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ALIPHATIC KETONES ARE ACETYLCHOLINESTERASE INHIBITORS BUT NOT TRANSITION STATE ANALOGS

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Summary

A number of C_4—C_9 aliphatic ketones are acetylcholinesterase (acetylcholine hydrolase, EC 3.1.1.7) inhibitors, with K_i values in the 0.7-5 mM range. Comparison to analogous substrates would suggest that these ketones are transition state analogs; e.g. 2-pentanone binds to the enzyme approx. 550 times more tightly than ethylacetate. However, a number of other criteria contradict this conclusions: (1) the binding is insensitive to ketone structure: isomeric ketones, cycloalkanones, and sterically hindered ketones have similar inhibitory potencies. (2) Analogous alcohols are also good inhibitors even though they cannot form hemiketals with the enzyme. (3) Representative ketones are relatively ineffective at blocking inactivation of the enzyme by methylsulfonyl fluoride, indicating that ketones do not bind principally at the hydrolytic site. (4) A competition experiment shows that binding of tetramethylammonium chloride excludes binding of 2-pentanone, suggesting that ketones bind to the anionic rather than the hydrolytic site. Thus, observation of tight binding relative to a substrate is not a sufficient criterion to establish that an inhibitor is a transition state analog.

Introduction

Transition state analogs are inhibitors which bind tightly to an enzyme because they resemble the substrate portion of the transition state for the enzyme-catalyzed reaction. Such analogs have now been reported for a considerable number of enzymes [1]. These inhibitors are potentially useful both

because of their specificity and potency and as tools to probe enzymatic mechanisms.

In earlier work, ketones were investigated as possible transition state analogs for acetylcholinesterase (acetylcholine hydrolase, EC 3.1.1.7) [2,3]. The first step in the mechanism of acetylcholinesterase-catalyzed hydrolysis of esters is nucleophilic attack of an enzymatic serine hydroxyl group on the ester carbonyl group to form a tetrahedral intermediate [4]; nucleophilic attack on a corresponding ketone should yield a hemiketal analogous in some respects to this intermediate. The ketone analogous to acetylcholine was shown to be a good transition state analog, binding about 125 times more tightly than the reference substrate acetylcholamine. The ketone corresponding to benzylacetate interacted somewhat more weakly but still specifically with the enzyme [2,3]. In both cases, the mode of binding was further examined by determining its pH dependence, by comparison to binding of the corresponding alcohol, and by demonstrating that the ketones protect against methylsulfonyl fluoride inactivation.

Even simpler aliphatic ketones were also of interest. Ethylacetate is a known substrate [5], although it binds very weakly, and acetone has been briefly reported to be an inhibitor [6]. Thus, larger ketones more closely resembling ethylacetate might be good inhibitors and transition state analogs. In addition, if induced fit is an important feature of the enzymatic mechanism for acetylcholinesterase [7], ions such as tetramethylammonium might enhance the binding of ketones, leading to highly synergistic inhibition.

The present work had two specific goals: to survey the inhibition of acetylcholinesterase by simple ketones, and to determine whether these ketones are transition state analogs.

Materials and Methods

General. Acetylcholinesterase from *Electrophorus electricus* was obtained from Sigma Chemical Co. All batches used had activity of greater than 1000 units/mg at 25° C. All ketones and alcohols used were of reagent grade, and most were redistilled before use. NMR spectra were determined with a Varian A-60 and infrared spectra with a Beckman Acculab 2. Melting points were determined with a Thomas-Hoover capillary melting point apparatus and are uncorrected.

Kinetic methods. The enzyme was assayed using a Radiometer pH Stat system based on a TTT-60 control module, ABU-11 titrator, PHM 62 meter, and SBR 3 recorder. Temperature was controlled with a Haake FS circulating water bath. All experiments were run at pH 7.5 and 25° C, in solution containing 0.1 M NaCl and 0.04 M $MgCl_2$, unless otherwise noted. No organic cosolvent was used. The ketones larger than C_5 were used at concentrations near their limits of solubility (5—20 mM). Details of the determination of inhibition constants and rates of methylsulfonyl fluoride inactivation were described previously [3]. Velocities were determined at 6—8 acetylcholine concentrations for each of 1—3 inhibitor concentrations. Data for competitive inhibition constants were analyzed using the least-squares procedure of Cleland [8] and a Wang Model 2272 computer. To test for irreversible inhibition by acetone, a

1 ml solution containing 0.1 M NaCl, 0.04 M $MgCl_2$, 0.001 M NaH_2PO_4, 0.72 M acetone, and 12 units/ml enzyme at pH 7.7, was maintained at 25°C. Aliquots (0.1 ml) were removed periodically and assayed using 1 mM acetylcholine as substrate. Michaelis-Menten parameters for hydrolysis of ethylacetate were determined in the presence of 0.1 M NaCl and 0.04 M $MgCl_2$ at pH 7.5, on the pH Stat. Parameters were obtained from a double-reciprocal plot of velocities measured at 0.07—0.67 M ethylacetate.

Preparation of 6-methyl-2-heptanone. A solution of 10.5 g (0.0353 mol) $Na_2Cr_2O_7 \cdot 2H_2O$ in 7.8 ml conc. H_2SO_4 and 44 ml H_2O was added dropwise to 8.7 g (0.075 mol) 6-methyl-2-heptanol and 21.8 ml H_2O in a 500 ml Erlenmeyer flask. The temperature of the reaction mixture was held at 55—60°C by occasional cooling in an ice bath. The reaction was allowed to continue for 15 min after the temperature began to drop spontaneously. The mixture was cooled to 25°C and extracted with three times 20 ml benzene. The benzene extracts were washed with water, dried over $MgSO_4$, and vacuum-distilled through a Vigreaux column. 6-Methyl-2-heptanone was collected at 55°C/87 Torr. Infrared (film): 1710 cm^{-1} (C=O), no band above 3000 cm^{-1}. NMR (C^2HCl_3/TMS): 0.86 δ (d, 6H); 1.0—1.9 δ (m, 5H); 2.15 δ (s, 3H); 2.45 δ (t, 2H). DNP derivative, m.p. 77.5—78.5°C (literature: 77°C) [9].

Results

A large number of simple aliphatic ketones were found to be competitive inhibitors of eel acetylcholinesterase, mostly with dissociation constants in the 1—5 mM range. These results are presented in Table I. Fig. 1 summarizes observed dissociation constants in terms of number of carbon atoms in the inhibitor. A general correlation between molecular size and dissociation constant is apparent, with heptanones being the best inhibitors. The compounds studied may be subdivided into several structural classes, including straight-chain 2-alkanones, straight-chain 3- and 4-alkanones, and branched-chain alkanones. Similar trends in dissociation constant as a function of size are apparent in both series of straight-chain ketones. Branched alkanones show a similar pattern as a function of overall size (number of carbon atoms) but more

TABLE I

DISSOCIATION CONSTANTS OF ALIPHATIC KETONE AND ALCOHOL INHIBITORS

Compound	K_I (mM)	Compound	K_I (mM)
Acetone	35 (79) *	2,5-Hexanedione	4.1
Butanone	5.0	2-Heptanone	1.3
3-Methyl-2-butanone	1.8	4-Heptanone	0.71 (3.2) *
2-Pentanone	1.8 (8.1) *	6-Methyl-2-heptanone	2.8
3-Pentanone	1.5	2-Octanone	3.8
Cyclopentanone	5.0	3-Octanone	1.9
2,4-Dimethyl-3-pentanone	0.86	2-Nonanone	4.8
2,2,4,4-Tetramethyl-3-pentanone	2.6		
2-Hexanone	2.2		
3-Hexanone	0.90		
Cyclohexanone	2.7		

* K_I values for corresponding alcohols.

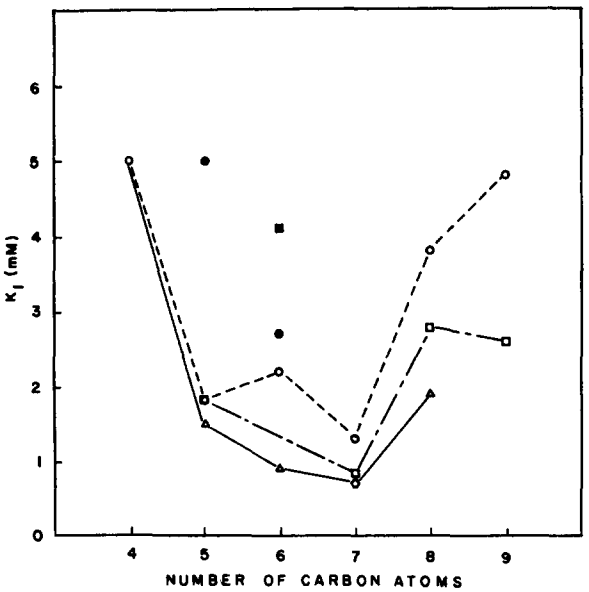

Fig. 1. Dissociation constants for aliphatic ketone acetylcholinesterase inhibitors as a function of number of carbon atoms in the inhibitor. ○, 2-substituted straight-chain alkanones; △, 3-substituted straight-chain alkanones; ◎, 4-heptanone; □, branched-chain alkanones; ●, cycloalkanones; ■, 2,5-hexanedione.

variation when dissociation constants are plotted against the length of the main chain. The cycloalkanones and 2,5-hexanedione are somewhat poorer inhibitors.

Other approaches were also desirable to elucidate the mode of binding of these ketones. Comparison to the corresponding alcohols is a simple method of determining whether the ketone carbonyl group is necessary for binding. Acetone, 2-pentanone, and 4-heptanone were picked as typical of the ketones tested; dissociation constants for the corresponding alcohols are also given in Table I. All alcohols tested are competitive inhibitors, binding 2—5 times less strongly than the corresponding ketones. For comparison, alcohols corresponding to the probable transition state analogs 4-oxo-*N*,*N*,*N*-trimethylpentanaminium chloride and 4-phenyl-2-butanone bind 375 and 18 times, respectively, less tightly than the corresponding ketones [3].

The binding site for aliphatic ketones can be further localized using reagents which interact with only one subsite of the active site. One such reagent is methylsulfonyl fluoride, which apparently reacts with the nucleophilic hydroxyl group in the esteratic site and irreversibly inactivates the enzyme [10]. A dissociation constant for reversible binding of a ketone at the esteratic site can be measured by the ability of the ketone to block this irreversible inactivation by methylsulfonyl fluoride [3]. A typical experiment illustrating protection by 4-heptanone is shown in Fig. 2. Dissociation constants for acetone and 4-heptanone measured by protection against methylsulfonyl fluoride are 2.3 M and 21 mM, respectively. Protection of the enzyme by 2-pentanone is barely discernible at its limit of solubility (approx. 0.1 M). Although an accurately reproducible dissociation constant could not be

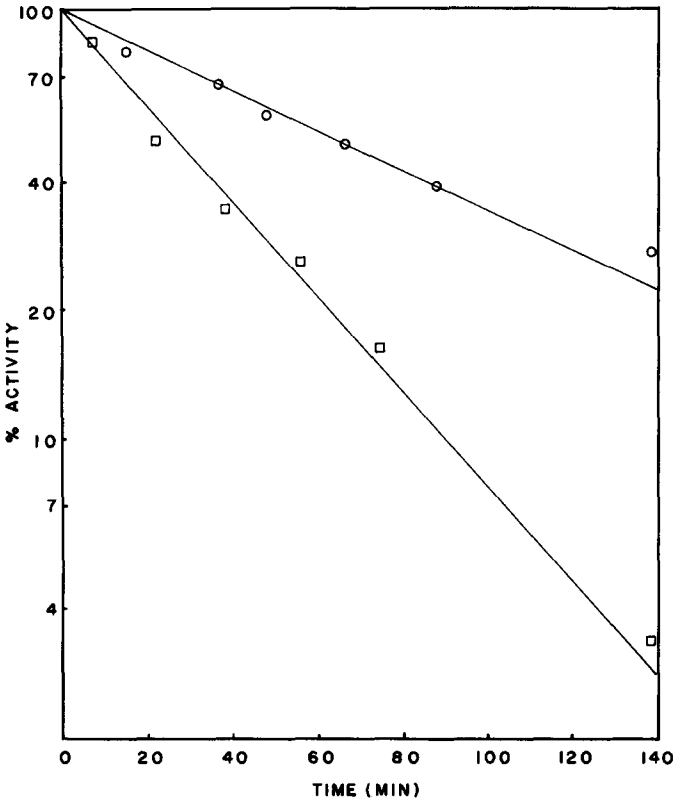

Fig. 2. Protection of acetylcholinesterase against methylsulfonyl fluoride by 4-heptanone. Remaining enzyme activity in solutions containing 0.2 mM methylsulfonyl fluoride is shown as a function of time in the presence (○) and absence (□) of 12 mM 4-heptanone. For this experiment, second-order rate constants were 0.93 and 2.1 $M^{-1} \cdot min^{-1}$, respectively.

obtained, a lower limit of 0.3 M was determined. The three ketones tested are poorer inhibitors of methylsulfonyl fluoride inactivation than of acetylcholine hydrolysis (Table I) by factors of 30—170. In contrast, the ketones studied earlier differ only by factors of 2 and 3, respectively, in ability to block the two reactions [3].

The results above are consistent with binding of simple ketones in the anionic subsite normally occupied by the quaternary ammonium group of acetylcholine, or in a peripheral subsite. Since such competitive inhibitors as tetramethylammonium ion apparently bind at the anionic subsite [4,11], the combined effects of tetramethylammonium chloride and 2-pentanone on acetylcholine hydrolysis were examined. 2-Pentanone was selected since it bears the closest structural resemblance to the substrate ethylacetate. The results are presented in Fig. 3 in the form of a Yonetani-Theorell plot [12]. A series of essentially parallel lines is obtained, indicating that binding of either inhibitor excludes binding of the other. The line at 12 mM tetramethylammonium chloride does appear to converge slightly with the other lines. This apparent convergence most likely results from experimental scatter, since

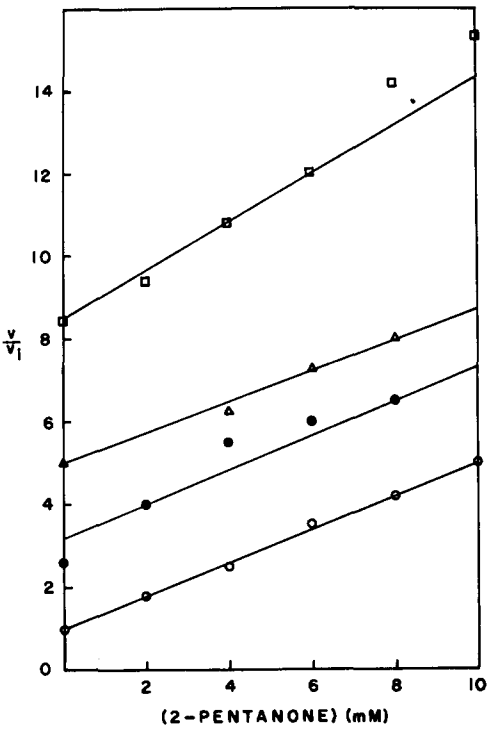

Fig. 3. Combined effects of 2-pentanone and tetramethylammonium chloride on acetylcholinesterase. The ratio of initial velocities for acetylcholine hydrolysis in the absence (v) and presence (v_i) of inhibitors is plotted as a function of 2-pentanone concentration at the following concentrations of tetramethylammonium ions. ○, 0 mM; ●, 3 mM; △, 6 mM; □, 12 mM.

velocities at this concentration are very slow and difficult to measure accurately, no convergence occurs at lower inhibitor concentrations, and no convergence was observed in an earlier experiment over the range 0—9 mM tetramethylammonium chloride. Extrapolation of the 0 and 12-mM lines leads to a dissociation constant of about 40 mM for 2-pentanone from the ternary complex compared to a K_i of 1.8 mM for simple competitive inhibition. Thus binding of tetramethylammonium ion and 2-pentanone are mutually exclusive (or very nearly so).

Since acetone was used at fairly high concentrations, it was necessary to show that the observed inhibition did not result from denaturation or irreversible inhibition. The enzyme was incubated with 0.72 M acetone and aliquots removed and assayed periodically. A slight loss of activity was observed within 10 min after mixing, but no further loss over 43 min.

V and K_m for ethylacetate hydrolysis were also redetermined under our conditions. From two runs, K_m = 1.0 M and V is 50% of V for acetylcholine.

Discussion

In order to be considered a transition state analog, an appropriate competitive inhibitor must bind to the enzyme substantially more tightly than the com-

parable substrate [1]. The best comparison among the ketones studied is between 2-pentanone and ethylacetate, since 2-pentanone could form a hemiketal with the active site serine hydroxyl group of the enzyme closely analogous to the transition state for ethylacetate hydrolysis. 2-Pentanone binds to the enzyme approx. 550 times more tightly than ethylacetate, and certainly qualifies as a transition state analog based on this criterion. In fact, the dissociation constants for 2-pentanone and other aliphatic ketones are comparable to dissociation constants for the good substrates phenylacetate and acetylcholine. These constants are probably 11 mM and 1 mM, respectively, as estimated by the inhibition constant for acetanilide [3] and the K_m for acetylcholamine hydrolysis [13].

Despite the fact that they are inhibitors and could form complexes resembling transition states, a number of lines of evidence indicate convincingly that these ketones do not bind strongly in the esteratic site where reaction occurs and thus are not transition state analogs. First, the enzyme appears to be rather nonspecific in binding of ketones, whereas a much higher specificity might be expected for binding of a transition state analog. By itself, this observation could be conclusive only if relative hydrolysis rates for esters corresponding to all of the ketones studied were measured and found to be different from relative binding of corresponding ketones. However, the enzyme is known to be quite specific in hydrolysis of acetylcholine but not butyrylcholine [4], and a very low specificity for hydrolysis of corresponding aliphatic esters would be somewhat surprising. In contrast, 4-heptanone binds better than 2-heptanone. Furthermore, diisopropyl and di-*t*-butyl ketones would be expected to be more resistant to hemiketal formation than linear ketones, yet bind to the enzyme almost as well.

More direct evidence that these ketones are not transition state analogs is the observation that corresponding alcohols are also good competitive inhibitors, in spite of the fact that they obviously cannot form hemiketals with the serine hydroxyl group of the enzyme.

These ketones must bind in the esteratic site near the nucleophilic serine hydroxyl group if they are transition state analogs, but two kinds of evidence indicate that they do not. First, unlike both the substrate acetylcholine [14] and previously studied ketone transition state analogs [3], aliphatic ketones have very little ability to protect the enzymatic hydroxyl group from inactivation by methylsulfonyl fluoride. Second, binding of 2-pentanone excludes binding of tetramethylammonium ion. The simplest interpretation of both experiments is that 2-pentanone binds primarily at the anionic site and blocks binding of both tetramethylammonium ion and acetylcholine, but has little effect on reactions of the esteratic site as monitored by methylsulfonyl fluoride. The slight protection against methylsulfonyl fluoride at high ketone concentrations may reflect either weak binding at the esteratic site or a steric effect caused by ketone bound in the anionic site.

Aliphatic ketones are an unusual class of inhibitors; the observation that they bind tightly relative to analogous substrates suggests that they are transition state analogs, but all other tests applied indicate that they are not. Some instances have been reported of compounds which were expected to be transition state analogs, but were found not to bind more tightly than substrate

[15,16]. Many more such instances may not have been reported. However, this is the first report of compounds which have structural features analogous to a substrate, contain a functional group which could form an enzyme-inhibitor complex resembling a transition state, and bin i much more tightly than that substrate, yet are not transition state analogs. This anomalous situation may result from the fact that ethylacetate binds very poorly, with a K_m of 1 M and a probable binding constant of approx. 2 M *, so that binding of another compound need not be very tight for the compound to appear to be a transition state analog. Nevertheless, there is no reason not to expect similar anomalies with analogs of specific substrates. These results should serve as a note of caution not to label inhibitors as transition state analogs except on the basis of persuasive evidence regarding their actual mode of binding.

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* The binding constant can be roughly estimated using the observed K_m, V relative to acetylcholine and Eqns. 3a and b, p. 160, of Ref. 4.