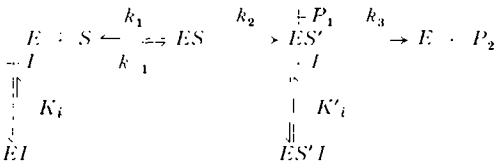
BBA 63495

Acetylcholinesterase: New evidence for an acetyl-enzyme intermediate

Elucidation of the kinetic parameters of ester hydrolysis catalyzed by acetylcholinesterase (Acetylcholine hydrolase, E.C. 3-1-1-7) has been the object of numerous investigations in recent years. Most of these studies have been carried out using acetylcholine as a substrate[1-4] or a variety of acetyl esters known to be poor substrates of acetylcholinesterase[5-8]. Some studies using carbamates as acetylcholinesterase substrates have also been carried out[3,9,10]. The results can be presented in the generalized Scheme I for both hydrolysis and inhibition set forth by WILSON AND ALEXANDER[3], where ES' is an acetyl enzyme intermediate (or carbamyl-enzyme, depending on the substrate).



(I)

This mechanism predicts that inhibitors may interact non-competitively if $k_2 > k_3$, and K'_i is significant. (This is the case for the hydrolysis of acetylcholine). It also implies that for a series of acetyl ester substrates, v_{max} should be the same in all cases where $k_2 > k_3$*, but differing v_{max} values would be expected for each substrate when $k_2 < k_3$. The observation of the former conditions would offer good evidence for an acetyl-enzyme intermediate. In view of the evidence indicating an acetyl-enzyme intermediate for acetylcholinesterase[1,5-7,9,11], it was of interest to synthesize several acetyl ester substrates of acetylcholinesterase which contained all the properties of the natural substrate: An oxyacetyl ester, a positively charged

quaternary nitrogen atom, and a $N^+ \rightarrow C$ distance of 5 Å. If several acetyl ester substrates of acetylcholinesterase with the above mentioned properties could be prepared, they would allow for a comparison with acetylcholine and should result in a common intermediate being formed in Scheme I. In addition, such substrates should be inhibited in the same fashion as the natural substrate. Although hundreds of substrates have been prepared[11], up to now, there have been almost none which have met these requirements. Finally, the synthesis of the isoelectronic analogs of these specific substrates would allow for some qualitative estimates of whether the charge requirements were unique to binding or were also involved in the catalytic process. Results have now been obtained using several synthetic substrates of acetylcholinesterase which are consistent with Scheme I and which also indicate that the presence of a charged group may be important in catalysis as well as binding. These results will be discussed in detail below.

Experimental: The preparation of 3- and 4-acetoxypyridine-1-oxide from the corresponding 3- or 4-hydroxypyridine was accomplished by treatment with 30% hydrogen peroxide followed by acetylation with acetic anhydride: m.p. 98–99°, *meta*

* Under comparable conditions of $[E_t]$, pH and temperature.

TABLE I

KINETIC CONSTANTS OF SUBSTRATES OF ACETYLCHOLINESTERASE

Substrate	$N^+ - C$	K_m (M^{-1})	v_{max} (min$^{-1} \cdot M$)**	pK_a***
3-Acetoxy-pyridine-*N*-methiodide	4.2 - 4.85	$3.8 \cdot 10^{-5}$	$8.47 \cdot 10^{-5}$	5.1
3-Acetoxy-pyridine-*N*-oxide	4.2 - 4.85	$5.50 \cdot 10^{-4}$	$1.75 \cdot 10^{-5}$	6.4
m-(trimethyl-ammonium) Phenyl-acetate iodide	5.4 - 6.1	$5.70 \cdot 10^{-5}$	$1.10 \cdot 10^{-4}$	8.05
p-(trimethyl-ammonium) Phenyl-acetate iodide	6.29	$3.50 \cdot 10^{-4}$	$4.00 \cdot 10^{-8}$	8.25
Acetylcholine	3.84 - 4.9	$1.20 \cdot 10^{-4}$	$1.14 \cdot 10^{-4}$	

* From Buchi models and from scale drawings.
** All values corrected to [*E*] = 0.0050 g/20 ml.
*** pK_a of corresponding phenol.

derivative; (Found: C, 55.0%; H, 4.46%; N, 9.18%. Calculated for $C_7H_7NO_3$: C, 54.9%; H, 4.57%; N, 9.15%); m.p. 84.5–85.5, *para* derivative.** The 3- and 4-acetoxypyridine-1-methiodides were prepared from the respective hydroxypyridines by treatment with methyl iodide followed by acetylation with acetic anhydride: m.p. 110–112°, *meta* derivative (Found: C, 34.02%; H, 3.70%; N, 5.04%. Calculated for $C_8H_{10}NO_2I$: C, 34.40%; H, 3.58%; N, 5.01%).

Finally, 3-(trimethylammonium iodide)phenylacetate (m.p. 175–175.5°) and 4-(trimethylammonium iodide)phenylacetate (m.p. 209.5–210°) were prepared by acetylation of the corresponding trimethylammonium iodide phenols using acetic anhydride (0°, pH 9.0). The acetylcholinesterase was from bovine erythrocytes (Mann Biochemicals, Inc.).

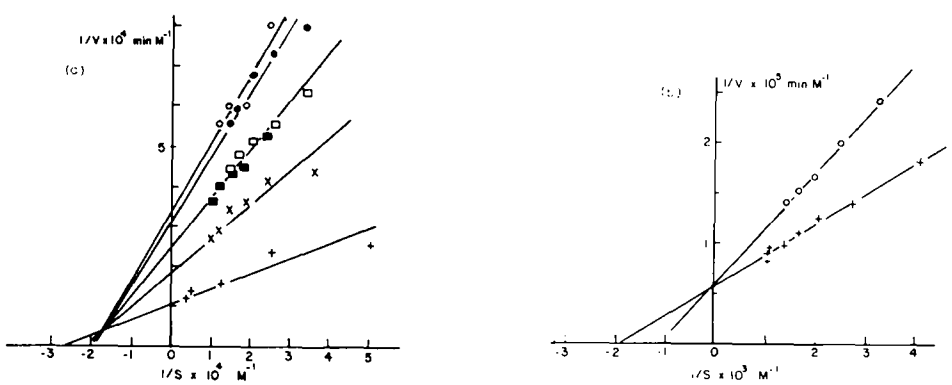


Fig. 1. (a) Inhibition of 3-acetoxypyridine-*N* methiodide at various concentrations of trimethylammonium ion: ○–○, $9.8 \cdot 10^{-3}$ M; ●–●, $9.4 \cdot 10^{-3}$ M; □–□, $4.9 \cdot 10^{-3}$ M; ■–■, $4.7 \cdot 10^{-3}$ M; ×–×, $2.45 \cdot 10^{-3}$ M; +–+, 0.0 M. (b) Inhibition of 3-acetoxypyridine-1-oxide by trimethylammonium ion: ○–○, $2.6 \cdot 10^{-3}$ M inhibitor; +–+, 0.0 M inhibitor.

** The *p*-acetoxypyridine-1-oxide and 1-methiodide hydrolyzed spontaneously in water at a rate too fast to measure. Because of this, neither was used subsequently in this study.

The acetylcholinesterase hydrolysis of each of these acetyl esters was measured by standard pH-stat techniques. The observed kinetic constants in water at 25° (pH 7.0) in the presence of 0.1 M NaCl are given in Table I. Control experiments measuring the rate of spontaneous hydrolysis of the substrates demonstrated that in each case, enzymatic hydrolysis was dependent on enzyme concentration. The results of inhibition of the acetylcholinesterase hydrolysis of the *m*-pyridine acetylesters by trimethylammonium iodide are given in Fig. 1.

RESULTS AND DISCUSSION

Acetylcholine analogues: As can be seen in Table I both *m*-(trimethylammonium) phenylacetate iodide and 3-acetoxypyridine-*N*-methiodide are hydrolyzed at a rate equal to or slightly less than acetylcholine. These are the first two synthetic substrates of acetylcholinesterase to be hydrolyzed under conditions where deacylation is rate limiting and which contain all of the essential features of acetylcholine. These two substrates also have K_m values smaller than acetylcholine, acetylthiocholine or phenyl acetate[10] (5 to 10^3 times smaller (Table I)). The only previously reported substrates, in addition to acetylcholine, where k_3 is rate limiting in hydrolysis are phenylacetate and acetylthiocholine[11], the former of which does not have the charge similarity to acetylcholine. In fact, as will be discussed below, phenylacetate may be an anomaly rather than a "good" substrate of acetylcholinesterase. Acetylthiocholine also seems to occupy a unique position. Recent results obtained by HILLMAN AND MAUTNER[13] place the hydrolysis of acetylthiocholine in a position between acetylcholine (k_3 rate limiting) and other substrates where k_2 is rate limiting. The two other substrates, *p*-(trimethylammonium)phenylacetate iodide and 3-acetoxypyridine-*N*-oxide are both hydrolyzed at rates much slower (10^3 and 10 times, respectively) than acetylcholine. The low v_{max} for *p*-(trimethylammonium)phenylacetate iodide can be

understood upon examination of the $N^+ \cdots C$ distance, 6.29 Å, which exceeds the critical 5 Å postulated as the distance between anionic and catalytic sites[1]. We conclude that while this substrate is clearly bound to the enzyme, the ester linkage is poorly oriented with regard to the catalytic site. Consistent with this result is the fact that K_i for the corresponding phenol is 10^2 times poorer than for the *meta* isomer. This can be attributed to the poor orientation of the *para* phenol[14]. The results for the pyridine-*N*-oxide, which is isoelectronic at the nitrogen atom with the pyridine-*N*-methiodide, will be discussed in detail below. It should be noted though that the *N*-oxide and *N*-methiodide are almost identical except for the presence of a partial charge in the former and a full formal charge in the latter on the quaternary nitrogen.

The results reported here for *m*-(trimethylammonium)phenylacetate iodide and 3-acetyxypyridine-*N*-methiodide clearly support the acetyl enzyme hypothysis. Both substrates are bound 5 30 times better than acetylcholine, yet the rate of hydrolysis observed is equal to that of acetylcholine for *m*-(trimethylammonium)-phenylacetate iodide and slightly less for 3-acetoxypyridine-*N*-methiodide. While it might be expected that the phenolic esters would hydrolyze faster than the choline ester (all other things being equal) in neither case is the enzymatic rate of acetyl-

choline hydrolysis exceeded (although it is equalled). If $k_3 \leq k_2$ (as is the case for these substrates) one would expect a common value for v_{max} as $v_{max} \cong k_3$ (since E_t is the same in all cases). In addition, these substrates have all the essential features of a good substrate of acetylcholinesterase and they obey the kinetic Scheme I describing acetylcholine hydrolysis. The most direct conclusion from these results is that all three substrates of acetylcholinesterase undergo hydrolysis in a similar manner, through a common intermediate, that being an acetyl-enzyme.

Inhibition with trimethylammonium hydrochloride: In order to verify that 3-acetoxypyridine-*N*-methiodide and *N*-oxide were true substrates of acetylcholinesterase and to further test the validity of Scheme I, their hydrolysis in the presence of the known inhibitor, trimethylammonium ion, was studied (Fig. 1). Trimethylammonium ion was chosen, as the complex *ES'I* (Scheme I) does not break down to give products[5,11]. The *N*-methiodide undergoes noncompetitive inhibition while the *N*-oxide shows competitive inhibition (Fig. 1). The inhibition constant, $K'_i = 4.7 \cdot 10^{-3}\ M^{-1}$ for the inhibition of the *N*-methiodide by trimethylammonium ion, is in good agreement with values obtained for this inhibitor with acetylcholine[3,11]. These results support the conclusion that k_3 is rate limiting for the *N*-methiodide and k_2 is rate limiting for the *N*-oxide. It is clear that these two compounds are acetylcholinesterase substrates and that their hydrolysis is catalyzed by the enzyme. The spontaneous rate of hydrolysis for these two substrates at pH 7.0 in water was at least 10 times less than v_{max} in both cases*. That results of hydrolysis of the *N*-methiodide when inhibited by trimethylammonium ion are very similar to acetylcholine lends further credence to Scheme I. These results then give additional support of a direct kinetic nature to the acetyl-enzyme hypothesis.

Effect of Charge on Catalysis: Classically, acetylcholinesterase has been depicted as containing an anionic site concerned with binding and a catalytic site concerned with hydrolysis of substrates[1]. These two centers of the active site have been depicted as acting in a manner independent of each other, although both are deemed necessary for full catalytic activity[1]. Recently, KRUPKA[5-7] concluded that phenylacetate is a substrate where k_3 is rate limiting, analogous to the case for acetylcholine[2]. It is interesting that in this case, no quaternary nitrogen is present in the substrate. Further, KRUPKA[6,7] has shown for neutral substrates, that charged inhibitors can, under certain conditions, accelerate the catalytic steps of the reaction between substrate and enzyme. The present study using 3-acetoxypyridine-*N*-methiodide (k_3 rate limiting) and 3-acetoxypyridine-*N*-oxide (k_2 rate limiting) further illustrates the fact that the presence of a charged group may alter the catalytic steps of hydrolysis. In comparing these results to those observed using phenylacetate as a substrate[11], it should be remembered that the *N*-oxide is bound as well as acetylcholine and 5-10 times

better than phenylacetate (where $k_3 \leq k_2$)[11]; the $N^+ \rightarrow C$ distance in the *N*-oxide and *N*-methiodide are identical and both agree well with this distance in acetylcholine

(Table I). (There is no $N^+ \rightarrow C$ distance in phenylacetate).

* Since $v_{max} = [E_t]\, k_3$ and if we estimate $[E_t]$ at 10^{-8} M then $k_3 \sim 10^3\ min^{-1}$, 10^8 times faster than spontaneous hydrolysis.

In light of these results, the most logical conclusion is that a positively charged group aids in the catalytic as well as binding steps. Krupka[5-7] has implicated a group of pK_a 6.3 as important for binding and most recently[6] has suggested that this group may have a role in the acylation step of hydrolysis although its main function is to bind substrate[5,6]. If this is correct, we propose that the uncharged *N*-oxide cannot orient the group of pK 6.3 in its proper steric position to assist in acylation, thereby making this step rate limiting. A corollary to this is that phenylacetate now appears to be an anomaly as an uncharged substrate where $k_3 < k_2$, and perhaps results with this substrate[5-7] should be examined more critically. This study demonstrates that the presence of a positively charged group is important in catalysis as well as binding, and the role charged groups have in acetylcholinesterase catalyzed-hydrolysis will have to be examined with care in future studies.

The authors gratefully acknowledge the support of this work by the National Science Foundation (Grant GB-795).

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