Oxime Reactivation of Diethylphosphoryl Human Serum Cholinesterase

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SUMMARY

Reactivation of diethyl p-nitrophenyl phosphate inhibited human serum cholinesterase by pyridine-2-aldoxime methiodide and isonitrosoacetophenone has been investigated as a function of reactivator concentration, pH, and temperature in boric acid-borax buffer and in salt solution. Constants shown were the dissociation constants of the diethylphosphoryl cholinesterase reactivator complex for the reactivation by pyridine-2-aldoxime methiodide and the bimolecular rate constants for that by isonitrosoacetophenone. The protonation constants of the diethylphosphoryl cholinesterase were evaluated from the relationship between pH and the dissociation constants of the complex; they indicated a value of 8.4 in buffer medium and 7.8 in salt solution. Changes in reactivation rate and in apparent activation energy of reactivation due to the medium changes may be explained by the nature of the activated complex formed in the respective reactivation processes.

In the present work, the reactivation was carried out with PAM\(^1\) and isonitrosoacetophenone, with the use of diethyl p-nitrophenyl phosphate-inhibited human serum cholinesterase as the enzyme source. Studies on the reactivation of inhibited human serum cholinesterase have been relatively few (8, 9). The dissociation constants and the rate constants for the breakdown of the complex by PAM reactivation and the bimolecular rate constants for isonitrosoacetophenone reactivation are reported here for this enzyme. The rate measurements were performed as a function of concentration of the reactivators, pH, and temperature in boric acid-borax buffer. The salt effect on reactivation was also studied.

EXPERIMENTAL PROCEDURE

Materials

Commercial pyridine-2-aldoxime methiodide (Calbiochem) and isonitrosoacetophenone (Aldrich) were twice recrystallized from aqueous ethanol and their melting points were 218° (with decomposition, reported m.p., 218-220° (10)) and 125.5-127° (reported m.p., 124-126° (10)), respectively. The stock solutions of PAM prepared in distilled water were kept at 4° and of isonitrosoacetophenone prepared in 10% ethanol were kept at room temperature; both stock solutions, examined spectrophotometrically, were stable for 1 week. The o-nitrophenyl butyrate substrate (11) was freshly prepared for each set of experiments from a 0.5 M stock solution with phosphate buffer (50 mM, pH 7.6) containing 15% (v/v) isopropyl alcohol. The substrate concentration was 1.55 mM.

Human serum used in this work was obtained from the blood of volunteers. It was stored in a deep freeze without anticoagulant.

Methods

Inhibition of Human Serum Cholinesterase—Human serum, 1.00 ml, was treated with 10 µl of diethyl p-nitrophenyl phosphate (0.01 mM in isopropyl alcohol) at 3°, and the mixture was allowed to stand about 1 hour at room temperature before being stored in a deep freeze. It was about 70% inhibited. After having been stored for 96 hours in a deep freeze, the inhibited serum showed no indication of a change in its nature toward reactivation.

\(^1\)The abbreviations used are: PAM, pyridine-2-aldoxime methiodide; DFP, diisopropyl phosphorofluoridate.
Reactivation—To 0.10 ml of inhibited human serum were added 2.60 ml of boric acid-borax buffer (30 mM) of appropriate pH and 0.30 ml of reactivator of suitable concentration to make up a total volume of 3.00 ml and the pH of samples was measured with a Beckman model GS pH meter. The reactivation was carried out in a water bath at 25 ± 0.1°C. The residual free inhibitor, if present, does not interfere with reactivation.

Determination of Enzyme Activity—The procedure used was essentially the same as that described by Main (11), except that the substrate, o-nitrophenyl butyrate, was prepared in a phosphate buffer containing 15% (v/v) isopropyl alcohol, and that the subsequent reaction mixture was diluted with 15% (v/v) aqueous ethanol. At intervals, 0.20 ml of reaction mixture was added to two 5.00-ml substrate solutions, one for the reference solution and the other for the sample. At the end of a 30-min period of incubation at 25 ± 0.1°C, the enzymatic reaction was stopped by addition of 2 drops (about 0.05 ml) of DFP (25 mM in isopropyl alcohol). The DFP was added to the reference solution before addition of the reaction mixture. The solutions were then diluted to 10.0 ml with 15% (v/v) aqueous ethanol. The absorbances (A) of the reference and the sample were measured with a Hilger spectrophotometer at 414 mp in 1-cm silica cells against water and the difference of the two represented the amount of o-nitrophenyl butyrate hydrolyzed by the enzyme.

For each set of reactivation experiments, it was necessary to measure the activity of normal enzyme (Ao), the reactivated sample (A), and the inhibited enzyme (A1). The fraction of the inhibited enzyme remaining in the system was calculated as

\[
F = \frac{A - A_1}{A_0 - A_1}
\]

RESULTS

The kinetics of the reactivation process may be represented by the scheme

\[
EI + R \overset{k_R}{\longrightarrow} EIR \overset{k_R}{\rightarrow} E + P
\]

where EI is the inhibited enzyme, R is the reactivator, E is the reactivated enzyme, P is the products as yet unidentified, EIR is the intermediate complex, and KR and kR are the dissociation constant and the rate constant for breakdown of the complex, respectively. The first stage in this scheme involves an equilibrium step

\[
K_R = \frac{[EI][R]}{[EIR]} \tag{1}
\]

Since \([EI]_o = [EI] + [EIR] + [E] \), one obtains, from Equation 1,

\[
[EIR] = \frac{[EI]_o - [E]}{1 + \frac{K_R}{[R]}}
\]

where \([EI]_o\) represents the initial concentration of the inhibited enzyme present. It follows that the rate of reactivation is

\[
v = \frac{dE}{dt} = k_R[EIR] = \frac{k_R([EI]_o - [E])}{1 + \frac{K_R}{[R]}} \tag{2}
\]

Letting the initial concentration of the inhibited enzyme equal the final enzyme concentration, i.e. \([EI]_o = [E]_o\) and \([R] \gg [EI]_o\), Equation 2 gives rise to a simple first order rate equation

\[
\frac{dE}{dt} = k_\text{app}([E]_o - [E]) \tag{3}
\]

where

\[
k_\text{app} = \frac{k_R}{1 + \frac{K_R}{[R]}} \tag{4}
\]

Integration of Equation 3 gives

\[
2.303 \log \frac{[E]_o - [E]}{[E]_o} = -k_{\text{app}}t \tag{5}
\]

where \([E]_o\) represents the amount of the inhibited enzyme reactivated in time t. The ratio \(\frac{[E]_o - [E]}{[E]_o}\) represents the fraction of the inhibited enzyme remaining in the system after a period of reactivation and can be calculated as described under "Experimental Procedure."

Equation 4 can be inverted to give the equation of a straight line

\[
\frac{1}{k_{\text{app}}} = \frac{1}{k_R} + \frac{K_R}{k_R[R]} \tag{6}
\]

The slope of the line gives \(K_R/k_R\) and the intercept on the ordinate gives \(1/k_R\); so both \(K_R\) and \(k_R\) can be calculated. At very low concentration of the reactivator, i.e. \([R] \ll K_R, k_R/K_R\) can be set equal to a bimolecular rate constant.

Reactivation with Pyridine-β-Alanine Methiodide—The reactivation was carried out over the range of PAM concentration from 0.1 to 1 mM and pH 7.6 to 9.0 in a 40 mM boric acid-borax buffer at 25 ± 0.1°C. Within a 15-min period of reactivation, the initial rate followed pseudo-first order kinetics (Fig. 1). When the reciprocals of \(k_{\text{app}}\) and \(R^+\) (the molar concentration of dipolar ion of PAM) were plotted, a straight line was obtained which did not pass through the origin (Fig. 2A). It is concluded that a saturation effect of the reactivator occurs at high concentrations and measurable amounts of reversible complex are formed. The reason for the use of a concentration of dipolar ion in the plot is discussed later. Dipolar ion concentrations were computed with \(pK_a = 7.84\) (reported values by potentiometric method: 7.82 (10) and 8.0 (12)) for the reactivation carried out in buffer medium and 8.10 in salt solution. Both \(pK_a\) values were determined spectrophotometrically at \(\lambda_{\text{max}} = 340 \mu\text{m}\) in respective media at 26 ± 1°C.

The effect of pH on \(k_{\text{app}}\) and \(pK_a\) is shown in Figs. 2 and 3. The \(pK_a\) values were calculated from the slope of straight lines and \(k_R = 0.067 \text{ min}^{-1}\) in accordance with Equation 6. It should be pointed out that the values of \(pK_a\) obtained in this way are the true for the reaction \(EIH^+ + R^+ = EIR\). It follows that the change of \(pK_a\) with respect to pH changes depends on the extent to which the \(EIH^+\) ionizes. It is for this reason that the concentration of dipolar ion of PAM, rather than that of unionized species, was used in a plot of reciprocals. Accordingly, the inflection points at 7.8 and 8.4 in Fig. 3 were taken as the dissociation constants for the inhibited enzyme \(EIH^+\) \((pK_{EIR^+})\) in a buffer medium with and without salt present, respectively. As a comparison, the \(pK_a\)-pH curve for the interaction between horse serum cholinesterase and acetylcholine (taken from Dixon (13)) is also shown. The inflection point at pH 7.7 in the curve
Fig. 1. Development of reactivation produced by reaction of diethyl p-nitrophenyl phosphate-inhibited human serum cholinesterase with four different concentrations of PAM: a, 1 mM; b, 0.5 mM; c, 0.25 mM; and d, 0.1 mM, in 40 mM boric acid-borax buffer at pH 8.55 and 25 ± 0.1°C.

The rate constant for the breakdown of the complex, \(k_b\), has a value of 0.067 mM⁻¹, being independent of pH and free from salt effect. It makes no difference whether the concentration of the dipolar ion or unionized species is chosen for the reciprocal plot. Hence the affinity between the inhibited enzyme and the reactivator may be of primary importance in the rate process, as seen in Equation 1.

Reactivation with Isonitrosoacetophenone—The rate of reactivation was studied over the pH range of 7.6 to 9.0 and the concentration of reactivator 0.27 to 1 mM. The saturation effect was ascribed to the dissociation constant of the enzyme pKₐ (13). This suggests that a phosphorylation of cholinesterase by a diethylphosphate radical increases the pKₐ value by 0.7 unit. The validity of pKₑᴴ⁺ is discussed below under “Reactivation with Isonitrosoacetophenone.”

The pKₑ₋ₐpH curve appears to be the same type of curve to which the pKₑ₋ₐpH curves belong. In the region in which pKₑ₋ₐ is independent of pH, the dissociation of complex gives no change of charge, i.e. \(EIH⁺R²⁺ = EIH⁺ + R⁺\), and zero slope prevails. On the other hand, if the dissociation proceeds as \(EIH⁺R²⁺ = E⁻ + R⁺\), in which a proton ionizes away from \(EIH⁺\) in a medium having a pH greater than pKₑᴴ⁺, a change of -1 unit charge gives rise to a -1 slope (Curves a and b in Fig. 3). Such difference, however, is expected in view of the fact that the former two (Curves a and b) represent nucleophilic reaction, while the latter represents an electrophilic one.

The results obtained in salt solution followed a pattern similar to that reported by Wilson (12) in which there is a decrease in \(k_{app}\) and a shift of pH optimum to a lower value. The reciprocal plot in accordance with Equation 6 gave straight lines, just as in the case of competitive inhibition (Fig. 2C). This appears to satisfy the explanation given by Wilson (12) that the decrease in optimal rate in salt solution is due to competition between sodium ion and quaternary nitrogen for the anionic site of the inhibited cholinesterase. This argument, however, is in conflict with the finding conducted with an uncharged reactivator and is discussed later. A shift of pH optimum, or pKₑ₋ₐ, to a lower value in salt solution is apparently a matter of secondary salt effect, resulting from a change of chemical equilibrium in a reactivation process (14).

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Reactivation with Isonitrosoacetophenone—The rate of reactivation was studied over the pH range of 7.0 to 9.0 and the concentration of reactivator 0.27 to 1 mM. The saturation effect...
of reactivator did not occur up to 1 mM in either buffer or salt medium, so that a plot of the reciprocals of $k_{app}$ and $R$ in accordance with Equation 6 yielded a straight line which passed through origin. In this case, $k_{app} = k_R/R$. Attempts to raise the reactivator concentration higher than 1 mM failed because of low solubility of isonitrosoacetophenone under the experimental conditions studied. The optimal rate was 4.92 liter mole$^{-1}$ min$^{-1}$ in buffer at pH 8.3 and 15.9 in salt medium at pH 7.9 (Fig. 4). The inhibited human serum cholinesterase was about 3 times more difficult to reactivate than erythrocyte cholinesterase under similar experimental conditions (3.52 against 10.7 liter mole$^{-1}$ min$^{-1}$) (4). The increase in optimal cholinesterase under similar experimental conditions (3.52 against 10.7 liter mole$^{-1}$ min$^{-1}$) was about 3 times more difficult to reactivate than erythrocyte and pK = 8.03.

Isonitrosoacetophenone 8.23; (b) in salt solution, with $k_{obs} = 14.1$ at pH 7.7, pK$_{nm+}$ = 7.8, nitrosoacetophenone reactivation. Points, experimental values. Curves, constructed according to Equation 7 (a) in boric acid-borax buffer, with $k_{obs} = 4.92$ at pH 8.3, pK$_{Em+}$ = 8.4, and pK$_a$ = 8.23; (b) in salt solution, with $k_{obs} = 14.1$ at pH 7.7, pK$_{Em+}$ = 7.8, and pK$_a$ = 8.03.

**Fig. 4.** Effect of pH on the bimolecular rate constant for isonitrosoacetophenone reactivation. Points, experimental values. Curves, constructed according to Equation 7 (a) in boric acid-borax buffer, with $k_{obs} = 4.92$ at pH 8.3, pK$_{Em+}$ = 8.4, and pK$_a$ = 8.23; (b) in salt solution, with $k_{obs} = 14.1$ at pH 7.7, pK$_{Em+}$ = 7.8, and pK$_a$ = 8.03.

**TABLE 1**

<table>
<thead>
<tr>
<th>Reactivator</th>
<th>pK$_a$</th>
<th>pK$_{Em+}$</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hydroxylamine</td>
<td>6.62</td>
<td>6.9</td>
<td>16</td>
</tr>
<tr>
<td>Nicotinohydroxamic acid methiodide</td>
<td>6.48</td>
<td>7.0</td>
<td>3</td>
</tr>
<tr>
<td>Nicotinohydroxamic acid</td>
<td>8.26</td>
<td>8.0</td>
<td>3</td>
</tr>
<tr>
<td>Acetohydroxamic acid</td>
<td>9.43</td>
<td>8.3</td>
<td>3</td>
</tr>
<tr>
<td>Pyridine</td>
<td>5.2</td>
<td>6.6</td>
<td>3</td>
</tr>
<tr>
<td>Monoisonitrosoacetone</td>
<td>7.3</td>
<td>7.8</td>
<td>4</td>
</tr>
<tr>
<td>Isonitrosoacetophenone</td>
<td>8.25</td>
<td>8.3</td>
<td>Present work</td>
</tr>
</tbody>
</table>

$^a$ The reactivation was conducted with diethyl p-nitrophenyl phosphate-inhibited α-chymotrypsin.
$^b$ The reactivation was conducted with diethyl p-nitrophenyl phosphate-inhibited electrophorus electricus acetylcholinesterase.
$^c$ The reactivation was conducted with tetraethyl pyrophosphate-inhibited human erythrocyte cholinesterase.

Table II

<table>
<thead>
<tr>
<th>Reactivator</th>
<th>$E_0$</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>In buffer</strong></td>
<td><strong>In salt solution</strong></td>
</tr>
<tr>
<td>Pyridine-2-aldoxime methiodide</td>
<td>12.6</td>
</tr>
<tr>
<td>Isonitrosoacetophenone</td>
<td>15.6</td>
</tr>
</tbody>
</table>

$^a$ The temperature range was 12–37°.
$^b$ The temperature range was 12–25°.
$^c$ The temperature range was 25–37°.

**TABLE III**

<table>
<thead>
<tr>
<th>Solvent</th>
<th>Pyridine-2-aldoxime methiodide (1 mM)</th>
<th>Isonitrosoacetophenone (1 mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH</td>
<td>$k_{app}$</td>
<td>pH</td>
</tr>
<tr>
<td></td>
<td>m / mm</td>
<td></td>
</tr>
<tr>
<td>0.04 M phosphate buffer containing 15% (v/v) isopropyl alcohol</td>
<td>8.2</td>
<td>2.32</td>
</tr>
<tr>
<td>0.04 M phosphate buffer</td>
<td>8.2</td>
<td>6.10</td>
</tr>
<tr>
<td>0.04 M phosphate buffer containing 0.52 M sodium chloride</td>
<td>8.2</td>
<td>0.49</td>
</tr>
</tbody>
</table>

Effect of solvents on rate of reactivation at 25 ± 0.1°
This treatment gives the location of pH optimum in fact at the region where two ionization curves cross. Table I showed the results so obtained. The close agreement between the experimental and calculated values indicates that the value 8.4 for pHK+ is basically correct, not only for diethylphosphoryl human serum cholinesterase, but also for diethylphosphoryl acetylcholinesterase and \( \alpha \)-chymotrypsin (see Table I). The reason that these diethylphosphoryl enzymes displayed the same value of \( \mathrm{pK}_{\text{IEH}^+} \) may be because their \( \mathrm{pK}_a \) values are nearly identical, i.e. 6.7 for human serum cholinesterase (17), 6.5 for electrophorus electricus acetylcholinesterase (18), and 6.6 for \( \alpha \)-chymotrypsin (17); a phosphorylation of the same basic group on the esteratic site of the respective enzymes is not likely to cause a change of \( \mathrm{pK}_{\text{IEH}^+} \) value.

The present value 8.4 for \( \mathrm{pK}_{\text{IEH}^+} \) is close to 8.2 predicted for the tetraethyl pyrophosphate-inhibited acetylcholinesterase by Wilson and his co-workers (3) and 8.0 for diethyl p-nitrophenyl phosphate-inhibited \( \alpha \)-chymotrypsin by Cunningham (16), but substantially greater than 7.6 for tetraethyl pyrophosphate-inhibited acid-chymotrypsin (17); a phosphorylation of the same basic group on the anionic site and the positively charged phosphorylated esteratic site of the inhibited enzyme. An increase in polarity of solvent, in this case in going from normal buffer to the same buffer containing sodium chloride, would, therefore, retard the reaction (14). This view is supported by the fact that the apparent activation energy of reactivation with PAM in salt solution is about 5.4 kcal mol\(^{-1}\) larger than that obtained in buffer. In a less polar medium, such as a buffer containing 15\% (v/v) isopropl alcohol, one would expect an increase in rate; but in fact the rate remains approximately the same (Table III). It is not yet known whether this complication is caused by the fact that PAM is more soluble in water than in alcohol because of its ionic nature.

On the other hand, the activated complex formed with isonitrosoacetophenone may be of an ionic nature, for the anionic site of the inhibited enzyme is not involved in the reaction. Thus the presence of salt facilitates the formation of activated complex, lowers the apparent activation energy of reactivation (Table II), and increases the rate (Table III). In an alcoholic buffer, the rate decreases as expected (Table III).

A comparison of apparent activation energies of reactivation obtained with PAM (12.8 kcal mol\(^{-1}\)) and with isonitrosoacetophenone (15.6 kcal mol\(^{-1}\)) in the same buffer medium is also consistent with the nature of activated complex formed as well as the magnitude of the rate just discussed above. The same trend prevails in salt medium.

DISCUSSION

The salt effect in reactivation is of interest since it decreases the rate with positively charged PAM and substantially increases the rate with uncharged isonitrosoacetophenone. In the former case, the decrease in rate may be regarded as due to the formation of a neutral activated complex, resulting from a charge neutralization which involves the dipolar ion of PAM and both

\(^2\) These values were obtained from the pH rate curves and may become higher if \( \mathrm{pK}_a \) is used in the plot (13).

REFERENCES

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