MECHANISM OF HYDROLYSIS

II. NEW EVIDENCE FOR AN ACYLATED ENZYME AS INTERMEDIATE*

by

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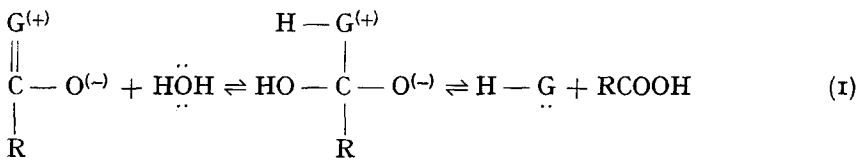
The mechanism by which acetylcholinesterase catalyzes chemical reactions has been presented in previous papers[1-4]. The enzyme catalyzes reactions of esters and acids with nucleophilic reagents such as alcohols (especially alkylated amino alcohols), water and hydroxylamine. The enzyme was shown to contain an anionic site capable of binding the cationic portion of acetylcholine and similar molecules, and also an esteratic site characterized by an acidic and basic group upon which the enzymic activity is directly dependent. The basic group reacts with the electrophilic carbon atom found in esters and other compounds.

The acylated enzyme proposed as intermediate and whose formation is the rate controlling step reacts with H_2O in the following way:



(1)

where H—G symbolizes the esteratic site, H representing the acid, and the electron pair the basic group. This intermediate, which can be formed from either esters or acids, reacts with nucleophilic reagents. Acids, however, are not favorable reactants since only the undissociated molecules contain the electrophilic carbon atom necessary for forming the initial complex.

Apparently reaction (1) must produce oxygen exchange between carboxylate ion and water. It is thus a necessary condition for the correctness of the theory that the enzyme catalyzes oxygen exchange. That exchange does in fact occur can be shown indirectly by using thiolacetic acid as a substrate. In this case reaction (1) indicates that hydrogen sulfide will be eliminated with the simultaneous formation of acetylated enzyme which then reacts with water to regenerate the enzyme and produce acetic acid, *i.e.* thiolacetic acid will be hydrolyzed. While the hydrolysis of thiolacetic acid is not identical with oxygen exchange, its occurrence is strong evidence that exchange does take place. The anionic site would appear to play no role in the hydrolysis of thiolacetic

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References p. 525.

acid since this compound contains no grouping which might be bound to the site. The relative smallness of the sulfhydryl group suggests that the reaction of the anionic site with a small molecule such as trimethyl ammonium ion would not completely interfere with the hydrolytic process when thiolacetic acid is the substrate, whereas the process might be completely abolished when ethyl acetate is the substrate. The effect of trimethyl ammonium ion, 1:10-decane bis(trimethylammonium) bromide (C_{10}), and prostigmine upon these processes has therefore been investigated.

METHOD

Thiolacetic acid solutions are relatively stable with respect to hydrolysis. The addition of enzyme produces H_2S which can be recognized by odor. Although both thiolacetic acid and hydrogen sulfide can be oxidized with iodine, mole for mole the latter requires twice as much iodine. The reaction can therefore be followed quantitatively by iodimetric titration. The disulfide formed by the oxidation of thiolacetic acid is sufficiently soluble to yield a clear solution at the end of the titration but if hydrogen sulfide is present, a white precipitate of sulfur is obtained.

The enzyme solution was a highly purified preparation of acetylcholinesterase from electric tissue of *Electrophorus electricus* having an activity of 11 g of acetylcholine hydrolyzed per hour per ml.

7.50 ml of $7.40 \cdot 10^{-3}$ *N* I_3^- solution (0.2 *M* KI) were introduced into a 50 ml Erlenmeyer flask, and then a small cup containing 0.100 ml of a solution of thiolacetic acid (0.23 *M*) in phosphate buffer (0.3 *M*) adjusted to the required p_H.

0.100 ml of enzyme solution were added to the cup and the flask tightly stoppered. Controls contained water instead of the enzyme. After one half hour the flasks were opened, two drops of 0.1 *M* prostigmine solution were added to the cups, and 4 drops of 1 *M* acetate buffered at p_H 4.5 were added to the iodine solution. After one minute the cups were overturned, starch added, and the excess iodine back titrated with $8.00 \cdot 10^{-3}$ *N* thiosulfate solution.

For the inhibition measurements, 0.100 ml of enzyme and 0.100 ml of inhibitor solution (or water) were incubated for 10 mins. Addition of 0.100 ml of 0.34 *M* thiolacetate solution at a favorable p_H (5.5 final) starts the reaction.

The ethyl acetate hydrolysis and its inhibition was conducted as follows: 0.100 ml of enzyme and 0.100 ml of inhibitor (or water) were incubated for 10 mins, and the reaction started by the addition of 0.100 ml of 0.04 *M* ethyl acetate solution in 0.3 *M* phosphate buffer at p_H 7. After 5 minutes the amount of ester was measured by the hydroxamic acid ester test[5].

RESULTS

During the reaction period a film developed on the surface of the iodine solution in those flasks in which enzyme had been added, but in the other flasks the solution remained clear. The curd is sulfur formed from the oxidation of H_2S which distills from the cups to the iodine solution.

The reaction rate as a function of p_H in terms of excess iodine is presented in Table I. The experimental error in these figures is about $\pm$ 0.04 ml.

TABLE I

THIOLACETIC ACID HYDROLYSIS BY ACETYLCHOLINESTERASE AS A FUNCTION OF p_H

The figures indicate the number of milliliters of iodine (0.00740 *N*) used in the titration of the test mixture in presence of enzyme in excess of that required to oxidize the thiolacetic acid. Details are described under methods.

The values were zero in all controls.

	Excess iodine	
p_H	½ h	1 h
4.9	0.62	1.20
5.6	0.65	1.08
6.2	0.40	0.79
7.1	0.12	0.21

References p. 525.

A summary of the inhibitory effects of trimethyl ammonium chloride, 1:10-decane bis(trimethylammonium)bromide, and prostigmine bromide on thiolacetic acid hydrolysis at p_H 5.5 and on ethyl acetate hydrolysis at p_H 7 is shown in Fig. 1. The units of concentration are the 50% inhibition concentrations for each substance when the substrate is acetylcholine. These values are $1.5 \cdot 10^{-2}$, $2.5 \cdot 10^{-5}$, and $7 \cdot 10^{-7}$ respectively.

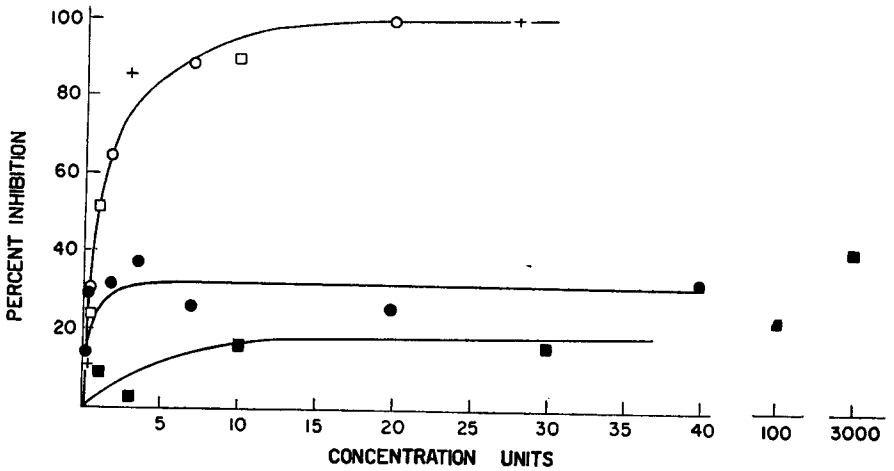

Fig. 1. Effect of cationic inhibition upon the hydrolysis of thiolacetic acid by acetylcholinesterase. The unit of the concentration is that required for 50% inhibition with acetylcholine as substrate. These values are $1.5 \cdot 10^{-2}$, $2.5 \cdot 10^{-5}$ and $7 \cdot 10^{-7}$ for trimethylammonium chloride, 1:10-decane bis (trimethylammonium) bromide and prostigmine bromide respectively. The action of prostigmine is included to demonstrate the effect of an inhibitor which reacts with both the anionic and the esteratic sites. The inhibition of ethyl acetate hydrolysis illustrates the effect of increase in molecular size. CH_3COSH: + prostigmine, • trimethylamine, ■ C_{10}; $CH_3COOC_2H_5$: O trimethylamine, □ C_{10}

DISCUSSION

The hydrolysis of thiolacetic acid by acetylcholinesterase strengthens the theory that the enzyme during its catalysis is acylated by the internal elimination of the non-acyl residue of the substrate from the enzyme substrate complex. The p_H dependence confirms studies with other acids indicating that it is the undissociated acid molecule which is the direct reactant. The dissociation constant of the acid is about $4 \cdot 10^{-4}$ so that as the p_H is decreased from 7 to 5, there is about a hundredfold increase in undissociated acid. The affinity of the enzyme and substrate is probably low as in the case of acetic acid since there is no attraction to the anionic site[3]. The rate will therefore be proportional to the concentration of substrate. On the other hand, the enzymic activity decreases in acid solution by conversion of the basic group to its conjugate acid. If thiolacetate ion were the reactant the rate would fall very rapidly with decreasing p_H, which is quite apparently not the case.

Mathematical analysis of the equilibria and rate process based upon the supposition that undissociated acid is the reactant leads to the rate equation:

$$v = \frac{k E^\circ S^\circ}{\left(\frac{H^+}{K_{EH_2^+}} + 1\right)\left(\frac{K}{H^+} + 1\right)}$$

References p. 525.

where k is a constant, E° the stoichiometric concentration of enyme, S° the stoichiometric concentration of thiolacetic acid, $K_{EH_2}{}^+$ the dissociation constant of the conjugate acid of the basic group, H^+ the hydrogen ion concentration, and K ($4 \cdot 10^{-4}$) the dissociation constant of thiolacetic acid. This equation may be used to evaluate the enzyme constant $K_{EH_2}{}^+$ from the p_H dependence of the reaction rate. The value so obtained is about $3 \cdot 10^{-7}$ which is in satisfactory agreement with the value $2 \cdot 10^{-7}$ obtained from the non-competitive prostigmine inhibition of acetylcholine hydrolysis and with $0.7 \cdot 10^{-7}$ obtained from the p_H dependence of acetylcholine hydrolysis.

When acetylcholine is used as substrate the concentration of enzyme and of the non-enzyme proteins is only about one thousandth of that used with thiolacetic acid. In order to eliminate any possible consequence of this very different protein concentration the effect of the cationic inhibitors upon thiolacetic acid hydrolysis was compared to their effect upon ethyl acetate hydrolysis, since both reactions can be carried out with the same enzyme concentration. During the enzymatic hydrolysis ethyl acetate must be oriented relative to the enzyme surface in a manner similar to the orientation of acetylcholine during the hydrolysis of that compound, *i.e.*, the ethyl chain must be oriented in the general direction of the anionic site. Similarly, the sulfhydryl group of thiolacetic acid must also be oriented in the direction of the anionic site. Consequently, one might anticipate that the spatial disposition would be such that a trimethylammonium ion bound at the anionic site would very greatly interfere with the binding or orientation of ethylacetate, while interfering little (or not at all) with the binding or orienting of the smaller thiolacetic acid molecule. The data indicate that this is in fact the case. From Fig. 1 it is seen that even in the presence of very large concentrations, 40 units, of trimethyl ammonium ion only about 33% inhibition of the thiolacetic acid reaction can be achieved, whereas 70% inhibition of the ethyl acetate reaction is obtained with only 2 units, and 90% with ten. The ethyl acetate data form a normal inhibition curve. It is also important to note that whatever inhibition of the thiolacetic acid reaction can be achieved is obtained at low concentrations and that further increase in concentration does not raise the inhibition, thus indicating that the binding of trimethyl ammonium ion is complete at the lower concentration but that this produces only a moderate interference with the hydrolytic process.

Prostigmine was used as an inhibitor to demonstrate that thiolacetic acid hydrolysis could be completely inhibited by a molecule which is bound at both the esteratic and the anionic site. With this inhibitor and thiolacetic acid as substrate the inhibition falls upon the normal curve, i.e. the trimethylammonium ion-ethyl acetate curve. Thus when both sites are occupied, inhibition is complete as expected.

The results with C_{10} are essentially the same. With ethyl acetate the normal inhibition curve is obtained but with thiolacetic acid 3000 units produce only 41% inhibition. There is, however, some departure from the trimethyl ammonium curve. The inhibitor does not reach a maximum at a low concentration, but there is rather an initial rapid rise and then a gradual increase in inhibition. C_{10} is bound very much more strongly than trimethyl ammonium ion so that it must be bound by not only the anionic site but by other portions of the enzyme molecule[6]. Evidently the fixation of the inhibitor on the enzyme surface does not very closely approach the esteratic site so that it is impossible to achieve complete interference in the thiolacetic acid hydrolysis.

The inhibition data indicate a spacial and functional separation of the anionic and esteratic sites of the enzyme. The mere existence of an anionic site demands a spacial

References p. 525.

distinction and hence a separation from any other characterized portion of the enzyme. The data thus are a new support for the concept of two distinct sites. It is also known that the anionic site is close to the esteratic site since otherwise trimethylammonium ion could not interfere with the hydrolysis of ethyl acetate. Obviously the anionic site must be close to the esteratic site, since otherwise acetylcholine could not be bound simultaneously at both sites. However, these data are an interesting confirmation of ideas which were necessarily obtained in an indirect manner.

The concept that cationic inhibitors complete with the enzymatic binding of acetylcholine and that inhibition was therefore merely a sterical effect was satisfactory for explaining all the observations with these substances. It was still possible, however, that these inhibitors produced more profound changes perhaps affecting the state of aggregation or altering the structure of the hydrolytic region. The fact that the thiolacetic acid hydrolysis cannot be more than partially inhibited by trimethylammonium ion indicates that these alternate explanations cannot be of primary importance. Evidently the anionic site is functionally independent of the esteratic site.

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SUMMARY

Acetylcholinesterase catalyzes the hydrolysis of thiolacetic acid. The undissociated acid molecules are the direct reactants. The dissociation constant of the conjugate acid of the basic group of the enzyme calculated from the p_H dependence of this reaction is about $3 \cdot 10^{-7}$.

This reaction is very strong evidence that the enzyme catalyzes oxygen exchange between acids and water, a necessary condition for the correctness of the proposed theory of enzymic activity, involving an acylated enzyme as intermediate.

Trimethylammonium ion and 1:10-decane bis(trimethylammonium) bromide even in enormous excess only partially inhibit the reaction, whereas ethyl acetate hydrolysis is completely inhibited, thus indicating a spacial and functional separation of the anionic and esteratic enzyme sites. Prostigmine which reacts with both sites completely inhibits thiolacetic acid hydrolysis.

RÉSUMÉ

L'acétylcholinestérase catalyse l'hydrolyse de l'acide thiolacétique. Les molécules d'acide non-dissocié entrent directement en réaction. La constante de dissociation de l'acide conjugé du groupe basique de l'enzyme calculée à partir de la dépendance du p_H de cette réaction, est environ de $3 \cdot 10^{-7}$.

Cette réaction est un argument puissant en faveur de l'idée que l'enzyme catalyse l'échange d'oxygène entre des acides et l'eau, condition nécessaire pour la justesse de la théorie proposée de l'activité enzymatique qui fait intervenir un enzyme acylé comme intermédiaire.

L'ion triméthylammonium et le bromure de décane 1.10-bis-(triméthylammonium), même en excès énorme, inhibent seulement partiellement la réaction, tandis que l'hydrolyse de l'acétate d'éthyl est complètement inhibée, montrant ainsi que les centres anionique et estérasique sont séparés dans l'espace et en leur fonction. La prostigmine qui réagit avec les deux centres de l'enzyme inhibe complètement l'hydrolyse de l'acide thiolacétique.

ZUSAMMENFASSUNG

Acetylcholinesterase katalysiert die Hydrolyse der Thiolessigsäure. Die undissoziierten Säuremoleküle treten direkt in Reaktion. Die Dissoziationskonstante der konjugierten Säure der basischen Gruppe des Enzyms, berechnet aus der p_H-Abhängigkeit dieser Reaktion, beträgt ungefähr $3 \cdot 10^{-7}$.

References p. 525.

Diese Reaktion zeigt deutlich, dass das Enzym den Sauerstoffaustausch zwischen Säuren und Wasser katalysiert, eine notwendige Bedingung für die Richtigkeit der vorgeschlagenen Theorie der Enzymaktivität, welche ein acyliertes Enzym als Zwischenprodukt annimmt.

Das Trimethylammonium-Ion und Decan-1.10-bis-(trimethylammonium)-bromid, sogar in enormen Überschuss, hemmen die Reaktion nur teilweise, während die Hydrolyse des Äthylacetates vollständig gehemmt wird; dies weist auf eine räumliche und funktionelle Trennung der anionischen und Ester spaltenden Zentren des Enzyms hin. Prostigmin, welches mit beiden Zentren reagiert, hemmt die Hydrolyse der Thiolessigsäure vollständig.

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