A KETOPENTYL TRANSITION STATE ANALOG FOR ACETYLCHOLINESTERASE

Alan Dafforn and Paul Kerr
Department of Chemistry
Bowling Green State University
Bowling Green, Ohio 43403

and

Robert R. Murray
Department of Chemistry
University of Texas
Austin, Texas 78712

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4-oxo-N,N,N-trimethylpentanaminium chloride is a competitive inhibitor of eel acetylcholinesterase with $K_I = 8 \times 10^{-6}$ M at 25°, 0.1 M NaCl, 0.04 M $MgCl_2$, pH 7.5. Its binding decreases at low pH with $pK_a = 6.0$. N,N,N-trimethylpentanaminium bromide has $K_I = 4 \times 10^{-4}$ M under the same conditions. Its binding also decreases with pH with $pK_a = 5.35$. Comparison with literature data indicates that the ketone binds much more strongly than substrates and that its binding shows the pH dependence expected for a transition state analog.

Transition state analogs represent a new and promising approach to the study of enzymatic mechanisms (1). In a general sense, these analogs are inhibitors which bind unusually tightly to an enzyme because they resemble in some respect the transition state for the normal enzymatic reaction. The structural features of a successful analog might yield information about the structure of the transition state and thus the mechanism of the reaction. Acetylcholinesterase has been intensively studied because of its important role in nerve transmission and the consequent pharmacological and industrial importance of its inhibitors. It offers a good system for the testing of new possible transition state analogs and of the usefulness of the theory.

One report of a borinic acid analog for acetylcholinesterase has appeared (2), as well as a number of descriptions of boronic acid (3) and aldehyde (4) analogs for the mechanistically similar serine and sulfhydryl proteases. We wish to report evidence that 4-oxo-N,N,N-trimethylpentanaminium chloride (I) (ketopentyltrimethylammonium chloride) is a good transition state analog for acetylcholinesterase. This compound might be expected to bind to

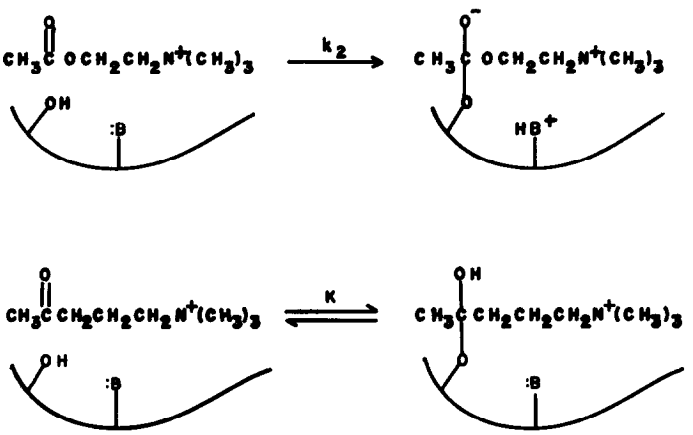

Figure 1. The analogy between acetylcholine hydrolysis and hemiketal formation from ketone I. Nucleophilic attack of an enzymic serine hydroxyl on an ester (top) gives a tetrahedral intermediate which resembles in structure the tetrahedral hemiketal formed by nucleophilic attack on a ketone inhibitor.

the enzyme with its ketone group near the enzymatic serine hydroxyl. Nucleophilic attack on the carbonyl should yield a hemiketal with a structure analogous to the tetrahedral intermediate for hydrolysis of acetylcholine (Figure 1). X-ray evidence that a chloromethyl ketone binds to subtilisin as the hemiketal has recently been reported (5). Koehler and Hess (2) have reported K_I values for inhibition of acetylcholinesterase by compound I over a limited pH range. Their values differ somewhat from ours, and they did not discuss whether I was a transition state analog.

Materials and Methods

General: Acetylcholinesterase Electrophorus Electricus (E 3.1.1.7) was obtained from Aldrich Chemical Company. All batches used had activity of >1000 units/mg and had been purified by gel filtration. All other materials were reagent grade. Solutions were prepared from deionized distilled water. NMR spectra were obtained on a Varian A-60 nmr. Elemental analyses were performed by Galbraith Laboratories, Knoxville, Tennessee.

4-oxo-N,N,N,-trimethylpentanaminium chloride (I): Attempts to repeat the reported preparation from 5-chloro-2-pentanone(6) and trimethylamine yielded an intractable mixture containing a high proportion of trimethylammonium chloride. The corresponding iodide (K and K Laboratories) consisted of brown lumps of highly variable composition as judged by nmr. Pure material was obtained by dissolving 6 g of 5-chloro-2-pentanone ethylene ketal (0.0365 mole) in 23.7 g of 25% aqueous trimethylamine (0.1 mole) and 7 ml of 95% ethanol. The mixture was sealed in a thick-walled glass tube and heated for 15 hours in a steam bath. Trimethylamine was evaporated from

the product with an aspirator, the mixture was extracted twice with ether, and the water layer was stirred overnight with Norit charcoal. Evaporation of water at aspirator pressure left 8.4 g of yellow oil which solidified on standing. The ketal was hydrolyzed by standing overnight in 2% aqueous trifluoroacetic acid; water and acid were then removed with a vacuum pump. The resulting oil was shaken with 3 50 ml portions of 50:50 (v/v) acetone-ether to remove ethylene glycol. Thorough drying under vacuum gave a white solid which was recrystallized from 78% anhydrous ether: 22% absolute ethanol to give hygroscopic white crystals, m.p. 137-139°. Anal: Calc: C, 53.47%; H, 10.10%; N, 7.80%; Cl, 19.73%. Found: C, 53.22%; H, 10.23%; N, 7.86%; Cl, 19.62%

N,N,N-trimethylpentanaminium bromide(II). 60g of a 13.5 wt. % aqueous solution of trimethylamine (0.137 mole) and 14.9 g (0.099 mole) of 1-bromopentane were mixed in a stoppered flask. Two phases were no longer present after two days standing and two days slow stirring at room temperature. After nine days, the solution was extracted once with ether. The water layer was evaporated with an aspirator to leave an off-white powder. Two recrystallizations from ethanol-ethyl acetate gave white crystals with m.p. 191-192° (lit. 174-175°) (7). Anal: Calc: C, 45.72%; H, 9.59%. Found: C, 45.29%; H, 9.63%.

Kinetic Methods: Kinetic parameters were obtained from double reciprocal plots of initial velocities obtained with Sargent or Radiometer Recording pH-stats. All solutions contained 0.1 M NaCl and 0.04 M $MgCl_2$. Typically, 10 ml of solution containing substrate and inhibitor was equilibrated to 25° under nitrogen. Reaction was initiated by adding 50 λ of enzyme dissolved in distilled water, and the acetic acid product was titrated with 0.006 M NaOH. Substrate concentration was varied from 10^{-4} to 10^{-3} M. Reported K_I values are the average of results at three different inhibitor concentrations. Results at pH 7.5 were obtained independently by two of the authors.

Results

The ketone I was found to be a competitive inhibitor of acetylcholinesterase with $K_I = 8 \times 10^{-6}$ M at 25°, pH 7.5. A typical double reciprocal plot is shown in Figure 2. Koehler and Hess (2) report a K_I of 5.1×10^{-5} M at pH 7.5, 25°, 0.2 M NaCl for the iodide. The source of the discrepancy in results is unclear, although we have found that an impure commercial preparation of the iodide gives higher and more variable K_I values than does the chloride used in this study. The pH dependence of binding was also determined. A Dixon-Webb plot (8) of -log K_I vs pH, shown in Figure 3, indicates decreased binding at low pH and a pK_a of 6 for the ionizing group affecting binding. Within experimental scatter, this data is consistent with Koehler and Hess' conclusion that binding is independent of pH above 6.0.

For comparison, the binding constant and its pH dependence were also determined for the competitive inhibitor N,N,N-trimethylpentanaminium chloride

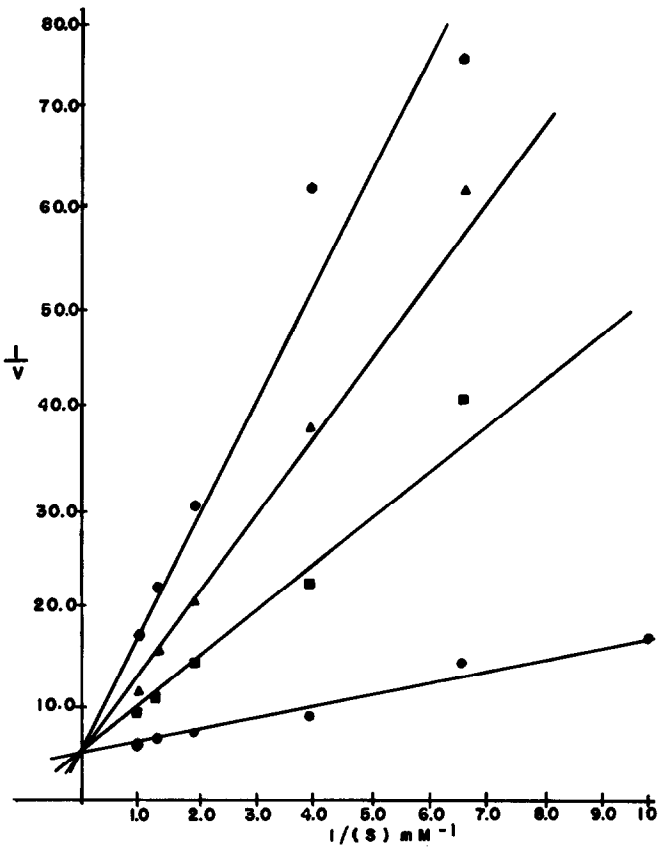

Figure 2. Inhibition of acetylcholine hydrolysis by 4-oxo-N,N,N-trimethylpentanaminium chloride (I); pH 7.5, 25°, 0.1 M NaCl, 0.04 M $MgCl_2$. (●) no inhibitor; (■) 0.04 mM ketone; (▲) 0.08 mM ketone; (●) 0.16 mM ketone.

(pentyltrimethylammonium chloride) (II). Results are plotted in Figure 3. Our value of K_I = 4.0 x 10^{-4} M at pH 7.5 is in reasonable agreement with the value 2.0 x 10^{-4} M found by Wilson and Alexander (9) under slightly different conditions.

Discussion

The simplest and most widely applied criterion for a transition state analog is that it must bind to the enzyme more tightly than does the substrate. The simplest estimate for K_S, the substrate dissociation constant, is K_m = 2.5 x 10^{-4} M under our conditions. However, K_m for acetylcholine hydrolysis

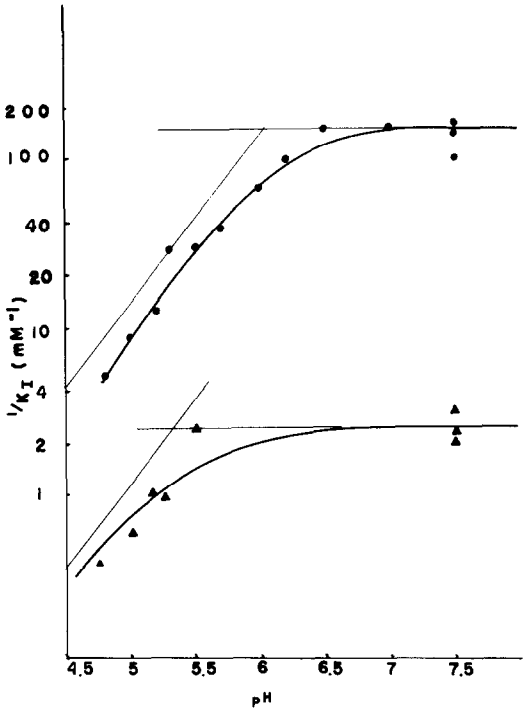

Figure 3. Binding of N,N,N-trimethylpentanaminium bromide (▲) and its 4-oxo-derivative, compound I (●), to acetylcholinesterase as a function of pH. The light lines represent limiting slopes of 0 and 1 on a logarithmic scale; their intersection occurs at the pK_a of the group causing decreased binding.

gives only a lower limit for K_s because the rate-determining step for the reaction is probably hydrolysis of an acetyl-enzyme intermediate (k_3 in Equation 1) (10):

$$E + S \underset{}{\overset{K_s}{\rightleftharpoons}} ES \xrightarrow{k_2} ES' \xrightarrow{k_3} E + P_2 \qquad (1)$$
$$+ P_1$$

K_s may be estimated more accurately by using the observed value of K_m = 1×10^{-3} M for hydrolysis of acetylcholamine (11). In the latter case, acetylation of the enzyme (k_2) is rate-determining and $K_m = K_s$ if the simple scheme of equation 1 applies. K_s may also be estimated as 4.0×10^{-4} M, the observed inhibition constant for N,N,N-trimethylpentanaminium chloride (II). Using the more probable estimate, $K_s \sim 10^{-3}$ M, the analog I binds approximately 120 times

more tightly than acetylcholine and certainly qualifies as a transition state analog.

If ketone I is a transition state analog, the pH dependence of its binding should be predictable from the mechanism for enzymatic hydrolysis of acetylcholamine (The ketone obviously is not a good analog for the rate-determining deacylation observed for acetylcholine). K_{TX}, the transition state binding constant, is given by equation 2,

$$K_{TX} = k_{ne}K_M/k_{cat} \qquad (2)$$

and its pH dependence follows from that of the other parameters (1, 12). In practice, the choice of the most appropriate model rate k_{ne} is not trivial (13). For acetylcholinesterase, the choice is simplified because K_M and k_{cat}/K_M apparently depend primarily on the same ionization. The data of Moore and Hess (11) may be re-plotted using Dixon-Webb plots to give $pK_a \sim 5.85$ for K_M and $pK_a \sim 6.1$ for k_{cat}/K_M for acetylcholamine hydrolysis.

Binding of I is decreased at low pH by ionization of a group of $pK_a = 6.0$, as shown in Figure 3. The interpretation of this observation is complicated by the fact that simple quaternary ammonium compounds also bind less strongly at low pH (14). Accordingly, the pH dependence of the binding of the pentyltrimethylammonium compound II was also determined, as shown in Figure 3. The resulting pK_a of 5.35 is clearly different from the pK_a of 6.0 for the ketopentyl compound I. Thus the binding of I resembles that expected from the enzymatic mechanism, but is different from that of a simple quaternary ammonium compound.

The observation of both tight binding and the expected pH dependence indicates that there is a specific interaction between the ketone carbonyl group and the enzyme active site and strongly suggests that ketone I may be regarded as a transition state analog.

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