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Conference Paper

The Reaction Mechanism of Butyrylcholinesterase

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The kinetics of butyrylcholinesterase, present in human and horse plasma, cannot be described by the Michaelis-Menten equation, demonstrated with enzyme preparations of various degrees of purity. This deviation from a simple kinetic model is best described by a mathematical model for the rate equation of a single enzyme with two catalytic sites. The structure of the Hill plots is an additional indication that the substrate interactions are of the homotropic co-operative type.

The effect of certain specific cholinesterase inhibitors resulted in linear Dixon plots with butyrylthiocholine as substrate and a more complex effect with thiophenyl acetate or butyrate. Inhibitors as choline might form dead-end complexes with free and monoacylated enzyme.

A mechanistic model with butyrylthiocholine as substrate is proposed and can be extended to fit also with thiophenyl esters. This model involves a co-operative pair of catalytic sites, but no allosteric substrate binding site, and includes a mono- and diacylated enzyme intermediate.

The hydrolysis of acetylcholine or butyrylcholine by butyrylcholinesterase, present in human and horse blood plasma, cannot be described by the Michaelis-Menten equation except for a limited range of substrate concentrations, as that used for instance in the Warburg technique. This has been demonstrated clearly when new substrates and techniques were introduced for measuring esterase activity. The new results obtained regarding the steady-state kinetics have been valuable in understanding the reaction mechanism and have thrown light on our knowledge of the structure of this enzyme.

EXPERIMENTAL

Enzyme, substrates and inhibitors

Preparations of butyrylcholinesterase from horse serum of various degrees of purity were dissolved in 50 mM Tris-HCl buffer, pH = 7.4. They were all free from other esterase activities.

A partly purified enzyme preparation was purchased from Nutritional Biochemicals Corporation, Cleveland, Ohio, USA. This preparation was subjected to further purification by three steps using (1) DEAE A-25 Sephadex, (2) Sephadex G-200, and (3) Hydroxylapatite. The results of the first two steps are presented in Figs. 3 and 4.

The choline and thiocholine esters, in the form of iodide salts and choline chloride were obtained from Sigma Chemical Co., St. Louis, Mo., USA. Thiophenyl acetate and butyrate were purchased from Polysciences Inc., Washington. »Astra 1397« [10-(α-diethylaminopropionyl)-phenothiazine HCl] was a gift from prof. Richard Dahlbom (formerly at AB Astra, Södertälje, Sweden).

Assay of esterase activity

Esterase activity was measured by a spectrophotometric method described in detail elsewhere[1,2]. It is based on the use of thiol esters as substrates and the reaction of the thiol derivative (*e. g.*, thiocholine) formed with 5,5′-dithiobis-(2-nitrobenzoate) to give 5-thio-2-nitrobenzoate, which has an extinction maximum at 405—420 nm.

Esterase activity was expressed in nanokatals, *i. e.* nanomoles of substrate hydrolysed per second.

RESULTS AND DISCUSSION

Single-substrate

Previous reports have demonstrated[2] that butyrylcholinesterase does not obey the Michaelis-Menten equation when butyrylthiocholine or a thiophenyl ester is used as the substrate. Fig. 1 illustrates the results obtained with thio-

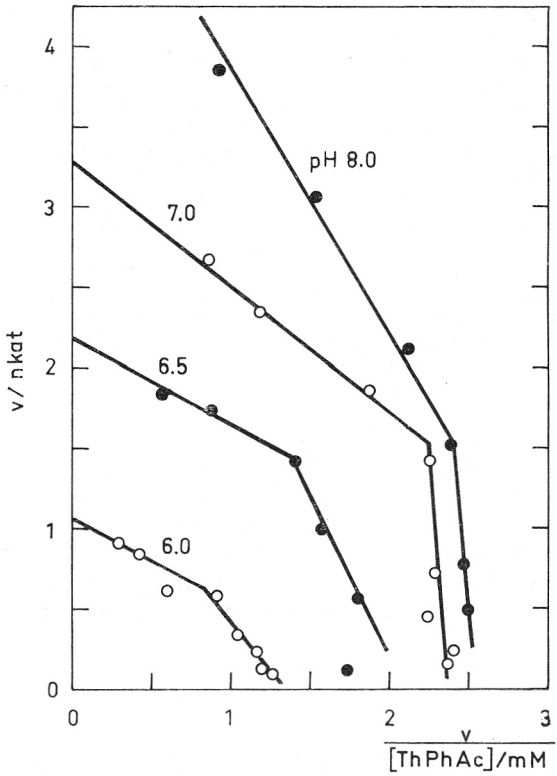


Fig. 1. Relationship between the initial hydrolysis rate of thiophenyl acetate and substrate concentration illustrated by an EAH (Eadie-Augustinsson-Hofstee) plot. Results obtained with a purified preparation of butyrylcholinesterase at various pH values (Tris-buffer).

phenyl acetate. The best mathematical model for the description of the data has been selected out of several models for one single enzyme and for two enzymes acting on the substrate by non-linear regression analysis[2]. Among the models tested statistically, the following model (1) offers the best description of the kinetics of the

$$v = \frac{V_1 [A] + V_2 [A]^2}{1 + K_1 [A] + K_2 [A]^2} \quad (1)$$

hydrolysis of butyrylthiocholine and corresponds to the equation for one single enzyme with two catalytic sites. It should be noted that the substrate concentrations used covered a 40 000-fold range in our experiments with butyrylthiocholine. This substrate in concentrations > 30 mM, and thiophenyl butyrate in concentrations > 2—3 mM, showed substrate inhibition, while the corresponding acetates did not show this effect[3,4].

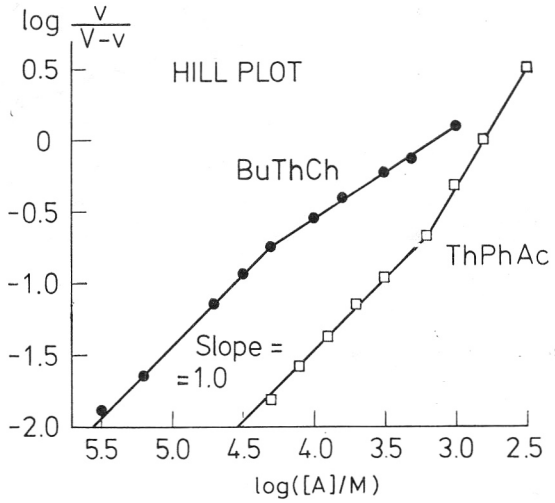


Fig. 2. Hill plot for butyrylcholinesterase with butyrylthiocholine (BuThCh) and thiophenyl acetate (ThPhAc) as substrates.

The model (1) is also supported by the structure of the Hill plot (Fig. 2) which suggests that the rate equation is an $n/n - 1$ function, probably a 2/1 function. This was recently demonstrated with both butyrylthiocholine and thiophenyl acetate as substrates[4]. We regard these results as an additional indication that the substrate interactions with butyrylthiocholine are of the negative homotropic co-operative type as the slope of the second line segment in the Hill plot was significantly less than unity. A similar result was recently published with acetylthiocholine[8].

The same kinetic model was found to be valid for enzyme preparations of various purity. The results of the purification procedures are illustrated in Figs. 3 and 4. Although the activity by polyacrylamide electrophoresis was divided into several components even in our purest preparation the kinetics observed were the same with all preparations. We therefore conclude that the electrophoretic pattern as well as the kinetics of butyrylcholinesterase were not significantly changed during the purification procedures. In addition, these multiple forms might function in kinetic analysis as a single enzyme unit with multiple catalytic sites.

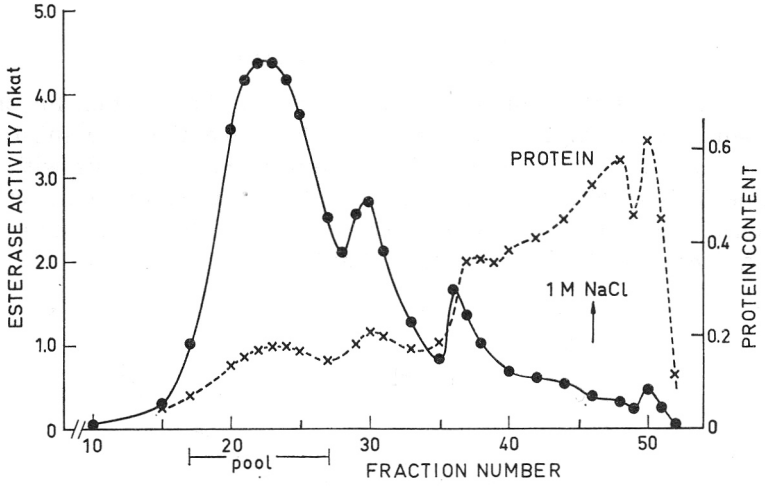


Fig. 3. Elution of butyrylcholinesterase from a DEAE-Sephadex A-25 column (2 cm × 30 cm) by a linear NaCl gradient. (0—0.2 M) in a 50 mM Tris-HCl buffer, pH = 7.4, containing 1 mM $MgCl_2$. Activity (●) and protein (x) profiles of 12 ml fractions.

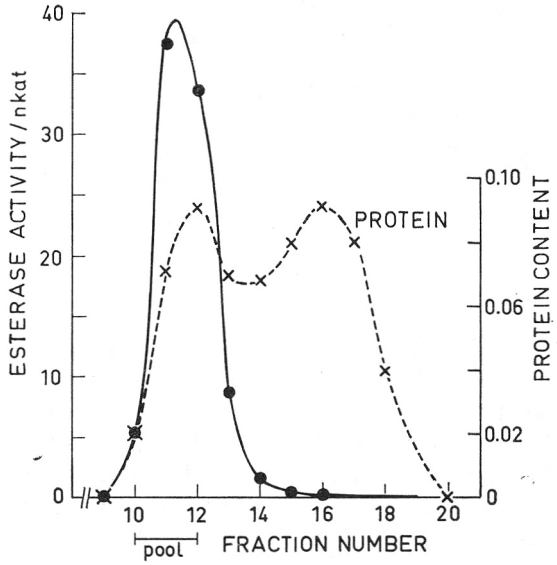


Fig. 4. Elution of butyrylcholinesterase from a Sephadex G-200 column (1 cm × 90 cm) by the same buffer as in Fig. 3 without NaCl. Activity (●) and protein (×) profiles of 4 ml fractions.

Alternative-substrates

Two types of experiments were carried out with two substrates simultaneously present[2]. In one of these, two thiol esters were used and the *observed* velocity was consequently due to the hydrolysis of both substrates. The results of one of these experiments are illustrated in Fig. 5. In the other type of experiments an oxygen ester and a thiol ester were simultaneously used, *e. g.*, butyrylthiocholine of varying concentrations and phenyl acetate of fixed concentration (Fig. 6). In such experiments, the spectrophotometric assay used

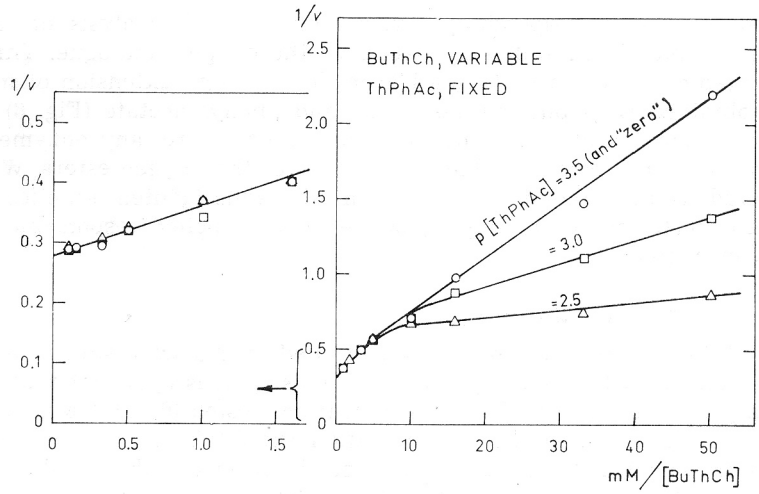


Fig. 5. Effect of simultaneous presence of butyrylthiocholine (BuThCh) and thiophenyl acetate (ThPhAc) on the initial hydrolysis rate illustrated by a Lineweaver-Burk plot. BuThCh concentration was varied and ThPhAc was used at fixed concentrations (expressed in — log molar concentration in the graph).

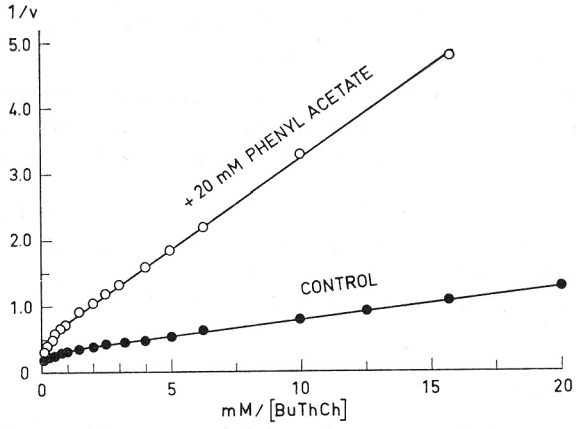


Fig. 6. Effect of varying the butyrylthiocholine (BuThCh) concentrations in the absence (●) and presence of a fixed concentration (20 mM) of phenyl acetate (○) illustrated by a Lineweaver-Burk plot.

records only the hydrolysis of the thiol esters. However, the oxygen ester will influence the kinetics by competition (inhibition) with the thiol ester substrate.

Experiments with two thiol esters (Fig. 5) showed that increasing the butyrylthiocholine concentration extinguishes the contribution of thiophenyl acetate hydrolysis to the observed activity. Excess of thiophenyl acetate, however, did not completely extinguish the hydrolysis of butyrylthiocholine. We conclude that only one enzyme species catalyses the hydrolysis of each substrate, and consequently one single enzyme was responsible for the activity recorded with both substrates. This conclusion was supported by the results obtained in the other type of experiments where one of the substrates was

exchanged for the corresponding oxygen ester. The hydrolysis of the thiol ester was completely inhibited by excess of the oxygen analogue. This result could be predicted from a mathematical model being an extension of model (1) and was obtained with butyrylthiocholine and phenyl acetate (Fig. 6) as well as with thiophenyl acetate and butyrylcholine. Therefore, any enzyme species active with the thiol esters is able to bind any of the oxygen esters. We therefore concluded[2] that the deviations from Michaelis-Menten kinetics can be interpreted in terms of a single enzyme species being responsible for all activities measured.

Specific competitive inhibitors

The inhibitory effect of choline was studied over a wide substrate concentration range. From the Dixon plots ($1/v$ *versus* [I] it is apparent that a linear relationship was obtained with all concentrations tested (0 — 0.1 M), suggesting that the choline terms in the rate equation should be of first order in the denominator. The same conclusions can be drawn from the results obtained with another cholinesterase inhibitor, Astra 1397[4].

The slopes of the lines in the Dixon plots could be expressed algebraically, being discussed in detail elsewhere[4]. The rate equation derived was an extension of model (1):

$$\frac{1}{v} = \frac{1 + K_1[\mathrm{A}] + K_2[\mathrm{A}]^2 + (K_3 + K_4[\mathrm{A}])[\mathrm{I}]}{V_1[\mathrm{A}] + V_2[\mathrm{A}]^2} \qquad (2)$$

The data obtained in the inhibition experiments confirm the conclusion from the alternative-substrates data that the deviation from Michaelis-Menten kinetics can be interpreted in terms of a single enzyme species rather than a mixture of two or more enzymes.

Choline can probably bind to both the free enzyme and the monoacylated enzyme[5,6], forming dead-end complexes. In addition, it is obvious from our mathematical models that only one choline molecule can bind to the enzyme molecule at a time, and that choline can prevent deacylation of the monoacylated enzyme.

Non-choline esters as substrates

The mechanistic model discussed below might be extended to fit also with non-choline esters, *e. g.*, thiophenyl esters. The results obtained with thiophenyl acetate were shown above in Fig. 1. The effect of choline on the hydrolysis of these esters differed from that observed with choline esters. Fig. 7 demonstrates that the inhibition of the hydrolysis of thiophenyl acetate is independent of the substrate concentration except at high concentrations of the ester. With thiophenyl butyrate of high concentration (5 mM) a small, but significant, activation was observed at an optimum concentration of 50 mM choline.

These effects of choline may be explained by an alternative catalytic pathway leading from an EAI complex to the FI complex, competing with an EAA complex in the case of thiophenyl butyrate (see further below and Fig. 8).

One of the reasons for the different results obtained with the non-choline esters in comparison with those obtained with the choline esters might be due to a less pronounced co-operative effect produced by the former substrates.

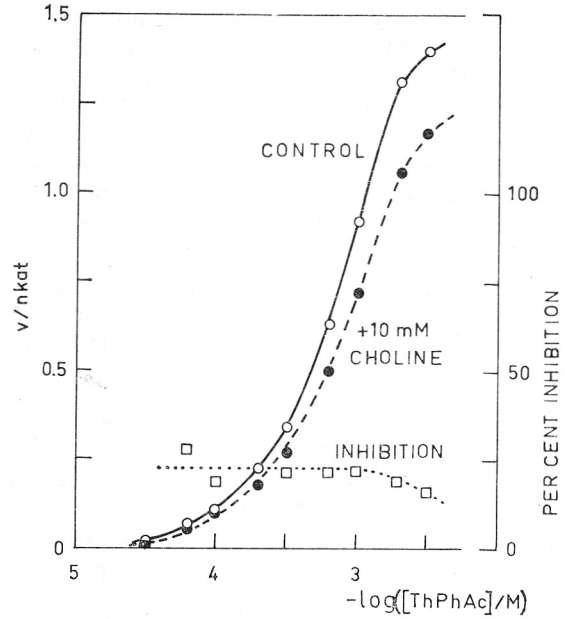
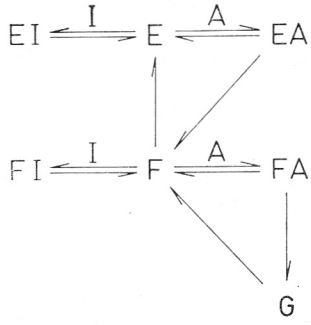


Fig. 7. Effect of choline (10 mM) on the hydrolysis of thiophenyl acetate as a function of — log molar substrate concentration.

A mechanistic model

From the experimental data obtained and from the mathematical models proposed, the following reaction mechanism (Fig. 8) was presumed[4].

The substrate (A) or inhibitor (I) molecule binds to one of two active sites of the enzyme and induces a conformational change at the other site



E: ENZYME A: SUBSTRATE
EA: ENZYME-SUBSTRATE COMPLEX
F: ACYLATED ENZYME INTERMEDIATE
FA: ACYLATED ENZYME-SUBSTRATE COMPLEX
G: DIACYLATED ENZYME INTERMEDIATE
EI: ENZYME-INHIBITOR COMPLEX
FI: ACYLATED ENZYME-INHIBITOR COMPLEX

Fig. 8. A mechanistic model of butyrylcholinesterase.

which prevents further binding of substrate or inhibitor. When the substrate is bound to the enzyme (EA) the latter is acylated (F) and the second site returns to the original conformation. The enzyme can now bind another substrate or inhibitor molecule at the non-acylated site, forming two new complexes (FA or FI). This induces a conformational change which prevents deacylation, but further acylation or dissociation is possible. The diacylated enzyme (G) can now deacylate. It is worth noting that choline probably does not react with the acyl intermediate to form an alternate product (as was proposed previously[6]).

This model differs from those suggested previously for acetylcholinesterase and butyrylcholinesterase[7] by including two co-operative catalytic sites and no allosteric substrate binding site. Another difference is that choline can prevent deacylation.

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REFERENCES

1. K.-B. Augustinsson and H. Eriksson, *Biochem. J.* **139** (1974) 123.
2. K.-B. Augustinsson, T. Bartfai, and B. Mannervik, *Biochem. J.* **141** (1974) 825.
3. A. R. Main, E. Tarkan, J. L. Aull, and W. G. Soucie, *J. Biol. Chem.* **247** (1972) 566.
4. H. Eriksson and K.-B. Augustinsson, to be published.
5. R. M. Krupka and K. J. Laidler, *J. Amer. Chem. Soc.* **83** (1961) 1445.
6. J. K. Balcom and W. M. Fitch, *J. Biol. Chem.* **245** (1970) 1637.
7. W. N. Aldridge and E. Reiner, *Enzyme Inhibitors as Substrates, Frontiers of Biology,* Vol. 26, North-Holland, Amsterdam 1972.
8. V. Simeon, *Croat. Chem. Acta* **46** (1974) 137.

DISCUSSION

W. N. Aldridge:

Have you any evidence that there are two sites per mol which may be phosphorylated by DFP and have you measured the form of the curves relating activity to the time of inhibition by several organophosphorus compounds?

K.-B. Augustinsson:

We have not tested our model with organophosphates as inhibitors. A study is planned with DFP, and, if possible, with its methyl analogue or related derivatives.

E. Reiner:

How many dissociation constants can you derive for your kinetic model? When you insert these constants into the theoretical equation, does the equation fit the experimental results?

K.-B. Augustinsson:

It is possible to derive all the dissociation constants for the enzyme-inhibitor complexes although we have not done it yet.

U. Brodbeck:

In your *Biochem. J.* paper you were mentioning that butyrylcholinesterase might be a half-of-the-site enzyme. Today you have not talked about this mechanism. Does this mean that this enzme does not act like a half-site enzyme?

K.-B. Augustinsson:

At least the fact that the binding of ligands to one of the sites prevents binding at the other might be interpreted in terms of half-of-the-sites reactivity. The complete mechanism might thus be considered also as a special case of half-of-the-sites reactivity.

U. Brodbeck:

The deviation from Michaelis-Menten kinetics of butyrylcholinesterase described by Dr. Augustinsson, parallels observation we have recently made on form G_p of the eel enzyme (B. Wermuth, R. Ott, R. Gentinetta and U. Brodbeck, *Cholinergic Mechanisms*, P. G. Waser, (Ed.), Raven Press, New York 1975, pp. 299—308). Moreover, Mr. Gentinetta recently carried out kinetic experiments with acetylcholine at low substrate concentration and found that the Hill coefficient for acetylcholine increased from 0.55 to 0.93 with increasing ionic strength. The inhibition by DFP as well as the rate of incorporation of ^{3}H-DFP into acetylcholinesterase were biphasic, the latter being slower than the former. At low DFP concentrations the light subunit of form G_p was phosphorylated whereas higher DFP concentrations and prolonged exposure to the OP-inhibitor increased the amount of label in the heavy subunit. These data suggest that the two subunits are labelled at different rates and that phosphorylation of one subunit decreases the activity of the other suggesting possible subunit interactions (U. Brodbeck and R. Gentinetta, *9th FEBS Meeting 1974, Abstr. Commun.* s2i9).

SAŽETAK

Reakcijski mehanizam butirilkolinesteraze

K.B. Augustinsson i H. Eriksson

Michaelis-Mentenovu zakonitost, koja vrijedi za enzimske preparacije različitih stupnjeva čistoće, nije moguće primijeniti na kinetiku butirilkolinesteraze nazočne u plazmi ljudi i konja. Takvo odstupanje od jednostavnoga kinetičkog modela najbolje se može opisati matematičkim modelom za brzinu reakcije jednog enzima s dva katalitička mjesta. Struktura Hillovih dijagrama također upućuje na to da su interakcije supstrata homotropno-kooperativnog tipa.

Učinak nekih specifičnih kolinesteraznih inhibitora rezultira linearnom zavisnošću u grafičkom prikazu po Dixonu, dok su učinci tiofenilacetata ili -butirata složeniji. Inhibitori poput kolina mogli bi dovesti do stvaranja nereaktivibilnih kompleksa sa slobodnim i monoaciliranim enzimom.

Predložen je model mehanizma za butiriltiokolin kao supstrat koji je moguće primijeniti također i na tiofenilne estere. Model uključuje dva kooperativna katalitička mjesta, a nijedno alosteričko mjesto vezivanja supstrata, te mono- i diacilirani enzimski međuprodukt.

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