MECHANISM OF ENZYMIC HYDROLYSIS

I. ROLE OF THE ACIDIC GROUP IN THE ESTERATIC SITE OF ACETYLCHOLINESTERASE*

by

IRWIN B. WILSON

Department of Neurology, College of Physicians and Surgeons, Columbia University, New York, N.Y. (U.S.A.)

INTRODUCTION

Acetylcholinesterase is not only a highly effective catalyst for the hydrolysis of acetylcholine but also catalyzes certain other acylation reactions. The source of the acyl radical may be either an ester or fatty acid. In previous papers a single mechanism was developed for all the reactions subject to catalysis by this enzyme[1-4]. An essential feature of the mechanism is that the esteratic site contains a basic and acidic group upon which the catalysis depends. Thus

$$\underset{\text{inactive}}{EH_2^+} \overset{+H^+}{\rightleftharpoons} \underset{\text{active}}{EH} \overset{+OH^-}{\rightleftharpoons} \underset{\text{inactive}}{E^-} + H_2O.$$

The first step in the process is the formation of a thermodynamically stable enzyme substrate complex characterized by bond formation between the electrophilic acyl carbon atom of the substrate and the basic group of the enzyme. If a tertiary amine, quaternary ammonium, or geometrically similar group is available, additional binding will occur at an electrically negative, *i.e.* anionic site. Then a rearrangement follows in which a proton is transferred from the acid group of the enzyme to a nucleophilic portion of the substrate resulting in the internal elimination of a small molecule such as an alcohol or water molecule. The basic group of the enzyme is thereby acylated. The next step is a reaction of the acylated enzyme with nucleophilic reagents such as H_2O, OH^-, NH_2OH, $[N(CH_3)_3C_2H_4OH]^+$, etc. In the scheme outlined below only reactions involving the esteratic site are depicted. The symbol H—G represents the esteratic site. G is assumed to have the ability to transmit electrons as illustrated by conjugate double bonds.

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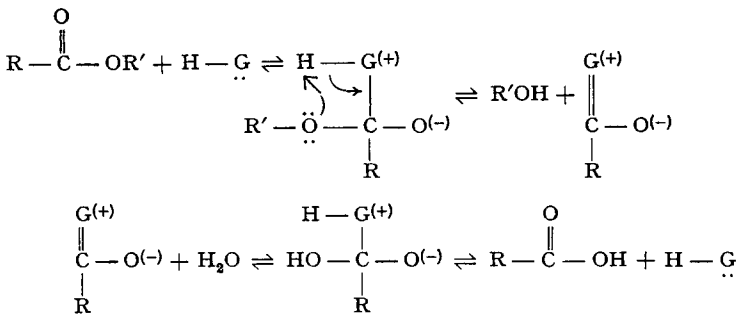

The formation of the acylated enzyme, a reactive intermediate, is the rate controlling step. In this scheme the essential function of the acid group is in the formation of the acylated enzyme and not in the formation of the enzyme substrate complex. Hydrogen bonding with either oxygen is possible but measurements with choline do not indicate that it is of great importance (unpublished data). This role of the acid group can be tested experimentally with the aid of a suitable inhibitor such as prostigmine. Compound formation of the enzyme with this inhibitor is essentially the same as that with acetylcholine. Prostigmine contains a quaternary ammonium group and an electrophilic carbon atom in a carbonyl group separated from the cationic nitrogen by approximately the same distance as in acetylcholine. Since inhibition by this compound involves only formation of a complex without hydrolysis, it can be determined whether or not the acid and basic groups are important in this formation by comparing the inhibition at various p_H levels. If the inhibition should decrease in alkaline medium, this would suggest that the acidic group of the enzyme was essential for the complex formation, since the acid group in alkaline medium is converted to its conjugate base. On the other hand, if no change occurs it would indicate that the acid group is not necessary for the binding.

Any change in binding will be less manifest under competitive than under non-competitive conditions since complex formation with both acetylcholine and inhibitor will be similarly affected.

Let us consider p_H levels more acid than the optimum p_H, *i.e.* < 8. Rate equations can be derived for various circumstances. Let us derive equations for the case that the inhibitor may combine with the basic group of the enzyme but not with its conjugate acid.

For competitive inhibition (at substrate concentrations, $4 \cdot 10^{-3}$ M or less, which is sufficiently low to prevent the formation of an inactive supercomplex) the following equilibria are pertinent:

$$EH_2^+ \rightleftharpoons EH + H^+$$
$$EH + I \rightleftharpoons EHI$$
$$EH + S \rightleftharpoons EHS$$

yielding

$$\frac{v^\circ}{v} = 1 + \frac{(I)}{K_I\left[\frac{(H^+)}{K_{EH_2^+}} + 1 + \frac{K_I}{(S)}\right]} \qquad (1)$$

where v = is the reaction velocity with inhibitor, v° without inhibitor, K_I is the inhibitor-enzyme dissociation constant, K_I is the substrate-enzyme dissociation constant,

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$K_{EH_2^+}$ is the dissociation constant of the conjugate acid of the basic group of the enzyme, (I) is the concentration of inhibitor, (S) the concentration of substrate and (H^+) is the concentration of hydronium ion. For non-competitive inhibition the inhibitor simply decreases the amount of available enzyme as determined by the first two equilibria, yielding

$$\frac{v^\circ}{v} = 1 + \frac{(I)}{K_I\left(\frac{(H^+)}{K_{EH_2^+}} + 1\right)} \qquad (2)$$

Since under convenient experimental conditions $(S)/K_I$ is large compared to the p_H variable term, it is apparent that non-competitive inhibition is far more sensitive to p_H change. Under non-competitive conditions there will be no p_H effect if the inhibitor may combine with both the enzyme form containing the basic group and that containing its conjugate acid.

Non-competitive conditions can be approached with some competitive inhibitors by incubating the enzyme first with inhibitor and then adding substrate, but using only the first 3 or 4 minutes for kinetic measurements. With prostigmine, for example, some 15 or more minutes are required for "equilibrium" to be reached with respect to enzyme, substrate and inhibitor[5]. All the enzyme which is in the form of a complex with prostigmine is thus withheld from the system for the first few minutes.

METHOD

Enzyme and inhibitor were incubated for 10 minutes in phosphate or borate buffers as described previously[2]. Acetylcholine ($4 \cdot 10^{-3}$ M final concentration) was then added and samples withdrawn for analysis after one and two minutes at p_H between 6 and 10 and after two and four minutes at other p_H levels. The hydrolysis of the ester was determined by the colorimetric hydroxamic acid procedure[6]. At each p_H a measurement was made without inhibitor but in these cases one-half as much enzyme was used.

The enzyme was acetylcholinesterase purified from electric tissue of *Electrophorus electricus*.

RESULTS

The inhibition of prostigmine was measured as a function of p_H under non-competitive conditions. Prostigmine inhibition decreases markedly in acid media (Table I) indicating that the basic group is necessary for the formation of the enzyme-prostigmine complex.

The constancy of inhibition in alkaline media indicates that the acid group is not significantly involved in complex formation.

DISCUSSION

The findings with prostigmine indicate that only the basic group is necessary for the binding. This conclusion must be applicable to other similarly constituted molecules including acetylcholine. If the acid group is not involved in the binding of acetylcholine and yet essential for its hydrolysis, it must be involved in the cleavage process which follows the complex formation. These results thus confirm the conclusions deduced from the proposed mechanism of hydrolysis.

It is possible to calculate the basic ionization constant from equation (2), but the

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TABLE I

NON-COMPETITIVE INHIBITION OF ACETYLCHOLINESTERASE BY PROSTIGMINE BROMIDE AS A FUNCTION OF p_H

The final molar concentration of inhibitor is indicated. The control solution at p_H 7 hydrolyzed 0.6 micromoles of acetylcholine per ml per min. Readings were made at 1 and 2 minutes for p_H between 6 and 11 and at 2 and 4 minutes for other p_H. For acid p_H the data of 2 representative experiments are given.

p_H	Per cent inhibition	
	$4 \cdot 10^{-7}$ M	$1.3 \cdot 10^{-7}$ M
5.2	7	
5.4	28	
5.6	30	
5.9	53	
6.2	49	
6.3	68	
7.0	80	56
7.5		
8.0		65
9.0		64
10.0		55
11.0		57

value so obtained is not to be regarded as very precise in view of the difficulties of experimentation and calculation. For example, if the inhibition is not completely non-competitive, $K_{EH_2^+}$ would tend to come out high. Moreover, $K_{EH_2^+}$ is calculated from $v^\circ - 1$ (the difference between two comparable quantities). The value obtained is about $2 \cdot 10^{-7}$ which is in satisfactory agreement with the value of $7 \cdot 10^{-8}$ determined from the p_H dependence of the hydrolysis of acetylcholine.

The acid dissociation constant $5 \cdot 10^{-10}$ obtained from the p_H dependence of acetylcholine hydrolysis must be the acid dissociation constant not of the free enzyme but of the enzyme substrate complex. This is apparent since the acid group comes into play after the complex is formed. Evidently the measured basic dissociation constant will be the same for all reactants but the acid dissociation constant will depend upon the substrate. In the consideration of various structures as possible enzyme "surfaces", it would be advantageous to know the acid dissociation constant of the free enzyme. Apparently this will be quite difficult to measure. We may, however, consider its relationship to the value of the acid dissociation constant of the enzyme-substrate complex. From the mechanism of hydrolysis it is seen that the formation of the complex involves a separation of charge. The electrolytic system symbolized by G acquires a formal + charge. The effect of this charge would be to repel the proton of the acid group and thus increase the acidic dissociation. This effect is clearly illustrated by the respective carboxyl dissociation constants of acetic acid and glycine. The effect on the enzyme, however, would be considerably less pronounced since a dipole rather than a free charge is here involved. The acid dissociation constant of the enzyme is therefore certainly considerably less than $5 \cdot 10^{-10}$.

Prostigmine inhibition as a function of p_H was reported in a previous paper. In that work the lowest p_H was 6. The reaction time was a half hour period so that conditions

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probably approximated competitive inhibition. That the inhibition was found not to vary under those conditions down to $p_H = 6$ is therefore in no way inconsistent with the work reported in this paper, since as has been discussed above competitive inhibition is relatively intensitive to p_H change.

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SUMMARY

Measurements of the inhibition of acetylcholinesterase by prostigmine as a function of p_H under non-competitive conditions reveal that the basic group in the esteratic site of the enzyme is essential for the formation of the enzyme substrate complex but that the acid group is not significantly involved. The acid group is essential in the hydrolytic process which follows the formation of the enzyme-substrate complex. These conclusions are in accordance with a previously developed mechanism of enzyme catalyzed hydrolysis.

The measured dissociation constant of the acid group is that of the enzyme substrate complex and not that of the free enzyme.

RÉSUMÉ

Des mesures de l'inhibition sans concurrence de l'acétylcholinestérase par la prostigmine en fonction du p_H montrent que le groupe basique de la partie estérasique à la surface de l'enzyme est essentiel pour la formation du complexe enzyme-substrat mais que le groupe acide ne joue pas de rôle significatif. Le groupe acide est essentiel pour le processus d'hydrolyse qui suit la formation du complexe enzyme-substrat. Ces conclusions sont en accord avec un mécanisme précédemment développé expliquant l'hydrolyse catalysée par un enzyme.

La constante de dissociation mesurée du groupe acide est celle du complexe enzyme-substrat et non de l'enzyme libre.

ZUSAMMENFASSUNG

Messungen der Hemmung von Acetylcholinesterase durch Prostigmin als Funktion des p_H unter nicht konkurrierenden Bedingungen zeigen, dass die basische Gruppe des esteratischen Teils in der Oberfläche des Enzyms für die Bildung des Enzym-Substrat-Komplexes notwendig ist, die saure Gruppe aber keine bedeutende Rolle spielt. Die saure Gruppe ist für den hydrolytischen Prozess, welcher auf die Bildung des Enzym-Substrat-Komplexes folgt, notwendig. Diese Folgerungen stimmen mit einem früher entwickelten Mechanismus der enzymatisch katalysierten Hydrolyse überein.

Die gemessene Dissoziationskonstante der sauren Gruppe ist diejenige des Enzym-Substrat-Komplexes und nicht des freien Enzyms.

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