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KINETIC EVIDENCE FOR THE ALLOSTERIC SUBSTRATE INHIBITION OF HUMAN ERYTHROCYTE ACETYLCHOLINESTERASE

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Summary

Kinetic experiments described in this study were carried out with an electrophoretically and immunologically homogeneous acetylcholinesterase (acetylcholine hydrolase, EC 3.1.1.7) preparation isolated from human erythrocyte membranes. At low concentration of substrates, the acetylcholinesterase-catalyzed reaction follows Michaelis-Menten kinetics. In comparative studies using acetylthiocholine and acetylcholine as substrates, the corresponding K_m values were 150 ± 30 μM and 200 ± 20 μM, respectively. At low concentrations of both substrates, Hill plots indicated the existence of either a single or multiple independent active site(s). The inhibition mechanism of acetylcholinesterase by high concentration of substrates were studied by utilizing a new kinetic parameter, Δ, which allows discrimination between the competitive and uncompetitive types of substrate inhibitions (Wang, C.-S. (1977) Eur. J. Biochem. 78, 568—574). This kinetic approach provided evidence that the inhibition of acetylcholinesterase by excess substrate was effected by its interaction with multiple allosteric sites on the enzyme.

Introduction

Several authors have suggested that the substrate inhibition of acetylcholinesterase (acetylcholine hydrolase, EC 3.1.1.7) may be due to conformational changes in the enzyme rather than to formation of reversible, inactive enzyme-substrate complexes [1—5]. To examine the mechanism of substrate inhibition, we have carried out a kinetic analysis of human erythrocyte acetyl-

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cholinesterase by utilizing a new kinetic parameter, Δ, which allows discrimination between the competitive, non-competitive or uncompetitive types of substrate inhibitions [6]. These experiments were performed with an electrophoretically and immunologically homogeneous acetylcholinesterase preparation isolated by affinity chromatography from human erythrocyte membranes [7].

Results of this study indicate that inhibition of human erythrocyte acetylcholinesterase by excess substrate is mediated by an allosteric mechanism.

Materials and Methods

Enzyme preparation. Purified acetylcholinesterase preparations were isolated from the ghosts [8] of freshly drawn human blood erythrocytes according to a previously described procedure [7]. The protein content of acetylcholinesterase preparations was determined by the method of Wang end Smith [9] using bovine serum albumin as standard. The enzyme had a specific activity of 420 units/mg protein.

Enzyme assays. Acetylcholinesterase activity was measured with both acetylcholine and acetylthiocholine as substrates. Enzyme activity was expressed as μmol of substrate hydrolyzed/min.

The spectrophotometric assay with acetylthiocholine was carried out by the method of Ellman et al. [10]. The potentiometric assay with acetylcholine as a substrate was based on the method of Michel [11].

The linearity of all plots was checked graphically and the data were analyzed in a Compucorp 344 Micro Statistician computer (Computer Design Corp., Los Angeles, CA). The data were analyzed by least-squares linear regression and Pearson's correlation.

Results and Discussion

Kinetic characteristics of acetylcholinesterase

Acetylcholinesterase exhibited optimal activity at pH 8.0 (0.1 M phosphate buffer, 37°C) in the presence of either acetylthiocholine or acetylcholine. This value is in good agreement with those found by others [12—15]. The K_m value for acetylthiocholine was 150 ± 30 μM and the K_m value for acetylcholine was 200 ± 20 μM. These values are also in good agreement with those reported by other investigators [16—18]. Sihotang [12] reported a higher K_m value of 380 μM for acetylcholinesterase using acetylthiocholine as substrate. This discrepancy in results may be due to different concentrations of sodium deoxycholate used as the solubilizing agent. In fact, Sihotang [19] demonstrated that the phospholipid content of acetylcholinesterase depends on the concentration of sodium deoxycholate used for the isolation of the enzyme and that optimal activity is associated with a particular phospholipid content.

The Hill plots in Fig. 1A (acetylthiocholine) and Fig. 1B (acetylcholine) show slopes of 0.991 and 1.022, respectively, indicating essentially no interaction between active sites at low substrate concentrations.

Mechanism of substrate inhibition

At low substrate concentrations, the enzyme reaction follows Michaelis-

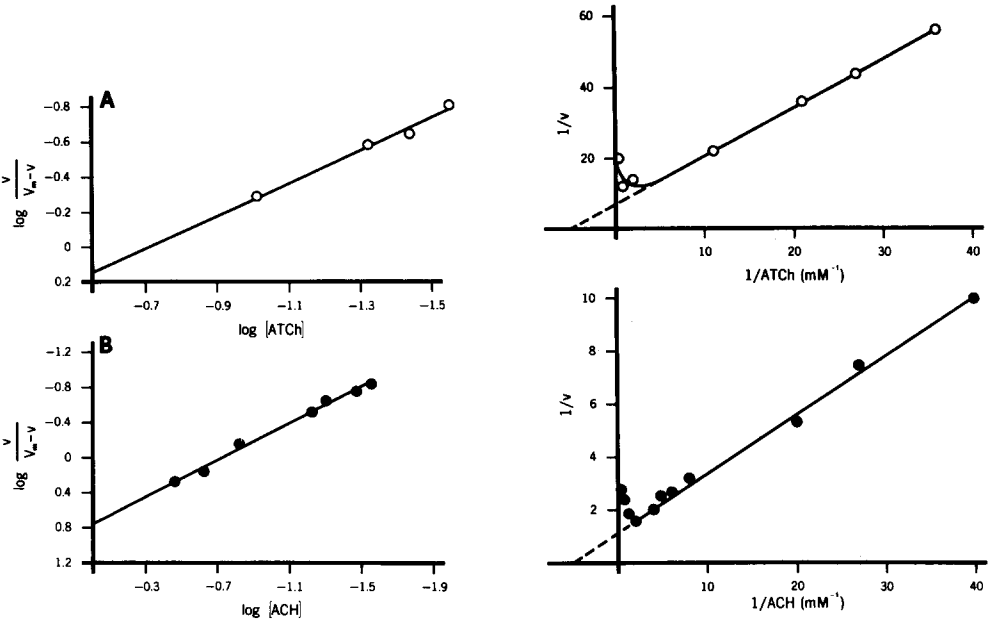

Fig. 1. Hill plots of acetylcholinesterase activity at low substrate concentrations. (A) Substrate, acetylthiocholine [ATCh]; slope, $n = 0.991$; enzyme concentration, 0.25 μg/test; buffer system: 0.1 M phosphate, pH 8.0, 37°C. (B) Substrate, acetylcholine; slope, $n = 1.036$; enzyme concentration, 0.26 μg/test; buffer system: 0.15 M NaCl, 1.3 mM $MgCl_2$, 1.0 mM sodium phosphate, pH 7.9, 25°C.

Fig. 2. Lineweaver-Burk plots of acetylcholinesterase activity with acetylthiocholine (ATCh), and acetylcholine (ACh) as substrates.

Menten kinetics as shown by the linear Lineweaver-Burk plot. However, the bell-shaped curve of a typical plot of rate versus logarithm of the substrate concentration demonstrates that acetylcholinesterase exhibits the phenomenon of substrate inhibition. As shown in Fig. 2, the Lineweaver-Burk plot of the data displays a non-linear curve over the substrate concentration range beyond initial inhibition. In order to examine the mechanism of substrate inhibition, a new kinetic parameter, Δ, was used to allow discrimination between the competitive, non-competitive and uncompetitive types of substrate inhibition [6]. The parameter Δ is defined as the difference between the reciprocals of the initial reaction rate obtained from the experimental measurements and the rate estimated by extrapolating the linear part of Lineweaver-Burk plots. The relationship of Δ and substrate concentrations for competitive, non-competitive and uncompetitive substrate inhibition can be shown in Eqns. 1—3.

$$\Delta = \frac{1}{V}\left(\frac{K_m}{K_I}\right) \tag{1}$$

$$\Delta = \frac{1}{V}\left(1 + \frac{K_m}{[S]}\right)\left(\frac{[S]}{K_I}\right) \tag{2}$$

$$\Delta = \frac{1}{V}\frac{[S]}{K_I} \tag{3}$$

where K_m is the Michaelis-Menten constant, V is the maximal reaction rate, and

K_I represent the corresponding dissociation constants for the inactive enzyme-substrate complexes. The substrate may bind to the active site in an improper orientation and result in a competitive substrate inhibition. However, such an inhibition would nevertheless give rise to a linear Lineweaver-Burk plot in which the apparent V and K_m are displaced from their true values by an undeterminable amount (Eqn. 1). Therefore, the non-linear Lineweaver-Burk plot (Fig. 2) is not due to competitive substrate inhibition. Eqns. 2 and 3 describe linear relations between Δ and [S]. However, since plots of Δ versus [S] for both acetylthiocholine and acetylcholine (Fig. 3) were non-linear, the inhibition could not conform to the non-competitive and uncompetitive models of Eqns. 2 and 3. Therefore, the following possible explanations for substrate inhibition of acetylcholinesterase were considered: (1) if the substrate can combine with the enzyme to form a reversible, inactivated enzyme-substrate complex, the non-linear plot of the Δ versus [S] would be due to the presence of multiple substrate inhibition sites on the enzyme, and (2) the substrate may bind to allosteric site(s) causing a partial inhibition brought about by conformational change of the enzyme. In the first case, the enzyme reaction can be described using Michaelis-Menten kinetics and the two parameters K_m and V can be defined. It can be shown that Δ relates to [S] in the following manner:

$$\Delta = \sum_{i=0}^{n} a_i S^i \tag{4}$$

where $a_i \geqslant 0$ and are constants, and n is the number of substrate inhibition sites per molecule. This equation depicts a non-linear concave upward curve with the absence of an inflection point. Since the curves of Δ versus [S] plots of Fig. 3 contain concave upward and convave downward regions with the presence of inflection points, the possible occurrence of multiple substrate inhibition sites on the enzyme can be excluded as the explanation for substrate inhibition of acetylcholinesterase. It seem, therefore, that allosterism is the most likely mechanism for the inhibition. In such a case, the curves of Δ versus [S] plots can be concave upward or downard, and the inflection points can be present or absent (Fig. 3). These various possibilities will depend on the mode of interaction between enzyme and substrate and the resulting changes in the conformation and catalytic rate of the enzyme. Thus, when substrate molecules are in excess, they are apparently reacting with some site(s) sensitive to substrate, other than the catalytic sites. This interpretation compares favorably with those made by other investigators. Brestkin et al. [3] suggested that the inhibition of bovine erythrocyte acetylcholinesterase by high substrate concentration was due to conformational changes induced by the presence of excess substrate. By using eserine to block the active site, Changeux et al. [4] demonstrated that acetylcholine also binds to non-catalytic sites on the enzyme isolated from electric tissue of *Electrophorus electricus*, and interpreted this finding as evidence for the presence of catalytic and regulatory sites on the surface of acetylcholinesterase. Kato et al. [5] also suggested that the inhibition of acetylcholinesterase from siquid ganglia by excess substrate may be mediated by an allosteric mechanism. Rosenberry found no evidence for sub-

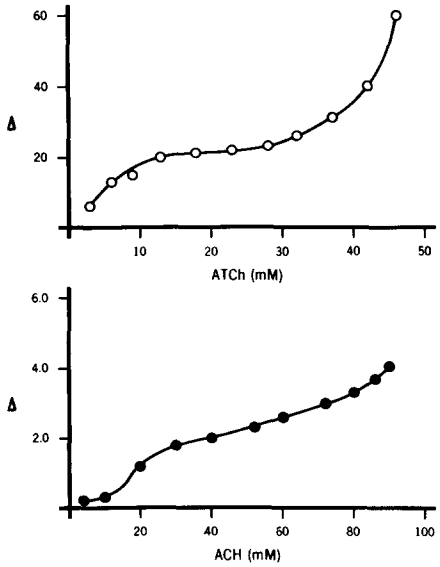

Fig. 3. Plots of the inhibition of acetylcholinesterase activity by excess concentrations of substrates. The parameter Δ is defined as the difference between the reciprocals of initial reaction rates of the observed and extrapolated linear plots of enzyme activities in the region of substrate inhibition. The values for Δ are taken from the respective Lineweaver-Burk plots (Fig. 2). ATCh, acetylthiocholine; ACh, acetylcholine.

unit cooperativity in acetylcholinesterase but suggested that ligand binding at peripheral sites may induce allosteric effects [20]. The present study based on the use of a simple kinetic parameter, Δ, provides additional evidence for the occurrence of allosteric binding site(s) on the acetylcholinesterase molecule. Inspection of the inflection points of the non-linear curves in Fig. 3 might lead one to suggest the existence of multiple regulatory sites. It seems that if only one regulatory site were present on the enzyme molecule, the data would depict a hyperbolic curve representative of saturation at high concentrations of substrate. Therefore, we suggest that the substrate inhibition of human erythrocyte acetylcholinesterase is due to the presence of multiple allosteric sites on the enzyme.

Although the allosteric theory as proposed by Monod et al. [21] is useful for explaining experimental data in studies on enzymes with sigmoidal relationship between initial rate and [S], the model is not concerned with the phenomenon of substrate inhibition. Evidence presented in this and previous studies indicates that the kinetic parameter, Δ, represents a new and simple means for studying the mechanism of substrate inhibition. We have already utilized this parameter for demonstrating the competitive substrate inhibition effect of pyruvate on the lactic acid dehydrogenase [6] and that of glyceraldehyde 3-phosphate on the glyceraldehyde-3-phosphate dehydrogenase-catalyzed reactions. [22]. In the present study, the application of this kinetic parameter showed that the substrate inhibition of acetylcholinesterase is mediated by an allosteric mechanism.

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