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Research Article

Criterion of Stability of the Mechanism of Proceeding Reactions Catalyzed by Enzymes

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Abstract

Possible use of the criterion of stability of the mechanism of proceeding of enzyme catalyzed reactions for studying the effect of increasing concentration of inhibitors on the enzyme is analysed. It is shown that in the three-dimensional $K'_m V' I$ coordinate system the length of $\mathbf{L}_i$ vectors for enzyme inhibition characterizes the intensity of enzyme inhibition ($\mathbf{L}_a$ vectors – of the intensity of enzyme activation, Figure 1). This opens up additional possibility of studying the mechanism of proceeding of enzyme catalyzed reactions. Examples of using the dependencies of change in the length of $\mathbf{L}_i$ vectors of enzyme inhibition are given.

Keywords: Mechanism; Enzymes; Vector

Introduction

When using enzymes in practice, one has to know the limit of addition of this or that inhibitor, activator as well as a temperature range of reaction proceeding, etc., without change in the mechanism of proceeding of the reaction that may lead to decrease in the yield of the product of reaction. Such situation most frequently occurs at increasing concentration of inhibitors (*i*), activators (*a*) and other effectors, which are introduced into a reacting system, for example, for controlling of drug metabolism process. The question is also important from a theoretical point of view as it concerns the stability of the mechanism of proceeding of enzyme catalyzed reactions, which is of interest for enzymologysts.

The studies revealed that when the amount of any inhibitor added to the enzyme increased, the strength of their binding to the enzyme characterized by the constant of inhibition (K_i) enhanced K_i– the values got lower [1-3]. weakened – the values got higher (Table 2) [1,2,4,5] or was independent of increasing concentration of *i* – the K_i values remained unchangeable (Table 1) [6-8]. Before the development of a vector method of representation of the mechanism of proceeding of enzyme catalyzed reactions, it was difficult to understand how all this is connected with retention of stability of the mechanism of proceeding of enzyme inhibition, e.g. at increasing concentration of inhibitor (or activator) in the reacting system.

Numerous attempts to employ the K_i constant of inhibition for characterization of not only the strength of binding of the enzyme to the inhibitor [9-13], but also the estimation of intensity of enzyme inhibition [4-17] failed in practice, because it was impossible to explain the case when K_i values got higher or remained unchangeable at increasing concentration of *i*. It was also impossible to use K_i values for estimation of intensity of enzyme inhibition as in most cases the initial reaction rate decreased ($v_i < v_0$) at increasing concentration of *i*, indicating the enhanced effect of enzyme inhibition, while the course of change in the K_i values did not correspond with this.

The vector method of representation of enzymatic reactions in the three-dimensional $K'_m V' I$ coordinate system (Figure 1) [18-23].

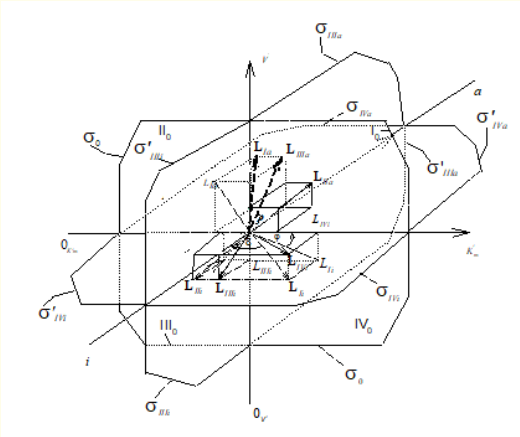

Figure 1: Three dimensional (branched) $K'_m V' I$ system of rectangular coordinate system with separately P_i and P_a semiaxes of molar concentrations of [*i*] inhibitor and [*a*] activator. $\mathbf{L}_{Ii}$, $\mathbf{L}_{IVi}$, $\mathbf{L}_{IIIi}$, $\mathbf{L}_{IIi}$, $\mathbf{L}_{Ia}$, $\mathbf{L}_{IIIa}$, $\mathbf{L}_{IIa}$ symbols vectors, of enzyme catalyzed reactions, $\mathbf{L}_{Ii}$, $\mathbf{L}_{IVi}$, $\mathbf{L}_{IIIi}$ and $\mathbf{L}_{Ia}$ symbols of theirs projection on basic σ_0 plane I_o, II_o ... quadrants of this plane, the magnitude of φ angle about 340^0.

showed that the length of $\mathbf{L}_i$ vectors of inhibited (or activated) reaction determined by a ratio of the K'_m, K^0_m, V', V^0, and i (or a) parameters characterizes intensity of the effect of increasing concentration of (or a) on the enzyme:

$$l_i = \left((K'_m - K^0_m)^2 + (V' - V^0)^2 + (i - 0)^2\right)^{0.5} \quad \text{.....(1)}$$

where K'_m and V' – the effective Michaelis constant and the maximum reaction rate determined in the presence of increasing concentration of i (or a); K^0_m and V^0 – the same parameters of initial (uninhibited, i = 0 and non-activated, a = 0) reaction.

As follows from the equation (1), the greater is a difference between K'_m and K^0_m, V' and V^0, i and 0, the longer is a length of the respective $\mathbf{L}_i$ vector of inhibited reaction (Figures 3, 5, 7). For description of the effect of i on the enzyme, two parameters ought to be analysed: one must compare the values of K_i constant of enzyme inhibition characterizing the strength of binding of i to the enzyme and the length of $\mathbf{L}_i$ vectors for enzyme inhibition characterizing intensity of the effect of i on the enzyme. Construction of $\mathbf{L}_i$ vectors for enzyme catalyzed reactions in the $K'_m V' I$ coordinate system (Figure 1) gives another possibility for analysis of stability of the mechanism of effect of increasing concentration of different inhibitors (or other effectors) on enzymes.

By marking the consecutive position of $\mathbf{L}_i$ vectors for intensity of enzyme inhibition and connecting their mobile ends with a circular line, one can obtain several positions of a characteristic curve individual for each concrete reaction that can be either a single straight line (Figure 3) or a two straight lines (Figure 5) or several consecutive short rectilinear segments (Figure 7).

The dependence of vector length upon a change in the value of intervals

$$K'_m - K^0_m,\ V' - V^0,\ i - 0 \quad \text{.....(2)}$$

on the axes in the three-dimensional coordinate system was examined (Figure 1). It was found that until an increase in the concentration of leading to as great increase in the length of a respective $\mathbf{L}_i$ vector as possible is accompanied by a proportional increment in the length of above segments on the coordinate axes that exhibits itself by constancy of the slope angles of respective rectilinear segments of characteristic curves:

$$\text{tg } \varphi = \text{const and tg } \in = \text{const}, \quad \text{.....(3)}$$

the mechanism of proceeding of such inhibited reaction remains unchangeable (Figure 3). And conversely, the presence of a bent on such curves will mean a transition to another mechanism of reaction proceeding more stable under changed conditions, and the presence of two (Figure 5) or several short rectilinear segments (Figure 7) indicates a series of consecutive changes in this mechanism, i.e., instability of the mechanism of reaction proceeding at increasing concentration of *i*, (*a* or other conditions) of reaction proceeding.

This gives another possibility of studying the effect of inhibitors, activators and other effectors on enzymes by plotting the dependencies of a course of change in the length of vectors for enzyme inhibition upon increasing concentration of effectors, etc [18-23].

Examples of stability and instability of the dynamics of enzyme inhibition are given.

Materials and Methods

- **Chemicals**: Calf intestinal alkaline phosphatase (EC 3.1.3.1) – a product of Fluka – (Switzerland), Bovine pancreatic – specific RNase A (EC 3.1.44.22) – a product of Sigma (USA).
- **Substrate**: p-Nnitrophenylphosphate 2CHA salt (pNPP) – a product of Serva (Germany) and cytidine-2′,3′-monophosphate (C>p) for RNase A – a product of Sigma.
- **Inhibitors**: Orthophosphoric acid (H_3PO_3) and disodium of tungstic acid ($Na_2WO_4.2H_2O$) ....(1), Adenosinetetraphosphate (APPPP) – the domestic preparations of high purity grade.

A process of pNPP cleavage was recorded by a CF-4 DR two-beam spectrophotometer (Optica Milano, Italy). Reactions were carried out in 0.05 M Tris-HCl buffer (pH 9.0) with ionic strength of 0.1 by NaCl at constant stirring [18] in a thermostat (37^0C). The kinetic curves were estimated by increase in the absorption $+\Delta D_{400}$ ($+\Delta D_{286}$ for RNase A) [24] of a solution containing the substrate, the enzyme and the inhibitor against a solution of the same composition, but without the enzyme.

The selection of substrate was stipulated by an interval of minimum error in the determination of K_m and V parameters [25].

Determination of enzyme activity

The initial reaction rates (v) were determined by a slope angle of tangents to initial segments of curves representing substrate cleavage in at least five parallel experiments.

The kinetic K_m and V parameters were calculated by respective plots in the (v^{-1}, S^{-1}) coordinates of Lineweaver-Burk using the computer program Sigma Plot Version 10 (USA). Root-mean-square deviation at five-fold determination was as follows: v = 2.5%, Km and V = ± 7.5%, l = ± 10%.

To calculate the length of vectors Eq. (1), the following parameters (Figure 3) were unified: K^0_m and K'_m = 0.1 mM = one conventional unit (c.u.), i = 0.05 mM = one c.u.; V' and V^0 = 1 μmol/min μg enzyme = one c.u. (the appropriate data in parentheses in each Table).

Results and Discussion

The results are presented in Figures. 2-7 and Tables 1-3.

Example of stability in the dynamics of enzyme inhibition

Effect of orthophosphoric acid H_3PO_3 on the initial rates of pNPP cleavage by calf alkaline phosphatase shows that increasing concentrations of the acid in a solution lead to increased values of the Michaelis effective constants, though the maximum reaction rates remain unchangeable ($K'_m > K^0_m$, $V' = V^0$, $i > 0$). It is an example of the associative (IV_i type of enzyme inhibition Figure 2 and Table 1) [18-23,26-28].

H_3PO_3 (mM)	K'_m (10^{-5} M)	K_{IVi} (mM)	l_{IVi} (c.u.)
0	4.58		
1.75 (0.175 c.u.)	8.16 (0.816 c.u.)	2.24	0.398
3	10.64	2.27	0.676
5	14.62	2.28	1.122

Table 1: The kinetic parameters of calf alkaline phosphatase inhibition by orthophosphoric acid.

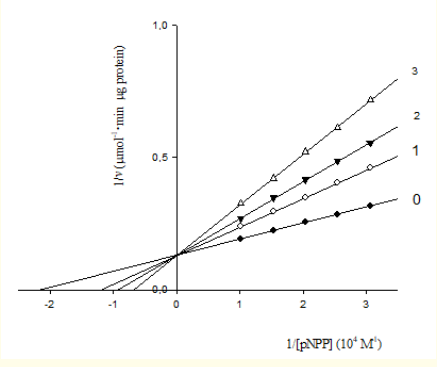

Figure 2: Inhibitory effect of increasing concentration of H_3PO_3 on the initial rate of pNPP cleavage by calf alkaline phosphatase. The concentrations of H_3PO_3 (10^{-3} M): lines 1, (2), 3 - 1.75, (3) and 5, respectively. Line 0 – the inhibitor is absent.

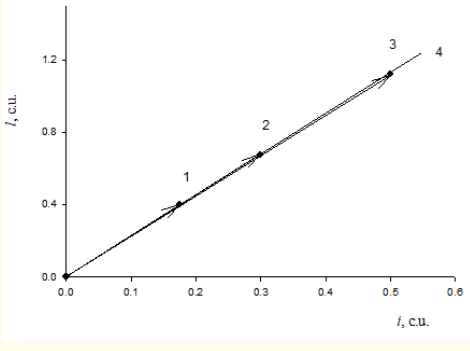

Figure 3: Dependence of a change in the l_{IVi} length of vectors for calf alkaline phosphatase inhibition upon increasing concentration of H_3PO_3. The other conditions are given in the text and Figure 2.

Substitution of the data from Table 1 in Eq. (1) indicates that the inhibitory of the associative (IV_i type, Figure 2) effect of anions of orthophosphoric acid on enzyme [18-23,26-28] and enhances proportional to the content of H_3PO_3 in a whole range of concentrations (Figure 4). This fully meets the criterion of stability of the mechanism of enzyme inhibition enhanced by H_3PO_3 (Eq. 3) and testifies to incorrectness of using here the constants of enzyme inhibition to characterize the intensity of inhibition, as in this experiment the values of K_{IVi} constant remain unchangeable despite permanent strengthening of the inhibitory effect at increasing concentration of H_3PO_3 (Table 1), i.e. we have all grounds to say about a stable mechanism of enzyme inhibition in the whole range of tested concentrations of orthophosphoric acid.

Example of occurring once exchange of mechanism of enzyme inhibition

The results are given in Figures 4, 5 and Table 2.

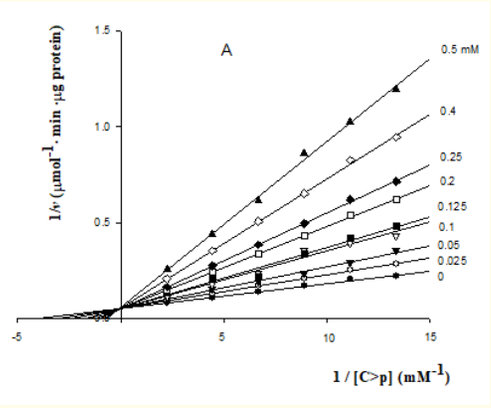

Figure 4: Inhibitory effect of APPPP on the initial rates of C>p cleavage by RNase A in the (v_0^{-1}, S^{-1}) coordinate system. The concentrations of APPPP (mM) are indicated near the respective lines. Line 0 – the inhibitor is absent.

APPPP (c.u.)	K^0_m (c.u.)	K'_m (c.u.)	K_{IVi} (mM)	l_{IVi} (c.u.)
0 (0)	0.0247 mM (2.47 c.u.)			
0.05		3.31	0.0742	0.977
1		4.15	0.0741	1.955
2		5.93	0.0718	3.996
2.5		6.08	0.0858	4.391
4		8.12	0.0877	6.922
5		9.64	0.0863	8.717
8		12.17	0.1019	12.573
10		14.06	0.1066	12.581

Table 2: The inhibitory effect of APPPP on RNase A.

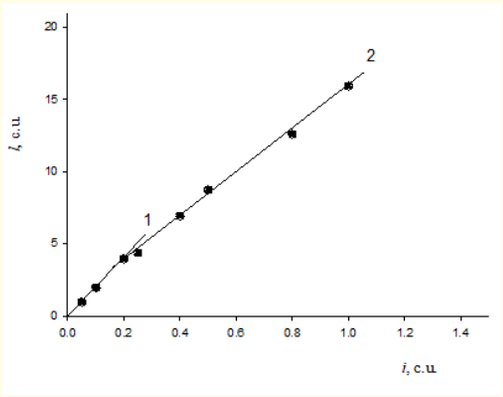

Figure 5: Dependence of a change in the l_{1Vi} length of $\mathbf{L}_{1Vi}$ vectors for RNase A inhibition upon increasing concentration of APPPP. The other conditions are given in the text and Figure 4 and Table 2.

Adenosinetetraphosphate exhibits the features of associative (IV_i type) inhibitor towards RNase A, (Figure 5) [18-23,26-28] and, the inhibition enhances with increasing concentration of APPPP.

As seen from (Figure 5) the intensity of the inhibitory effect of APPPP strengthens at their increasing concentration but have two linear intervals: the first interval (0 – $0.2 \cdot 10^{-4}$ M APPPP, line 1) and the second interval (0.2 – 1 10^{-4}M APPPP, line 2). This results to the idea that mechanism of inhibition of RNase A was exchanged at $0.2 \cdot 10^{-4}$M APPPP.

Example of instability of mechanism of enzyme inhibition

Effect of anions on the initial rates of pNPP cleavage by calf alkaline phosphatase shows that the Michaelis effective constants increase with a concomitant decrease in maximum reaction rates ($K'_m > K^0_m$, $V' < V^0$, $i > 0$), if concentration of these anions rises (Table 3). It is an example of the biparametrically coordinated (I_i type of enzyme inhibition, Figure 6) [18-23,26-28].

Inhibitor (10^{-4} M)	K'_m (10^{-5} M)	V' (μmol/min· μg protein)	K_I (10^{-5} M)	l_{1Vi} (c.u.)
0.0	4.45	2.56		
0.0625	5.28	2.51	2.94	0.834
(0 . 0 6 2 5 c.u.)	(5.28 c.u.)	(2.51 c.u.)		
0.125	5.39	2.30	3.59	0,983
0.250	5.97	2.15	4.17	1.59
0.50	6.56	1.74	4.27	2.32
1.0	7.43	1.32	4.45	3.36

Table 3: Inhibitory effect of increasing concentration of WO_4^{-2} anions on calf alkaline phosphatase.

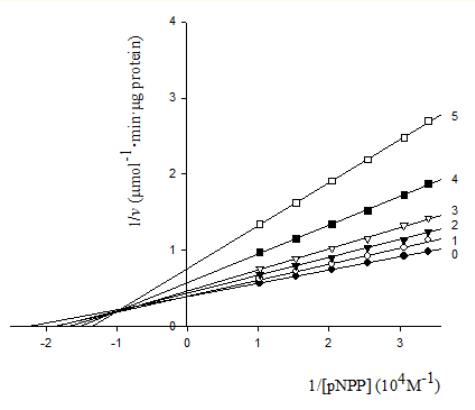

Figure 6: Inhibitory effect of WO_4^{2-} increasing concentration of WO anions on the initial rates of pNPP cleavage by calf alkaline phosphatase. The concentrations of WO_4^{2-} (10^{-5} M): line 1 – 0.625, 2 – 1.25, 3 – 2.5, 4 – 5 and 5 – 10. Line 0 – the inhibitor is absent.

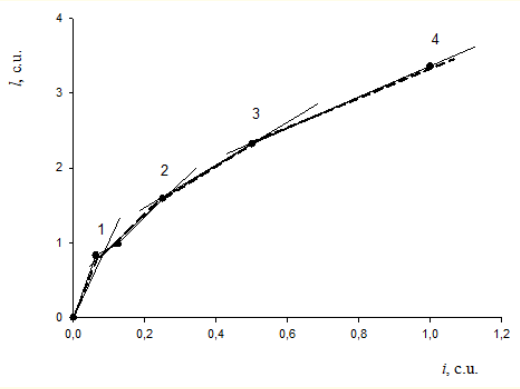

Figure 7: Dependence of a change in the l_{1i} length of $\boldsymbol{L_{1i}}$ vectors for calf alkaline phosphatase inhibition upon increasing concentration of WO_4^{2-} anions. The other conditions are given in the text and Figure 6.

Analysis of the obtained data (Figure 7) indicates that intensity of the inhibitory effect of WO_4^{2-} anions strengthens at their increasing concentration but gives no idea as to how this process is developing as a function. As seen from Table 3, the values of $\boldsymbol{K_{Ii}}$ constant get higher at increasing concentration of WO_4^{2-}, thus characterizing weakness of the binding of anions to the enzyme. At the same time, a slope of graphs gradually gets larger, which testifies to continuous enhancement of enzyme inhibition by these anions.

Effect of increasing concentration of WO_4^{2-} anions on calf alkaline phosphatase inhibition changed at least four times in the interval 0 – $1{\cdot}10^{-4}$ M (Figure 7). This is due to destructive inhibitory effect of increasing concentration of WO_4^{2-} anions on the enzyme.

It would be much more difficult to make such conclusion on the basis of only conventional analysis of experimental data regarding a course of change in the K'_m, V' and K_{Ii} parameters (Figure 6 and Table 3).

Obviously, that Eqs: 1 - 3 and experiments (analogously to Figures 3-7) may be used and for treatment of data activation of enzymes.

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