THEORETICAL STUDY OF THE RATE DETERMINING PROTON TRANSFER AT THE MECHANISM OF SERINE PROTEASES ACTION

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The proton transfer step at the breakdown of the tetrahedral intermediate of chymotryptic catalysis was studied using the simple quantum-statistical theory of unimolecular proton transfer reactions. The physical basis of the enzymatic high catalytic efficiency is discussed from the view-point of experimental and theoretically obtained kinetic parameters. The experiment is suggested to test the possible importance of vibrational excited states at the enzyme action.

Despite the intensive studies carried out in the field of enzymology and the sophisticated experimental techniques used, relatively slow progress has been made in understanding the enyzmes mechanism. This situation concerns not only enzymes with the highest functional hierarchy, but also the simplest enzymes fulfilling just the chemical degradative function. Serine proteases are the best example of such enzymes. Chymotrypsin, a member of this group of enzymes is probably the best studied enzyme in general. It catalyses the hydrolysis of "hydrophobic", mainly aromatic, peptide bonds of proteins as well as low molecular amide or ester bonds of aromatic L-aminoacid derivatives.

At present, the structure of this enzyme, the structure of ES(EI) complexes, formal chemical mechanism of catalytic action and kinetic parameters of particular reaction steps are known[1,2]. The formal reaction mechanism can be written as follows:

$$E + S \rightleftharpoons ES \rightleftharpoons TI_1 \rightleftharpoons AE + P_1 \xrightleftharpoons{H_2O} TI_2 \rightleftharpoons E + P_2$$

$$ES \xleftarrow{k_2} AE$$

For amide substrates hydrolysis it was shown that the breakdown of TI_1 connected with proton transfer from imidazole ring of His 57 of the enzyme to N atom of substrate amide bond to be broken, is the slowest step in the entire catalytic process[2,3-6]. Experimentally measured primary kinetic isotope effect for acylation step[2,7-10] in the range of 2—5, indicates that the proton transfer at the breakdown of TI_1, is the rate limiting elementary reaction act.

Besides the old "lock and key" mechanism which gave a qualitatively reasonable explanation for enzyme specificity but not for high catalytic efficiency, several theories have been postulated in an effort to explain the basis of high catalytic efficiency of the enzymes. Individual theories are not the subject of the present paper, they have been discussed elsewhere (see *e.g.* Lumry's papers[11]). Nevertheless, it should be said that the thermodynamical theories are more successful at present, at least as far as the general discussion of the cardinal question of the enzymatic mechanism is concerned[12]; how is the part of free energy of ES cmplex formation (which is not realized

through binding) converted into a decreasing of the free energy of activation of decisive reaction step.

Recently a hypothesis was suggested[13], according to which a part of free energy of ES complex formation is used to populate some excited vibration modes in the enzyme, including the imidazole His 57 — NH bond stretching vibration. High catalytic efficiency of the enzyme is then explained by tunnelling mechanism of the proton from imidazole — NH bond vibrationally excited state to substrate bond to be cleaved. The true activation energy of proton transfer step was supposed to be much higher than the experimental value ~ 40 kJ/mol. The suggested hypothesis was formulated on the basis of theoretically calculated proton transfer frequencies (microcanonical rate constants) from imidazole molecule to amide or ester substrate. To discuss the problem of the enzyme mechanism on the basis of microcanonical rate constants appears to be not very effective, since a very important entropy part as well as the influence of environment are missing. A theoretical method for proton transfer canonical rate constant calculation readily applicable to the studied problem is not available at the present, and more over the situation in the theory of proton transfer reactions is rather complicated.

Besides the well-known problems in arriving at theoretical potential function accurately describing the H^+ transfer[14-16], the serious difficulties exist in the theories describing the dynamics of the process. The transition state/transmission coeficient corrected phenomenological theory of H^+ transfer [15] has recently been critically discussed by Cribb and coworkers[17]. The consistent quantum-statistical theories of H^+ transfer in condensed media[18-20] can not be used for studied enzymatic process, because of difficulties in exact specification of external relaxation mechanism.

In the present work a simple quantum-statistical method for the calculation of canonical rate constant of unimolecular proton transfer reactions has been formulated. This proves to be a more convenient approach in discussion of the studied problem than discussion based on the values of microcanonical rate constants.

Canonical Rate Constant of Proton Transfer Process — Simple Quantum Statistical Formulation

For the rate constant of the unimolecular proton transfer process we can write

$$k = \alpha \sum_E k(E) \,.\, n(E) \,. \qquad (1)$$

In this equation, α means the degeneracy of the reaction pathway, $k(E)$ represents microcanonical rate constant of the proton transfer from the energy level E and $n(E)$ is an occupation probability of the Eth energy level.

Assuming the equilibrium process, the statistics of Gibss' canonical ensemble can be used and the Eq. (1) takes the form:

$$k = \alpha \sum_E k(E) \,.\, g(E) \,.\, \exp\left(-E/k_B T\right) / \sum_E g(E) \,.\, \exp\left(-E/k_B T\right) , \qquad (2)$$

where $g(E)$ is the degeneracy of the particular energy level.

We can, further assume that the reaction pathway of the proton transfer is controlled by stretching vibration mode of A-H bond. The reaction pathway is perturbed however, to some extent by other degrees of freedom (bending vibration, skeletal vibration, rotation, ...). The extent of perturbation by these degrees of freedom depends on the geometry of the reacting complex. A further factor which must be taken into account is solvent reorganization. Energy contributions of „perturbative" degress of freedom, together with the energy contribution of solvent reorganization, are independent of the stretching vibrational quantum number of the proton in the H-bonded reacting complex. This means that while the energy spectrum of the proton stretching vibration in reacting complex is quantised, the energy spectrum of „perturbative" degrees of freedom along with solvent reorganization energy, are assumed to form a continuum in the model presented. On the basis of this assumption, we can write:

$$E = E_v + E_c \,. \tag{3}$$

The degeneracy of the particular energy level E can be then factorised:

$$g(E) = g(E_v) \,.\, \varrho(E_c) \,. \tag{4}$$

In this equation $g(E_v)$ is degeneracy of the proton stretching vibrational energy level E_v in the reacting complex and $\varrho(E_c)$ is the density of states of the continuum. Under this assumption the expression for the canonical rate constant can be rewritten:

$$k = \alpha \sum_{E_v} k(E_v) \,.\, g(E_v) \,.\, \exp\left(-E_v/k_B T\right) / \sum_{E_v} g(E_v) \,.\, \exp\left(-E_v/k_B T\right) \,.$$
$$\,.\, \int_0^\infty \mathrm{d}E_c k(E_c) \,.\, \varrho(E_c) \,.\, \exp\left(-E_c/k_B T\right) \Big/ \int_0^\infty \mathrm{d}E_c \,.\, \varrho(E_c) \,.\, \exp\left(-E_c/k_B T\right) \tag{5}$$

or:

$$k = \alpha \,.\, FC \,.\, FC' \,.\, \sum_{E_v} k(E_c) \,.\, g(E_v) \exp\left(-E_v/k_B T\right) / \sum_{E_v} g(E_v) \,.\, \exp\left(-E_v/k_B T\right) \,.$$

where FC and FC' are parameters of the continuum.

The parameter FC comprises the influence of the environment (solvent) on the rate process. The influence of perturbative degrees of freedom (bending vibration, rotation) on the stretching vibration mode is expressed through FC'.

For the purpose of the present work, both FC and FC' will not be evaluated exactly and they will serve just as adjusting parameters. It is obvious that for $FC = FC' = 1$, the Eq. (5) gives the value of *in vacuo* canonical rate constant for proton transfer along the stretching vibration mode of the A-H bond in reacting complex with rigin linear H-bond geometry.

Assuming the adiabatic character of the reaction, in one-dimensional approximation, the Schrödinger's equation can be used to describe the proton motion in the rigid linear H-bond.

$$\mathbf{H}(x)\,\Psi_v(x) = E_v\Psi_v(x) \quad v = 0, 1, \ldots \tag{6}$$

$$\mathbf{H}(x) = \frac{1}{2m}\cdot\frac{\mathrm{d}^2}{\mathrm{d}x^2} + V(x)$$

The potential function $V(x)$ of the proton motion is in general an assymetric double well potential. It is suitable to express this potential as a sum of parabola and Gauss' function with translated extrema.

$$V(x) = a_2x^2 + a_3[\exp - a_4(x - x_0)^2] \tag{7}$$

Schrödinger's equation (6) with the potential (7) can be solved directly by variational method using Hermite's polynomials as a basis set. The calculation procedure is described elsewhere[13,14]. The calculated set of eigenvalues $\{E_v\}$ can be used directly in Eq. (5) and using the set of eigenfunctions $\{\Psi_v\}$, the proton penetration coefficients $\{P(E_v)\}$ can be calculated. The microcanonical rate constant – $k(E_v)$ for linear reacting complex can be expressed as a product of stretching vibrational frequency of the proton and its penetration coefficient $P(E_v)$ through the energy barrier on the particular energy level E_v.

$$k(E_v) = \nu(E_v)\,.\,P(E_v) \tag{8}$$

In order to simplify the rate equation, the value of $\nu(E_v)$ can be replaced by some mean frequency $\bar{\nu}$ determined by the second derivative of $V(x)|x_0$ or from stretching vibration frequency of A-H bond in harmonic approximation. This substitution is justified by the fact that the deviation of $\nu(E_v)$, for E_vs near the top of the energy barrier, from the value of $\bar{\nu}$ is negligible for the $k(E_v)$ calculation. The direct calculation shows that the changes in $k(E_v)$ are first of all determined by the changes of $P(E_v)$s going from one energy level to the other.

For the canonical rate constant we can write the expression:

$$k = \alpha\,.\,A\,.\sum_{E_v} P(E_v)\,.\,g(E_v)\,.\exp\left(-E_v/k_BT\right)/\sum_{E_v} g(E_v)\,.\exp\left(-E_v/k_BT\right), \tag{9}$$

where $A = FC\,.\,FC'\,.\,\bar{\nu} = FC\,.\,\nu_{eff}$.

In case that the proton transfer potential $V(x)$ is constructed based on the experimental data $(E^{\neq}_{exp}, \Delta H_{exp})$, the parameter FC comprises only the solvent reorganization en-

tropy contribution. The enthalpy part of the free energy connected with the solvent reorganization is already included in the model potential (hight of energy barrier, reaction heat, ...). Under this condition, the constant A is a frequency factor similar to the frequency factor from the absolute rate theory.

For the activation entropy, the usual expression can be written:

$$\Delta S^{\neq} = R[\ln A - \ln (k_B T/h)] \qquad (10)$$

or

$$\Delta S^{\neq} = R[\ln A - \ln \nu_{eff}] .$$

For the process of deuterium transfer, the same equations as for proton transfer process can be used. The difference will be just in the reduced mass of the transfered particle.

Studied Models

The model potentials which simulate the proton transfer from imidazole molecule of His 57 to amide or ester substrate bond during the breakdown of tetrahedral intermediate are the same as those used in our previous paper[13]. For the construction of the model potentials experimental data of stretching vibration frequencies of particular H bonds, equilibrium distances and activation parameters were used.

RESULTS

Figs 1–2 present model potentials of H^+ transfer in rigid linear interacting complex from imidazole molecule to amide type substrate. The value of activation energy (referring to zero-point vibrational energy) roughly corresponds to experimentally determined $\Delta H_2^{\neq}$ value of H^+ transfer (rate determining) at acylation step of chymotryptic catalysis. The potentials of H^+ transfer from imidazole molecule to ester type substrate are qualitatively of the same character.

The energy levels and wave functions were calculated by variational method using eigenfunctions of linear harmonic oscilator as a basis set.

In Tables I, II are both experimental as well as theoretically calculated kinetic parameters for ester type substrate hydrolysis. Tables III and IV present results for amide type substrate hydrolysis. Frequency factors (A) in Tables I–IV have been calculated as a product of the proton mean frequency $\bar{\nu}$ and the parameter FC while keeping $FC' = 1$ (assuming rigid linear complex and transfer along unperturbed stretching mode). The parameter FC has been obtained as a ratio of experimental rate constant (k_2 for particular type of substrate and temperature at pH = 7) and theoretically calculated canonical rate constant of H^+ transfer without perturbations ($FC = FC' = 1$ – *in vacuo* process, rigid linear geometry).

As we can see from the Tables I – IV the contribution of the energy levels, with the factor $\exp(-E_v/k_BT)$ smaller then 10^{-13}, to the value of the canonical rate constant is negligible. The same is true for the contribution of the "hopping" mechanism over the energy barrier. For studied potentials ($E^* \sim 40$ kJ/mol) and temperature about 300 K the energy levels close to the top of the barrier ($k_BT \sim$ kJ/mol) are extremly low populated.

Based on the obtained results we can say that the rate constant is determined by the following main factors: *1*) underbarrier delocalization of the proton – tunnel effect, *2*) quantum-statistical population of energy levels, *3*) solvent reorganization and perturbative degrees of freedom. Two effects are worth to mention; the decisive role of the ground state energy level to the value of canonical rate constant and underbarrier delocalisation. Even in the case when the ground state energy level (left-hand side well) is below the energy minimum of the right-hand side well, this level decisively determines the value of canonical rate constant. The influence is realized through the statistical sum in which the ground state is the most important contributor. The second effect is delocalization. We can see from the calculated wafe functions (Fig. 1, 2) that the proton originally localised on the ground state energy level of A-H bond vibration, this state corresponds roughly to the energy level A_0 in double well potential, can pupulates in the reacting complex [A-H···B] not only the excited states "correspond-

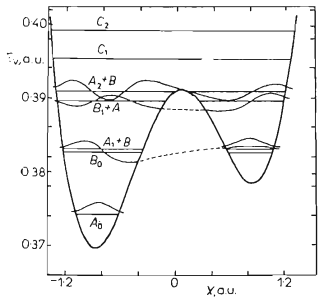

FIG. 1

Calculated energy levels and wave functions of the proton for the model endothermic potential of the proton transfer from imidazole molecule to amide type substrate. $E^* = 40{\cdot}5$ kJ/mol, $\Delta V = 57{\cdot}2$ kJ/mol, $\Delta V = 23{\cdot}3$ kJ/mol

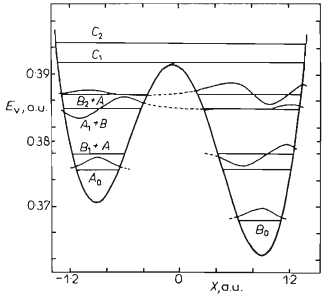

FIG. 2

Calculated energy levels and wave functions of the proton for the model exothermic potential of the proton transfer from imidazole molecule to amide type substrate. $E^* = 38{\cdot}3$ kJ/mol, $V_{max} = 55{\cdot}0$ kJ/mol, $\Delta V = -20{\cdot}9$ kJ/mol

TABLE I

Kinetic parameters for H^+ transfer from imidazole molecule to ester type substrate; Endothermic potential[a]

Energy level	Inner turning points *a.u.* a	Inner turning points *a.u.* b	E_v J	[e] P^{H^+}	[e] P^{D^+}	$\exp(-E_v/kT)$	$\tilde{\nu} P \exp(-E_v/kT)$ H^+	$\tilde{\nu} P \exp(-E_v/kT)$ D^+
A_0	—	∞	$2{\cdot}34\ 10^{-20}$	0·0	0·0	$2{\cdot}77\ 10^{-3}$	0 0	0 0
[b] B_0	—	—	$6{\cdot}19\ 10^{-20}$	0·0	0·0	$1{\cdot}73\ 10^{-7}$	0 0	0 0
$A_1 + B$	−0·26	0·44	$6{\cdot}82\ 10^{-20}$	$2{\cdot}7\ 10^{-3}$	$2{\cdot}34\ 10^{-4}$	$3{\cdot}72\ 10^{-8}$	$6{\cdot}65\ 10^{3}$	$5{\cdot}75\ 10^{2}$
$B_1 + A$	−0·03	0·19	$9{\cdot}81\ 10^{-20}$	$5{\cdot}2\ 10^{-1}$	$4{\cdot}09\ 10^{-1}$	$1{\cdot}95\ 10^{-11}$	$6{\cdot}44\ 10^{2}$	$4{\cdot}96\ 10^{2}$
[c] C_1	—	—	$1{\cdot}08\ 10^{-19}$	1·0	1·0	$1{\cdot}73\ 10^{-12}$	$1{\cdot}17\ 10^{2}$	$1{\cdot}17\ 10^{2}$
[c] C_2	—	—	$1{\cdot}3\ 10^{-19}$	1·0	1·0	$5{\cdot}76\ 10^{-15}$	$3{\cdot}58\ 10^{-1}$	$3{\cdot}58\ 10^{-1}$
T K	k^H, s^{-1} $FC = FC' = 1$	k^D, s^{-1} $FC = FC' = 1$	*k.i.e.*	A^{H^+}, s^{-1}	A^{D^+}, s^{-1}	$\Delta S^{\neq H^+}$ *e.u.*	$\Delta S^{\neq D^+}$ *e.u.*	[d] k_2, s^{-1} exp.
288	$2{\cdot}67\ 10^{6}$	$4{\cdot}28\ 10^{5}$	6·25	$1{\cdot}60\ 10^{9}$	$4{\cdot}55\ 10^{9}$	−16·3	−14·3	64·1
298	$4{\cdot}03\ 10^{6}$	$7{\cdot}24\ 10^{5}$	5·56	$1{\cdot}98\ 10^{9}$	$5{\cdot}01\ 10^{9}$	−15·9 [d] (−16·0)	−14·1	119·3

[a] $E = 45{\cdot}2$ kJ/mol $\tilde{\nu}_{AH} = 6{\cdot}69\ 10^{13}$, $\tilde{\nu}_{BH} = 6{\cdot}22\ 10^{13}$, $V_{max} = 61{\cdot}9$ kJ/mol, $V = 24{\cdot}5$ kJ/mol, $T = 288$ K; [b] wave function localised in right-hand well; [c] over barrier wave function; [d] experimental data[23]; [e] calculated according[21].

TABLE II

Kinetic parameters for H^+ transfer from imidazole molecule to ester type substrate; Exothermic potential[a]

Energy level	Inner turning points a.u. a	b	E_v J	$e\,P^{H^+}$	$e\,P^{D^+}$	$\exp(-E_v/kT)$	$\tilde{\nu}P\exp(-E_v/kT)$ H^+	D^+
B_0	$-\infty$	—	—	0·0	0·0	—	—	—
A_0	−0·54	0·37	$2{\cdot}47\ 10^{-20}$	$2{\cdot}3\ 10^{-5}$	$2{\cdot}7\ 10^{-7}$	$2{\cdot}01\ 10^{-3}$	$3{\cdot}15\ 10^{6}$	$3{\cdot}75\ 10^{4}$
[b] $B_1 + A$	—	—	$3{\cdot}45\ 10^{-20}$	0·0	0·0	—	0·0	0·0
$A_1 + B$	−0·3	0·17	$6{\cdot}95\ 10^{-20}$	$4{\cdot}2\ 10^{-2}$	$1{\cdot}2\ 10^{-2}$	$2{\cdot}57\ 10^{-8}$	$7{\cdot}83\ 10^{4}$	$2{\cdot}15\ 10^{4}$
$B_2 + A$	−0·25	0·11	$7{\cdot}79\ 10^{-20}$	$1{\cdot}6\ 10^{-1}$	$7{\cdot}5\ 10^{-2}$	$3{\cdot}03\ 10^{-9}$	$3{\cdot}67\ 10^{4}$	$1{\cdot}72\ 10^{4}$
[c] C_1	—	—	$1{\cdot}03\ 10^{-19}$	1·0	1·0	$5{\cdot}47\ 10^{-12}$	$3{\cdot}77\ 10^{2}$	$3{\cdot}81\ 10^{2}$
[c] C_2	—	—	$1{\cdot}21\ 10^{-19}$	1·0	1·0	$5{\cdot}76\ 10^{-14}$	4·37	4·37
T K	k^{H^+}, s^{-1} $FC = FC' = 1$	k^{D^+}, s^{-1} $FC = FC' = 1$	k.i.e.	A^{H^+}, s^{-1}	A^{D^+}, s^{-1}	$\Delta S^{\pm H^+}$ e.u.	$\Delta S^{\pm D^+}$ e.u.	k_2, s^{-1} exp.
288	$1{\cdot}49\ 10^{9}$	$3{\cdot}50\ 10^{7}$	42·6	$2{\cdot}96\ 10^{6}$	$5{\cdot}73\ 10^{7}$	−28·8	−22·9	64·1
298	$1{\cdot}51\ 10^{9}$	$4{\cdot}39\ 10^{7}$	34·4	$5{\cdot}43\ 10^{6}$	$8{\cdot}49\ 10^{7}$	−27·7	−22·2	119·3

[a] $E^* = 37{\cdot}8$ kJ/mol, $V_{max} = 54{\cdot}5$ kJ/mol, $\Delta V = -24{\cdot}9$ kJ/mol, $T = 288$ K, $\tilde{\nu}_{AH} = 6{\cdot}88\ 10^{13}$, $\tilde{\nu}_{BH} = 7{\cdot}58\ 10^{13}$; [b] wave function localised in right-hand well; [c] over barrier wave function.

TABLE III

Kinetic parameters for H^+ transfer from imidazole molecule to amide type substrate; Endothermic potential[a]

Energy level	Inner turning points a.u. a	Inner turning points a.u. b	E_V J	[e] P^H	[e] P^D	$\exp(-E_v/kT)$	$\nu P \exp(-E_v/kT)$ H^-	$\nu P \exp(-E_v/kT)$ D^+
A_0	—	$+\infty$	$2{\cdot}07\ 10^{-20}$	0·0	0·0	$5{\cdot}48\ 10^{-3}$	0 0	0·0
[b] B_0	—	—	$5{\cdot}73\ 10^{-20}$	0·0	0·0	$5{\cdot}5\ 10^{-7}$	0 0	0·0
$A_1 + B$	−0·35	0·56	$5{\cdot}95\ 10^{-20}$	$4{\cdot}20\ 10^{-5}$	$1{\cdot}68\ 10^{-5}$	$3{\cdot}2\ 10^{-7}$	$7{\cdot}98\ 10^{3}$	$3{\cdot}18\ 10^{2}$
$B_1 + A$	−0·19	0·26	$8{\cdot}82\ 10^{-20}$	$1{\cdot}93\ 10^{-1}$	$9{\cdot}79\ 10^{-2}$	$2{\cdot}52\ 10^{-10}$	$2{\cdot}29\ 10^{3}$	$1{\cdot}16\ 10^{3}$
$A_2 + B$	−0·005	0·15	$9{\cdot}38\ 10^{-20}$	$8{\cdot}19\ 10^{-1}$	$7{\cdot}55\ 10^{-1}$	$5{\cdot}65\ 10^{-11}$	$2{\cdot}77\ 10^{3}$	$2{\cdot}55\ 10^{3}$
[c] C_1	—	—	$1{\cdot}13\ 10^{-19}$	1·0	1·0	$4{\cdot}33\ 10^{-11}$	$2{\cdot}21\ 10^{1}$	$2{\cdot}21\ 10^{1}$
[c] C_2	—	—	$1{\cdot}30\ 10^{-19}$	1·0	1 0	$6{\cdot}44\ 10^{-15}$	$3{\cdot}84\ 10^{-1}$	$3{\cdot}84\ 10^{-1}$
T K	k^{H^+}, s^{-1} $FC = FC' = 1$	k^{D^+}, s^{-1} $FC = FC' = 1$	*k.i.e.*	A^{H^+}, s^{-1}	A^{D^+}, s^{-1}	$\Delta S^{\neq H^+}$ *e.u.*	$\Delta S^{\neq D^+}$ *e.u.*	[d] k_2, s^{-1} exp.
288	$2{\cdot}38\ 10^{6}$	$7{\cdot}38\ 10^{5}$	3·22 [d] (2—5)	$3{\cdot}76\ 10^{7}$	$5{\cdot}5\ 10^{7}$	−23·3	−22·5	1·5

[a] $E^{\neq} = 40{\cdot}5$ kJ/mol, $V_{max} = 57{\cdot}2$ kJ/mol, $\Delta V = 23{\cdot}3$ kJ/mol, $T = 288$ K, $\tilde{\nu}_{AH} = 5{\cdot}96\ 10^{13}$, $\tilde{\nu}_{BH} = 5{\cdot}08\ 10^{13}$; [b] wave function localized in right-hand well; [c] over barrier wave function; [d] experimental data[1]; [e] calculated according[21].

TABLE IV

Kinetic parameters for H^+ transfer from imidazole molecule to amide type substrate; Exothermic potential[a]

Energy level	Inner turning points *a.u.* a	b	E_v J	[e] P^{H+}	[e] P^{D+}	$\exp(-E_v/kT)$	$\tilde{\nu}P\exp(-E_v/kT)$ H^+	D^+
B_0	$-\infty$	—	—	0·0	0·0	—	—	—
A_0	−0·65	0·49	$2{\cdot}22\ 10^{-20}$	$1{\cdot}07\ 10^{-6}$	$3{\cdot}58\ 10^{-9}$	$3{\cdot}81\ 10^{-3}$	$2{\cdot}52\ 10^{5}$	$8{\cdot}47\ 10^{2}$
[b] $B_1 + A$	—	—	$3{\cdot}29\ 10^{-20}$	0·0	0·0	$2{\cdot}57\ 10^{-4}$	0·0	0·0
$A_1 + B$	−0·39	0·29	$6{\cdot}29\ 10^{-20}$	$4{\cdot}96\ 10^{-3}$	$5{\cdot}51\ 10^{-4}$	$1{\cdot}42\ 10^{-7}$	$4{\cdot}39\ 10^{4}$	$4{\cdot}88\ 10^{3}$
$B_2 + A$	−0·33	0·22	$7{\cdot}15\ 10^{-20}$	$2{\cdot}87\ 10^{-2}$	$6{\cdot}66\ 10^{-3}$	$1{\cdot}55\ 10^{-7}$	$3{\cdot}02\ 10^{4}$	$6{\cdot}94\ 10^{3}$
[c] C_1	—	—	$9{\cdot}37\ 10^{-20}$	1·0	1·0	$5{\cdot}77\ 10^{-11}$	$3{\cdot}59\ 10^{3}$	$3{\cdot}59\ 10^{3}$
[c] C_2	—	—	$1{\cdot}06\ 10^{-19}$	1·0	1·0	$2{\cdot}68\ 10^{-12}$	$1{\cdot}66\ 10^{2}$	$1{\cdot}66\ 10^{2}$
T K	k^{H+}, s^{-1} $FC = FC' = 1$	k^{D+}, s^{-1} $FC = FC' = 1$	*k.i.e.*	A^{H+}, s^{-1}	A^{D+}, s^{-1}	$\Delta S^{\neq H+}$ *e.u.*	$\Delta S^{\neq D+}$ *e.u.*	[d] k_2, s^{-1} exp.
288	$8{\cdot}11\ 10^{7}$	$3{\cdot}99\ 10^{6}$	20·3	$1{\cdot}15\ 10^{6}$	$1{\cdot}06\ 10^{7}$	−30·1	−25·7	1·5

[a] $E^{\neq} = 38{\cdot}3$ kJ/mol, $V_{max} = 55{\cdot}0$ kJ/mol, $\Delta V = -20{\cdot}9$ kJ/mol, $T = 288$ K, $\tilde{\nu}_{AH} = 6{\cdot}21\ 10^{13}$, $\tilde{\nu}_{BH} = 6{\cdot}79\ 10^{13}$; [b] wave function localised in right-hand well; [c] over barrier wave function; [d] experimental data[1]; [e] calculated according[21].

ing" to A-H bond vibration, its own states, but also new-mixed states {B + A} which originate from stretching vibration of B-H bond. From the wave functions (Fig. 1, 2) it is obvious that the probability of finding the proton on "mixed" energy levels in the region of the left-hand side well of the potential is nonzero, and thus such states for calculation of rate constant should be included. Tables I–IV show that contribution of these states is important.

Tables I–IV present, besides the calculated values of canonical rate constants, also calculated values of kinetic isotope effect (*k.i.e.*). Calculations were performed for isotope independent potentials. As we can see from Tables I–IV, contribution of vibration excited states to proton transfer process decreases the value of *k.i.e.* The reason for this is very simple. The distance between the inner turning points on excited energy levels is smaller than the one for ground state energy level, and thus the difference between the vaules of P^{H^+} and P^{D^+} for excited states is much smaller. In the case that proton transfer is mainly controlled by ground state vibration, the difference in P^{H^+}/P^{D^+} will be greater and so will the value of *k.i.e.* In general, it can be expected that *k.i.e.* will be larger for exothermic than for endothermic type of double-well potentials. The largest value can be expected for symmetric double-well potential (assuming the same distances of energy minima).

In the framework of the suggested model, *k.i.e.* can be calculated without assumption that effective distance for H^+ and D^+ transfer is different contrary to the model suggested by[20]. As it follows from our results, *k.i.e.* is fully determined for a particular type of reaction (potential) by the position of proton energy levels which are "active" at the proton transfer process in the reacting complex.

DISCUSSION

Calculated canonical rate constants $k(FC = FC' = 1)$ for studied model potentials which represent *in vacuo* H^+ transfer in the rigid linear reacting complex are, as can be expected, very high. By comparing calcualted rate constants $k(FC = FC' = 1)$ with experimental ones, we obtain the parameter FC (at fixed $FC' = 1$) which for the models studied range from 10^{-5} to 10^{-8}. Corresponding values of frequency factors are then very low: $A = 10^6 - 10^9\ s^{-1}$. Relatively large negative values of activation entropy, see Tables I–IV, correspond to such frequency factors. Nevertheless, in the case of endothermic model potential for ester type substrate, activation entropy calculated in this way is very close to the experimental value (Table I) and of course, $k(FC = 2{\cdot}0\ 10^{-5}, FC' = 1)$ then perfectly fits the experimental value of k_2. Also the calculated value of *k.i.e.* for this potential is comparable to the experimental one. Unfortunately, we have no access to experimental value of $\Delta S_2^{\neq}$ for amide type substrate catalysis and thus the comparison can not be performed for this type of substrate.

Let us now return to the present results and experimental data. In spite of relatively good agreement between theoretical and calculated kinetic parameters for ester type substrate (Table I) it still appears that experimental activation parameters ($\Delta H_2^{\neq}$, $\Delta S_2^{\neq}$) do not represent true activation values of elementary chemical act. If we accept that negative entropy produced at ES complex formation is effectively used in decreasing reaction activation energy by bringing the structure of tetrahedral intermediate close to the structure of activated complex, then this advantage is only apparent because of the relatively large negative entropy (-16 to -20 e.u.) of proton transfer step (rate determining for amide type substrate hydrolysis), which contrary to the former case of entropy loss increases the activation energy. In such a case, high catalytic efficiency of the enzyme must be controlled mainly by an enthalpy factor. This means that the electronic structure of tetrahedral intermediate, including the influence of electrostatic field of ES complex, cuase the weakness of the substrate bond to be broken. The experimental value of acylation step: $\Delta H_2^{\neq} \sim 40$ kJ/mol seems to support this assumption. In our opinion, however, this value can be equally true as it can be merely a phenomenological parameter which fits temperature dependence of the rate constant (k_2). In the latter case, it is a rather confusing parameter which can hardly clarify the mechanism.

Let us return once again to the activation entropy of k_2 step. Scheiner and Lipscomb[22] have published a theoretical study on the serine proteases catalytic pathway. They have found that proton transfer from imidazole His 57 to eletronegative atom of substrate bond to be broken at the breakdown of tetrahedral intermediate is realized along the almost linear H-bond. In the case of specific substrate, the structure of tetrahedral intermediate must be fairly rigid, because the substrate carbonyl oxygen forms two H-bonds with the enzyme oxyanion hole and the position of substrate bond is fixed in the vicinity of the enzyme active center by the interaction of the substrate side chain with the enzyme sorption region. Moreover, in the case of amide substrates, the position of amido N is also fixed by H-bond between amido H and carboxyl O of Ser 214. The position of imidazole ring His 57 is also stabilised by H-bond to carboxyl oxygen of Asp 102.

Thus, let us try to estimate the effective proton frequency ν_{eff} for such a structure of tetrahedral intermediate. Effective frequency for proton transfer is a simple product of the proton mean frequency in rigid linear H-bond and steric corrections imposed on this structure by „perturbative" degrees of freedom (FC'). In the case of amide type substrate, the declination from linear structure of H bond can be caused by imidazole His 57 —N—H bond bending vibration, and by substrate C—N— bond bending vibration. In harmonic approximation, a harmonic oscillator in its ground vibrational state spends about 8% of its time at or beyond its inner turning points. This means that the two bending vibrations contribute to the effective frequency at least by the factor of $(0{\cdot}08)^2$. The effective frequency for model amide ento (exo) potential is then: $\nu_{eff} = (0{\cdot}08)^2 \,.\, \bar{\nu} = 3{\cdot}7\ 10^{11}\ s^{-1}$ ($3{\cdot}9\ 10^{11}\ s^{-1}$). For ester substrates hydrolysis

the same perturbative degrees of freedom can be expected as for amide substrates but for esters there is no stabilization of ester O atom (in amide, N is stabilized by its H to Ser 214 carboxyl oxygen) and for ester substrate we can suppose additional degree of freedom – rotation around substrate C—O bond. Let us suppose that proton transfer is possible for —O—O bond rotation in the range of $\pm 15°$. Then, for model ester substrate we obtain the following values of effective frequency: $\nu_{eff} = (30/360)(0.08)^2 \cdot \bar{\nu} = 3{\cdot}7\ 10^{10}\ (3{\cdot}9\ 10^{10})\ s^{-1}$. Now, for frequency factor we can write: $A = \nu_{eff} \cdot \exp(\Delta S^{+}/R)$ As it appears in this expression, effective frequency of the proton in the bond to be broken differs from one reacting system to the other. On the other hand, the frequency part (effective frequency) of the frequency factor from the Absolute rate theory does not depend on the reacting system, and for all systems it has the same value $-k_B T/h$. It is obvious that activation entropy calculated by means of effective frequency ν_{eff} will differ from the value obtained when using the absolute rate theory.

The recalculated values of $\Delta S_2^{\neq}$ by means of estimated values of ν_{eff} are in Table V. In this approximation, the activation entropy comparable with experimental value was obtained for exothermic model ester potential. For this potential, however, the calculated value of *k.i.e.* as well as the difference in activation entropy for H^+ and D^+ transfer are very high.

Inspite of the relatively good agreement between theoretical results and experimental data it is very problematic to draw conclusions on the physical basis of the enzyme high catalytic efficiency. At this place a very important fact should be recalled. The published experimental activation parameters as well as presented theoretical results have been determined or calculated using the equations derived assuming equilibrium process. On the other hand, however, the studied reaction step in the enzymatic catalysis is without any doubts a nonequilibrium process. In order to be

Table V
Recalculated values of $\Delta S_2^{\neq}$ by means of ν_{eff}

Potential	Parameter *e.u.*	Substrate	
		amide	ester
Endothermic	$^{H^+}\Delta S_2^{\neq}$	−18·28	−6·12
	$^{D^+}\Delta S_2^{\neq}$	−17·52	−4·09
Exothermic	$^{H^+}\Delta S_2^{\neq}$	−25·29	−18·68
	$^{D^+}\Delta S_2^{\neq}$	−20·88	−12·74

able tu study the enzymatic process more realisticaly, the present method needs to be reformulated. To do this, at least the coupling between imidazole His 57 N-H bond stretching vibration mode and „relevant" vibrational modes of the rest of the system must be taken into account. In such case, the proton population probability, $n(E)$, will not obey Gibbss' statistics but it will be an interaction and the time dependent function. The question is, which are „relevant" modes. From the physical point of view, it seems accetable that „relevant" modes could be only the modes created in the connection with physical oj chemical changes undergoing in the system. There exists the evidence that chymotrypsin efficiency can be enhanced by the external – impulse like perturbation[23,24]. How can be created, however, the „relevant" vibrational mode (modes) at natural catalytic process – without external perturbation? Davydov has recently shown [25] that the free energy released at chemical processes or nobounded interactions in biosystems can be transferred along the polypeptides chain in the form of vibrational solitons – corresponding to amide I vibrational mode. In the case of specific substrates, the released free energy at the ES complex formation is large enough and the process fulfill the necessary conditions for solitons formation. If the described process realy takes place, it can be expected that the coupling between solitons and imidazole N-H bond could be strong. Under this assumption the physical basis of the studied enzymatic process can be described as the phonon (soliton) -asisted proton tunneling and the contribution of imidazole N-H bond (His 57) stretching vibrational I. excited state can be expected to be important.

The importance of the vibration excited states in the catalytic process can be verified partially by the experiment we are suggesting here. Kolias and Melander have published results of chymotrypsin enhanced efficiency by laser irradiation[24]. The measurements were done for the hydrolysis of benzoyl-L-tyrosine ethyl ester which is a rather good substrate for chymotryptic catalysis. Our suggestion is to perform the same experiment but with nonspecific substrates: N-acetyl-L-glycine (alanine)-ethyl ester and N-acetyl-L-glycine (alanine)-ethylamide. If the contribution of vibrational excited states play an important role in the enzyme action, then the k_2 rate constant in this experiment could reach the value comparable to the value of k_2 for hydrolysis of specific substrates (nonirradiated experiment). The results of the experiment need not to be unambiguous and the reason for uncertainity is a simple one. In the case of the hydrolysis of nonspecific substrates, the position of substrate bond to be broken has not to be fixed at the proper orientation in the vicinity of the enzyme active center in contrast to specific substrate (interaction of substrate side chain with the enzyme sorption region). This orientational drawback represents additional and very important perturbative degree of freedom, which can cause very low value of frequency factor. Nevertheless, it is realistic to expect $10^1 - 10^3$ times rate enhancement.

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