# The interaction of acetylcholinesterase with carbodiimides

## Irreversible inhibition with 1-cyclohexyl-3-(2-morpholinoethyl)-carbodiimide metho-*p*-toluenesulfonate

Dina Segal and Yechiel Shalitin*

*Department of Biology, Technion-Israel Institute of Technology, Haifa 32000, Israel*

Received 27 July 1982

*Acetylcholinesterase* *Water-soluble carbodiimides* *Competitive inhibition* *Irreversible inhibition* *Ternary complex*

## 1. INTRODUCTION

'Active' phosphates, phosphonates and carbamates are effective covalent inhibitors of acetylcholinesterase (AChE) [1,2] and therefore find use in research, pharmacological treatment and especially in pest control. It was of interest to examine whether other types of active reagents can also affect the enzyme. As carbodiimides are known to react with a variety of functional groups such as carboxylic acids, alcohols, amines, imidazoles [3] and have been found to react with several enzymes [4–8], we studied the interaction of a number of carbodiimides with AChE.

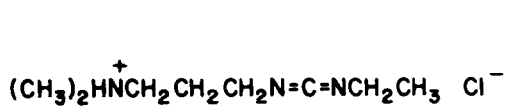
The water-soluble 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (DEC) and 1-cyclohexyl-3-(2-morpholinoethyl)carbodiimide-metho-*p*-toluenesulfonate (CMC) were found to be competitive inhibitors of AChE with $K_i$ = 0.08 mM and 0.12 mM, respectively. In incubation with the enzyme CMC, but not DEC, causes inactivation. Analysis of the kinetics of this covalent inhibition suggests that binary as well as ternary enzyme–inhibitor complexes are formed prior to inactivation. Competitive inhibitors protect the enzyme from inactivation and several strong nucleophiles are capable of reactivating the covalently inhibited enzyme, suggesting that CMC blocks the enzyme at the active site. Dicyclohexylcarbodiimide (DCC) was devoid of either competitive or covalent inhibitory capacity.

## 2. MATERIALS AND METHODS

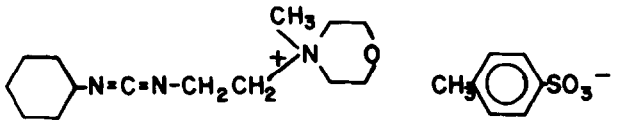
Acetylcholinesterase from electric eel was purchased from Worthington and Sigma. DEC, DCC and CMC (fig.1) were from Aldrich. Enzymic activity was followed pH-statically. The reaction mixture contained 1.2 mM acetylcholine, 0.1 M NaCl, 0.02 M $MgCl_2$ and 10 nM enzyme at pH 8.0, 25°C and 0.05 N NaOH was the titrant. For measuring reversible inhibition by carbodiimide different concentrations of the inhibitor were added to the reaction vessel containing constant concentration of substrate, enzyme was then added and the reaction recorded. From the Dixon plot [9] the inhibition constant was calculated. For measuring the kinetics of irreversible inhibition, different concentrations of carbodiimide in the 0.1–40 mM range were added to solution of 1 μM AChE in 0.1 M NaCl, 0.02 M $MgCl_2$, pH 8.0, 25°C and at timed intervals an aliquot of the solution was assayed for enzyme activity. The inactivation rate constant was obtained by plotting the log of residual activity *vs* time. The kinetic parameters of the inhibition reaction were deduced from a Kitz–Wilson plot [10]. The reactivation of CMC-inhibited enzyme was followed by incubating it in a solution containing nucleophile and at different

* To whom correspondence should be addressed

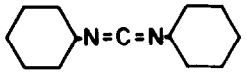
*Published by Elsevier Biomedical Press*
00145793/82/0000–0000/$2.75 



DEC



CMC



DCC

Fig.1. Formulae for DEC, DCC and CMC.

times an aliquot of the reaction mixture was assayed for activity.

The enzyme concentration was determined by active site titration with phenothiazine-10-carbonyl chloride [11].

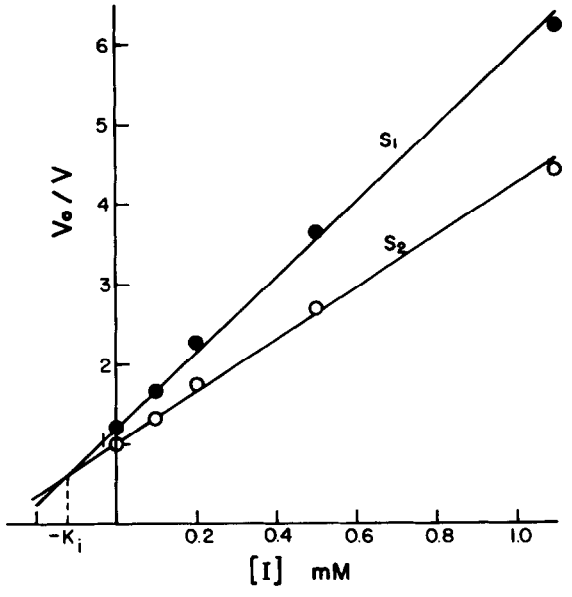



Fig.2. Dixon plot for the competitive inhibition of AChE with CMC; $S_1$ = 0.3 mM and $S_2$ = 0.6 mM acetylcholine solution; enzyme was 10 nM; the reaction was carried out at pH 8.0, 25°C; $v_o$ is the initial rate in the case of 0.6 mM substrate and no CMC.

## 3. RESULTS AND DISCUSSION

Fig.2, the Dixon plot of the inhibition of AChE with CMC, indicates that CMC is competitive inhibitor of the enzyme. The intersection of the lines yields $K_i$ = 0.11 ± 0.01 mM. Similar analysis of the interaction of AChE and DEC also yields competitive inhibition with $K_i$ = 0.08 ± 0.01 mM. The uncharged DCC (in 1% acetonitrile solution in water) did not display any inhibition. When AChE was followed to react with CMC at pH 8.0, 25°C for longer periods a progressive loss of enzymic activity took place with time which followed first order kinetics according to:

$$v_i = \frac{d[E]}{dt} = -k[E] \quad (1)$$

where:

$k$ = the pseudo first-order inhibition constant

For an irreversible inhibition which involves a reversible enzyme–inhibitor complex formation prior to the inhibition event [eq.(2)] $k$ is expressed by eq. (3) according to [10].

$$E + I \overset{K_i}{\rightleftharpoons} EI \overset{k_i}{\rightarrow} EI' \quad (2)$$

and

$$k = k_i[I]/K_i + [I] \quad (3)$$

where:

$K_i$ = the dissociation constant of EI, the enzyme–inhibitor complex;

$k_i$ = the first-order rate constant for the formation of EI', the irreversibly inhibited enzyme.

For such a case the Kitz–Wilson plot of $1/k$ *vs* $1/[I]$ yields a straight line with intercepts at $1/k_i$ and $-1/K_i$ [10].

For CMC inactivation of AChE plot of $1/k$ *vs* $1/[I]$ yields a curved line, (fig.3) indicating a more complex mechanism than that depicted by eq. (2). Extrapolation of the linear right part of the curve, corresponding to high $1/[I]$ values, namely low inhibitor concentration, intercepts the abscissa at $[I] = 0.12 \pm 0.01$ mM, the $K_i$ value of the reversible binding step, which is equal to the competitive inhibition constant in fig.2. The intercept on the ordinate corresponds to $k_i = 4.3 \pm 0.3 \times 10^{-3}$ $min^{-1}$. However, at higher concentrations of I (low $1/[I]$ values) the line curves down and the inactivation rate increases much faster than expected. This behavior can be interpreted by assuming that the enzyme can bind two inhibitor molecules with different affinities. The high affinity has $K_i$ = 0.12 mM which is also the competitive inhibition constant and it manifests itself already at relatively low inhibitor concentration. The reversibly bound inhibitor reacts covalently with an essential group of the enzyme thus inhibiting it irreversibly. At higher concentrations of inhibitor the low affinity site binds another inhibitor molecule and $EI_2$, the ternary complex formed, is inactivated with a larger rate constant than that of the binary complex. Such a mechanism is represented by:

$$E + I \underset{k_i}{\overset{K_i}{\rightleftharpoons}} EI + I \underset{k_i'}{\overset{K_i'}{\rightleftharpoons}} EI_2 \qquad (4)$$

$$\downarrow \qquad\qquad \downarrow$$

$$EI' \qquad\qquad EI_2'$$

$EI'$ and $EI_2'$ are the irreversibly inhibited enzyme forms, $K_i$ and $K_i'$ represent the dissociation constants of the binary and ternary enzyme–inhibitor complexes and $k_i$ and $k_i'$ are the first-order rate constants of irreversible inhibition of the enzyme–inhibitor complexes. Assuming steady state for EI and $EI_2$, scheme (4) leads to the following kinetic equation:

$$v_i = \frac{d[E]}{dt} = -[E]\frac{k_i K_i'[I] + k_i'[I]^2}{K_i K_i' + K_i'[I] + [I]^2} = -k[E] \qquad (5)$$

Inserting $K_i = 0.12$ mM, $k_i = 4.3 \times 10^{-3}$ $min^{-1}$, $K_i' = 18$ mM and $k_i' = 10 \times 10^{-3}$ $min^{-1}$ into eq. (5) results in the solid line (fig.3 and inset) which fits the experimental results.

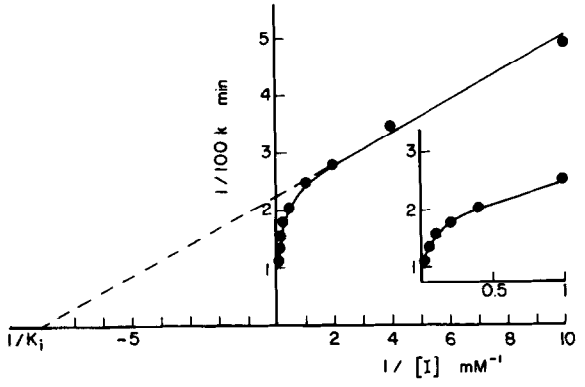



Fig.3. Kitz–Wilson plot for inactivation of AChE with CMC. $k$ is the apparent first-order constant of inactivation. The points are experimental and the solid line was calculated from eq. (5) with $K_i = 0.12$ mM, $k_i = 4.3 \times 10^{-3}$ $min^{-1}$, $K_i' = 18$ mM and $k_i' = 10 \times 10^{-3}$ $min^{-1}$. The inset is expanded abscissa for the left part of the graph.

It is established that AChE can form ternary complexes by binding ligands to the specificity site and to an additional site [12–16], and it is likely that the enzyme–$(CMC)_2$ complex which is formed by binding ligand molecules to two sites undergoes enhanced covalent inhibition. A similar case was reported in [17] where supposed binding of ligand to a low affinity site greatly enhanced the inhibition of AChE by arsenite. It is interesting that DEC did not exert irreversible inhibition although it was even a slightly more effective competitive inhibitor than CMC. In [6] bovine erythrocyte AChE was not inhibited by 1 mM DEC, but the diimide eliminated the $Ca^{2+}$-induced activation enhancement of the enzyme and rendered its activity to the basal level. When we studied the effect of the uncharged DCC on the enzyme it was found to be devoid of any noticeable inhibition.

The ability of the water-soluble carbodiimides to inhibit competitively stems from their charged ammonium group, (similar to a large variety of ammonium derivatives [1]) suggesting that the inhibitor is bound to the active site of the enzyme. Also, addition of 10 mM tetramethylammonium chloride, a reversible inhibitor, to a solution of enzyme containing 10 mM CMC decreased the inactiva-

tion rate by 33%, and in the presence of 100 mM of salt inactivation rate was decreased by 80%.

The discrimination between CMC and DEC in their inactivation ability emanates most probably from the difference in their structure.

Attempts were made to reactivate the covalently inhibited enzyme by using good nucleophiles. Pyridinealdoxime methiodide (2-PAM), a potent reactivator of phosphorylated AChE [1] failed to show any reactivation. However, simple nucleophiles like hydroxylamine and hydrazine completely reactivated the CMC-inhibited enzyme. Hydroxylamine (1 M) at pH 8 reactivated with $t_{0.5}$ = 180 min and hydrazine (1 M) with $t_{0.5}$ = 100 min.

At this point we cannot locate the enzyme groups attacked by CMC. However, analogy with other covalent inhibitions and resemblance to chymotrypsin inhibition by CMC [5] suggest that the active site serine of AChE is modified and a derivatized isourea is formed:

$$\mathrm{EOH} + \mathrm{R_1N{=}C{=}NR_2} \rightarrow \mathrm{EO{-}\underset{\displaystyle \mathrm{HNR_1}}{\underset{|}{C}}{=}NR_2} \xrightarrow{\mathrm{YH}} \mathrm{EOH} + \mathrm{\underset{\displaystyle \mathrm{HNR_1}}{\underset{|}{YC}}{=}NR_2} \quad (6)$$

EOH denoted the active enzyme and YH is a nucleophilic reactivator.

A less likely possibility is that another residue at or near the active site has been modified [17].

The fact that hydroxylamine and hydrazine can reactivate the covalently inhibited enzyme whereas 2-PAM cannot, suggests that the binding site in the enzyme is occupied by the bulky bound inhibitor and is not available for PAM or kindred bulky reactivators. However, the blocked active site is accessible to small molecules which can react with the modified enzyme group and liberate the diimide moiety resulting in reactivation.

## REFERENCES

[1] Froede, H.C. and Wilson, I.B. (1971) in: The Enzymes, 3rd ed. (Boyer, P.D. ed) vol. 5, pp. 87–114, Academic Press, New York.
[2] Rosenberry, T.L. (1975) Adv. Enzymol. 43, 103–218.
[3] Williams, A. and Ibrahim, I.T. (1981) Chem. Rev. 81, 589–636.
[4] Beechey, R.B., Robertson, A.M., Holloway, C.T. and Knight, I.G. (1967) Biochemistry 6, 3867–3879.
[5] Banks, T.E., Blossey, B.K. and Shafer, J.A. (1969) J. Biol. Chem. 244, 6323–6333.
[6] Roufogalis, B.D. and Wickson, V.M. (1973) J. Biol. Chem. 248, 2254–2256.
[7] Perfetti, R.B., Anderson, C.D. and Hall, P.L. (1970) Biochem. 15, 1735–1743.
[8] George, A.L. and Border, C.L. (1979) Biochem. Biophys. Res. Commun 87, 59–65.
[9] Dixon, M. (1953) Biochem. J. 55, 170–171.
[10] Kitz, R.J. and Wilson, I.B. (1962) J. Biol. Chem. 237, 3245–3249.
[11] Naveh, M., Bernstein, Z., Segal, D. and Shalitin, Y. (1981) FEBS Lett. 134, 53–56.
[12] Changeux, J.P. (1966) Mol. Pharmacol. 2, 369–375.
[13] Mooser, G. and Sigman, D.S. (1974) Biochemistry 13, 2299–2307.
[14] Rosenberry, T.L. and Bernhard, S.A. (1972) Biochemistry 11, 4308–4321.
[15] Taylor, P. and Lappi, S. (1975) Biochemistry 14, 1989–1997.
[16] Wilson, I.B. and Silman, I. (1977) Biochemistry 16, 2701–2708.
[17] Blumberg, S. and Silman, I. (1978) Biochemistry 17, 1125–1130.