

where D is the penetration coefficient

$$D = \exp\left(-\frac{2}{\hbar}\left|\int_{U_a}^{U_b} dx\,\{2\mu[E_i - V(x)]\}^{1/2}\right|\right) \quad (10)$$

ω_0 is frequency of A-H bond stretching vibration (noninteracting molecule) and U_a, U_b are the classical turning points of the proton on the left and the right of the potential barrier, i.e., the points of intersection of the energy eigenvalue E_i with the potential function $V(x)$. Tunneling time τ, means life time of the protomeric structure I in the following reaction scheme:

$$\underset{\text{I.}}{AH + B} \rightleftharpoons \underset{\text{II.}}{A^- + BH^+} \quad (11)$$

The reciprocal of the tunneling time τ is the proton transfer frequency; $\nu = \tau^{-1}$.

The computation program used in this work is based on the program described in [29]. Calculation facilities are, however, expanded also for the computation of excited states. The program is in FortranIV and it is adapted for the computer Siemens 4004/150. As basis set functions we have used Hermite's polynomials up to order 38. The calculations were performed in double precision accuracy at Computing Centre of Comenius University in Bratislava.

3. Results

For the studied enzymatic process, i.e., for proton transfer from $N^{\epsilon 2}$ atom of imidazole His57 to the N atom of the amide substrate scissile bond we have constructed 14 ADWPs. The same number of ADWPs were constructed for ester substrates (H^+ transfer to O atom of ester scissile bond). The maximum of experimental data were used for construction of these potentials – table 1. The curvature of potential (left and right hand well) was calculated in harmonic approximation from the stretching vibration frequency of NH bond of imidazole and NH stretching vibration frequency of primary amines (primary amine is released as P_1 after TI_1 breakdown) or aliphatic alcohols in case of ester substrate hydrolysis. The distance of minima for a particular type of ADWP – Δx (amide or ester hydrolysis) was calculated from the distance between the $N^{\epsilon 2}$ atom of imidazole and the N or O atom of substrate of TI_1 and from the equilibrium bond lengths for N-H imidazole and N-H or O-H of sub-

Table 1
The experimental and computed data used for construction of the ADWPs

	Imidazole N–H bond	Primary amine N–H bond	Aliphatic alcohol O–H bond
Stretching * vibration	2804 [cm^{-1}]	3387 [cm^{-1}]	3639 [cm^{-1}]
Force constant	0.299 [a.u.]	0.436 [a.u.]	0.504 [a.u.]
Zero point energy	6.38×10^{-3} [Hartree]	7.72×10^{-3} [Hartree]	8.29×10^{-3} [Hartree]
Equilibrium ** bond length	0.105 [nm]	0.1 [nm]	0.096 [nm]
Mean distance of N atoms in *** H-bond between imidazole and peptide bond: 0.31 [nm]		Mean distance of N and O atom in *** H-bond between imidazole and ester: 0.285 [nm]	

* – [39] 1 $cm^{-1} = 4.556 \times 10^{-6}$ Hartree
** – [40] 1 Hartree = 2.627×10^{3} kJ $mole^{-1}$
*** – [41] 1 a.u. of length = 5.292×10^{-2} nm

strate (table 1). The height of the energy barrier ($\Delta V^{\ddagger}$) was determined from the experimental value of activation enthalpy $\Delta H^{\ddagger}_{AE}$ and from calculated correction to the zero-point energy of the imidazole NH stretching mode (E_{ZPE}). Because, experimentally is available only $\Delta H^{\ddagger}_{AE} \approx 42$ kJ $mole^{-1}$ for ester substrate hydrolysis, we have constructed ADWPs starting from $\Delta H^{\ddagger}_{AE} \approx 42$ kJ $mole^{-1}$ upwards. For each value of $\Delta V^{\ddagger}$ was constructed an exo and an endothermic potential. ADWPs for proton transfer from imidazole His57 to substrate molecule constructed in this way, were then expressed in analytical form, eq. (3). The parameters a_2–a_5 for particular potential were calculated by non-linear method of least squares using the Fletcher-Powell optimisation procedure [30].

The detailed results for proton transfer from imidazole to amide substrate with activation energy ~40 kJ $mole^{-1}$ are presented in figs. 4–7 and tables 2, 3. The table 4 presents calculated penetration coefficients (D), life times (τ) and proton transfer frequencies (ν) from ground and first excited vibrational state of imidazole NH bond to amide substrate for all studied model ADWPs. In table 5 are the same results for ester substrates hydrolysis.

4. Discussion

At physiological temperatures the energy of the proton in NH bond of imidazole molecule is not sufficient to overcome the energy barrier $E^{\ddagger} \approx 40$ kJ $mole^{-1}$. For the classical proton transfer over the energy barrier even direct contribution of ES complex formation energy is not sufficient because the free energy of ES complex formation for specific substrates is still much smaller than 40 kJ $mole^{-1}$. On the other hand, however, the proton can pass through the potential barrier by the quantum mechanical tunnel effect. This fact is demonstrated by calculated values of penetration coefficients (D) and proton tunneling frequen-

Table 2
The first seven eigenvalues of the proton in the endo and exothermic ADWPs with $E^{\ddagger} \approx 40$ kJ $mole^{-1}$. The proton transfer to amide substrate (figs. 4, 5). The number of basis set function $\chi_k = 38$

Eigenfunctions	Eigenvalues [Hartree]	
	potential [a] $\Delta V^{\ddagger}/\Delta V = 57.2/23.3$	potential [a] $\Delta V^{\ddagger}/\Delta V = 55.0/-20.9$
ϕ_1	0.374 (A_0)	0.368 (B_0)
ϕ_2	0.382 (B_0)	0.375 (A_0)
ϕ_3	0.383 (A_1)	0.378 (B_1)
ϕ_4	0.389 (B_1)	0.384 (A_1)
ϕ_5	0.391 (A_2)	0.387 (B_2)
ϕ_6	0.395 (C_1)	0.392 (C_1)
ϕ_7	0.399 (C_2)	0.395 (C_2)

[a] $\Delta V^{\ddagger}/\Delta V$ = [kJ $mole^{-1}$/kJ $mole^{-1}$]

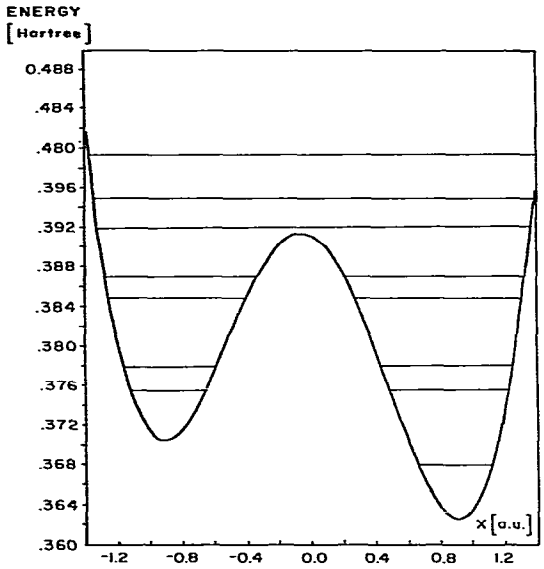

Fig. 4. The exothermic ADWP of proton transfer from imidazole to N atom of amide substrate cleaved bond and calculated energy levels. $\Delta V^{\ddagger}/\Delta V = 57.2/23.3$; $E^{\ddagger} = 40.5$ kJ mole^{-1}, $V(x) = 0.12431\,x^2 + 0.39096\exp[-0.44214\,(x - 0.02068)^2]$.

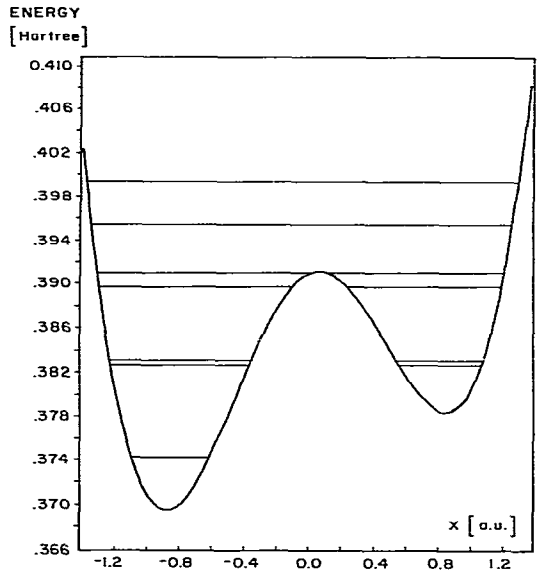

Fig. 5. The endothermic ADWP of proton transfer from imidazole to N atom of amide substrate cleaved bond and calculated energy levels. $\Delta V^{\ddagger}/\Delta V = 55.0/-20.9$; $E^{\ddagger} = 38.3$ kJ mole^{-1} $V(x) = 0.128\,x^2 + 0.39121\exp[-49149\,(x + 0.01709)^2]$.

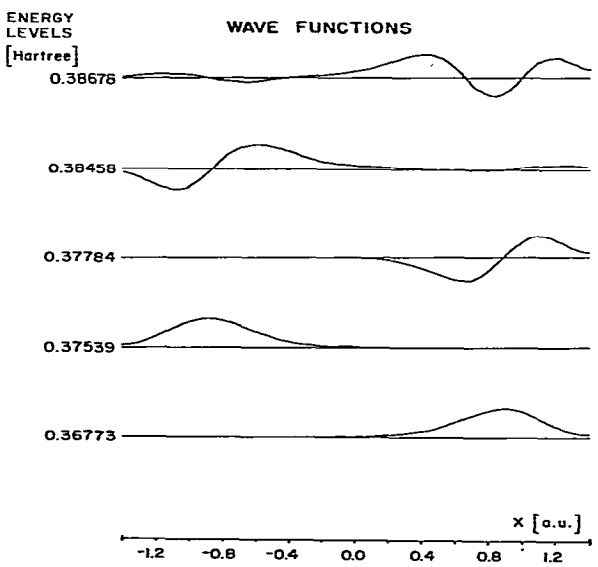

Fig. 6. The first five eigenfunctions of the proton in exothermic ADWP – fig. 4. The eigenfunctions correspond to energy levels of the proton under the top of the energy barrier.

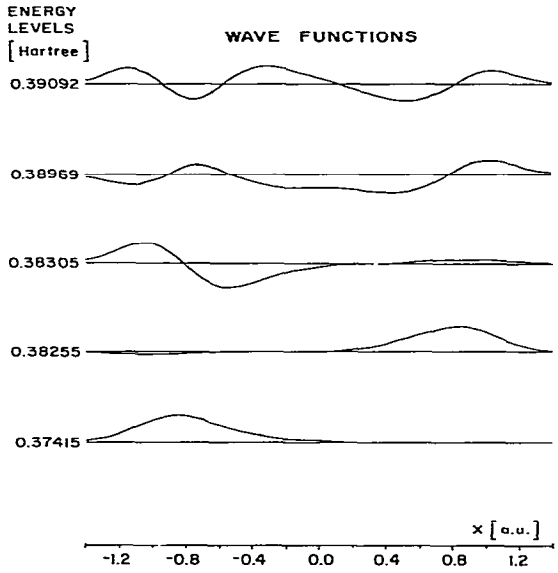

Fig. 7. The first five eigenfunctions of the proton in endothermic ADWP – fig. 5. The eigenfunctions correspond to energy levels of the proton under the top of the energy barrier.

Table 3
The expansion coefficients of the proton initial state for the endo and exothermic proton transfer to amide substrate with $E^{\ddagger} \approx 40$ kJ mole^{-1} (figs. 4, 5). The initial state in the both cases is approximated by first excited vibrational state of imidazole NH bond stretching vibration. The number of basis set functions $\phi_i(x) = 38$

Base functions	Expansion coefficients	
	potential [a] $\Delta V^{\ddagger}/\Delta V = 57.2/23.3$	potential [a] $\Delta V^{\ddagger}/\Delta V = 55.0/-20.9$
ϕ_1	$C_1^{(1)} = -0.119$	$= .0.000$
ϕ_2	$C_2^{(1)} = 0.065$	$= -0.113$
ϕ_3	$C_3^{(1)} = -0.904$	$= 0.002$
ϕ_4	$C_4^{(1)} = -0.199$	$= 0.913$
ϕ_5	$C_5^{(1)} = 0.108$	$= -0.096$
ϕ_6	$C_6^{(1)} = 0.108$	$= 0.323$
ϕ_7	$C_7^{(1)} = 0.064$	$= 0.169$

[a] $\Delta V^{\ddagger}/\Delta V$ = [kJ mole^{-1}/kJ mole^{-1}]

cies (tables 4, 5). Moreover, the proton tunneling ability is visualised in figs. 6, 7. The calculations show, that the eigenfunctions of the proton are delocalised on both sides of the energy barrier.

As indicated in tables 4, 5 calculated proton tunneling frequencies for potentials with activation energy about 40 kJ mole^{-1} are about 10^6–10^7 times higher than experimental values k_{AE} for amide or ester substrates hydrolysis. Calculated proton tunneling frequencies comparable with experimental values (10^0 s^{-1} for amide substrates and 10^2 s^{-1} for ester substrates) were obtained for potentials with activation energy approximately 4 times higher than 40 kJ mole – tables 4, 5.

At first sight it seems that theoretical results contradict the experimental data. Let us turn our attention, however, to the following reaction;

$$H_3O^+ + H_2O \rightleftharpoons H_2O + H_3O^+.$$

Mean life time of the H_3O^+ in ice, determined experimentally is about 10^{-13} s [31]. The high rate of the proton transfer in this system was explained by Caldin [32] on the basis of proton tunneling along the water H-bonds. The frequency of proton tunneling about 10^{11} s^{-1} was obtained [33] for Eckart's type energy barrier with $\Delta V^{\ddagger} \approx 42$ kJ mole^{-1} and with distance of minima $\Delta x = 0.08$ nm. The proton transfer potential for this system was calculated [34] by nonempirical SCF ab initio method using extended basis set and the

Table 4
The calculated values of characteristic quantities of proton tunneling from imidazole to N atom of amide substrate bond for model ADWPs. The proton initial state is approximated by ground and first excited vibrational state of imidazole NH bond stretching vibration

Proton transfer from imidazole to amide substrate							
$\Delta V^{\ddagger}/\Delta V$ kJ mole^{-1}/kJ mole^{-1}	$E^{\ddagger}$ kJ mole^{-1}	Ground state			First excited state		
		$D^{(0)}$	$\tau^{(0)}$ [s]	$\nu^{(0)}$ [s^{-1}]	$D^{(1)}$	$\tau^{(1)}$ [s]	$\nu^{(1)}$ [s^{-1}]
57.2/23.3	40.5	–	–	–	2.2×10^{-4}	1.8×10^{-10}	5.4×10^{9}
55.0/–20.9	38.3	5.5×10^{-7}	6.7×10^{-8}	1.5×10^{7}	1.6×10^{-3}	2.3×10^{-11}	4.3×10^{10}
83.7/23.3	67.0	–	–	–	6.9×10^{-7}	5.2×10^{-6}	1.9×10^{5}
83.7/–20.9	67.0	3.7×10^{-9}	9.8×10^{-6}	1.0×10^{5}	1.2×10^{-5}	3.1×10^{-9}	3.2×10^{8}
104.6/23.3	87.9	–	–	–	6.0×10^{-9}	6.7×10^{-6}	1.5×10^{5}
104.6/–20.9	87.9	2.1×10^{-10}	1.7×10^{-4}	5.8×10^{3}	4.4×10^{-7}	8.3×10^{-8}	1.2×10^{7}
125.6/23.3	108.9	–	–	–	1.4×10^{-10}	2.9×10^{-4}	3.5×10^{3}
125.6/–20.9	108.9	6.8×10^{-12}	5.3×10^{-3}	1.9×10^{2}	5.3×10^{-9}	6.9×10^{-6}	1.4×10^{5}
146.5/23.3	129.8	–	–	–	9.5×10^{-12}	4.3×10^{-3}	2.3×10^{2}
146.5/–20.9	129.8	3.5×10^{-13}	1.0×10^{-1}	9.6	2.1×10^{-10}	1.7×10^{-4}	5.7×10^{3}
167.4/23.2	150.7	–	–	–	8.2×10^{-13}	4.9×10^{-2}	2.0×10^{1}
167.4/–20.9	150.7	3.7×10^{-14}	9.8×10^{-1}	1.0	2.4×10^{-11}	1.5×10^{-3}	6.6×10^{2}
188.4/23.3	171.7	–	–	–	8.7×10^{-14}	4.7×10^{-1}	2.1 × 10
188.4/–20.9	171.7	4.6×10^{-15}	7.9	1.2×10^{-1}	3.3×10^{-12}	1.1×10^{-2}	9.2×10^{1}

Table 5
The calculated values of characteristic quantities of proton tunneling from imidazole to O atom of ester substrate bond for model ADWPs. The proton initial state is approximated by ground and first excited vibrational state of imidazole NH bond stretching vibration

Proton transfer from imidazole to ester substrate

$\Delta V^{\ddagger}/\Delta V$ kJ mole^{-1}/kJ mole^{-1}	$E^{\ddagger}$ kJ mole^{-1}	Ground state			First excited state		
		$D^{(0)}$	$\tau^{(0)}$ [s]	$\nu^{(0)}$ [s^{-1}]	$D^{(1)}$	$\tau^{(1)}$ [s]	$\nu^{(1)}$ [s^{-1}]
61.9/24.5	45.2	–	–	–	3.0×10^{-3}	1.4×10^{-11}	7.1×10^{10}
54.5/–24.9	37.8	2.4×10^{-5}	4.8×10^{-9}	5.5×10^{8}	4.2×10^{-2}	1.0×10^{-12}	9.5×10^{11}
83.7/24.5	67.0	–	–	–	1.2×10^{-5}	3.5×10^{-9}	2.8×10^{8}
83.7/–24.9	67.0	7.9×10^{-8}	5.5×10^{-7}	1.8×10^{6}	7.8×10^{-5}	5.6×10^{-10}	1.8×10^{9}
104.6/24.5	87.9	–	–	–	5.0×10^{-7}	8.5×10^{-8}	1.7×10^{7}
104.6/–24.9	87.9	4.6×10^{-9}	9.4×10^{-6}	1.0×10^{5}	2.5×10^{-6}	1.7×10^{-8}	5.7×10^{7}
125.6/24.5	108.9	–	–	–	1.5×10^{-8}	2.9×10^{-6}	3.5×10^{5}
125.6/–24.9	108.9	9.1×10^{-10}	4.8×10^{-5}	2.1×10^{4}	4.1×10^{-7}	1.0×10^{-7}	9.5×10^{6}
146.5/24.5	129.8	–	–	–	1.5×10^{-9}	2.8×10^{-5}	3.6×10^{4}
146.5/–24.9	129.8	1.3×10^{-10}	3.4×10^{-4}	2.9×10^{3}	6.5×10^{-8}	6.7×10^{-7}	1.5×10^{6}
167.4/24.5	150.7	–	–	–	1.9×10^{-10}	2.2×10^{-4}	4.6×10^{3}
167.4/–24.9	150.7	2.1×10^{-11}	2.1×10^{-3}	4.8×10^{2}	1.2×10^{-8}	3.6×10^{-6}	2.8×10^{5}
188.4/24.5	171.7	–	–	–	3.0×10^{-11}	1.4×10^{-3}	7.0×10^{2}
188.4/–24.9	171.1	3.8×10^{-12}	1.1×10^{-2}	8.8×10^{1}	2.6×10^{-9}	1.6×10^{-5}	6.1×10^{4}

value $\Delta V^{\ddagger} = 39.7$ kJ mole^{-1} has been obtained.

In this connection our calculated results for the studied enzymatic process seems to be correct. In our case the distance of minima is 0.105 nm for amide substrates hydrolysis and 0.084 nm for ester substrates hydrolysis. The most critical parameter for interpretation of the enzyme catalytic efficiency is therefore the value of the activation energy (enthalpy). Is the experimentally determined value $E^{\ddagger}_{AE} \approx 42$ kJ mole^{-1} indeed the true activation energy (enthalpy)?

According to Blumenfeld [35], for such a complex process as enzymatic catalysis we cannot use the Arrhenius equation for determination of true activation energy. From the analysis performed in [35] it follows that also in the case when experimental data satisfy Arrhenius equation, we do not measure the true activation parameters but some effective quantities $\Delta E^{\ddagger}_{eff} = \Delta E^{\ddagger}_{tr} - bT$ and $\Delta S^{\ddagger}_{eff} = \Delta S^{\ddagger}_{tr} - b$, which may greatly differ from $\Delta E^{\ddagger}_{tr}$ and $\Delta S^{\ddagger}_{tr}$.

From this point of view, we can interpret our theoretical results in the framework of Blow's reaction mechanism and postulate a new hypothesis for physicochemical basis of chymotrypsin enzymatic efficiency;

1) The proton transfer from the imidazole group of His57 to N atom of scissile substrate bond is rate determining elementary reaction act of whole catalytic process of amide substrates hydrolysis (breakdown of TI_1)

2) The true activation energy of this reaction step is very high (about 170 kJ mole^{-1}) – comparable with uncatalysed process.

3) The proton transfer in this reaction step is realised by a tunneling mechanism through the energy barrier from the first excited vibrational state of the NH bond of imidazole group of His57.

The presence of the excited vibrational state of imidazole NH bond is the essential point of the suggested reaction mechanism. The evidence for the metabolic excitation of vibrational states in bacteria has been obtained recently by the Raman effect [42,43]. In this connection, very interesting experimental work concerning directly chymotrypsin action has been published by Kollias and Melander [47]. The authors observed enhancement of chymotrypsin activity by laser irradiation. The effect persists for several minutes after removal of the laser beam, which indicated long-living vibrational excited states. The authors concluded that the physical basis of this laser-induced activation is unclear. Unfortunately, this experiment was not combined with measurement of Raman scattering.

The different theories concerning nonthermal vibrational excitations in biological systems with implication for enzymatic catalysis have been published in the past few years [44–46, 36]. In spite of the different standpoints (long-range coherence, bottle-neck effect, vibrational solitons) of these theories the same conclusion is reached, however: the nonthermal vibrational excitation is specific for polypeptide macromolecules after the energy supply (for example energy released after ES complex formation) and excited states have long-life times. It is out of the scope of the present work to study the mechanism of energy transfer from the "sorption" region to the "active" site of the enzyme and thus the following is a hypothetical interpretation.

The high enzymatic efficiency of chymotrypsin (serine proteases in general) is probably based on presence of nonthermal excited vibrational states, particularly of the NH bond of the imidazole group of the histidine residue in the active site of the enzyme. The imidazole molecule as a part of the active site of the enzyme is a strong acidic catalyst, because of vibrational excitation, in contrast to the imidazole molecule in general hydrolysis in solution. In that case nonthermal excitations are impossible. It is interesting that the calculated energy (figs. 4,5) for the imidazole NH bond stretching vibration excitation in H-bond to substrate leaving group is 20–24 kJ $mole^{-1}$, which is very close (20 kJ $mole^{-1}$) to the energy transferred by vibrational solitons in polypeptides [36]. Based on the results of our model calculations (harmonic approximation), the Raman scattering on chymotrypsin (experiment [47]) can be expected about 2000 cm^{-1} – in any case at smaller wavenumber than 2800 cm^{-1}. For specific substrates, the energy released after ES complex formation is enough for this excitation in contrast to nonspecific substrates.

This interpretation is based, however on the results for an endothermic type of model proton transfer potentials. In the case of an exothermic type of model proton transfer potentials, the frequency of proton transfer comparable with the experimental rate constant (10^0, resp. 10^2 s^{-1}-amide/ester) is calculated for proton tunneling from ground vibrational state for the activation energy ~152 kJ $mole^{-1}$, which is still very high. If we will accept, that the proton transfer process in the stage of TI_1 breakdown is an exothermic process, then in this case proton tunneling is possible from the ground vibrational state. In this case, however, the role of ES complex formation energy will be unclear and moreover the neutral imidazole molecule must be as strong an acidic catalyst in the general hydrolysis in solution as the imidazole group in the active site of the enzyme. Evidently this is not true. On the other hand, the breakdown of TI_1 in the case of ester substrates hydrolysis has been determined as an exothermic step ($\Delta H_{AB} \sim -8.5$ kJ $mole^{-1}$) by means of the Vant'-Hoff equation. The direct calorimetric measurements of heat of reactions and Blumenfeld's analysis show, that Vant'-Hoff equation does not hold for protein reactions (for details see [35] and references therein) and ΔH determined in this way has no strict physical meaning.

In order to make unambiguous conclusions the problem studied here requires more extensive theoretical and special experimental study.

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