Biochimica et Biophysica Acta, 484 (1977) 375—385

BBA 68252

THE MODE OF BINDING OF POTENTIAL TRANSITION-STATE ANALOGS TO ACETYLCHOLINESTERASE

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(Received March 9th, 1977)

Summary

Phenylacetone, 4-phenyl-2-butanone, and 4-oxopentyltrimethylammonium chloride were tested as potential transition state analogs for eel acetylcholinesterase (acetylcholine hydrolase, EC 3.1.1.7). Phenylacetone is a competitive inhibitor of the enzyme but not a transition state analog, since its binding constant is similar to that for the substrate phenyl acetate. 4-Phenyl-2-butanone binds 6—18 times more tightly than the inhibitors 4-phenyl-2-butanol and *N*-benzylacetamide and the substrate benzyl acetate and also blocks inactivation of the enzyme with methanesulfonyl fluoride. However, its binding is independent of pH in the range 5—7.5, whereas both V and V/K_m for benzyl acetate hydrolysis decrease with decreasing pH in this range. These data indicate a specific but weak interaction between the ketone carbonyl and the enzyme, but probably do not justify considering this compound a transition state analog. 4-oxopentyltrimethylammonium iodide has previously been shown to bind about 125 times more strongly than the substrate acetylcholamine. It also binds about 375 times more strongly than the alcohol 4-hydroxypentyltrimethylammonium iodide. Furthermore, the ketone protects the enzyme from inactivation by methansulfonyl fluoride, while the corresponding quaternary ammonium alcohol accelerates this inactivation reaction. This additional information confirms that the ketone is a transition state analog.

Introduction

Studies of potential transition state analogs are becoming increasingly common because such compounds often are very good enzyme inhibitors and may yield information about enzymatic mechanisms [1—3]. To qualify as a transition state analog, an inhibitor with an obvious resemblance to a proposed

transition state must at least bind substantially more tightly than the corresponding substrate.

Earlier work in this laboratory showed that 4-oxopentyltrimethylammonium chloride is a transition state analog for eel acetylcholinesterase (acetylcholine hydrolase, EC 3.1.1.7) since it binds to the enzyme about 125 times more tightly than do the substrates acetylcholine and acetylcholamine [4]. It was also shown that the pH dependence for binding of the ketone is the same as that of k_{cat}/K_m for acetylcholamine hydrolysis [4]. The present work was undertaken in order to determine whether there is a specific interaction between the ketone carbonyl group and the esteratic site. The most plausible such interaction would be attack of the nucleophilic serine hydroxyl group on the ketone carbonyl to form a hemiketal analogous to the tetrahedral intermediate in the hydrolysis of acetylcholine [5].

Uncharged transition state analogs resembling the substrate phenyl acetate were also of interest for two reasons. First, the pH dependence for their binding might not be affected by ionization of the anionic site, thus simplifying interpretation of the results obtained. Second, it has been suggested that the rate-limiting step for phenyl acetate hydrolysis is an induced conformation change [6], and a suitable transition state analog should prove useful in observing this change.

Several criteria might be considered in determining the extent to which an enzyme inhibitor complex resembles the transition state for the normal enzymatic reaction. A good analog should bind tightly not only with respect to the substrate, but also compared to structurally analogous inhibitors with different functional groups. For example, ketones might be compared to alcohols. For enzymes with multiple subsites, the transition state analog must inhibit by binding to that subsite where the enzymatic reaction occurs. In addition, binding should be specific for isomers of the analog resembling good substrates. Finally, the pH dependence for binding of the analog should be predictable from that for reaction of the normal substrate. Accordingly, as many of these criteria as possible have been applied in the present work to both charged and neutral ketones analogous to good substrates.

Materials and Methods

General

All enzyme assays were performed with a Sargent-Welch Recording pH Stat. NMR spectra were determined with a Varian A-60 or a CFT-20, infrared spectra with a Beckman Acculab 2, and ultraviolet spectra with a Beckman ACTA IV. Melting points were determined with a Thomas-Hoover capillary melting point apparatus and are uncorrected. Temperature control for assays was by means of the integral unit in the Sargent-Welch pH Stat. Temperature of reaction with methanesulfonyl fluoride was controlled with a Haake FS water bath. Solutions were prepared with distilled and deionized water. All other chemicals were of reagent grade. Elemental analyses were performed by Galbraith Laboratories, Knoxville, Tennessee.

Acetylcholinesterase from *Electrophorus electricus* (EC 3.1.1.7) was

obtained from Sigma Chemical Co. All batches used had activity of >1000 units/mg at 25°C.

Kinetic methods

Determination of Michaelis-Menten constants and dissociation constants for reversible inhibition. All solutions contained 0.1 M NaCl and 0.04 M $MgCl_2$. All solutions of neutral inhibitors or substrates also contained 1% (v/v) methanol. Separate stock solutions of substrate and inhibitor were mixed with salt solution to give a total volume of 20 ml of solution in the pH Stat reaction vessel. The solution was equilibrated to 25°C and the pH adjusted with 6 mM NaOH or 1 mM HCl. Reaction was initiated by addition of 0.1 ml of acetylcholinesterase in distilled water and monitored by automatic titration with 6 mM NaOH under a stream of N_2. For competitive inhibition studies, velocities were determined at six concentrations of acetylcholine over the range 0.1—1 or 0.2—2 mM in the absence of inhibitor and in the presence of three different inhibitor concentrations. The inhibitor dissociation constants K_I were calculated from double reciprocal plots either as the average of values obtained at each inhibitor concentration or as the intercept of a plot of slope vs. inhibitor concentration. V and K_m for benzyl acetate were determined as above, using assays over the concentration range 1—10 mM. Values of V obtained below pH 5.5 were corrected for ionization of acetic acid. The K_m reported is an average of three values. K_m for phenyl acetate is a single value based on 7 points over the range 0.5—5 mM.

Solubility presented problems for most of the neutral compounds used. The most concentrated stock solutions that could easily be prepared were as follows: 4-phenyl-2-butanone, 10 mM; 4-phenyl-2-butanol, 20 mM; *N*-benzylacetamide, 40 mM; and benzyl acetate, 10 mM.

Reaction of acetylcholinesterase with methanesulfonyl fluoride and protection by reversible inhibitors. All solutions contained 0.1 M NaCl, 0.04 M $MgCl_2$ and 0.001 M NaH_2PO_4 and were adjusted to pH 7.0. One reaction solution contained 0.1—0.2 mM methanesulfonyl fluoride and a reversible inhibitor; the other contained an equal concentration of methanesulfonyl fluoride. A separate control contained only salts and buffer. Typically, 1.9 ml of each solution was equilibrated to 25°C in test tubes immersed in a water bath, and reaction was initiated by adding 0.1 ml of acetylcholinesterase solution (150 units/ml in distilled water) to each tube. 0.1-ml aliquots were removed periodically from each tube and acetylcholinesterase assayed on the pH Stat as described above, with 1 mM acetylcholine as substrate. Runs were limited to about 100 min after preparation of methanesulfonyl fluoride solution since this reagent was found to hydrolyze slowly, with 10% reaction occurring over about 134 min under these conditions. The control containing only enzyme and buffer generally showed little or no loss of activity over this time period. Some loss of activity was observed with one batch of enzyme and was applied as a correction to data for inactivation by methanesulfonyl fluoride. For the solutions containing methanesulfonyl fluoride, plots of log enzyme activity vs. time gave excellent first order lines. First order rate constants were obtained graphically, and second order rate constants were calculated based on the known concentration of methanesulfonyl fluoride.

The reaction between enzyme and methanesulfonyl fluoride consistently had a rate constant within about 10% of 2.5 $M^{-1} \cdot s^{-1}$, in agreement with the value reported by Kitz and Wilson under slightly different conditions [7]. For all reversible inhibitors except 4-hydroxypentyltrimethylammonium iodide, the rate was slower in the presence of the inhibitor. The dissociation constant for 4-oxopentyltrimethylammonium was obtained as described in the Results. Because of their limited solubility, inhibition constants for 4-phenyl-2-butanone and 4-phenyl-2-butanol were obtained over a very limited concentration range. Constants were calculated for each run by comparing the rate constants k in the presence of reversible inhibitor I and k_0 in its absence according to the equation:

$$K_I = [I]/((k_0/k) - 1)$$

The value for the ketone is an average of K_I values from fifteen runs; the value for the alcohol is an average from eight runs.

N-Benzylacetamide as a Potential Substrate. In a typical experiment, a solution was prepared containing 40 mM *N*-benzylacetamide and 150 units of enzyme/ml dissolved in 0.1 M NaCl, 0.04 $MgCl_2$, and 0.001 M NaH_2PO_4 and adjusted to pH 7.5. 0.10-ml aliquots were pipetted into test tubes and incubated at 25°C in a water bath. Reaction was quenched in individual tubes by addition of 1.0 ml of ninhydrin solution [8] at various intervals, color was developed by heating in boiling water for 20 min, and the absorbance of the solution in each tube was measured at 570 nm. The absorbance increased from 0.577 to 0.747 over a period of 8 h and showed little change over the next 16 h. A blank containing 5 mM benzylamine had an absorbance of 1.14 after 10 : 1 dilution, indicating that no appreciable amount of benzylamine was present in any of the reaction vessels. Other experiments under very similar conditions showed no consistent increase in absorbance at all.

Syntheses

N-Benzylacetamide. Water (60 ml) and benzylamine (15 g = 0.14 mol) were mixed in a 250 ml round-bottom flask equipped with reflux condenser, dropping funnel, and magnetic stirrer. Acetic anhydride (29.6 g = 0.25 mol) was added dropwise with stirring. The mixture became hot and was allowed to reflux for 30 min, then cooled and extracted with ether. The ether layer was washed with dilute NaOH and dried over $MgSO_4$. After filtering, cyclohexane was added to induce crystallization. The product was obtained as white needles with m.p. 60.5—61°C (lit. 57—58°C) [9]. Analysis: calculated for $C_9H_{11}NO$: C, 72.69%, H. 7.43%. Found: C, 72.69%; H, 7.26%.

4-Oxopentyldimethylamine. 9 g (0.075 mol) of pure 5-chloro-2-pentanone was mixed with 25 ml of 40% aqueous dimethylamine (0.225 mol) in a glass tube and sealed in a pressure bomb for 48 h. The resulting solution was saturated with NaCl and extracted 6 × 100 ml with ether. The ether solution was dried with $MgSO_4$, decanted, and aspirated to remove the bulk of the ether. The product was obtained as a colorless oil by distillation at aspirator pressure.

4-Hydroxypentyltrimethylammonium Iodide. The neutral amino ketone above (3.5 g = 0.026 mol), was reduced by adding it dropwise to a continuously stirred solution of 0.98 g (0.026 mol) of $NaBH_4$ in 95% ethanol (approxi-

mately 30 ml). After stirring 1 h in an ice bath, 10 ml of concentrated HCl, diluted to 50 ml, were added dropwise. 50 ml of 4.3 M NaOH was then added to make the solution basic to litmus. The solution was saturated with NaCl and extracted 6 × 100 ml with ether. The ether extract was dried with $MgSO_4$, aspirated to remove the ether, and then distilled to yield 1.3 g (0.00992 mol) of the neutral amino alcohol. This alcohol was then methylated with stirring at 0°C by the dropwise addition of an equivalent amount of CH_3I (1.41 g dissolved in 5 ml of ether. After stirring for 1 h at 0°C, the white precipitate was filtered and dried to give 0.65 g (0.0024 mol) of hygroscopic white crystals. m.p. 102—103°C. Infrared (KBr pellet): strong band 3440 cm^{-1}, no band at 1600—1800 cm^{-1}. NMR: (^2H_2O/ext. TMS) 1.2 δ(d, 3H, CH_3), 1.7 δ(m, 2H, CH_2), 3.2 δ(s, 9H, NMe_3), 3.4 δ(m, 2H, NCH_2), 4.0 δ(m, 1H, CH).

4-Phenyl-2-Butanol. In 45 ml of 95% ethanol were dissolved 8.0 g (0.054 mol)4-phenyl-2-butanone. Sodium borohydride (6.4 g = 0.17 mol) was added slowly to the solution with stirring. Approximately 0.5 h after the addition of the sodium borohydride, 100 ml distilled H_2O and 10 ml of 10% HCl were added to the solution. After evolution of H_2 ceased, the solution was heated to boiling and then cooled in an ice bath. The cool solution was extracted twice with 45-ml portions of ether. The ether extracts were combined and dried over anhydrous $MgSO_4$. The dry solution was then distilled, with the fraction from 135—140°C being identified as 4-phenyl-2-butanol. The yield was 5.6 g NMR ($CDCl_3$/TMS) 1.2 δ(d, 3H, J = 6Hz), 1.7 δ(m, 2H), 2.25 δ(s, 1H), 2.7 δ(m, 2H), 3.8 δ(sextet, 1H, J = 6Hz), 7.25 δ(m, 5H). Analysis calculated for $C_{10}H_{14}O$: C, 79.95%; H, 9.39%. Found: C, 80.17%; H, 9.59%.

3-*Oxopentyltrimethylammonium Iodide.* Sealed in a glass-lined steel bomb were 12 g (0.1 mol) of 1-chloro-3-pentanone and 60 g (0.15 mol) of 40% aqueous dimethylamine. After 72 h the solution was saturated with sodium chloride and extracted 5 times with 100 ml portions of ether. The combined extracts were dried over $MgSO_4$ for 24 h and filtered, and ether was stripped off with an aspirator. Vacuum distillation gave 6.5 g of colorless oil collected over the range 30—50°C at approx. 1 Torr. NMR (neat/ext. TMS): 0.7 δ(t); 1.8 δ(s); 2.1 δ(m); 2.25 δ(s).

The product above (0.05 mol) was dissolved in 40 ml of anhydrous ether and 18.2 g (0.128 mol) of methyl iodide in 10 ml of ether was added dropwise with magnetic stirring for 1 h. The solution was cooled in solid CO_2/acetone bath during addition, then allowed to warm very gradually to room temperature as described by Ing, Kordik and Tudor Williams [10]. White needles formed as the solution warmed. These needles were filtered, washed with ether, re-crystallized twice from absolute ethanol and dried under vacuum. Yield was 12.6 g or 93% of material with m.p. = 144—146°C (lit. m.p. = 146°C) [10]. Analysis: calculated for $C_8H_{18}NOI$: C, 35,44%; H, 6.69. Found: C, 34.56%; H, 6.58%. NMR: (^2H_2O/ext. TMS): 1.05 δ(t, 3H), 2.7 δ(quartet, 2H), 2.9 δ(s); 3.2 δ(s), 3.65 δ(m, 4H).

Addition of Na_2CO_3 to the n.m.r. tube caused a downfield shift of the peak at 2.9 δ, suggesting that this peak represents hydroiodide rather than a quaternary ammonium compound. Addition of trimethylamine hydrochloride to another sample confirmed its identity as trimethylammonium.

A number of purification attempts failed to remove this peak from the

NMR. Included were multiple recrystallization from ethanol, recrystallization from chloroform, and titration with Amberlyst-21. Titration with NaOH led to extensive decomposition.

During one of these experiments, a slight change with time in the ratio of the peaks at 2.9 and 3.2 δ was noted, suggesting that the trimethylammonium might be formed by a reverse Michael reaction approaching equilibrium. The presence of such an equilibrium was confirmed by determining the ratio of these peaks as a function of concentration using a Varian CFT-20 NMR. Ratios obtained (product/trimethylammonium) at various concentrations (M) were: 3.2 at 0.073 M; 6.3 at 0.22 M; and 11.8 at 0.375 M. Both experiments at lower concentrations give dissociation constants for the quaternary ammonium compound of about 5 mM; the experiment at highest concentration shows somewhat less dissociation. A small multiplet at approx. 6.0 δ was also observed, confirming the presence of some alkene. Addition of trimethylammonium chloride to one sample decreased the size of these peaks.

Results

Several compounds structurally related to phenyl acetate, benzyl acetate, and acetylcholine were found to be competitive inhibitors of acetylcholine hydrolysis by acetylcholinesterase. Inhibition constants determined from double reciprocal plots are shown in Table I. 4-phenyl-2-butanone also caused slight non-competitive inhibition with K_I 10 mM, but an accurate value could not be obtained because of limited inhibitor solubility.

Benzyl acetate was found to be a good substrate, with K_m = 9 mM. V is 73% of the value observed for acetylcholine under identical conditions. Phenyl acetate was found to hydrolyze with a K_m of 2 mM under our conditions.

The ability of several of these inhibitors to protect the enzyme against methanesulfonyl fluoride was also demonstrated, since such protection implies that the inhibitor binds near the esteratic site of acetylcholinesterase. The dissociation constant K_I for binding of 4-oxopentyltrimethylammonium chloride was determined by plotting the observed second order rate constant k_2 for methanesulfonyl fluoride inactivation according to eqn. 1,

$$\frac{1}{k_2} = \frac{1}{k_0 K_I}[I] + \frac{1}{k_0} \quad (1)$$

where I represents ketone concentration and k_0 is the rate in the absence of ketone. This equation is readily derived based on observations of Kitz and Wilson [7] that methanesulfonyl fluoride does not bind measurably to the enzyme. A good straight line was obtained with ketone concentrations covering the range 0.02—0.16 mM. Dissociation constants obtained for several compounds using methanesulfonyl fluoride are included in Table I.

4-hydroxypentyltrimethylammonium iodide accelerates the reaction between enzyme and methanesulfonyl fluoride. The ratios k_2/k_0 of rates with and without accelerator were 1.51 at 4 mM and 2.18 at 5 mM accelerator in two experiments. Similar behavior is typical of quaternary ammonium compounds such as pentyltrimethylammonium bromide, and has been attributed to

TABLE I

COMPETITIVE INHIBITORS OF ACETYLCHOLINESTERASE

Binding constants determined by inhibition of acetylcholine hydrolysis (ACh) or by ability to block irreversible inactivation by methanesulfonyl fluoride (MSF).

Compound	K_I (mM), ACh	K_I (mM), MSF
$PhCH_2CH_2\overset{O}{\overset{\|}{C}}CH_3$	2	6
$PhCH_2CH_2\overset{OH}{\overset{\|}{C}}HCH_3$	36	28
$PhCH_2NH\overset{O}{\overset{\|}{C}}CH_3$	13	—
$PhCH_2\overset{O}{\overset{\|}{C}}CH_2CH_3$	5	—
$PhCH_2\overset{O}{\overset{\|}{C}}CH_3$	5	—
$PhNH\overset{O}{\overset{\|}{C}}CH_3$	11	—
$PhCH_2CH_2\overset{O}{\overset{\|}{C}}—H$	6	—
$CH_3\overset{O}{\overset{\|}{C}}CH_2CH_2CH_2\overset{+}{N}(CH_3)_3Cl^-$	0.008 *	0.020
$CH_3\overset{OH}{\overset{\|}{C}}HCH_2CH_2CH_2\overset{+}{N}(CH_3)_3I^-$	3	—

* From Ref. 4.

a change in conformation of the enzyme induced by the accelerator [11]. Insufficient material was available to obtain the saturating concentration needed to determine a dissociation constant for the accelerator.

pH dependences for binding of 4-phenyl-2-butanone, and N-benzylacetamide and hydrolysis of benzyl acetate were also determined in order to clarify the mode of interaction of the ketone with the catalytic functional groups of the enzyme. The Dixon-Webb plot shown in Fig. 1 illustrates the results obtained for benzyl acetate hydrolysis. Both V and V/K_m decrease at low pH, depending on ionizations of the enzyme with apparent pK_a values of 6.25 and 5.6 respectively. Similar pH dependences are observed for other neutral substrates of the enzyme [6]. The pH dependences for competitive inhibition by 4-phenyl-2-butanone and by benzylacetamide are shown in Fig. 2. Binding of the ketone is clearly independent of pH over the range pH 5—7.5. Binding of *N*-benzylacetamide appears to be slightly tighter at low pH, but the observed change with pH is much less than the scatter in the measurements at the 95% confidence level. Considerable scatter was observed with both compounds, probably because of their limited solubility. It is clear, however, that the two inhibitors have a similar pH dependence, and that both dependences are different from that for benzyl acetate hydrolysis. Benzylacetamide was also tested as a poten-

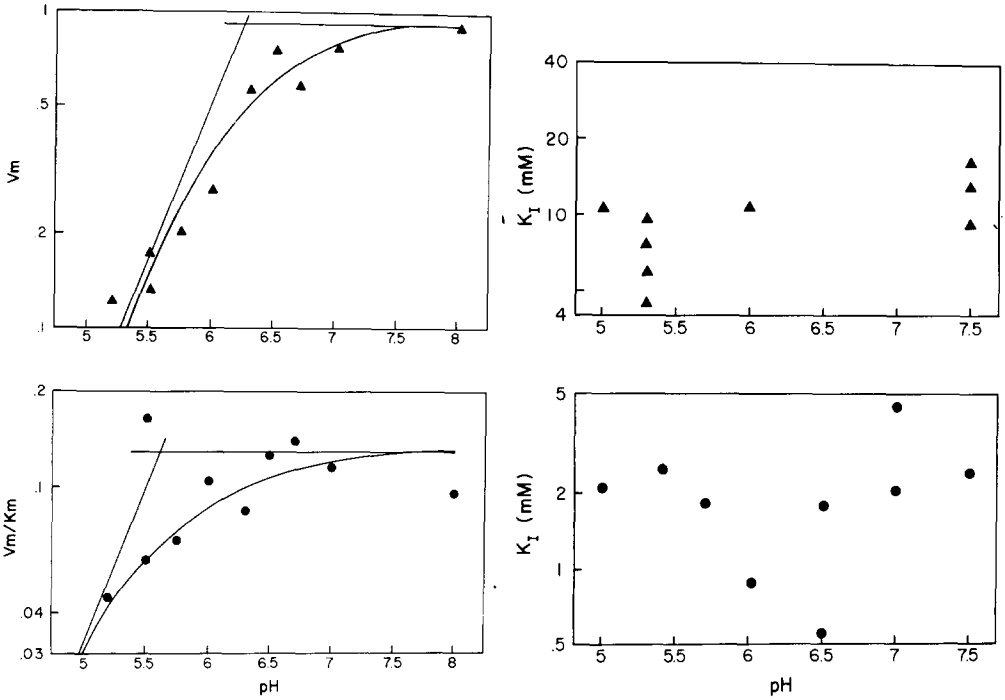

Fig. 1. Hydrolysis of benzyl acetate by acetylcholinesterase as a function of pH. All runs in 0.1 M NaCl, 0.04 M $MgCl_2$, 1% (v/v) methanol at 25° C. Velocities are in arbitrary units.

Fig. 2. Competitive Inhibition of acetylcholinesterase by *N*-benzylacetamide (▲) and 4-phenyl-2-butanone (●) as a function of pH. Conditions as for Fig. 1.

tial substrate. Very little or no hydrolysis occurred even with high concentrations of enzyme and benzylacetamide over 8 h; consequently, enzyme-catalyzed hydrolysis is not a complicating factor in the competitive inhibition experiments described above.

Discussion

The fundamental criterion determining whether an inhibitor with a clear structural resemblance to the transition state may be regarded as a transition state analog is whether it binds to the enzyme more tightly than does the substrate. Based on this criterion, 4-oxopentyltrimethylammonium qualifies as such an analog, binding to acetylcholinesterase approximately 125 times more tightly than acetylcholamine or acetylcholine. The neutral ketone phenylacetone was next tested because of its obvious resemblance to the known good substrate phenyl acetate. Although phenylacetone is a good inhibitor with K_I = 5 mM, the K_m for phenyl acetate hydrolysis under our conditions is 2 mM. Since acylation of the enzyme is not rate-determining for phenyl acetate, K_m does not equal the true dissociation constant K_s [5]. In order to estimate this constant more accurately, acetanilide was considered as a substrate analog. It was found to be too poor a substrate for quantitative investigations, but was

shown to be a competitive inhibitor of acetylcholine hydrolysis with K_I = 11 mM. Thus the ketone phenylacetone probably binds slightly more tightly than does the substrate, but any additional binding interaction between the ketone carbonyl and the enzyme must be very weak and phenylacetone is not a promising transition state analog.

4-Phenyl-2-butanone was also shown to be a competitive inhibitor of acetylcholinesterase with K_I = 2 mM, and benzyl acetate was found to be a good substrate with K_m = 9 mM. *N*-benzylacetamide, used as a substrate analog, was a competitive inhibitor with K_I = 13 mM. Although the increased binding of the ketone relative to substrate is not as great as that often associated with transition state analogs, a definite attractive interaction involving the ketone occurs.

Other criteria are clearly desirable to clarify the mode of binding of a potential transition state analog. In the present case, it is important to show that the increased binding is specific to the ketone carbonyl which presumably forms a hemiketal with the enzyme. Consequently, the analogous alcohols 4-hydroxypentyltrimethylammonium iodide and 4-phenyl-2-butanol were prepared and examined as inhibitors. Both compounds are competitive inhibitors of acetylcholinesterase at pH 7.5. The quaternary ammonium ketone binds 375 times more tightly than the corresponding alcohol, and the neutral ketone binds 18 times more tightly. Thus the tight binding observed requires the ketone carbonyl rather than simply a polar functional group.

A third criterion is the ability of the proposed transition state analogs to protect the enzyme against irreversible inactivation by reaction of methanesulfonyl fluoride with the nucleophilic serine hydroxyl group. This test is particularly useful for the quaternary ammonium compound, since similar compounds such as pentyltrimethylammonium actually accelerate inactivation of the enzyme by methanesulfonyl fluoride [11]. Both the charged and neutral ketones were shown to protect against metanesulfonyl fluoride, as did the neutral alcohol. Binding constants for these compounds as measured by protection against methanesulfonyl fluoride are given in Table I. For both ketones, the binding constants are similar to those measured by inhibition of acetylcholine hydrolysis. The differences observed are probably real, and may indicate binding in more than one mode, but it is clear that both ketones bind strongly in such a manner as to protect the active serine from inactivation. Such protection may indicate either hemiketal formation or simply binding in the esteratic site. This observation is particularly important for the charged ketone; since pentyltrimethylammonium and the corresponding alcohol accelerate reaction of this site, protection by the ketone must indicate a highly specific interaction.

An additional probe used with the neutral ketones was specificity. If the neutral ketone inhibits by forming a hemiketal resembling the transition state, then minor changes in structure of the ketone should affect binding. The isomeric ketone 1-phenyl-2-butanone was found to bind 2.5 times less tightly than 4-phenyl-2-butanone. Although aldehydes have been used as transition state analogs for serine proteases, 3-phenylpropanal binds about 3 times less tightly than the neutral ketone. These effects are not large, but they are in the expected direction and indicate some specificity for the proposed analog. An attempt to demonstrate a similar specificity for the charged ketones was

unsuccessful, since the isomer 3-oxopentyltrimethylammonium iodide was found to form an equilibrium mixture with ethyl vinyl ketone and trimethylammonium in aqueous solution. A number of studies have found marked differences in pharmacological effects of the 3 and 4 keto isomers: these differences may well result from almost complete dissociation of the 3 isomer at low concentrations [10,12,13].

A final criterion sometimes applied is the pH dependence for binding of an inhibitor. We have shown earlier that binding of the charged ketone has a pH dependence similar to that of k_{cat}/K_m for acetylcholamine hydrolysis [4]. In contrast, binding of the neutral ketone is independent of pH, whereas both k_{cat} and k_{cat}/K_m for benzyl acetate hydrolysis are pH-dependent, as shown in Figs. 1 and 2. pH dependence for binding of the ketone more closely resembles that for binding of the substrate analog *N*-benzylacetamide. The theoretical pH dependence for a transition state analog depends on that of the non-enzymatic model reaction chosen as well as on k_{cat}/K_m for the enzyme, but the choice of a suitable model reaction is not clear-cut [3]. On a simpler level, the decrease of k_{cat}/K_m for benzyl acetate with pH indicates that only the form of the enzyme predominant at pH 7.5 can form the transition state. In contrast, the neutral and protonated forms of the enzyme must interact equally strongly with the ketone to account for the observed pH independence. Clearly, the enzyme-inhibitor complex is not an exact analog of the transition state. As Jencks has pointed out, such an exact match is unlikely for enzymes such as chymotrypsin where the transition state presumably is an unstable zwitterion rather than a neutral tetrahedral adduct resembling a hemiacetal [3]. The same conclusion should hold for acetylcholinesterase insofar as it is mechanistically similar to chymotrypsin. On the other hand, it is important to note that the equilibrium between a hemiketal and a ketone bound near a serine hydroxyl is independent of pH. Thus the observed lack of pH dependence for ketone binding does not rule out formation of a hemiketal; it only implies that protonation of the basic catalytic group in the enzyme does not affect this equilibrium, as would be expected.

In summary, several kinds of evidence support the conclusion that 4-oxopentyltrimethylammonium chloride may be regarded as a transition state analog. As shown earlier [4], it binds tightly and with a pH dependence different from that of a normal quaternary ammonium compound. It also binds much more tightly than the corresponding alcohol and binds specifically in the esteratic site. The evidence that 4-phenyl-2-butanone is a transition state analog is much more equivocal. It binds only somewhat more tightly than the substrate, a substrate analog, or the corresponding alcohol, but it does bind in or near the esteratic site. Similarly, only a modest specificity is observed compared to other ketones or aldehydes. The pH dependence rules out a precise analogy to the transition state, but does not exclude hemiketal formation. The bulk of the evidence would seem to favor a weak but specific interaction between the carbonyl of 4-phenyl-2-butanone and some feature of the active site. This interaction may be too weak for the ketone to be considered a transition state analog, but the compound should still prove useful for probing the mechanism of acetylcholinesterase.

The contrast between charged and neutral analogs has possible mechanistic

implications. The charged ketone is a model for acetylcholamine hydrolysis, in which the rate-determining step is presumably attack on an amide carbonyl [14]. On the other hand, Rosenberry has suggested that the rate-determining step in phenyl acetate hydrolysis is an induced conformation change (6), based to a considerable extent on the observation that V and V/K_m show different pH dependences. Data presented here for benzyl acetate hydrolysis would lead to a similar conclusion. Hemiketal formation from a ketone would not be an appropriate model for a rate-limiting conformation change. Thus, phenyl acetone and 4-phenyl-2-butanone would not be expected to be transition state analogs, although phenyl acetate is as good a substrate as acetylcholine (and far better than acetylcholamine). Other explanations for the weakness of the specific interactions between neutral aromatic ketones and acetylcholinesterase are also possible, but the explanation provided here correlates very well with a possible mechanism and illustrates nicely the uses of transition state analogs as mechanistic probes.

Acknowledgments

Acknowledgment is made to the Donors of the Petroleum Research Fund, administered by the American Chemical Society, and to the Faculty Research Committee, Bowling Green State University, for support of this research. We are grateful to Dr. Larry Byers for helpful discussions.

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