CROATICA CHEMICA ACTA CCACAA 59 (4) 925—931 (1986)

YU ISSN 0011-1643
UDC 543.866
CCA-1700
Original Scientific Paper

Inhibition of Acetylcholinesterase by 4,4'-Bipyridine and its Effect upon Phosphylation of the Enzyme

Elsa Reiner

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

Received April 30, 1986

The reversible inhibition of acetylcholinesterase (AChE) by 4,4'-bipyridine (BP) was measured with acetylthiocholine as substrate. The enzyme/inhibitor dissociation constant for binding of BP to the catalytic site was $K_a = 1.0$ mM and the non-competitive dissociation constant for binding to an allosteric site was $K_i =$ $= 9.0$ mM.

BP protected AChE against phosphylation by sarin, soman, tabun and VX. The protective effect was also calculated theoretically from a model which assumed that BP reacts either with the catalytic site of the enzyme or with an allosteric site, but does not form a ternary complex with acetylcholinesterase. The calculated and experimentally determined protective indices agreed well.

INTRODUCTION

The purpose of the study was to evaluate the type of reversible binding of 4,4'-bipyridine (BP) to acetylcholinesterase (AChE). The evaluation was done in a two-step approach. First, the inhibition of AChE by BP was measured using different concentrations of BP and substrate (acetylthiocholine) wherefrom the enzyme/inhibitor dissociation constants were determined. The second step consisted in measuring the effect of BP upon phosphylation of AChE by organophosphorous compounds (OP). This effect will depend upon the binding sites of BP on the enzyme as laid out under Theoretical equations.

The OP compounds were sarin, soman, tabun and VX. The results obtained were discussed in terms of which type of reversible binding would best protect the enzyme against phosphylation.

EXPERIMENTAL

All experiments were done in 0.10 M phosphate buffer (NaH_2PO_4/Na_2HPO_4) pH 7.4 at 37 °C. The enzyme activities were measured by the spectrophotometric method of Ellman et al.[1]

The source of acetylcholinesterase (AChE; EC 3.1.1.7) was native human erythrocytes obtained from heparinized blood. The erythrocytes were washed twice with 0.9 M NaCl and diluted with 0.9 M NaCl to the volume of blood. The washed erythrocytes (0.5 ml) were added into buffer (85 ml; 0.20 M buffer) and 1.50 ml of the erythrocyte suspension was used per enzyme assay.

4,4'-bipyridine (BP; Riedel — de Haen A. G., Seelze — Hannover, West Germany). — Stock solutions (0.10 M) were prepared in 20% ethanol; further dilutions were made in water.

Organophosphorous compounds (OP). — The compounds are listed in Table I. Stock solutions (2.0 mg/ml or 4.0 mg/ml) were prepared in propylene glycol and further diluted with water. The purity of the OP was about 97%.

TABLE I

List of Studied Compounds and Their Constants for the Reaction with Acetylcholinesterase

Name	Formula	Constant ± std. error of mean	(n)
4,4'-bipyridine (BP)	(bipyridine ring structure)	$K_a = 1.0$ mM $K_i = 9.0$ mM	(11)
Sarin	iso-C_3H_7O—P(=O)(—CH_3)—F	$k_a = (2.7 \pm 0.1) \times 10^7$	(8)
Soman	$(CH_3)_3C$—CH(CH_3)O—P(=O)(CH_3)—F	$k_a = (2.0 \pm 0.1) \times 10^8$	(9)
Tabun	C_2H_5O—P(=O)($N(CH_3)_2$)—CN	$k_a = (3.4 \pm 0.2) \times 10^7$	(6)
VX	C_2H_5O—P(=O)(CH_3)—S—CH_2—CH_2—N(iso-C_3H_7)$_2$	$k_a = (7.2 \pm 0.3) \times 10^7$	(4)

K_a, K_i: reversible enzyme/inhibitor dissociation constants (see schemes I—IV)

k_a: second order rate constant of inhibition in absence of BP expressed in M^{-1} min^{-1} (see eqn. 1)

n: number of observations

Substrate. Stock solutions (0.10 M) of acetylthiocholine iodide were prepared in water and further diluted with water.

DTNB. — The thiol reagent 5,5'-dithiobis-(2-nitrobenzoic acid) was prepared in buffer (stock solution 10 mM). The final concentration during enzyme assay was 0.33 mM.

Reversible inhibition by BP. — The reaction mixture contained 1.50 ml erythrocyte suspension, 100 μl DTNB, 0.30 ml BP, 0.80 ml water and 0.30 ml substrate. The control samples contained 0.30 ml water instead of BP. The increase in absorbance was read in 1.0 cm cuvettes at 412 nm at 15 s intervals up to 2 min. The first reading was done 15 s after the addition of substrate.

Progressive inhibition by OP compounds. — The OP compound (0.30 ml) was added to the reaction mixture which contained 1.50 ml erythrocyte suspension, 100 μl DTNB and 0.80 ml water. After a given time of inhibition the substrate (0.30 ml) was added and the activity measured as described above. The control samples contained 0.30 ml water instead of the OP compound. The final substrate concentration was either 1.0 or 5.0 mM.

Effect of BP upon progressive inhibition by OP compounds. — The OP compound (0.30 ml) was added to the reaction mixture which contained 1.50 ml erythrocyte suspension, 100 μl DTNB, 0.30 ml BP and 0.50 ml water. After a given time of inhibition the substrate was added and the activity measured as described above. The control samples contained 0.30 ml water instead of the OP compound. The final substrate concentration was as above.

All activities were measured against a blank which contained 1.50 ml erythrocyte suspension, 100 μl DTNB and 1.40 ml water. The activities of the enzyme preparation (undiluted erythrocytes) were between 2 and 4 μmol min^{-1} (ml erythrocyte)$^{-1}$. During enzyme assay the increase in absorbance was linear with time in both, inhibited and non-inhibited enzyme samples.

Non-enzymic substrate hydrolysis: The non-enzymic hydrolysis was measured in the same way as the enzymic hydrolysis except that the reaction mixture contained 1.50 ml buffer (0.20 M) instead of the enzyme suspension. Corrections for the non-enzymic hydrolysis were required only when substrate concentrations were 5.0 and 10 mM; BP increased only slightly the non-enzymic substrate hydrolysis.

THEORETICAL EQUATIONS FOR THE EFFECT OF A REVERSIBLE INHIBITOR UPON PHOSPHYLATION

Four mechanisms are theoretically possible for the reaction of an enzyme (E) and a reversible inhibitor (I), if the enzyme has two binding sites for the inhibitor.

Scheme I:

$$E + I \overset{K_a}{\leftrightharpoons} EI$$

Scheme II:

$$E + I \overset{K_i}{\leftrightharpoons} IE$$

Scheme III:

$$E + I \overset{K_a}{\leftrightharpoons} EI$$

$$E + I \overset{K_i}{\leftrightharpoons} IE$$

Scheme IV:

$$E + I \overset{K_a}{\leftrightharpoons} EI$$

$$E + I \overset{K_i}{\leftrightharpoons} IE$$

$$EI + I \overset{K_i}{\leftrightharpoons} IEI$$

$$IE + I \overset{K_a}{\leftrightharpoons} IEI$$

EI and IE are enzyme/inhibitor complexes formed in the catalytic and an allosteric site respectively, and IEI is a ternary complex. The above reaction schemes are based upon theoretical considerations laid out earlier.[2] The basic assumption is the independence of the binding sites, i. e. the dissociation constant for a given site has the same numerical value irrespective of whether the other site is free or occupied. In Scheme I the reversible inhibitor binds only to the catalytic site, in Scheme II only to the allosteric site, in Scheme III the inhibitor can react either with the catalytic or with the allosteric site, while in Scheme IV the inhibitor can also form a ternary complex by binding to both sites simultaneously.

If an OP compound reacts only with the free enzyme:

$$E + OP \xrightarrow{k_a} E \text{ — phosphylated} \tag{1}$$

the effect of the reversible inhibitor upon the rate of phosphylation will be defined by one of the following equations:

$$\ln \frac{v_o}{v_t} = (k_a \cdot op \cdot t)/(1 + i/K_a) \tag{2}$$

$$\ln \frac{v_o}{v_t} = (k_a \cdot op \cdot t)/(1 + i/K_i) \tag{3}$$

$$\ln \frac{v_o}{v_t} = (k_a \cdot op \cdot t)/(1 + i/K_a + i/K_i) \tag{4}$$

$$\ln \frac{v_o}{v_t} = (k_a \cdot op \cdot t)/[(1 + i/K_a)(1 + i/K_i)] \tag{5}$$

v_o and v_t are the enzyme activities in the absence and in presence of the inhibitor, k_a is the second order rate constant of phosphylation in absence of the reversible inhibitor, i is the concentration of the reversible inhibitor, op is the concentration of OP, and t is the time of inhibition.

Equations (2) through (5) correspond to reaction Schemes I through IV. The reversible inhibitor will protect the enzyme against phosphylation and the protective efect will depend upon the ratio of i/K_a and i/K_i. The protective effect can be expressed in terms of a protective index:

$$\text{Protective index} = PI = k_a/k_a' \tag{6}$$

where k_a' is the second order rate constant of phosphylation in the presence of the reversible inhibitor. Four expressions can be derived for the protective indices corresponding to equations (2) through (5):

$$PI = 1 + i/K_a \tag{7}$$

$$PI = 1 + i/K_i \tag{8}$$

$$PI = 1 + i/K_a + i/K_i \tag{9}$$

$$PI = (1 + i/K_a)(1 + i/K_i) \tag{10}$$

The protective indices in eqn. (6) can also be calculated from first order rate constants (at a given OP concentration) instead of from k_a and k_a', provided that phosphylation follows eqn. (1).

RESULTS AND DISCUSSION

The reversible inhibition of AChE by BP was evaluated over a concentration range of BP from 1.0 to 9.0 mM, and over a range of acetylthiocholine concentrations from 0.10 to 10 mM. At each substrate concentration 3—5 different BP concentrations were tested, and the obtained results are presented

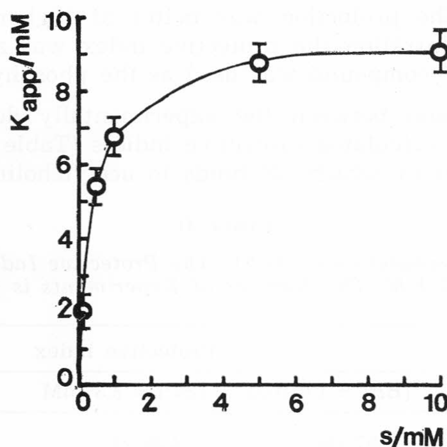

Figure 1. Reversible inhibition of AChE by BP. At each substrate concentration (s) three to five different BP concentrations were used. K_{app} is the apparent enzyme/ /inhibitor dissociation constant defined by eqn. (11). Each point is the mean value of 11 experiments. The bars are S.E.M.

in Figure 1 using the Hunter-Downs plot. The ordinate on that plot represents the apparent enzyme/inhibitor dissociation constant

$$K_{app} = v_i \cdot i/(v_o - v_i) \tag{11}$$

v_o and v_i are the enzyme activities in the absence and in the presence of inhibitor, and i is the inhibitor (BP) concentration. The Hunter-Downs plot was curved and this indicates that BP binds to two different sites on the enzyme.[2] An extrapolation of the curve to zero substrate concentration gives the dissociation constant K_a for binding of BP to the catalytic site. This constant was 1.0 mM obtained from graphic extrapolation. At substrate concentrations between 5.0 and 10 mM the inhibition approached a plateau indicating that BP was also a non-competitive inhibitor with respect to acetylthiocholine. The non-competitive dissociation constant K_i of BP was 9.0 mM (mean value of K_{app} at 5.0 and 10 mM acetyltiocholine). No theoretical equation was derived for the competition between BP and acetylthiocholine, however a qualitative conclusion can be made about the mechanism of the reaction. BP did not compete with acetylthiocholine at acetylthiocholine concentrations which inhibit the enzyme. This indicates that K_i cannot represent binding of BP to the substrate-inhibition-site, but is likely to represent binding to another allosteric site of acetylcholinesterase.

The effect of BP was also tested as protector against phosphylation of AChE by OP compounds. The OP compounds were sarin (40, 60 and 200 μM), soman (5.0 and 25 nM), tabun (50 and 100 nM) and VX (30, 60, 75 and 150 nM). At each OP concentration the time course of inhibition was measured over a period from zero up to 5 min. The BP concentrations covered a range which corresponded from its K_a to its K_i constant. The results of these measurements were expressed in terms of protective indices (eqn. 6) and are listed in Table II. The second order rate constants of inhibition in absence of the reversible inhibitor are listed in Table I. BP protected the enzyme against

phosphylation, and the protection was better at higher BP concentrations. At a given BP concentration the protective index was about the same irrespective of which OP compound was used as the phosphylating agent.

From a comparison between the experimentally determined (Table II) and the theoretically calculated protective indices (Table III) it is possible to assign the mechanism by which BP binds to acetylcholinesterase.

TABLE II

Effect of BP *upon Phosphylation of AChE. The Protective Index was Obtained from eqn. (6).* ± *is the S.E.M. The Number of Experiments is Given in Brackets*

OP Compound	Protective index		
	$[BP] = 1.0$ mM	$[BP] = 3.3$ mM	$[BP] = 10.0$ mM
Sarin	1.97 (3)	4.06 (3)	10.9 ± 0.8 (6)
Soman	2.31 (3)	4.11 (3)	14.2 ± 0.9 (6)
Tabun	1.54 (3)	3.80 (3)	11.8 ± 0.6 (6)
VX	1.75 (3)	3.55 (3)	11.0 ± 1.0 (7)

TABLE III

Theoretically Calculated Protective Indices for the Effect of a Reversible Inhibitor (I) upon Phosphylation of an Enzyme. The Protective Indices were Calculated from eqns. (7) Through (10) Which Correspond to Reaction Schemes I Through IV Defined in the Text. Protective Indices were Calculated from the Dissociation Constants of BP *Which are* $K_a = 1.0$ *mM and* $K_i = 9.0$ *mM*

Scheme (Eqn.)	Protective Index		
	$[I] = 1.0$ mM	$[I] = 3.3$ mM	$[I] = 10.0$ mM
Scheme I (7)	2.00	4.30	11.0
Scheme II (8)	1.11	1.37	2.11
Scheme III (9)	2.11	4.67	12.1
Scheme IV (10)	2.22	5.88	23.2

Schemes I and II described under Theoretical equations can be ruled out for the reaction of BP with AChE, because the results obtained from reversible inhibition have shown that BP must have two binding sites for the enzyme (Figure 1). It is however of interest to note that eqns. (7) and (8), which are based upon Schemes I and II, are mathematically identical. This means that for a given ratio of i/K_a or i/K_i the protective index will be the same irrespective of which mechanism holds good. In a previous study these two schemes were verified experimentally for the effect of several reversible ligands (tetramethylammonium, edrophonium, coumarin) upon phosphylation of AChE by VX.[3]

Schemes III and IV each assume that the enzyme has two binding sites for the reversible inhibitor. Furthermore, these two schemes can be well

distinguished from each other at inhibitor concentrations which approach the K_i constant (Table III). At 10 mM BP the theoretical protective index should be 12.1 if Scheme III holds, and 23.2 if Scheme IV holds. The experimentally obtained protective index at 10 mM BP was 12.0 (mean value obtained with four OP compounds; Table II). Consequently, it seems that BP reacts according to Scheme III. This would mean that BP binds either to the catalytic site EI, or to an allosteric site IE, but does not form a ternary complex IEI with AChE. Reversible inhibitors which form ternary complexes (Scheme IV) would provide a better protection against phosphylation.

Finally, a comparison between Schemes I and III can show that the second binding site of BP ($K_i = 9.0$ mM) contributes only little to the protection of AChE against phosphylation. If BP had only one binding site (Scheme I; $K_a = 1.0$ mM) its protection against phosphylation would be about the same as it is now with two binding sites (K_a and K_i) (compare calculated protective index for schemes I and III in Table III). The contribution of the second binding site would become more apparent only at BP concentrations much higher than K_i.

The described approach of verifying or establishing binding sites for ligands has so far been applied only to compounds which reversibly bind to one site on the enzyme (cf. 3). In the present study this approach has been extended to a ligand which has two binding sites for acetylcholinesterase.

Acknowledgement. — The author's thanks are due to Dr. Vera Simeon for valuable discussions while preparing the manuscript. The reliable technical assistance of Mrs. Andjelka Buntić is greatly appreciated. Bipyridine was kindly supplied by Dr. Zlatko Binenfeld.

REFERENCES

1. G. L. Ellman, K. D. Courtney, V. Andres, and R. M. Featherstone, *Biochem. Pharmacol.* **7** (1961) 88—95.
2. W. N. Aldridge and E. Reiner, *Enzyme inhibitors as substrates. Interaction of Esterases with esters of Organophosphorus and Carbamic Acids.* North-Holland, Amsterdam 1972, XVI + 328 pp.
3. Z. Radić, E. Reiner, and V. Simeon, *Biochem. Pharmacol.* **33** (1984) 671—677.

SAŽETAK

Inhibicija acetilkolinesteraze 4,4'-bipiridinom i njegov utjecaj na fosfiliranje enzima

Elsa Reiner

Reverzibilna inhibicija acetilkolinesteraze u 4,4'bipiridinom (BP) mjerena je s acetiltiokolinom kao supstratom. Konstanta disocijacije za kompleks u katalitičkom mjestu enzima iznosila je $K_a = 1,0$ mM, a za nekompetitivno vezanje u alosteričkom mjestu $K_i = 9,0$ mM.

BP je štitio enzim od fosfoniliranja sarinom, somanom, tabunom i VX-om. Protektivni učinak je i teorijski proračunan prema modelu koji pretpostavlja da se BP veže ili u katalitičkom mjestu enzima ili u alosteričkom mjestu enzima, ali ne stvara ternarni kompleks s enzimom. Eksperimentalni i proračunani protektivni učinci dobro se slažu.