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Inhibition of Cholinesterases by the Oximes P2AM and Toxogonin

V. Simeon, Z. Radić, and E. Reiner

Institute for Medical Research and Occupational Health, 158 M. Pijade, Zagreb, Croatia, Yugoslavia

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The reversible inhibition of electric eel acetylcholinesterase (EC 3.1.1.7) by P2AM (2-(hydroxyimino)methyl-1-methyl-pyridinium chloride) and Toxogonin (1,1'-[oxybis(methylene)] bis(4-(hydroxyimino)methyl-pyridinium) dichloride) was studied using acetylthiocholine as substrate. Two techniques were applied for measuring acetylthiocholine hydrolysis, the conventional spectrophotometric and the stopped-flow (at 25 °C in 100 mM phosphate buffer pH = 7.4).

The correlation between the degree of inhibition, and acetylthiocholine and oxime concentrations fits a theoretical model which postulates that the substrate and the inhibitor bind to two sites on the enzyme: the catalytic site and an allosteric, substrate-inhibition, site. The calculated dissociation constants for the two sites are: 0.13 and 0.76 mM for P2AM, and 0.16 and 2.0 mM for Toxogonin. The suggested model is an alternative to the hypothesis that two types of binding occur within the catalytic site.

Horse serum cholinesterase and bovine erythrocyte acetylcholinesterase are also inhibited by P2AM and Toxogonin to about the same degree as the electric eel enzyme.

Acetylthiocholine reacts with P2AM and Toxogonin; assuming that the reaction is bimolecular the corresponding rate constants are 13.4 and 22.4 M^{-1} min^{-1}.

INTRODUCTION

It is known that pyridinium oximes are inhibitors of acetylcholinesterase (EC 3.1.1.7) and cholinesterase (EC 3.1.1.8). In this paper the reversible inhibition of cholinesterases by P2AM and Toxogonin was studied in order to evaluate the binding sites on the enzyme. Previous studies on pyridinium oximes have suggested two types of binding[1–8] both occurring at the anionic sub-site, either in the free enzyme or in the acetylated enzyme. The present study confirms that there are two types of enzyme interaction with P2AM and Toxogonin, but the theoretical interpretation is consistent with an alternative mechanism[9] implying that the binding sites on the enzyme are the catalytic site and an allosteric, substrate-inhibition, site.

MATERIALS AND METHODS

Enzyme Preparations

The enzimes were electric ell and bovine erythrocyte acetylcholinesterase (Sigma Chem. Co., St. Louis, Mo., U.S.A. and Winthrop Ltd., N. Y., U.S.A.) and horse serum cholinesterase (Sigma Chem. Co., St. Louis, Mo., U.S.A.). Stock solutions

were prepared in phosphate buffer and kept at 4 °C. Dilutions in buffer were prepared immediately before use. During the assay the concentrations of the electric eel preparation were 0.02 μg/ml and 1. μg/ml and those of bovine erythrocyte and horse serum 4—8 μg/ml and 200—400 μg/ml for the conventional and stopped-flow techniques respectively.

Substrate and Inhibitors

The substrate was acetylthiocholine iodide (Fluka AG, Buchs SG, Switzerland). The inhibitors were P2AM (2-(hydroxyimino)methyl-1-methyl-pyridinium chloride) supplied by courtesy of Dr. Z. Binenfeld, Laboratory of Organic Chemistry, Faculty of Science, University of Zagreb) and Toxogonin (1,1'-[oxybis(methylene)]bis(4-(hydroxyimino)methyl-pyridinium)dichloride) (Merck AG, Darmstadt, Germany). Stock solutions of substrate and inhibitors were prepared in water.

Enzyme Inhibition

All experiments were done in 0.1 M phosphate buffer pH = 7.4 at 25 °C. The enzyme activities towards acetylthiocholine were measured by the spectrophotometric method of Ellman et al.[10] using two techniques: the conventional and the stopped-flow technique.

The conventional procedure was as follows: the enzyme (0.3 ml) was added to buffer (2.0 ml), inhibitor (0.3 ml) and 0.1 ml (10 mM) DTNB (5,5'-dithiobis-2-nitrobenzoate). Acetylthiocholine (0.3 ml) was added to the reaction mixture and the increase in absorbance was read at 412 nm (Unicam SP 500) in 1.0 cm cells against a blank containing buffer and DTNB. The time of assay was 0.25—2 min.

The equipment for the stopped-flow technique consisted of two syringes supplying the reaction solutions, a mixing chamber, and observation chamber, a source of monochromatic light (40 W halogen lamp and a light filter, $\lambda = 403$ nm), a differential amplifier, a DC power supply and an oscilloscope. The procedure was as follows: one syringe contained substrate dissolved in water and the other DTNB (0.8 mM), enzyme and inhibitor in buffer (0.2 M). Both syringes were pushed together and the mixed solutions entered into the observation chamber. The increase in absorbance was recorded on the storage oscilloscope over a period from 5 to 25 s after mixing.

Non-enzymic Substrate Hydrolysis

The non-enzymic hydrolysis of acetylthiocholine is a result of two independent reactions: spontaneous hydrolysis in buffer and hydrolysis catalysed by oximes. The rates of these reactions were measured following the concentration of thiocholine by the spectrophotometric method described above. Both the conventional and the stopped-flow techniques were used. The concentrations of hydrolysed acetylthiocholine were calculated using the molar absorption coefficient of the yellow anion 5-thio-2-nitrobenzoic acid ($\varepsilon_M = 13600\ M^{-1}\ cm^{-1}$) formed in the reaction of thiocholine and DTNB[10]. The initial acetylthiocholine concentrations varied from 0.055 to 10 mM.

Spontaneous hydrolysis was measured in solutions containing buffer, acetylthiocholine and DTNB. The reaction was followed over a period of 18 min — 12 hours.

The hydrolysis of acetylthiocholine in the presence of pyridinium oximes was measured in solution containing buffer, acetylthiocholine, DTNB and P2AM (0.1—10 mM) or Toxogonin (0.1—10 mM). The reaction was followed over a period of 5 s — 12 hours.

Equilibrium Dialysis

Binding of P2AM to the enzyme was measured by equilibrium dialysis at room temperature. The preparation of horse serum cholinesterase dissolved in 5.0 ml water (800 μg/ml) was in the dialysis bag immersed into 5.0 ml P2AM (2.0 mM). After 24 hours, the concentration of P2AM inside and outside the bag was determined by the method of Creasey and Green[11]. A sample (0.1 ml) was diluted with water 60 times: 0.2 ml NaOH (20%) was added to 3.0 ml of diluted sample, and the absorbance was read at 335 nm. The P2AM concentration was evaluated from a calibration curve obtained by the same method.

Evaluation of Constants

The Michaelis contant K_m, the substrate inhibition constant K_{ss} and the maximum velocity V for electric eel acetylcholinesterase were calculated from the equation:

$$v_o = \frac{V}{1 + K_m/s + s/K_{ss}} \quad (1)$$

where v_o is the enzyme activity and s is the substrate concentration.

All constants were obtained by computerized curve fitting minimizing the unweighted sum of squared deviations. Minimum search was done by using Univac Library Routine F04FAF based on algorithm described by Peckham[12]. Computations were done on a Univac 1110 computer of the Zagreb University Computer Centre.

The inhibition by oximes was interpreted according to a hypothesis by Aldrige and Reiner (9) concerning the binding sites in cholinesterases. The following was put forward by the authors: substrate hydrolysis occurs at the catalytic site, but substrate inhibition at a substrate-inhibition site which is allosteric. Both sites can also bind reversible inhibitors. Binding of either substrate or inhibitor to the substrate-inhibition site prevents hydrolysis of the substrate at the catalytic site. Consequently, binding of a reversible inhibitor to either site is competitive with the substrate.

The equation which relates substrate and inhibitor concentrations (s and i) to enzyme activities is[9]:

$$\frac{v \cdot i}{v_o - v} = \frac{(1 + K_s/s)(1 + s/K_{ss})}{K_s/s\,(1/K_a + 1/K_i + i/K_i K_a) + 1/K_i + K_s/K_a K_{ss}} \quad (2)$$

where v and v_o are enzyme activities (at a given substrate concentration) in the presence and absence of the reversible inhibitor. K_a and K_i are the dissociation constants of the enzyme-inhibitor complex at the catalytic site and substrate-inhibition site respectively. K_s and K_{ss} are the dissociation constants of the enzyme--substrate complex at the catalytic site and substrate-inhibition site, respectively. In all calculations the Michaelis constant (K_m) was used instead of K_s. The constants K_a and K_i were calculated from Eqn. (2) by computerized curve fitting as described above.

RESULTS

Non-enzymic Hydrolysis of Acetylthiocholine

The rates of spontaneous and oxime-catalysed hydrolysis of acetylthiocholine were measured by the conventional and stopped-flow techniques. All calculated rate constants are given in Table I.

The rate constant for spontaneous acetylthiocholine hydrolysis was calculated by assuming first-order kinetics. The same constant was obtained regardless of the initial acetylthiocholine concentration. The rate constants for the hydrolysis of acetylthiocholine catalysed by oximes were calculated assuming that the reaction occurs in a 1 : 1 stoichiometry as suggested by other authors[13,14]. The same rate constants were obtained regardless of the initial concentration of the reactants. No corrections were required for spontaneous hydrolysis of acetylthiocholine which at any time amounted to $< 5\%$ of the total hydrolysis rate. The constant obtained for P2AM is similar to the constant which we calculated from the data of Bergmann and Govrin[14]. The constant for Toxogonin is larger than for P2AM, but no attempt was made to establish whether both or one oxime group in Toxogonin reacted with acetylthiocholine.

TABLE I

Rate Constants (k) *for the Spontaneous and Oxime Catalysed Hydrolysis of Acetylthiocholine (ATCh). The Hydrolysis was Measured at 8 Different ATCh Concentrations Ranging from 0.055 to 10 mM. The Range of Oxime Concentrations is Given in the Table.* n *is the Number of Experiments.*

Oxime	$k \pm$ S.E.M.	n
None	$(5.7 \pm 0.3) \times 10^{-5}$ min^{-1}	(20)
P2AM (0.10—10.0 mM)	(13.4 ± 0.4) M^{-1} min^{-1}	(92)
Toxogonin (0.10—10.0 mM)	(22.4 ± 0.9) M^{-1} min^{-1}	(38)

Inhibition of Electric Eel Acetylcholinesterase

The effect of P2AM and Toxogonin on electric eel acetylcholinesterase was studied using acetylthiocholine (0.1—10 mM) as substrate. The enzyme activities were measured by two techniques (conventional and stopped-flow) and all activities were corrected for non-enzymic substrate hydrolysis. At substrate concentrations above 1.0 mM the stopped-flow technique was more suitable, because it requires a short time of assay which is convenient in order to minimize the interference of oximolysis. The activities determined by the two techniques agree well irrespective of whether the oximes were present or not.

The constant K_m, K_{ss} and V for acetylthiocholine were derived from activities in the absence of the oximes and are given in Table II. The inhibition by oximes was measured at different substrate concentrations (cf. Figure 1) using P2AM and Toxogonin at concentrations listed in Table II. The degree of inhibition was expressed by the ratio $v \cdot i/(v_o - v)$ and plotted against the substrate concentration (Figure 1) according to Eqn. (2).

TABLE II

Reaction of Electric eel Acetylcholinesterase with Acetylthiocholine (0.1—10 mM), P2AM (0.25—2.0 mM) and Toxogonin (1.0 mM). The Constants K_m, K_{ss} *and* V *were Calculated from Eqn. (1), and the Dissociation Constants* K_a and K_i *for the Enzyme-Inhibitor Complexes from Eqn. (2);* n *is the Number of Experiments.*

Reactant	Constants
Acetylthiocholine ($n = 68$)	$K_m = 0.081$ mM $K_{ss} = 12.2$ mM $V = 704$ μmol min^{-1} mg^{-1}
P2AM ($n = 56$)	$K_a = 0.13$ mM $K_i = 0.76$ mM
Toxogonin ($n = 29$)	$K_a = 0.16$ mM $K_i = 1.99$ mM

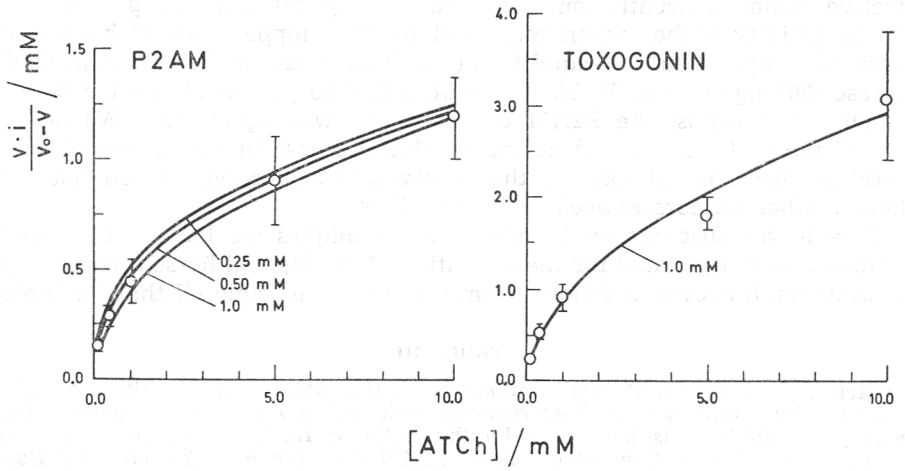

Figure 1. Reversible inhibition of electric eel acetylcholinesterase by P2AM and Toxogonin. The substrate was acetylthiocholine (ATCh). The points are mean values (4 to 13 experiments at each ATCh concentration) determined by both, conventional and stopped-flow techniques. The bars are standard deviations. The curves are theoretical and were calculated according to Eqn. (2) (for oxime concentrations indicated in the figure) using the constants given in Table II.

The derived dissociation constants K_a and K_i for the enzyme-inhibitor complexes are given in Table II. These constants were used to calculate the theoretical curves in Figure 1. Equation (2) predicts one inhibition curve for each inhibitor concentration, but this was not observed experimentally. However, Figure 1 shows that over the used range of oxime concentrations all theoretical curves fall within one standard deviation of the measured results, and this explains why the individual curves could not be distinguished experimentally. The theoretical curves closely resemble the experimental points and we therefore concluded that the model defined under »Evaluation of constants« describes well the inhibition of electric eel acetylcholinesterase by P2AM and Toxogonin.

Inhibition of Bovine Erythrocyte and Horse Serum Cholinesterases

The inhibition of bovine erythrocyte and horse serum cholinesterases by P2AM and Toxogonin was also studied with the two techniques. The enzyme activities in the absence of oximes measured by the two techniques agree well, but the degree of inhibition by the oximes was different being lower when measured by the stopped-flow technique (Table III). This technique requires a 50 times higher enzyme concentration than the conventional technique. When the same enzyme concentration was used for both techniques (horse serum cholinesterase, 8 μg/ml) the degree of inhibition (by 1.0 mM P2AM) was the same irrespective of the technique. This fact and a good agreement between the two techniques on the electric eel preparation (which has a 400 times higher specific activity than the erythrocyte and serum preparations) prove that the discrepancy in the degree of inhibition is not due to the techniques.

It was assumed therefore that high concentrations of erythrocyte and serum enzyme preparations unspecifically bind the oximes thus lowering the

effective oxime concentration and consequently making the percentage of enzyme activity higher when measured by the stopped-flow technique. However, the experiments of equilibrium dialysis made on horse serum cholinesterase (800 μg/ml) and P2AM (2.0 mM) failed to give evidence for it. After 24 hours of dialysis, the P2AM concentration was equal (1.0 mM) on both sides of the dialysis bag and no measurable decrease in P2AM was observed. Therefore, we can not explain the observed phenomenon and do not know whether other authors noticed a similar effect.

Due to the discrepancy between the techniques we have not calculated any dissociation constants for the inhibition of bovine and horse serum enzyme preparations. However, it follows from the results in Table III that the inhibit-

TABLE III

The Activities of Cholinesterases Against Acetylthiocholine and Inhibition by P2AM, Measured by Conventional Spectrophotometric (Conv.) and Stopped-flow (S. F.) Technique. Inhibition is Expressed by the Ratio of Inhibited (v) *and Uninhibited* (v_0) *Enzyme Activity. Concentrations of P2AM* (i) *were 0.10—2.0 mM for Bovine Erythrocyte and 0.50—2.0 mM for Horse Serum Cholinesterase. The Number of Experiments is Given in Parentheses.*

ATCh/mM	v_0/μmol min^{-1} mg^{-1}		$\frac{v \cdot i}{v_0 - v}$/mM	
	Conv.	S. F.	Conv.	S. F.
Bovine erythrocytes				
0.10	0.89 (2)	0.88 (11)	0.19 (24)	0.25 (8)
0.40	1.4 (6)	—	0.25 (26)	—
1.0	1.4 (17)	1.3 (14)	0.35 (33)	0.67 (15)
5.0	1.1 (7)	1.1 (9)	0.70 (20)	1.09 (14)
10.0	0.77 (2)	0.80 (12)	—	1.36 (11)
Horse serum				
0.10	0.96 (4)	0.78 (4)	0.55 (2)	1.01 (2)
0.40	1.83 (4)	1.83 (4)	0.73 (2)	1.11 (2)
1.0	2.54 (10)	2.31 (17)	0.87 (8)	1.62 (8)
5.0	3.46 (1)	3.19 (2)	—	2.75 (3)
10.0	3.67 (1)	3.42 (2)	—	3.36 (2)

ion by the oximes was similar to that observed for the electric eel acetylcholinesterase. Furthermore, the degree of inhibition, expressed as $v \cdot i/(v_0 - v)$, was a non-linear function of the substrate concentration and was independent of the inhibitor concentration, as was the case for the electric eel enzyme (cf. Figure 1).

DISCUSSION

Published data on inhibition of cholinesterases by P2AM and Toxogonin revealed two types of interaction with the enzyme, both of which were postulated to occur at the anionic sub-site of the catalytic centre[2-8]: one type is interaction with the anionic sub-site of the free enzyme (competitive inhibition) and the other with the anionic sub-site of the acetylated enzyme (non-competitive inhibition). Substrates were also postulated to interact in the same way[15]. To calculate the dissociation constants of the enzyme-inhibitor

complexes, it is required to know the rate constants of acetylation and deacetylation by substrates[15]. As it has not yet been possible to measure these rate constants, an assumption has been made concerning their relative values in order to evaluate the dissociation constants for the inhibitors[7].

The present study confirms that there are two types of interaction of P2AM and Toxogonin with acetylcholinesterase, but our results are consistent with an alternative mechanism[9] according to which binding occurs in the catalytic site and an allosteric, substrate-inhibition site, of the enzyme. The alternative mechanism does not distinguish between the esteratic and anionic sub-sites of the catalytic centre. Our results agree with the proposed mechanism, but this in itself does not exclude that other types of binding are also possible. The suggested mechanism implies an allosteric site in acetylcholinesterase, which has been demonstrated by other authors using the same or different approaches[8,16–20].The dissociation constants which we evaluated for P2AM and Toxogonin are of the same order of magnitude as those reported for the same or other sources of acetylcholinesterase[1,2,6–8]. We termed both types of binding as competitive inhibition, because the mechanism assumes that substrates and inhibitors bind to the same sites, and therefore compete with each other.

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SAŽETAK

Inhibicija kolinesteraza oksimima P2AM-om i Toxogoninom

V. Simeon, Z. Radić i E. Reiner

Studirana je reverzibilna inhibicija acetilkolinesteraze (EC 3.1.1.7) električnog organa jegulje, P2AM-om (2-(hidroksiimino)metil-1-metil-piridinium klorid) i Toxogoninom (1,1'-[oksibis(metilen)] bis(4-(hidroksiimino)metil-piridinium) diklorid), uz supstrat acetiltiokolin. Hidroliza acetiltiokolina mjerena je na dva načina; tehnikom zaustavljenog toka (stopped-flow) i konvencionalnom spektrofotometrijskom tehnikom. Mjerenja su vršena pri 25 °C, u 100 mM fosfatnom puferu pH = 7.4.

Ovisnost stupnja inhibicije enzima o koncentracijama acetiltiokolina i oksima slijedi model, koji pretpostavlja vezanje supstrata i inhibitora na dva mjesta na enzimu: jedno je katalitičko, a drugo je alosteričko mjesto koje je ujedno i mjesto inhibicije supstratom. Izračunate konstante disocijacije za ta dva mjesta vezanja iznose: 0.13 mM i 0.76 mM za P2AM i 0.16 mM i 2.0 mM za Toxogonin. Predloženi model je alternativan pretpostavci, da se dva tipa vezanja na enzim odvijaju u katalitičkom mjestu.

P2AM i Toxogonin na sličan način inhibiraju acetilkolinesterazu iz električnog organa jegulje i goveđih eritrocita, te kolinesterazu iz konjskog seruma.

P2AM i Toxogonin hidroliziraju acetiltiokolin i uz pretpostavku da je to bimolekularna reakcija, pripadajuće konstante brzine reakcije iznose 13.4 M^{-1} min^{-1} i 22.4 M^{-1} min^{-1}.

LABORATORIJ ZA BIOKEMIJU
INSTITUT ZA MEDICINSKA ISTRAŽIVANJA
I MEDICINU RADA, ZAGREB

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