MOLECULAR PHARMACOLOGY, 27:630–633

The Slow Rate of Inhibition of Acetylcholinesterase by Fluoride

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Received December 1, 1984; Accepted March 27, 1985

SUMMARY

We measured the rate of reaction of fluoride with acetylcholinesterase using a stopped flow apparatus for measurements on the millisecond time scale with phenylacetate as a chromogenic substrate. We found that the second order rate constant is 5×10^3 liters/mol/sec, which is very slow for a small symmetric ion; it is 3–4 orders of magnitude smaller than for the substrates acetylcholine, acetylthiocholine, and phenylacetate. The slowness of this reaction suggests that fluoride does not find a preexisting binding site but must create one, probably by breaking and reforming hydrogen bonds. With hydrolysis measurements made on the usual time scale, we found $k_{cat} = 7.5 \times 10^5$ min^{-1} and $K_M =$ 2.0 mM. We also found that fluoride enhances substrate inhibition and that with low phenylacetate concentration the per cent inhibition is independent of substrate concentration.

INTRODUCTION

Fluoride is a reversible inhibitor of the acetylcholinesterase-catalyzed hydrolysis of acetylcholine, acetylthiocholine, and other substrates (1–3). This is surprising because acetylcholinesterase does not contain a metal ion and no metal ion is needed for activity (4). Other enzymes specifically inhibited by fluoride, such as cytochrome oxidase, contain a metal (Fe) or, as in the case of enolase, need a metal ion (Mg^{2+}) for activity.

The acetylcholinesterase-catalyzed hydrolysis of substrates involves a number of distinct steps: the formation of the enzyme-substrate complex, the acetylation of the enzyme, and the deacetylation of the acetylenzyme. It is not known whether all of these steps are inhibited, but generally this is assumed and there is evidence for it (3). However, there is also evidence that deacetylation may not be affected by fluoride because fluoride does not affect the decarbamylation of the carbamylenzyme or the desulfonylation of the sulfonylenzyme (5, 6). Fluoride does inhibit carbamylation and sulfonylation (5, 6). Fluoride also inhibits the reaction of arsenite with the enzyme (7).

In order to obtain more information, we tried to measure the effect of fluoride on the steady state level of acetylenzyme using a rapid mixer-sampler with ^{3}H-labeled acetylcholine (8). However, it appeared that fluoride might not react rapidly enough with the enzyme to reach a steady state in the sampling time of 15 msec. Since it was rather interesting that a small symmetrical ion might react slowly with the enzyme, we decided to investigate the rate of the reaction on a millisecond time scale using a stopped flow apparatus with phenylacetate as a chromogenic substrate (9–11). We did in fact find that the binding of fluoride is slow: 3 to 4 orders of magnitude slower than the binding of substrates. A chromogenic substrate present in the reaction medium has been used previously to monitor the reaction of an inhibitor (irreversible) with acetylcholinesterase (11, 12).

MATERIALS AND METHODS

Materials. Acetylcholinesterase (18 S) (EC 3.1.1.7) from electric eel was purified by affinity chromatography using the methods of Brooks *et al.* (13) and Soucie *et al.* (14). Phenylacetate (Sigma) was distilled prior to use. All other chemicals were reagent grade.

Methods. The kinetic constants for the hydrolysis of phenylacetate were determined in 10 mM phosphate, 0.5 M NaCl, pH 7.0, 23.5°. A high salt concentration was used to prevent aggregation of the enzyme. The formation of phenol was monitored at 270 nm, $\Delta\epsilon = 1415$, using a Zeiss PMQ II spectrophotometer for measurements on a time scale of minutes. A Durrum stopped flow spectrophotometer interfaced with a Digital Equipment MINC-11 computer via a gain and offset controller was used for measurements on a time scale of milliseconds.

In the stopped flow work, fluoride was added to the substrate syringe, and the rate of binding of fluoride was determined from the rate of development of inhibition as measured by the decrease in the rate of formation of phenol compared to measurements without fluoride. The velocity of the reaction, v, at any time was calculated from 250 digitalized absorption measurements, A, at constant time intervals Δt of 1–15 msec over a total time of 0.25–3.735 sec using secants

$$v_i = \frac{A_{i+1} - A_{i-1}}{2\Delta t \Delta\epsilon l} \quad (1)$$

The concentration of substrate at any time was calculated from

$$S_i = S_0 - \frac{A_i}{\Delta\epsilon l} \quad (2)$$

where $\Delta\epsilon$ is the molar absorbance difference and l was 2 cm. These two

This work was supported by United States Public Health Service Grant 5R01NS07156-18.

0026-895X/85/060630–04$02.00/0

equations were used to make a Lineweaver-Burk plot and check k_{cat} and K_M as compared to the values obtained from measurements on the usual time scale. The quantities v_i and S_i in the presence of fluoride were used to calculate the concentration of active enzyme, ϵ_i, and enzyme with bound fluoride, $\epsilon \cdot F_i$, from

$$\epsilon_i = \frac{v_i}{k_{cat}}\left[1 + \frac{K_M}{S_i}\right] \quad (3)$$

$$\epsilon \cdot F_i = E_0 - \epsilon_i \quad (4)$$

where E_0 is the total enzyme concentration.

Eq. 3 is valid in the presence of a dead-end inhibitor regardless of its mode of inhibition, since only enzyme forms that do not contain F^- participate as catalysts. We also calculated the equilibrium constant, K_F, from

$$\frac{\epsilon_\infty \times F^-}{\epsilon \cdot F_\infty} = K_F = \frac{k_{-1}}{k_1} \quad (5)$$

This equation is valid because the inactive enzyme forms with bound F^- have the same dissociation constants. We know this from the observation that K_F, so evaluated, is independent of S ($S \leq 1$ mM; see later).

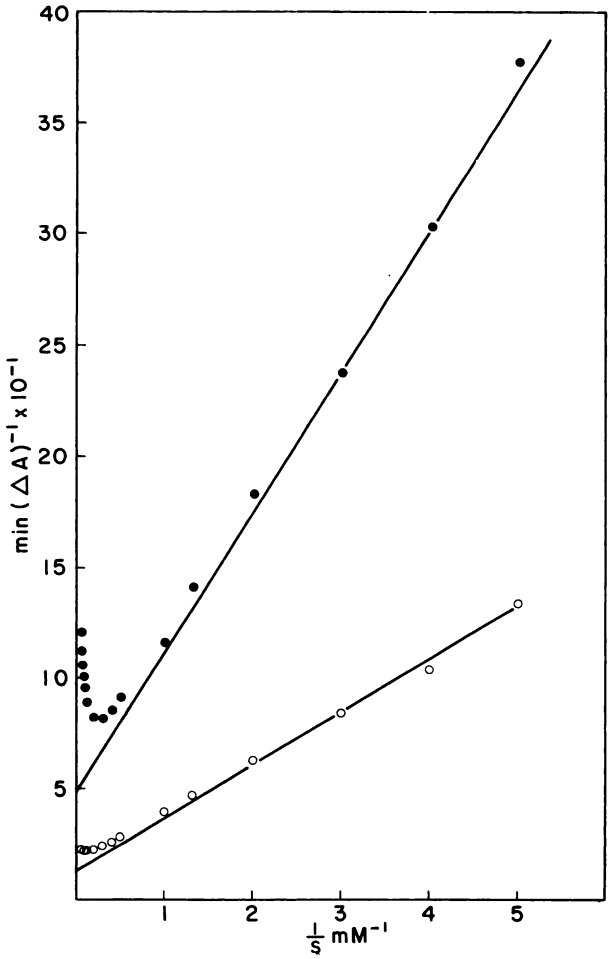

FIG. 1. *Acetylcholinesterase-catalyzed hydrolysis of phenylacetate; inhibition by fluoride*

The hydrolysis of phenylacetate was measured spectrophotometrically in the absence (O) and presence (●) of 1 mM fluoride at pH 7.0, 23.5° using assays of 3–5 min, starting a few seconds after mixing. The enzyme concentration was 36 pM. The highest substrate concentration was 20 mM. The Lineweaver-Burk line was drawn as described in the text.

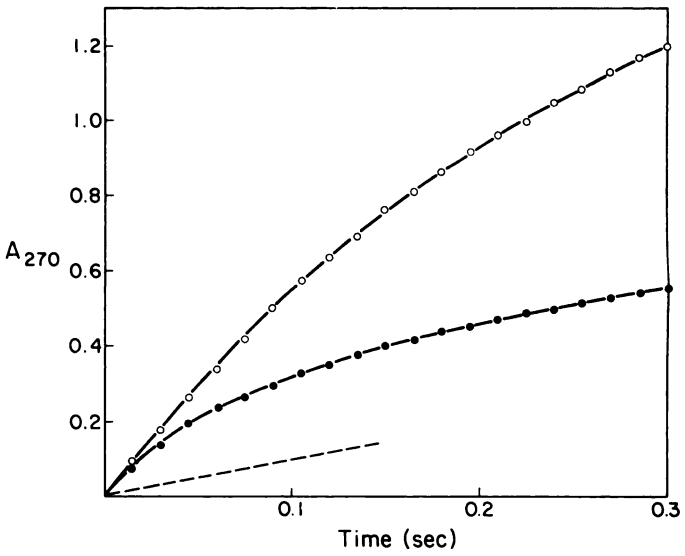

FIG. 2. *Acetylcholinesterase-catalyzed hydrolysis of phenylacetate; formation of phenol as a function of time*

The increase in absorbance, A_{270}, was measured during the hydrolysis of phenylacetate catalyzed by acetylcholinesterase without fluoride (O) and with 2 mM fluoride (●) at pH 7.0, 23.5°. The dashed line shows the increase in absorbance that should have been observed if fluoride reacted very rapidly with the enzyme. The enzyme concentration was 0.51 μM.

RESULTS AND DISCUSSION

Usual minute time scale measurements. Initial velocities of hydrolysis of phenylacetate were measured as a function of substrate concentration with and without fluoride and plotted as double reciprocals in accordance with the Lineweaver-Burk analysis (Fig. 1). It is apparent that there is substrate inhibition that is enhanced by fluoride. The mechanism of this enhancement of substrate inhibition by fluoride which also occurs with acetylthiocholine and propionylthiocholine[1] is not known. The occurrence of substrate inhibition makes it difficult to assign accurately values to k_{cat} and K_M. We have drawn the Lineweaver-Burk line as suggested by Cleland (15) so that its slope is one-half the slope of the line drawn through the intercept and the minimum of the curve. This graphic method is inaccurate because it is difficult to identify the coordinates of the minimum and also because it is based on the assumption that v is zero at infinite S. We do not need the values of k_{cat} and K_M for this report, but still it is interesting to give their values even though they may be less accurate than we would like. We obtain $k_{cat} = 7.5 \times 10^5$ min^{-1}, which is about the same as for acetylcholine. The value of K_M is 2 mM. These values are in agreement with the values reported by Bender *et al.* (9) and are similar to the values with the human erythrocyte enzyme (10).

If we ignore substrate inhibition and use only the data for $S \leq 2$ mM, we can draw a very good straight line corresponding to an empirical $k_{cat} = 5.1 \times 10^5$ min^{-1} and an empirical $K_M = 1.03$ mM. These values are the proper values to use for calculating v as a function of S for $S \leq 2$ mM. Turning to the curve with fluoride and considering

[1] Unpublished observation.

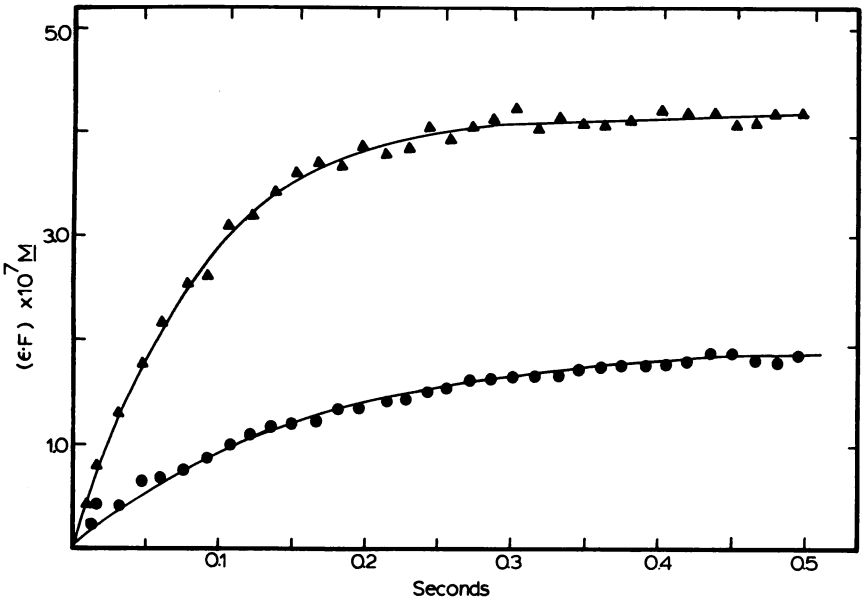

FIG. 3. *The formation of the fluoride-acetylcholinesterase complex as a function of time*

The upper curve shows the formation of the fluoride-enzyme complex for 2 mM fluoride and 0.51 μM enzyme and the lower curve for 1 mM fluoride and 0.32 μM enzyme, both in the presence of 1 mM phenylacetate. The points and curve were plotted by the computer.

TABLE 1

Kinetic constants for the inhibition of acetylcholinesterase by sodium fluoride using phenylacetate as substrate

Enzyme concentration: [a] 0.51 μM; [b] 0.32 μM; [c] 0.46 μM; [d] set. The error in k^* is about 7%. S is the initial concentration.

NaF	S	k^*	k_1 ($\times 10^{-3}$)	k_{-1}	K_F
mM	*mM*	sec^{-1}	$M^{-1}\ sec^{-1}$	sec^{-1}	*mM*
0.5[a]	1.0	5.0	4.9	2.5	0.52
1.0[b]	1.0	6.2	4.0	2.2	0.55
2.0[a]	1.0	12.2	4.9	2.4	0.49
2.0[c]	0.5	13.2	5.2	2.7	0.52[d]
2.0[c]	0.2	16.6	6.6	3.4	0.52[d]

only $S \leq 1$ mM, we find that the per cent inhibition is independent of S, yielding a value for $K_F = 0.52$ mM. We also measured v as a function of F^- at constant $S = 1.0$ and 0.2 mM. Again we found $K_F = 0.52$ mM. In this study, we used Eq. 5 in the form

$$\frac{v^0}{v} = 1 + \frac{F}{K_I}$$

where v^0 is the activity without F^- and v is the activity in the presence of F^-. We obtained very good straight lines ($F^- = 0.2$–4.0 mM), which indicates that F^- can completely block activity, i.e., F^- is a dead-end inhibitor.

Millisecond time scale measurements. The formation of phenol as a function of time is shown in Fig. 2 for a typical experiment. The data points are for three runs averaged by the computer. The slope declines because substrate is being consumed. We used this curve to calculate v as a function of S (using data out to 0.4 sec). The Lineweaver-Burk plot, which required a 2-fold extrapolation because our initial value of S was the same as the empirical K_M, was a good straight line ($r = 0.9954$). We obtained the empirical values, $k_{cat} = 5.0 \times 10^5\ min^{-1}$ and $K_M = 1.00$ mM, in very good agreement with the values obtained above from initial velocity measurements on the usual time scale.

The figure also shows that, when F^- is present, some time is required to reach the steady state degree of inhibition. The dashed line shows the rate of formation of phenol that would have been obtained if inhibition had been instantaneous. We treated the data for the formation of $\epsilon \cdot F$ according to the equation

$$\epsilon + F \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \epsilon \cdot F \qquad (6)$$

which yields

$$\epsilon \cdot F = \epsilon \cdot F_{\infty}(1 - e^{-k^* t}) \qquad (7)$$

where

$$k^* = k_1 F + k_{-1} \qquad (8)$$

$\epsilon \cdot F$ was calculated from Eqs. 3 and 4 and the values are displayed in Fig. 3. The value of k^* and of $\epsilon \cdot F_\infty$ was obtained by fitting the data points to Eq. 7 using the Marquardt algorithm nonlinear regression program. The values for k_1 and k_{-1} were then obtained from

$$k_1 = \frac{k^*}{F + K_F} \qquad (9)$$

$$k_{-1} = \frac{k^*}{1 + \frac{F}{K_F}} \qquad (10)$$

The data are presented in Table 1. At a constant initial substrate concentration of 1 mM and with fluoride varied from 0.5 to 2.0 mM, the same second order rate constant is obtained, indicating that the reaction of the enzyme with fluoride is second order. Also, the same value of k_{-1} is obtained, corresponding to a half-time for dissociation

of about 300 msec. The value of K_F is the same as we calculated from measurements made on the usual time scale.

There is a trend in the data when S is varied. In these experiments, F^- was substantially higher than K_F, which favors the accuracy of k_1. In these experiments with S = 0.2 mM and to some extent with $S = 0.5$ mM, the accuracy of the measurements is not as good as with $S = 1.0$ mM because $\Delta A/\Delta t$ is smaller to start and, of course, becomes smaller as the reaction with F^- proceeds. We therefore did not allow the computer to search out the best value of $\epsilon \cdot F_\infty$, but instead calculated $\epsilon \cdot F_\infty$ by assigning the value $K_F = 0.52$. The computer then calculated the best value of k^* using only the early data points, up to about 50% inhibition.

For $S = 0.2$ mM, the values of k_1 and k_{-1} are larger than for other values of S. This result can be explained by the fact that there are at least three enzymic species that might react with fluoride: the free enzyme, the enzyme-substrate complex, and the acetylenzyme. The last two are in constant ratio; but when combined, they decrease, and free enzyme increases as S is decreased. It appears then that fluoride reacts with free enzyme faster than with the enzyme-substrate complex or the acetylenzyme.

Rate constants for the binding of enzymes and substrates are usually very high and may approach a diffusion-controlled rate (16). A minimum value for this rate constant can be obtained from the requirement that it is greater than or equal to k_{cat}/K_M. For phenylacetate, it is greater than 6×10^6 M^{-1} sec^{-1} and for acetylcholine is greater than 5×10^7 M^{-1} sec^{-1}. The corresponding rate for F^- binding, 5×10^3 M^{-1} sec^{-1} is 3 to 4 orders of magnitude slower. Since fluoride is a small symmetrical ion, the slow reaction seems surprising. But with the advantage of hindsight, it seems that we might have expected the reaction to be slow. Given the nature of a protein, it seems reasonable to suppose that fluoride binds by ionic interaction and by hydrogen bonds as in ammonium fluoride crystals. We can explain the slow reaction by assuming that the hydrogen bond donors are already hydrogen bonded and, therefore, that F^- does not find a preexisting binding site but must create one by breaking up the existing hydrogen bonds and forming new ones. Such a process could have a significant energy of activation that would make the rate of binding slow. Another possibility is that for F^- to bind it must become protonated by reaction with, perhaps, an ammonium ion at the binding site. Since this involves the transfer of a proton from a weak acid to form a stronger acid, the reaction will be slow (17).

The binding of F^- is quite specific; it is not just one of a series of anions. We showed this by comparing the enzyme activity with 1 mM acetylthiocholine (18) in 0.1 M NaCl, $NaNO_3$, and NaBr and found no difference nor was there any difference in the amount of inhibition produced by F^-.

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