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Steady-State Kinetics of Rat Intestinal Butyrylcholinesterase

[Sıçan İnce Barsak Butirilkolinesterazının Yatışkın Durum Kinetikleri]

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ABSTRACT

Objectives: Butyrylcholinesterase is a major detoxification enzyme found in many tissues and body fluids of different organisms. Here, we investigated the steady state kinetics and the effects of heat on soluble butyrylcholinesterase purified from rat small intestine.

Methods: Rat intestinal butyrylcholinesterase kinetics were studied under steady state conditions by the method of Ellman using butyrylthiocholine as substrate.

Results: The calculated Vm (4.249±0.337 µmole/min/mg protein) for butyrylthiocholine was used to obtain the Hill plot. Hill plot displayed a biphasic character. At low substrate concentrations (0.05–0.5 mM), substrate activation (n_H=2.2) was observed whereas at high substrate concentrations (0.5–2.0 mM) substrate inhibition (n_H=0.375) was detected. Optimum temperature, energy of activation and the temperature coefficient were calculated as 37°C, 4915 cal/mol and 1.30 respectively from the Arrhenius plot. Enthalpy of activation was found to be 3432 cal/mol from enthalpy graph.

Conclusion: The hydrolysis of butyrylthiocholine iodide did not follow hyperbolic Michaelis-Menten kinetics. The temperature studies and their calculated constants also support the temperature dependency of rate constants and the allosteric property of butyrylcholinesterase.

Key Words: Butyrylcholinesterase, Hill coefficient, energy of activation, temperature coefficient, enthalpy of activation, rat intestine

ÖZET

Amaç: Butirilkolinesteraz farklı organizmaların birçok dokusu ve vücut sıvısında bulunan bir detoksifikasyon enzimidir. Bu çalışmamızda, sıçan ince barsağından saflaştırdığımız butirilkolinesterazın çözünür formuyla yatışkın durum kinetiğini ve ısı ile moleküler seviyedeki etkileşimlerini araştırdık.

Yöntem: Saf sıçan ince barsak butirilkolinesterazı yatışkın durum koşullarında Ellman yöntemi ile substrat olarak butiriltiyokolin kullanılarak incelendi.

Bulgular: Butiriltiyokolin iyodür için hesaplanan Vm değeri (4.249±0.337 µmol/dk/mg protein) Hill grafiklemesi çiziminde kullanılmıştır. Hill grafiği bifazik bir karakter gösterir. Düşük substrat derişimlerinde (0.05-0.5 mM) substrat aktivasyonu (n_H=2.2) gözlenirken yüksek substrat derişimlerinde (0.5-2.0 mM) substrat inhibisyonu (n_H= 0.375) saptanmıştır. Optimum sıcaklık, aktivasyon enerjisi ve sıcaklık katsayısı 37°C, 4915 cal/mol ve 1.3 olarak Arrhenius grafiğinden saptanmıştır. Aktivasyon entalpi değeri ise entalpi grafiğinden 3432 cal/mol olarak bulunmuştur.

Sonuç: Sıçan butirilkolinesterazı substratı olan butiriltiyokolin iyodür ile hiperbolik Michaelis-Menten kinetiğine uyum göstermemiştir. Sıcaklık ile ilişkili parametreler de enzimin allosterik özellikte olduğunu desteklemektedir.

Anahtar Kelimeler: Butirilkolinesteraz, Hill katsayısı, aktivasyon enerjisi, sıcaklık katsayısı, aktivasyon entalpisi, sıçan barsağı

Introduction

Butyrylcholinesterase (BChE; acylcholine acylhydrolase, E.C. 3.1.1.8) has important function in toxicology and pharmacology (1). It is found in small intestine as well as other body tissues and fluids of vertebrates (1-4). As a detoxifying enzyme, BChE bioscavenges many ester-containing drugs and toxic compounds such as succinyldicholine (5); organophosphate and carbamate esters (6); cocaine (7); aspirin (8); amitriptyline (9), benactyzine and drofenine (10); cisplatin, vinblastin and metothrexate (11); indole acetic acid (12) etc, and intestinal BChE constitutes the first line defense against orally introduced carboxylic or phosphoric acid esters.

BChE presents soluble or membrane bound monomeric, dimeric, tetrameric globular forms or asymmetric forms (the form contains one to three tetramers of subunits attached to membrane via collagen-like tail) in tissues and body fluids (1,4). By using sucrose gradient centrifugation, it is shown that rat intestine mucosal cells contain amphiphilic monomeric, dimeric or tetrameric globular forms (13). In our previous study, we purified and characterized the soluble isoform of BChE from rat intestine and found that the enzyme was composed of tetrameric globular form by non-reducing electrophoresis; the enzyme did not follow hyperbolic Michaelis-Menten kinetics with the positively charged substrates (acetyl-, propionyl-, butyrylthiocholine) like the human serum BChE; the pH optimum was determined as 7.2 after zero buffer extrapolation when butyrylthiocholine was used as substrate; the optimum temperature was examined as 37°C after zero time extrapolation using butyrylthiocholine as substrate (14). In this study, steady-state kinetics of rat intestinal butyrylcholinesterase is investigated in detail with butyrylthiocholine as substrate by using and formulating the data in a new theoretical approach which complements the previous study (14).

Materials And Methods

Chemicals

3-(N-Morpholino)propanesulfonic acid (MOPS), 5-5'-dithiobis(2-nitrobenzoic acid) (DTNB), ethopropazine were purchased from Sigma (USA). S-n-butyrylthiocholine iodide (BTCh) was from BDH (UK). All other chemicals used were of the best analytical grade. DTNB was prepared as 2.5 mM in 50 mM MOPS buffer, at pH 7.4.

BChE Purification

In this study, small intestines were obtained from female Wistar rats killed for students' laboratory coursework at Hacettepe University Medical School. Rat intestinal BChE was purified from the pooled small intestine of six female rats as reported previously (14) by Sephadex G-25 chromatography and procainamide affinity chromatography.

Enzyme Assay

Butyrylcholinesterase activity was measured at 37°C on Shimadzu UV-1601 spectrophotometer, by the method of Ellman, et al. (15) following the hydrolysis of thioesters of BTCh. Initial rates were measured in the activity mixture containing final concentrations of 100 mM MOPS buffer pH 7.4, 0.25 mM DTNB, 0-50 mM BTCh and 3.65 µg/ml pure BChE. Temperature studies were done in the range of 20-60°C. All rates were corrected for spontaneous degradation of substrates and reagents. All assays were carried out triplicate and activities were measured for up to 60 sec. One unit of BChE is expressed as the amount of enzyme, which catalyzes the formation of 1 µmole product per minute under the condition mentioned above.

Protein determination

Protein concentrations were determined by the micro method of Bradford (16) using BSA as standard.

Analysis of the kinetic data

The kinetic data were analyzed and the kinetic constants were calculated by means of the non-linear curve fitting module of Systat (version 9.0) software.

Results

BTCh Kinetics

The hydrolysis of BTCh by rat intestinal BChE did not follow the hyperbolic Michaelis-Menten kinetic (14) as observed in the other BChEs obtained from different sources (17-19). So, kinetic parameters such as Km, b, kcat etc were calculated from the equation for excess substrate activation/inhibition used from Boeck et al. (18) by using Systat (version 9.0) software and mentioned in our previous study (14). Vm was determined as 4.249±0.385 µmole/min/mg protein for BTCh by fitting the data to Michaelis- Menten equation.

Characterization of Substrate Binding

The calculated Vm was used to obtain nH (Hill coefficient) from the Hill plot (Figure 1). Hill plot showed biphasic character. At low substrate concentrations (0.05–0.5 mM BTCh), substrate activation (nH = 2.2) was observed, whereas at high substrate concentrations (0.5–2.0 mM BTCh), substrate inhibition (nH = 0.375) was seen (20).

Energy of Activation

Optimum temperature was examined as 37°C, after zero time extrapolation (13). Using the v values obtained for different temperatures, energy of activation (Ea) were examined to be 4915 cal/mole from the

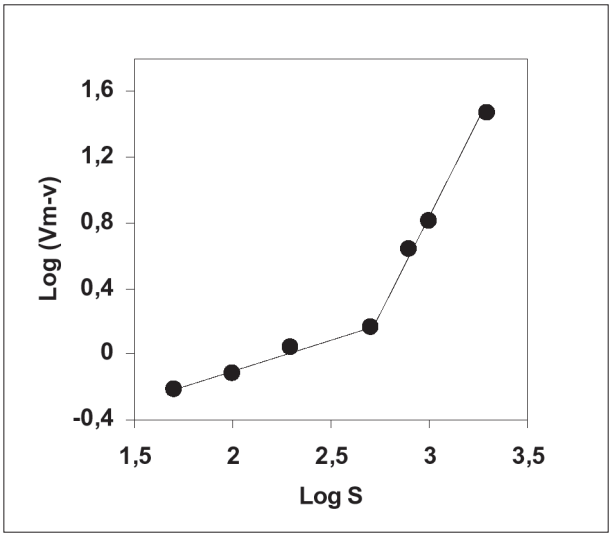

Figure 1. Hill plot of pure rat intestinal BChE with BTCh. Initial velocities were determined in the activity mixture containing 100 mM MOPS buffer, pH 7.4, 0.250 mM DTNB, 0–50 mM BTCh, and 3.65 µg/ml pure enzyme.

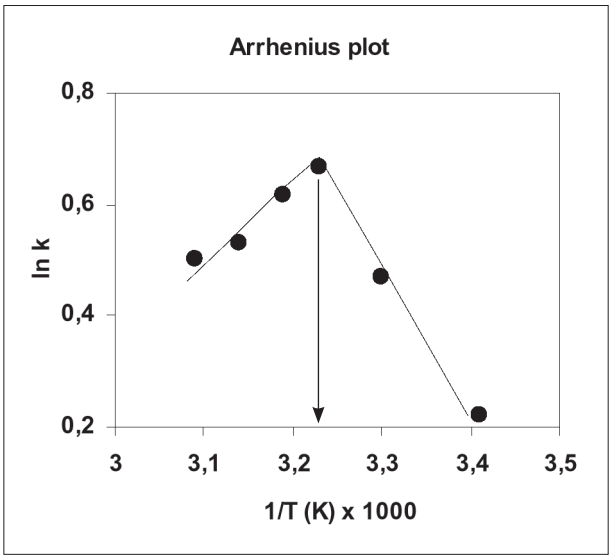

Figure 2. The graph of 1/ T versus ln k, the rate constant for different temperatures in which the slope is equal to – Ea / R. Sudden drop in the plot indicates enzyme inactivation. Arrow indicates that optimum temperature of the enzyme is 37°C (310 K). Initial velocities were determined in the activity mixture containing 100 mM MOPS buffer, pH 7.4, 0.250 mM DTNB, 4 mM BTCh, and 3.65 µg/ml pure enzyme.

slope of Arrhenius plot (Figure 2) graphed according to the linear form of Arrhenius equation (20):
Log k = -Ea / (2.3 R) (1/ T) + log A
Optimum temperature value is also strengthened as to be 37°C from Arrhenius plot.

Calculation of Temperature Coefficient

Temperature coefficient is the factor by which the rate constant is increased by raising the temperature from 303 K to 313 K (Q10) was calculated to use following equation (20):

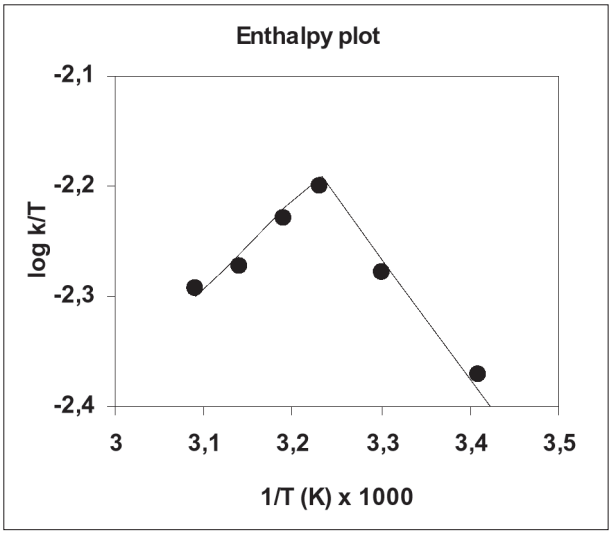

Figure 3. ΔH was determined by measuring the rate constant (k) at several different temperature and plotting log k / T versus 1/T. The slope = ΔH / 2.3 R. Initial velocities were determined in the activity mixture containing 100 mM MOPS buffer, pH 7.4, 0.250 mM DTNB, 4 mM BTCh, and 3.65 µg/ml pure enzyme.

Log Q10 = 10 * Ea / 2.303 * R* T * T2
Q10 was found to be 1.3. It means that the rate constant is increased by raising the temperature 10°C by the factor of 1.3.

Enthalpy of Activation

Enthalpy of activation (ΔH) was examined from the plot of following equation (19):
log k/T =(ΔH / 2.3 R) (1/ T) + log k/ h + ΔS / 2.3 R
When log k / T versus 1 / T are plotted, the slope is equal to ΔH / 2.3 R (3). ΔH was determined as 3432 cal /mole from the slope of enthalpy graph (Fig 3).

Discussion

The soluble isoform of rat intestinal BChE has four active centers because of the tetrameric globular structure similar to human serum BChE. At low BTCh concentrations (0.05–0.5 mM) BChE displays substrate activation, whereas at high BTCh concentrations (0.5–2.0 mM) substrate inhibition were observed. Binding of the first substrate molecule to first active centre on the enzyme molecule decreases the affinities of the other active sites to substrate molecules (negative cooperativity). Energy of activation (Ea), temperature coefficient (Q_{10}) and enthalpy of activation (ΔH) were calculated to be 4915 cal / mole, 1.3 and 3432 cal /mole, respectively.

BChE is a thermally stable enzyme, but in our previous studies with human serum BChE it was shown that heating the enzyme causes it to change its kinetic behavior towards BTCh. The enzyme protects its capacity up to 53°C (21) but when heat combined with increasing incubation period is prolonged BChE undergoes a conformational change. Thus human BChE heated at 45°C up

to 24 hrs changes conformation and displays Michaelis-Menten kinetics (22).

In this study with rat intestinal BChE, optimum temperature was found to be 37°C. As shown in Figure 3, a sudden drop in Arrhenius plot is observed. This type of behavior indicates enzyme inactivation. The change in slope also reflects a "transition temperature" where the dependency of Vm changes from one rate limiting step to another (20)

Rat BChE as well as human serum BChE displays non-Michaelan kinetics. At high substrate concentrations (BTCh > 0.5 mM) human and rat BChE displays substrate inhibition. Here in this study the optimum temperature assays were performed at 4 mM BTCh. Thus we can propose that the abundance of substrate BTCh at high temperature can trigger a conformational change. The observed enzymatic mechanism of BChE may be the result of a series of binding steps which occur within the active site. It can be said that this sudden drop in Arrhenius plot is indicative of its negative cooperativity at high substrate concentrations. At high temperatures Arrhenius plots of specificity constants gives a distinct maximum which is indicative of substrate dissociation and non-linearity in Arrhenius plots are indicative of the temperature dependence of Michaelis-Menten parameters (23,24). The activation energy calculated from our study is 4915 cal/mole which falls within the general range for enzyme catalysis (20). Although BChE is not defined as a classic allosteric enzyme, its abundance at the end of motor neurons and serum where it has important impact would make sense of its being under allosteric control.

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