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Additional Possibility of Data Analysis of Enzyme Inhibition and Activation. 4. Criterion of Stability of the Mechanism of Proceeding of Enzymatic Reactions

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Abstract: Possible use of the criterion of stability of the mechanism of proceeding of enzymatic reactions for studying the effect of increasing concentration of inhibitors on the enzyme were analysed. It was shown that the constant of inhibition (K_i) characterizes the strength of binding of the inhibitor to the enzyme. The length of L_i vectors for enzyme inhibition in three-dimensional K'_m VI coordinate system characterizes the intensity of inhibition. This opens up additional possibility of studying the mechanism of proceeding of enzymatic reactions at consecutive addition of inhibitors, activators and also under varying temperature conditions, etc., with the aim to increase the yield of a reaction product. Examples of using the dependencies of change in the length of L_i vectors of enzyme inhibition for characterization of the dynamics of a stable and unstable inhibitory effect on calf alkaline phosphatase are given.

Key words: Inhibition intensity, criterion of stability, of the mechanism, of proceeding of inhibitory reactions

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 interest. Such situation most frequently occurs at increasing concentration of inhibitors (I), activators (a) and other effectors, which are introduced into a reacting system for controlling the process with the aim to increase the yield of a reaction product. The question is also important from a theoretical point of view as it concerns the stability of the mechanism of proceeding of enzymatic reactions, which is of interest for biotechnology.

The studies revealed that when the amount of any of inhibitors added to the enzyme increased, the strength of their binding to the enzyme characterized by the constant of inhibition (K_i) enhanced-the K_i values got lower^[1-3], weakened - the K_i 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 enzymatic reactions, it was difficult to understand how all this is connected with retention of stability of the mechanism of enzyme inhibition, e.g. at increasing concentration of inhibitors in the reacting system.

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

H ₃ PO ₃ (mM)	K' _m (10 ⁻⁵ M)	K _{iVi} (mM)	I (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 2: Inhibitory effect of increasing concentration of WO₄²⁻ anions on calf alkaline phosphatase

Inhibitor (10 ⁻⁴ M)	K' _m (10 ⁻⁵ M)	V' (μmol/min- μg protein)	K _i (10 ⁻⁵ M)	I (c.u.)
0.0	4.45	2.56		
0.0625 (0.0625 c.u.)	5.28 (5.28 c.u.)	2.51 (2.51 c.u.)	2.94	0.834
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

Numerous attempts to employ the 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^[14-17] 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

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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 (Fig. 1)^[18-20] showed that the length of L_i vectors of inhibited or activated reaction determined by a ratio of the K'_m , K_m^0 , V' , V^0 and *i* (or *a*) parameters characterizes intensity of the effect of increasing concentration of *i* (or *a*) on the enzyme:

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

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_m^0 and V^0 -the same parameters of initial (uninhibited, $i = 0$ and nonactivated, $a = 0$) reaction.

As follows from the Eq. 1, the greater is a difference between K'_m and K_m^0 , V' and V^0 , *i* and 0, the longer is a length of the respective L_i vector of inhibited reaction. 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 *i* binding of to the enzyme and the length of L_i vectors for enzyme inhibition characterizing intensity of the effect of *i* on the enzyme. Construction of L_i vectors for enzymatic reactions in the $K'_m V/I$ coordinate system gives another possibility for analysis of stability of the effect of increasing concentration of different inhibitors or other effectors on enzymes.

By marking the consecutive position of 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 (Fig. 3) or a bent line or several consecutive short rectilinear segments (Fig. 5).

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

$$K'_m - K_m^0, V' - V^0, i - 0 \quad (2)$$

on the axes in the three-dimensional coordinate system was examined. It was found that until an increase in the concentration of *i* leading to as great increase in the length of a respective 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:

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

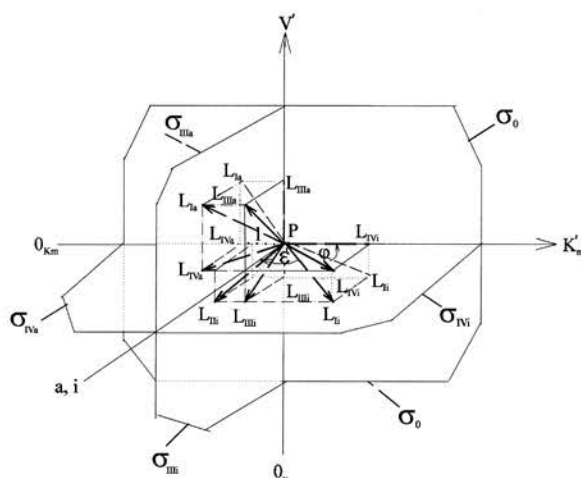


Fig. 1: Three-dimensional $K'_m V/I$ coordinate system with a coincident $P_{a,i}$ semi-axis convenient for description of the kinetic parameters: K'_m , K_m^0 ; V' vectors: L_{IVi} , ... L_{Ii} and their scalar projections: L_{IVi} , L_{Ii} , ... and also of planes σ_{IVi} , σ_{IIIi} , σ_{IIi} , ...

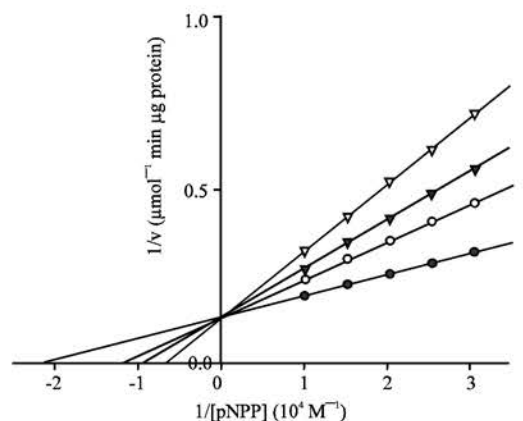


Fig. 2: Inhibitory effect of increasing concentration of H_3PO_3 on the initial rate of pNPP cleavage by calf alkaline phosphatase. The concentrations of H_3PO_3 (10^{-3} M): lines 1, 2, 3-1.75, 3, 5 (10^{-3} M), respectively. Line 0 - the inhibitor is absent

the mechanism of proceeding of such inhibited reaction remains unchangeable. 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 or several short rectilinear segments 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.

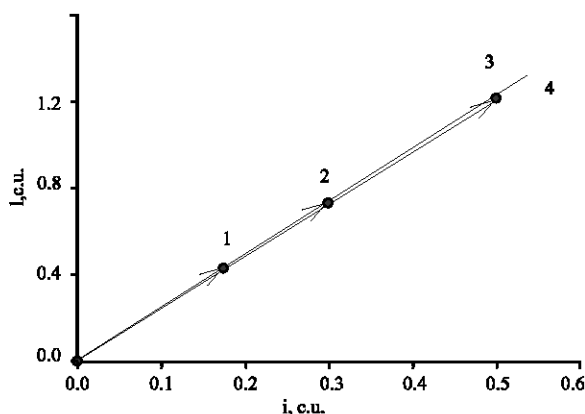


Fig. 3: Dependence of a change in the length of vectors for calf alkaline phosphatase inhibition upon increasing concentration of H_3PO_3 . The other conditions are given in the text and Fig. 2

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.,^[18-22].

Examples of stability and unstability in the dynamics of inhibition of calf alkaline phosphatase that illustrate the possibility of using the criterion of stability for characterization of such processes are given.

MATERIALS AND METHODS

Chemicals: Calf intestinal alkaline phosphatase (EC 3.1.3.1) - a product of Fluka - (Switzerland).

Substrate: p - Nitrophenylphosphate 2CHA salt (pNPP) - a product of Serva (Germany).

Inhibitors: Orthophosphoric acid (H_3PO_3) and disodium of tungstic acid ($Na_2WO_4 \cdot 2H_2O$) - 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 in a thermostat (37°C). The kinetic curves were estimated by increase in the absorption ($+AD_{400}$) of a solution containing the substrate, the enzyme and the inhibitor against a solution of the same composition, but without the enzyme.

The concentration of pNPP was changed from $0.294 \cdot 10^{-4}$ to $0.98 \cdot 10^{-4}$ M, the concentration of the enzyme was $0.978 \mu g mL^{-1}$, the concentrations of inhibitors are

given in the legends to Fig. 2 and 4. The selection of substrate concentration was stipulated by an interval of minimum error in the determination of K_m and V parameters^[23].

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 4.0 (USA). Root-mean-square deviation at five-fold determination was as follows: $v = 2.5\%$, K_m and $V = \pm 7.5\%$, $l = \pm 10\%$.

To calculate the length of $l_{0.175}$ vector (Eq. 1), of phosphatase inhibition by H_3PO_3 of concentration $1.75 \cdot 10^{-3}$ M the following parameters of Table 1 were unified and K_m^0 and $K_m^i = 0.1$ mM = one conventional unit (c.u.), $i = 1 \cdot 10^{-3}$ M = 0.1 c.u.; ($V^i = V^0$) having substituted which in Eq. 1, results to:

$$l_{0.175} = \sqrt{(0.816 - 0.458)^2 + (0.175 - 0)^2} = 0.398 \text{ c.u.} \quad (4)$$

RESULTS AND DISCUSSION

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 ($K_m^i > K_m^0$, $V^i = V^0$, $i > 0$). It is an example of the associative (IV_i) type^[18-20] of enzyme inhibition (Fig. 2 and Table 1).

Substitution of the data from Table 1 in Eq. (1) indicates that the inhibitory effect of anions of orthophosphoric acid enhances proportional to the content of H_3PO_3 in a whole range of concentrations (Fig. 2 and 3). 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.

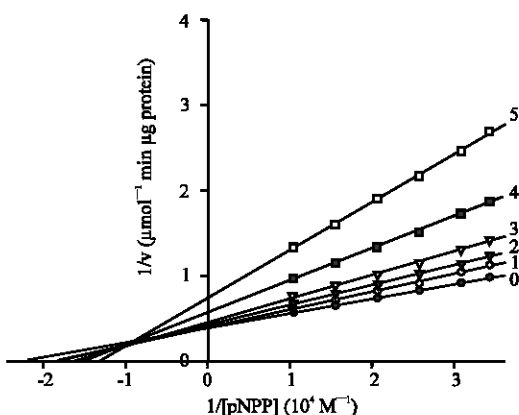


Fig. 4: Inhibitory effect of increasing concentration of WO_4^{2-} anions on the initial rates of pNPP cleavage by calf alkaline phosphatase. The concentrations of WO_4^{2-} (10^{-5} M): line 1-0.625, 2-1.25, 3-2.5, 4-5, 5-10 (10^{-5} M). Line 0 - the inhibitor is absent

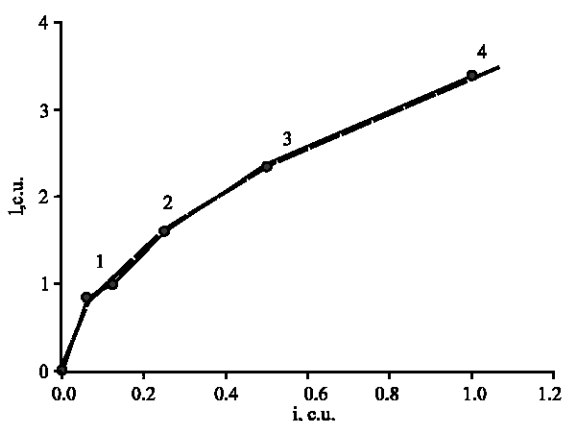


Fig. 5: Dependence of a change in the length of vectors for calf alkaline phosphatase inhibition upon increasing concentration of WO_4^{2-} anions

Example of instability in the dynamics of enzyme inhibition: Effect of WO_4^{2-} 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 ($K'_m > K_m^0$, $V' < V^0$, $i > 0$), if the concentration of these anions rises (Fig. 4, Table 2). It is an example of the biparametrically coordinated (I_i) type of enzyme inhibition^[18-20].

Analysis of the obtained data (Fig. 4 and 5) 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 2, the values of 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 three times in the interval 0-1. 10^{-4} M (Fig. 5 and Table 2, the segment of a respective curve between points: 0-1, 1-2 and 2-4). 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 (Fig. 4 and Table 2).

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