Acetylcholinesterase-Catalyzed Hydrolysis of an Amide†

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ABSTRACT: In this paper we report that acetylcholinesterase catalyzes hydrolysis of amides, an observation which had not been made previously. The amide used is an analog of acetylcholine, 2-acetoaminoethyltrimethylammonium iodide. The experiments were performed with an enzyme preparation obtained from electroplax of *Electrophorus electricus*. Inhibition of the enzyme by a specific organic phosphate inhibitor abolished both the esterase and the amidase activity of the enzyme. The effect of hydrogen ions between pH 5 and pH 10 on the steady-state kinetic parameters, K_m and k_{cat}, has been investigated. These parameters show essentially the same dependence on pH as is observed in catalytic hydrolysis of acetylcholine. k_{cat} is controlled by an ionizing group of the enzyme with an apparent pK of approximately 6.3, and reaches a pH-independent maximum value of 3.6 sec^{-1} above pH 8. The value for K_m of 1 mM at pH 7 and 25° is about five times greater than that for catalytic hydrolysis of the ester at the same pH and temperature. Preliminary electrophysiological experiments indicate that the amide analog binds to the receptor less well, by several orders of magnitude, than acetylcholine does.

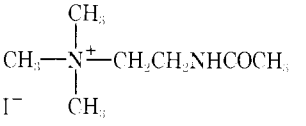
The structure and function of the membrane component to which acetylcholine binds, the acetylcholine receptor, are being investigated in a number of laboratories (Biesecker, 1973; Bulger and Hess, 1973; Eldefrawi and Eldefrawi, 1973; Karlsson et al., 1972; Klett et al., 1973; Meunier and Changeux, 1973; Moody et al., 1973; O'Brien et al., 1972). The use of acetylcholine itself in investigation of the membrane component is hampered by the intimate association of the receptor with acetylcholinesterase. The enzyme catalyzes the hydrolysis of acetylcholine very efficiently (Wilson, 1951) and thus rapidly removes this neurohumoral transmitter. In order to investigate the binding isotherms of the membrane-bound acetylcholine receptor we have searched for, and synthesized, highly specific bifunctional irreversible (Fu and Hess, 1975) and reversible (Koehler and Hess, 1974) inhibitors of the enzyme. In another approach we investigated the use of an amide analog of acetylcholine, 2-acetoaminoethyltrimethylammonium iodide. It was hoped that this compound would bind strongly to the receptor and not be catalytically cleaved by the enzyme since amidase activity of this enzyme had not been reported previously.

Here we report that acetylcholinesterase in fact does catalyze the hydrolysis of the amide analog of acetylcholine efficiently. The enzyme belongs to a group of enzymes called serine proteases (Hartley, 1960). Members of this class have a unique serine residue which reacts with organophosphorus compounds resulting in complete inhibition of enzymic activity (Aldrige, 1960; Jandorf et al., 1955; Wilson, 1951). Structural homologies among serine proteases have been noted (Hartley, 1970; Hartley and Shotton, 1971), and it has been suspected for some time that these enzymes have a common mechanism (Hartley, 1960; Bender et al., 1966). Amidase activity has been reported for all enzymes in this class except acetylcholinesterase. We have now made steady-state measurements of acetylcholinesterase-catalyzed hydrolysis of the amide analog of acetylcholine. The steady-state kinetic parameters k_{cat} and K_m were evaluated and the effect of hydrogen ions between pH 5 and pH 10 on these parameters was studied. A preliminary electrophysiological experiment using this compound is also reported.

Experimental Procedures

Materials. Partially purified acetylcholinesterase (*Electrophorus electricus*) (acetylcholine acetylhydrolase EC 3.1.1.7) was obtained from Worthington Biochemicals Corp. (ECHP, lot 1CV). A stock solution, prepared by dissolving 20,000 units (~20 mg) in 5.0 ml of 0.1 M sodium phosphate buffer (pH 7.0), was stored at 4°. The concentration of active enzyme was determined at intervals during the subsequent investigations by titration with Tetram (*O,O*-diethyl *S*-β-(diethylamino)ethyl phosphorothiolate) (Suszkiw, 1971). During 3 weeks the active enzyme concentration fell from 4.96 ± 0.25 μM to 3.50 ± 0.20 μM. Enzyme solutions used in the kinetic experiments were prepared by fivefold dilution of the stock solution.

"Acetylcholine amide" (2-acetaminoethyltrimethylammonium iodide) was prepared from *N,N*-dimethylethylenediamine (Aldrich Chemical Co.) as described by Price et al. (1965); mp 145° (lit. mp 139–140°). Anal. Calcd for $C_7H_{17}IN_2O$: C, 30.85; H, 6.25. Found: C, 30.51; H, 6.38.



Ninhydrin was obtained from Pierce Chemical Co. and stock solutions were prepared as described by Lenard et al. (1965). All other chemicals were reagent grade.

Methods and Apparatus. Acetylcholinesterase-catalyzed hydrolysis of the amide analog was followed automatically using a modified Technicon amino acid analyzer. The instrumentation and method, described in detail by Lenard et al. (1965), had been used previously to study the chymotrypsin-catalyzed hydrolyses of amides (Himoe et al., 1967a). A portion (0.10 ml) of buffered enzyme solution was added at zero time to a substrate solution (10.0 ml) in a

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Table I: Steady-State Kinetic Parameters for Acetylcholinesterase-Catalyzed Hydrolysis of the Amide Analog of Acetylcholine, 2-Acetaminoethyltrimethylammonium Iodide, at Various pH Values, $\mu = 0.18$, 25°.

pH	K_m (mM)	V_{max} (10^6 mol l.$^{-1}$ min^{-1})	[Enzyme] (nM)	k_{cat} (sec^{-1})	k_{cat}/K_m (10^{-3} l. mol^{-1}/ sec^{-1})
5.0	5.1 ± 0.2	0.7 ± 0.01	7.0 ± 0.5	1.6	0.32
5.5	3.3 ± 0.3	1.2 ± 0.04	8.6 ± 0.8	2.4	0.73
6.0	1.6 ± 0.1	1.8 ± 0.03	9.8 ± 0.7	3.1	1.96
6.0	1.5 ± 0.1	1.3 ± 0.02	7.0 ± 0.5	3.1	2.2
6.5	1.3 ± 0.1	1.7 ± 0.04	9.0 ± 0.7	3.1	2.3
7.0	0.9 ± 0.1	1.9 ± 0.04	9.8 ± 0.7	3.3	3.7
7.0	1.0 ± 0.1	1.6 ± 0.03	7.0 ± 0.5	3.9	3.9
7.5	1.1 ± 0.04	2.0 ± 0.02	9.0 ± 0.7	3.6	3.3
8.0	0.8 ± 0.04	2.1 ± 0.04	9.8 ± 0.7	3.5	4.4
9.0	1.0 ± 0.03	2.3 ± 0.02	9.8 ± 0.7	3.9	3.8
9.5	1.0 ± 0.2	1.6 ± 0.1	7.0 ± 0.5	3.7	3.7

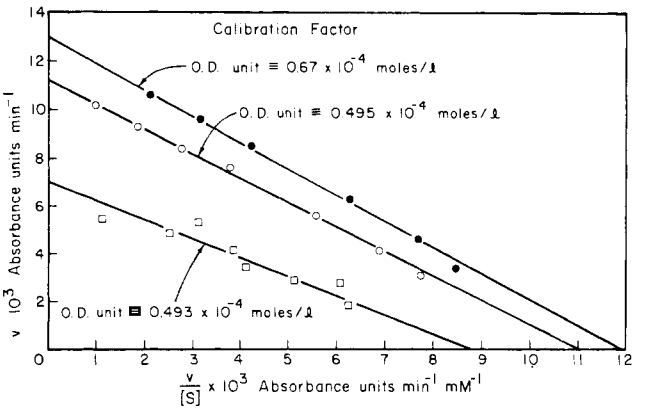

FIGURE 1: Eadie plots for acetylcholinesterase-catalyzed hydrolysis of the amide substrate at 25°, $\mu = 0.18$. (●) pH 7.5, $E_0 = 9.0 \times 10^{-9}$ M; (○) pH 9.0, $E_0 = 9.8 \times 10^{-9}$ M; (□) pH 9.5, $E_0 = 7.0 \times 10^{-9}$ M. For buffer composition and experimental conditions see Experimental Procedure.

covered reaction vessel thermostated at 25.0 ± 0.1°, and the concentration of amine produced was monitored continuously by a ninhydrin reaction. Measurements of the change in absorbance per minute were converted to moles of amide hydrolyzed per minute by reference to a standard curve, prepared using appropriately buffered solutions containing various amounts of ammonium chloride. The standards were measured immediately before and after each kinetic run. The standard curves agreed with those reported previously (Himoe et al., 1967a).

Each experiment consisted of a series of kinetic runs at constant pH and enzyme concentration, and at least seven different initial substrate concentrations in the range 4×10^{-2}–2×10^{-4} M. Initial concentrations of active enzyme ranged from 9.8 to 7.0 nM in the various experiments. In this range, the kinetic parameters were found to be independent of E_0, the initial concentration of enzyme. Control experiments in which either the enzyme or the substrate was omitted showed insignificant amine production over the range of concentrations and pH studied.

In the kinetic experiments, the final buffer compositions were as follows: pH 5.0 and 5.5, 0.02 M sodium acetate buffer; pH 6.0–8.0, 0.02 M sodium phosphate buffer; pH 9.0 and 9.5, 0.02 M Tris-HCl buffer; pH 9.5, 0.02 M sodium carbonate buffer. Sufficient sodium chloride was added to each buffer to give a final ionic strength of 0.18.

The pH of a reaction solution was checked at the beginning and end of each series of kinetic runs. Throughout, the pH did not vary by more than 0.05 unit. Enzyme stability was tested by preincubation of the enzyme in the appropriate buffer solution at 25°, for a time period equal to that of a kinetic run (20 min). Substrate was then added and the reaction followed in the usual way. The rate of these test reactions agreed with the normal runs, within experimental error.

The steady-state kinetic constants were calculated from the rate data using a digital computer program with data weighting and standard error calculation as discussed by Wilkinson (1961).

Results

Results typical of those obtained in the kinetic runs in the pH region 5.0–9.5 at 25°C, $\mu = 0.18$, are shown in Figure 1. The data were plotted according to the method of Eadie (1942) and linear relationships were obtained. The ordinate intercept of the graph gives the V_{max} value in absorbance units per minute, and the slope of the lines in the graph gives the K_m values. The change in absorbance units per minute was converted to moles of substrate hydrolyzed per minute by means of standard curves which were obtained at each pH value. The k_{cat} value was then obtained by dividing the V_{max} value by the molar concentration of enzyme active sites as determined by the Tetram method (Suszkiw, 1971). The steady-state kinetic parameters are listed in Table I. As can be seen from the data, both k_{cat} and K_m(app) are dependent on pH. A plot of k_{cat}/K_m vs. pH is shown in Figure 2. In the pH region measured, pH 5–pH 9.5, the value of k_{cat}/K_m increases with increasing pH at low pH and becomes independent of pH above 7.0. The inflection point of the curve is between pH 6.0 and 6.5.

At pH 9.5 what appeared to be product inhibition occurred as indicated by the nonlinearity of the absorbance vs. time curves for amine production, at substrate concentrations above 1.0 mM. Below 1.0 mM substrate, clearly defined initial rates could be measured and linear Eadie plots constructed (Figure 1).

That acetylcholinesterase does indeed catalyze amide hydrolysis was readily demonstrated by the addition to the reaction solution of the, for acetylcholinesterase, specific irreversible inhibitor Tetram, which at a concentration of 10^{-5} M immediately stopped further amine production.

Discussion

In this section the acetylcholinesterase-catalyzed hydrolysis of an amide analog of acetylcholine is compared to catalytic hydrolysis of acetylcholine, and to the catalytic hydrolysis of substrates by other serine proteases. The data summarized in Table I indicate that the enzyme catalyzes hydrolysis of the amide analog of acetylcholine with high efficiency. The pH-independent value for k_{cat} of 3.6 sec^{-1} at 25°, found in these studies, is comparable with the pH-independent k_{cat} values observed in the catalytic hydrolysis of specific amide substrates by another serine protease, α-chymotrypsin. The k_{cat} for α-chymotrypsin-catalyzed hydrolysis of N-acetyl-L-tryptophanamide is 0.05 sec^{-1} at 25° (Himoe et al., 1967b).

An ionizing group with pK(app) ~ 6.5 has been implicated in control of the rate of serine protease-catalyzed reactions and of acetylcholinesterase-catalyzed hydrolysis of acetylcholine (Wilson and Bergman, 1950; Krupka, 1966).

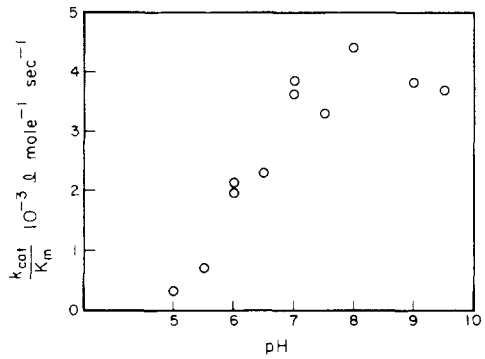

FIGURE 2: The pH dependence of k_{cat}/k_m(app) of acetylcholinesterase-catalyzed hydrolysis of the amide substrate. All experiments were performed at 25°. For buffer composition and experimental conditions see Experimental Procedure.

The ratio k_{cat}/K_m was plotted vs. pH for the acetylcholinesterase-catalyzed hydrolysis of the amide substrate (Figure 2). It is possible to estimate from the plot the dissociation constant of the ionizing groups of the free enzyme that are involved in the catalytic reaction, irrespective of the number of intermediates, providing there is only one state of ionization of a catalytically important ionizing group in which the catalysis can occur (Peller and Alberty, 1959). The inflection point of the plot in Figure 2 indicates that the ionizing group of the enzyme which is important in amide catalysis has an apparent pK of 6.3, the same value as the ionizing group important in ester hydrolysis.

Before considering the difference between the steady-state kinetic parameters of the acetylcholinesterase-catalyzed hydrolysis of acetylcholine and the analogous amide, it is instructive to consider the reaction pathway which has been proposed for the catalytic hydrolysis of substrates by this enzyme and other serine proteases. Equation 1 has been suggested for the acetylcholinesterase-catalyzed hydrolysis of acetylcholine (Froede and Wilson, 1971; Krupka and Laidler, 1961).

$$E + S \overset{K_s}{\rightleftharpoons} ES \overset{k_{23}}{\underset{P_1}{\rightarrow}} EP_2 \xrightarrow{k_{34}} E + P_2 \quad (1)$$

ES represents a reversible enzyme–substrate complex with dissociation constant K_s. EP_2 represents an acyl-enzyme, the formation of which involves the γ oxygen of a serine residue in the active site of the enzyme. P_1 and P_2 represent choline and acetic acid, respectively. The mechanism shown in eq 1 is well documented in reactions catalyzed by another serine protease, chymotrypsin (Hess, 1971; Fastrez and Fersht, 1973), and in these reactions P_1 represents an alcohol or an amide, and P_2 an aromatic amino acid. In the case of chymotrypsin-catalyzed reactions the individual rate constants pertaining to eq 1 have been measured for a series of specific substrates. It has been demonstrated in investigations of the presteady phase of the catalytic reactions, using stopped-flow techniques (Brandt et al., 1967; Hess et al., 1970; McConn et al., 1971), that in the catalytic hydrolysis of esters $k_{23} \gg k_{34}$ (eq 1). That is, acylation of the enzyme by ester substrates is the fast step and deacylation the rate-determining step. In chymotrypsin-catalyzed hydrolysis of amides it has been demonstrated that $k_{34} \gg k_{23}$ (Himoe et al., 1967a). K_m(app), for a reaction following eq 1, is equal to K_s when $k_{34} \gg k_{23}$, and is smaller than K_s when $k_{23} \gg k_{34}$. In the latter case K_m is $(k_{34}/k_{23})K_s$.

The enzyme–substrate dissociation constants for the analogous ester and amide substrate have been found to have very similar values in reactions catalyzed by chymotrypsin (Hess, 1971), another serine protease. If the same relationship holds for acetylcholinesterase-catalyzed reactions, the differences in the K_m(app) value in amide hydrolysis (1 mM) and ester hydrolysis (0.2 mM) may, therefore, reflect differences in the rate-limiting step in the two reactions. This assumption leads to the following conclusion. In the acetylcholinesterase-catalyzed hydrolysis of the amide, acylation is rate limiting and K_m(app) is K_s. In the catalytic hydrolysis of the ester, deacylation is rate limiting and k_{34} is about five times larger than k_{23}, (K_m(app) amide = 5 × K_m(app) ester). The conclusions regarding the rate-limiting steps in amide and ester hydrolysis are consistent both with the data obtained with other serine proteases and with the results of Wilson and Cabib (1956). These authors estimated from Arrhenius plots that in the acetylcholinesterase-catalyzed hydrolysis of acetylcholine, deacylation is rate limiting, and the ratio k_{23}/k_{34} has a value of 6. Preliminary electrophysiological experiments[1] indicate large differences between the dissociation constants of the ester and amide for the acetylcholine receptor.

The concentrations of the amide analog needed to produce equal electrical membrane potential changes in monocellular electroplax preparations are several orders of magnitude higher than those of acetylcholine.

It is of interest, but perhaps not surprising, to find that acetylcholinesterase catalyzes hydrolysis of an amide analog of acetylcholine. This finding, and the similarity of this reaction to the catalytic hydrolysis of other serine proteases, strengthens the hypothesis that serine proteases react by similar mechanisms.

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[1] For which we thank Dr. Bartels and R. Nachmansohn, Columbia University. Experimental details concerning the measurements are given in Schoffeniels (1957) and Schoffeniels and Nachmansohn (1957).

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Mechanism of Cytochrome *c* Peroxidase. *O*-Benzoylhydroxylamine as an Analog of Hydrogen Peroxide†

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ABSTRACT: A number of reagents, some of which are electronic analogs of hydrogen peroxide, will replace it in the reactions of cytochrome *c* peroxidase. These compounds include *N*-bromosuccinimide, sodium hypochlorite, and the novel oxidizing agent *O*-benzoylhydroxylamine. If fragments of the oxidant played a functional role in the structure of the oxidized form of the enzyme, it would be expected that the product formed from *O*-benzoylhydroxylamine would differ from that formed from hydrogen peroxide. The products formed on reaction of the two oxidizing agents with cytochrome *c* peroxidase are indistinguishable. This result carries implications for the structure of the so-called ES compound. The extension in the range of specific substrates for cytochrome *c* peroxidase allows identification of the structure which compounds must possess to be oxidizing substrates for the enzyme. A mechanism for the first step of the reaction is suggested. *O*-Benzoylhydroxylamine is also a reducing agent, and its reaction with the enzyme is analogous to that of hydrogen peroxide with catalase. The final product of the reaction is the inert nitric oxide complex of ferrous cytochrome *c* peroxidase.

Cytochrome *c* peroxidase (EC 1.11.1.5, ccp[1]) is a hemoprotein which catalyses the oxidation of cytochrome *c* by hydrogen peroxide (Yonetani, 1970). The enzyme has an obligatory order mechanism in which ferric ccp first reacts with peroxide. The so-called "ES compound" of the enzyme which is formed then oxidizes 2 mol of ferrous cytochrome *c* and is itself reduced to the native state.

$$\text{ccp} + H_2O_2 \longrightarrow \text{ES}$$

$$\text{ES} + 2\text{ferrocytochrome } c \longrightarrow \text{ccp} + 2\text{ferricytochrome } c$$

Physical measurements provide clues to the nature of ES, but its precise structure remains unknown. A chemical approach to the problem is to see how the properties of ES depend on the nature of the oxidizing substrate. Schonbaum (Schonbaum, 1970) has shown that stoichiometric quantities of ethanol can be distilled out of frozen solutions of ES prepared from ccp and ethyl hydroperoxide while the protein remains in its oxidized form and this implies that at least half of the oxidant molecule is freed from the protein on formation of ES.

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[1] Abbreviations used are: ccp, cytochrome *c* peroxidase; ES, product of reaction of ccp with H_2O_2: $BzONH_2$, *O*-benzoylhydroxylamine.