The Free Energy of Hydrolysis of Diethylphosphoryl Acetylcholinesterase

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SUMMARY

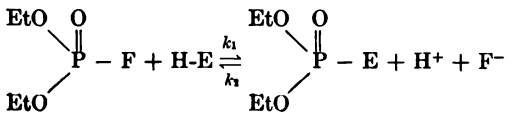
The equilibrium constant for the reaction of diethylphosphofluoridate with acetylcholinesterase was evaluated by measuring the second-order rate constant for the inhibition of the enzyme and the second-order rate constant for the restoration of enzyme activity, starting with inhibited enzyme and using fluoride as a reactivator. The value at pH 7.0, 25° in terms of total concentrations of reactants is 2.3×10^4.

The equilibrium constant for the hydrolysis of the diethylphosphoryl enzyme is estimated to be 2×10^5 in terms of total concentrations of reactants at pH 7.0; if diethylphosphoric acid is written in its acidic form, the equilibrium constant is 0.5. This last value is typical of low energy bonds. Assuming that a diethylphosphorylserine side chain is formed, the energy change in the reaction at the active site has the normal value for the bonds involved.

INTRODUCTION

Diethylphosphofluoridate inhibits acetylcholinesterase by reaction with the enzyme to form a kinetically stable diethylphosphoryl enzyme derivative. The site which is phosphorylated is believed to be the same as the site which is acylated during the course of enzymic hydrolysis (1–3).

The phosphorylation reaction is reversible, in principle, and is demonstrated to be reversible by the reaction (Eq. 1) of the phosphoryl enzyme with fluoride ion to produce an active enzyme:



(1)

where H-E is the enzyme.

Fluoride ion was previously shown to be a reactivator of sarin (isopropyl methyl phosphonofluoridate)-inhibited acetylcholinesterase (4). Its reactivity in dephosphorylating the inhibited enzyme is consonant with its high nucleophilicity toward phosphorus (5, 6).

The equilibrium constant for this reaction can be evaluated by measuring the kinetic constants k_1 and k_2. If, in addition, the equilibrium constant for the hydrolysis of the inhibitor is known, the equilibrium constant for the hydrolysis of the diethylphosphoryl enzyme can be calculated. This last quantity is related to the kind of chemical group that is phosphorylated in the enzyme and to the secondary interactions of the diethylphosphoryl group with the enzyme protein.

In this paper we describe the application of this method to the evaluation of the two equilbrium constants. We find that the

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equilibrium of Eq. 1 lies far to the right and that the diethylphosphoryl enzyme is a "low energy" compound with respect to hydrolysis.

METHODS

Enzyme. Acetylcholinesterase from electric tissue (electrophorus electricus) was partially purified by column chromatography (7). The preparation had a specific activity of about 100 mmoles of acetylcholine hydrolyzed per hour per milligram of protein. The enzyme activity was measured at 25° and pH 7.00, in 0.1 M NaCl, 0.02 M sodium phosphate, 1×10^{-5} M EDTA, and 0.01% gelatin with 3×10^{-3} M acetylcholine, using the hydroxamic acid method (8). A 0.1-ml sample of enzyme solutions was added to 1 ml of acetylcholine solution, and the reaction was stopped 2–8 min later by adding 2 ml of alkaline hydroxylamine.

Inhibition. Portions of 10^{-8} N enzyme solution were incubated at 25° and pH 7.0 with and without diethylphosphofluoridate in the previously described buffer, and enzyme activities in 0.1-ml samples were measured after various time intervals. Ten runs were made at five different concentrations of inhibitor in the range 2.6×10^{-7} to 2.2×10^{-6} M.

The inhibitor was dissolved in 0.01 M sodium acetate buffer at pH 5.0 to make a 1×10^{-3} M stock solution and stored in ice. Portions were diluted with pH 7.0 buffer just before being used.

Since the inhibitor was ten times less concentrated in the enzyme assay solution than in the solution producing inhibition and since over 90% of the enzyme was combined with substrate in this solution, no inhibition occurred during the measurement of enzyme activity.

Reactivation. Solutions of diethylphosphoryl enzyme were prepared by incubating portions of 6×10^{-7} N enzyme solution with 2.5×10^{-6} M, 1.8×10^{-6} M, and 9×10^{-7} M diethylphosphofluoridate in buffer for 1–1.5 hr. The enzyme was completely inhibited in the first two cases and 90% inhibited in the last.

Portions of the inhibited enzyme solutions were diluted 50 times with buffer and with buffer containing 1×10^{-3} M to 8×10^{-3} M sodium fluoride.

After various times at 25°, samples were withdrawn and the enzyme activity was measured. No significant recovery of enzyme activity occurred in controls without fluoride.

RESULTS

The inhibition reactions followed pseudo-first-order kinetics. Since the measured first-order constants were proportional to the concentration of inhibitor, the reaction is bimolecular. Measurements were made at five inhibitor concentrations in the range 2.6×10^{-7} to 2.2×10^{-6} M, and the average bimolecular rate constant, k_1, was $2.3 \pm 0.1 \times 10^{-5}$ l/mole·min (Table 1). Some typical curves are shown in Fig. 1.

TABLE 1
Inhibition of acetylcholinesterase by diethylphosphofluoridate

Concentration of inhibitor (M)	$t_{1/2}$ (min)	k_1 (l/mole · min $\times 10^5$)
2.16×10^{-6}	1.45	2.2
2.16	1.45	2.2
1.56	2.1	2.1
1.56	2.0	2.2
1.07	2.8	2.3
1.07	2.6	2.5
1.07	2.8	2.3
1.07	3.0	2.2
0.52	5.7	2.3
0.265	9.6	2.7

The reactivation reactions were more complicated in that recovery of enzymic activity was not complete in most cases (Table 2).

The semilogarithmic plots of inhibited enzyme versus time were not straight, but curved to the right and approached a limit corresponding to between 72 and 100% recovery. Straight lines were obtained by plotting log $(E\infty - E)/E\infty$ versus time, where E is the concentration of active

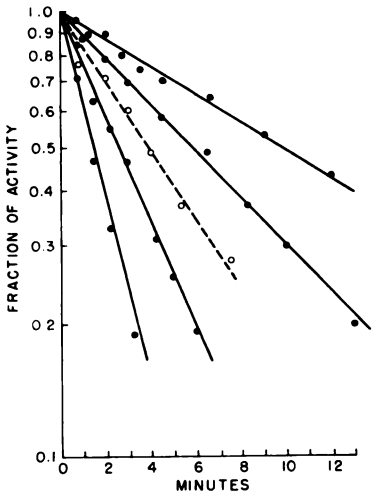


FIG. 1. *Inhibition of acetylcholinesterase by diethylphosphofluoridate at 25°, pH 7.0*

Data are plotted in semilog form in accordance with the pseudo-first-order rate equation. The concentrations of inhibitor are from left to right, filled, circles, 2.16×10^{-6} M, 1.07×10^{-6} M, 0.52×10^{-6} M, 0.265×10^{-6} M. The open circles are data for 2.16×10^{-6} M inhibitor in the presence of 1×10^{-3} M fluoride, showing that fluoride inhibits the reaction of diethylphosphofluoridate with acetylcholinesterase. Fluoride also inhibits the catalytic activity of this enzyme.

TABLE 2

The reactivation of inhibited enzyme by fluoride

In experiment 1, 2.5×10^{-6} M diethylphosphofluoridate was used to inhibit the enzyme; in experiment 2, 1.9×10^{-6} M; in experiment 3, 9×10^{-7} M. The values of k were calculated from k_{app} using the relationship $k_{app} = (E_t/E_\infty)k$. It is not certain whether k_{app} or k are the right values for the second-order rate constant, but for our purposes the difference is unimportant.

Expt. no.	Conc. of F^-	% Recovery of enzyme activity	k_{app} (l/mole · min)	k_2 (l/mole · min)
1	2×10^{-3}	72	14.0	10.1
2	4×10^{-3}	92	10.5	9.7
	2.5×10^{-3}	85	11.5	9.8
	2×10^{-3}	82	12.1	9.9
	1×10^{-3}	75	12.6	9.5
3	8×10^{-3}	100	9.1	9.1
	4×10^{-3}	100	9.6	9.6
	2.5×10^{-3}	90	8.9	8.0

enzyme and $E\infty$ is the concentration of active enzyme ultimately attained (Fig. 2).

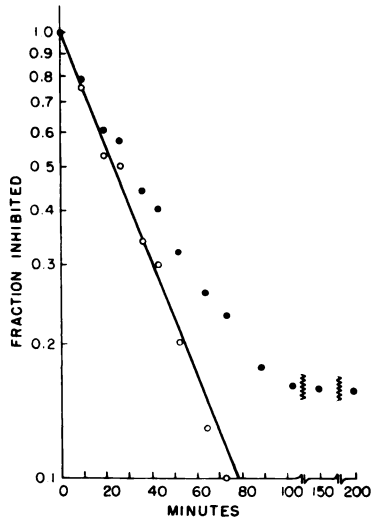


FIG. 2. *Reactivation of diethylphosphofluoridate-inhibited acetylcholinesterase by fluoride*

The data are for experiment 2, 2.5×10^{-3} M fluoride ion (Table 2) plotted as in Fig. 1. The original data are shown by filled circles; only about 85% of the activity was recovered. The values of the fraction of inhibited enzyme, calculated on the basis that only 85% of the initial enzyme can be recovered, are shown by the open circles. No reactivation occurred in the absence of fluoride.

The apparent pseudo-first-order rate constants obtained in this way were converted to the bimolecular rate constant by dividing them by the concentration of fluoride. The values for this apparent constant may show a trend, although the variation is not certainly beyond our experimental error.

Whether these values are to be "corrected" depends upon the reason for incomplete recovery. If the recovery is incomplete because the final enzyme activities correspond to equilibrium between the diluted inhibitor, fluoride, and enzyme, the apparent constants are given by $(E_t/E\infty)$ k_2 where E_t is the total enzyme concentration and k_2 is the real bimolecular rate constant. Equilibrium is a distinct possibility because after dilution into the

fluoride solution the concentration of diethyl phosphofluoride is calculated as 1.7×10^{-8}, 3.8×10^{-8}, and 5×10^{-8} M. These concentrations would be more than high enough to account for the incomplete recoveries, and the recoveries do appear to be more complete when less inhibitor is used, except that fluoride inhibits the phosphorylation reaction. Moreover, some hydrolysis of the inhibitor occurs during the 1–1.5 hr period used to inhibit the enzyme. When these effects are taken into account it appears that the inhibitor concentration might be only ⅓ to ½ as large as needed to account for the incomplete recoveries. Despite this uncertainty, we have tabulated the values of the bimolecular rate constant calculated on the assumption that incomplete restoration of enzyme activity is due to equilibrium. The difference in the value of k_2 is not significant for our purposes, and we have used the "round" value 10 liters per mole per minute in calculating the equilibrium constant. The rate constants reported here are for total concentrations of reactants, without regard to the various prototropic forms and without regard to H^+. Therefore, the values of the rate constants are appropriate only for pH 7.0.

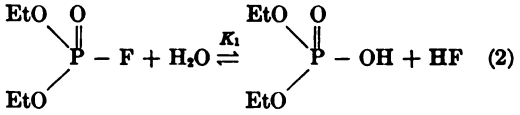
The equilibrium constant for the reaction of diethylphosphofluoridate with acetylcholinesterase, given by the ratio of the rate constants for the forward and reverse reactions is:

$$\frac{[(EtO)_2POE]_t(F^-)_t}{[(EtO)_2POF](HE)_t} = 2.3 \times 10^4$$

This constant is for an equilibrium expression not containing H^+ and in terms of total concentrations (subscript t) and is therefore appropriate only for pH 7.0 The reader is reminded that an equilibrium expression written to contain total concentrations applies only to one pH, but an equilibrium expression written to contain only one prototropic form applies to all pH values. Although arbitrary, the selection of the prototropic form determines the value of the equilibrium constant. Even if this constant were multiplied by 10^{-7} to obtain a new constant corresponding to an equilibrium expression containing H^+ as written in Eq. 1, and even though there is little undissociated hydrofluoric acid above pH 5, this new constant might still be pH dependent because the acidic groups in the free and diethylphosphoryl enzymes might have different dissociation constants. However, since these differences are probably not excessively large, a rough measure of the equilibrium at other pH values might be obtained in this way.

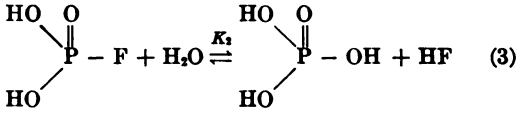
DISCUSSION

The equilibrium constant for the hydrolysis of diethylphosphofluoridate (Eq. 2)



(2)

is not known but the constant for the hydrolysis of fluorophosphoric acid (Eq. 3)

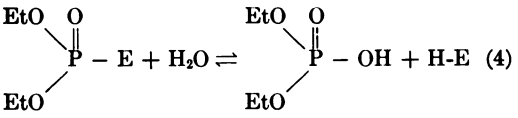


(3)

was found to be 3, in terms of the acidic species and including water as written above (9, 10). The value of K_1 should be about the same as K_2, perhaps slightly larger, since diethylphosphoric is slightly more acidic than phosphoric acid. We are interested in the equilibrium constant for reaction 2 in terms of total concentrations at pH 7.0. Assuming that the equilibrium constants for reactions 2 and 3 are the same and using 4.2×10^{-2} as the ionization constant of diethylphosphoric acid and 3.5×10^{-4} as the ionization constant for hydrofluoric acid, the calculated equilibrium constant for reaction 2 using *total* concentrations at pH 7.0 is

$$3\left(1 + \frac{4.2 \times 10^{-2}}{10^{-7}}\right)\left(1 + \frac{3.5 \times 10^{-4}}{10^{-7}}\right) = 5 \times 10^9$$

Subtracting Eq. 1 from Eq. 2 yields the equation for the hydrolysis of the diethylphosphoryl enzyme (Eq. 4):



The value of the equilibrium constant for this reaction is given by the ratio of the equilibrium constants for reactions 2 and 1, $5 \times 10^9/2.3 \times 10^4 = 2 \times 10^5$. This value corresponds to *total* concentrations of reactants at pH 7.0.

If diethylphosphoric acid is to be written in its acidic form in the equilibrium expression, the value 2×10^5 has to be divided by $(1 + [K_a/(H^+)])$ where $(H^+) = 10^{-7}$ and K_a, the ionization constant of the acid, is 4.2×10^{-2}. The resulting value is 0.5; $\triangle F^\circ = 0.42$ kcal/mole. This value still applies only to pH 7.0 because the enzyme forms must be written as total concentrations. However, since it is unlikely that any ionization constant in the enzyme is enormously altered, the value is probably appropriate for other pH values within a pH range where the enzyme structure does not change. This last value is typical of "low energy" phosphate bonds. The equilibrium constant for α-glycerophosphate is 0.33 (11) and for *O*-phosphorylethanolamine is 0.56 (12).

In comparing the equilibrium constant for the hydrolysis of the diethylphosphoryl enzyme with model compounds, we assume that the secondary interaction of the diethylphosphoryl group with the enzyme protein is not large. The structures of substrates and inhibitors afford no indication that a diethylphosphoryl group would interact extensively with this enzyme and diethylphosphate does not inhibit (reversibly) this enzyme. In further examining this question we found that diethylphosphoryl choline produces somewhat less inhibition (reversible) than choline but we did not pursue these studies in detail. The assumption that little interaction occurs between the diethylphosphoryl group and the enzyme appears to be reasonable. In making this comparison we further assume that the free energy of hydrolysis of a single ligand in a phosphate triester is not very different from the free energy of hydrolysis of a monoester when the reactions are written in terms of the acidic species. Since we made essentially the same assumption with respect to the hydrolysis of diethylphosphofluoridate, errors from this source tend to cancel in the comparison. The comparison indicates that a low energy bond is involved and that the free energy of hydrolysis is consistent with the phosphorylation of a hydroxyl group in a serine side chain. This result is consistent with the isolation of serine phosphate from the hydrolyzate of inhibited enzymes (13–17), and with the conclusion derived therefrom that the hydroxyl group of a serine side chain is the catalytic nucleophile of the active site. Since the diethylphosphoryl enzyme can be readily reactivated, it must still retain the same structure as the native enzyme or at least a structure that readily reverts to the native structure when the inhibited enzyme is dephosphorylated. The high reactivity of the diethylphosphoryl enzyme and the specificity involved in its reaction with nucleophilic agents indicate that the diethylphosphoryl enzyme is analogous to the acetyl enzyme, an enzyme derivative which is an intermediate in the catalytic process carried out by the enzyme. In other words, the diethylphosphoryl enzyme is a catalytic entity. If the group which is phosphorylated in this molecular species is a serine side chain, it is clearly a very special serine side chain, for its chemical reactivity is vastly different from other serine side chains or from simpler serine derivatives. Under these circumstances one should surely wonder if the value of the bond energy might not be very different from the values for simpler phosphate esters. Yet our results indicate that the free energy change in the hydrolysis of the diethylphosphoryl enzyme, a reaction involving the catalytic functional group of the active site, has the normal value for the bonds involved. The conclusion that the free energies of hydrolysis of catalytic enzyme intermediates have the normal values for a serine side chain has also been reached in cases involving acyl derivatives of chymotrypsin and phosphoryl alkaline phosphatase (12,

18, 19). It was previously suggested on the basis of the theory of substrate inhibition, that the acetyl derivative of acetylcholinesterase had a small free energy of hydrolysis (20).

ACKNOWLEDGMENTS

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