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Reactivation of ethyl methylphosphonylated eel acetylcholinesterase *in vitro* by 2PAM, H16, and a series of nonquaternary α-ketothiohydroximates

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Most toxic organophosphorus esters are irreversible inhibitors of acetylcholinesterase (AChE) [1–4]. The inhibition proceeds via phosphylation* of a serine hydroxyl at the enzyme active site. Conventional therapy of organophosphorus ester intoxication entails coadministration of atropine and AChE "reactivators" that restore activity to the enzyme [5–8]. The reactivators most frequently studied are pyridinium oximes such as 2-(hydroxyimino)methyl-1-methylpyridinium iodide (2PAM) and 1-[2-(hydroxyimino)methylpyridinio]-3-(4-carbamoyl-pyridinio)-2-oxapropane dichloride (HI6).

Our work has focused on nonquaternary reactivators that should surpass the pyridinium compounds with respect to distribution *in vivo* to sites containing inhibited AChE, particularly sites in the central nervous system. Thus, we reported earlier [9] the synthesis of several α-ketothiohydroximates, *1*:

$$RC_6H_4C(:O)C(:NOH)S(CH_2)_nNR'_2 \cdot HCl$$
1

and a series of heteroaromatic aldoximes and thiohydroximates given by the general formula, *2*:

$$RC(:NOH)Z$$
2

where R = oxadiazolyl, thiadiazolyl, and triazolyl, and Z = H or $SCH_2CH_2N(C_2H_5)_2 \cdot HCl$ [10].

For the type *1* and type *2* thiohydroximates [Z = $SCH_2CH_2N(C_2H_5)_2 \cdot HCl$], we anticipated that the protonated dialkylaminoalkyl moiety would enhance equilibrium binding to phosphylated AChE via coulombic interactions with anionic centers on the enzyme surface. However, our examination of the nonquaternary compounds as reactivators of eel AChE inhibited by ethyl *p*-nitrophenyl methylphosphonate (EPMP) suggested a somewhat different binding mechanism. Several type *2* thiohydroximates strongly compete with substrate (acetylthiocholine) for binding at the surface of the active enzyme. By contrast, the same type *2* thiohydroximates reversibly bind to ethyl methylphosphonyl-AChE only very weakly. Because type *2* oximes (Z = H) and thiohydroximates exhibited essentially equivalent equilibrium constants for binding to ethyl methylphosphonyl-AChE, we tentatively concluded [10] that the ethyl methylphosphonyl moiety shields anionic centers on the enzyme surface from close approach of reactivator protonated dialkylaminoalkyl groups. Moreover, hydrophobic forces apparently largely govern equilibrium binding of the relatively nonpolar nonquaternary compounds to ethyl methylphosphonylated AChE.

The objectives of the current study were to examine new α-ketothiohydroximates for activity relative to 2PAM and HI6 and to further elucidate the shielding phenomenon. To do so we prepared a series of type *1* compounds that differed only in the structure of the S-ester functionality:

$$4\text{-}BrC_6H_4C(=O)-CS(=NOH)R$$

1a, R = $-(CH_2)_2N(C_2H_5)_2 \cdot HCl$
1b, R = $-(CH_2)_3N(CH_3)_2 \cdot H_2C_2O_4$
1c, R = $-(CH_2)_2N(CH_3)_2 \cdot HCl$
1d, R = $-(CH_2)_2N(CH_3)_3 \cdot I$
1e, R = $-(CH_2)_2CH_3$

For each thiohydroximate, we determined kinetic constants for ethyl methylphosphonyl-AChE reactivation.

The five type *1* compounds were prepared from 4-bromobenzophenone using previously described methods [9]. The oxime acid dissociation constant (pK_a) values were: *1a* (7.4), *1b* (7.7), *1c* (7.7), *1d* (7.2), and *1e* (8.4).

The test compounds were incubated in large excess over EPMP-inhibited AChE, and reactivation kinetics were determined as described in Ref. 10. Under our experimental conditions, reactivation proceeds [10–13] according to reactions 1 through 3:

$$EI + OX \overset{K_r}{\rightleftharpoons} [EI \cdot OX] \quad (1)$$

$$[EI \cdot OX] \overset{k_r}{\rightarrow} E \quad (2)$$

$$HOX \overset{K_a}{\rightleftharpoons} OX + H^+ \quad (3)$$

where *E* is active AChE, *EI* is phosphonylated AChE, *OX* is the anionic (oximate) form of reactivator, *HOX* is the protonated form of added reactivator, and $[EI \cdot OX]$ is the reversibly-formed complex between reactivator and inhibited enzyme.

With *OX* present in excess over *EI*, pseudo first-order reactivation kinetics obtain and the observed rate constant for reactivation (k_{obs}) relates to K_r and k_r as in equation 4:

$$(k_{obs})^{-1} = K_r(k_r[OX])^{-1} + k_r^{-1} \quad (4)$$

To estimate the inherent activity of an oximate toward ethyl methylphosphonyl-AChE, k_{OX}, the bimolecular rate constant in the limit of low reactivator concentration ($[OX] \ll K_r$), is found from equation 5:

$$k_{OX} = k_r/K_r \quad (5)$$

Reactivation data for compounds *1a* through *e* and for the pyridinium oximes conformed well to equation 4, and kinetic plots were similar to those previously described [10]. Table 1 summarizes the relevant reactivator concentration ranges and kinetic constants of interest.

The table reveals a 15-fold range of k_{OX} values for the type *1* compounds, compound *1e* representing the poorest reactivator and *1d* the best. HI6 and (especially) 2PAM were significantly better reactivators than any of the type *1* compounds.

Mechanistically, K_r values for the type *1* compounds reveal a poor correlation between reactivator ionic character and binding affinity toward ethyl methylphosphonyl-AChE. If ionic forces were important for thiohydroximate binding, the single compound (*1e*) that is devoid of any cationic functionality should bind significantly more poorly (higher dissociation constant, K_r) to ethyl methylphosphonyl-AChE than other type *1* compounds (all of which feature protonated or quaternary alkylaminoalkyl groups). Table 1 clearly shows this to be untrue. Thus, the present data are consistent with earlier observations by ourselves [10] and others [14] that phosphonylating AChE alters the enzyme surface in such a way as to minimize the importance of electrostatic forces in controlling the reversible binding of certain reactivators. The data suggest that the thiohydroximates bind to a hydrophobic region on ethyl methylphosphonyl-AChE and that the organophosphorus moiety sterically shields these reactivators from approaching anionic centers near the enzyme active site.

* We use the term "phosphylation" when we do not distinguish between "phosphonylation" and "phosphorylation".

Table 1. Kinetic constants for reactivation of ethyl methylphosphonyl-AChE by various test compounds

Compound*	[HOX] (mM)	$[OX]^{-1}$ ($M^{-1} \times 10^{-3}$)	$(k_{obs})^{-1}$ (min)	Slope† (M-min × 10^2)	Intercept† (min)	Correlation coefficient	k_r† ($min^{-1} \times 10^3$)	K_r† (M × 10^4)	k_{OX}‡ (M^{-1} min^{-1})
1a	0.0300	56.4	614	1.05 ± 0.12	36.8 ± 29	0.971	27.2	2.85	95.3
	0.0600	28.2	403						
	0.100	16.9	142						
	0.200	8.46	183						
	0.300	5.64	56.2						
	5.00	3.38	84.7						
	10.0	1.69	46.3						
1b	0.0500	42.9	799	1.56 ± 0.061	132 ± 15	0.998	7.58	1.18	64.1
	0.100	21.5	478						
	0.500	4.29	178						
	1.00	2.15	181						
1c	0.0100	270,000	2,750	1.16 ± 0.0098	239 ± 75	0.993	4.18	0.485	86.0
	0.0250	87,700	1,410						
	0.0500	44,000	794						
	0.100	22,000	358						
1d	0.0125	110,000	752	0.659 ± 0.0066	25.9 ± 2.5	0.999	38.6	2.56	151
	0.0250	55,200	395						
	0.0500	27,600	208						
	0.100	13,800	113						
1e	0.500	14.6	2,730	11.0 ± 1.3	1060 ± 110	0.986	0.943	1.04	9.09
	1.00	7.31	1,720						
	2.00	3.65	1,500						
	3.00	2.44	1,400						
2PAM	0.00050	6,910	735	0.0104 ± 0.00064	27.8 ± 24	0.996	36.0	0.0374	9630
	0.00150	2,300	307						
	0.00400	864	109						
	0.0100	345	43.5						
HI6	0.00100	1,480,000	2,490	0.164 ± 0.0051	48.5 ± 26.6	0.999	20.6	0.339	608
	0.00300	493,000	806						
	0.0100	148,000	342						
	0.0300	49,300	120						

* See text for structures.
† Calculated from linear least-squares regression according to equation 4; error limits are ± S.D.
‡ Calculated according to equation 5.

Finally, it seems worthwhile to compare our kinetic constants for reactivation of ethyl methylphosphonylated eel AChE by 2PAM and HI6 with literature values [15] for the same oximes as reactivators of erythrocyte AChE inhibited by diethyl *p*-nitrophenyl phosphate (paraoxon). Reference 15 reports that for 2PAM $k_r = 40.6 \times 10^{-3}$ min^{-1}, $K_r = 0.24 \times 10^{-4}$ M, and $k_{OX} = 1690$ M^{-1} min^{-1}, whereas for HI6 $k_r = 46.9 \times 10^{-3}$ min^{-1}, $K_r = 3.82 \times 10^{-4}$ M, and $k_{OX} = 123$ M^{-1} min^{-1}. Thus, for both 2PAM and HI6, the diethyl phosphorylated erythrocyte enzyme is more difficult to reactivate than ethyl methylphosphonylated eel AChE by a factor of approximately 5. The reactivity differences derive primarily from higher K_r values for both pyridinium oximes versus paraoxon-inhibited erythrocyte AChE, but the available data cannot distinguish between the enzymes or the organophosphorus inhibitors as sources of the reactivity differences.

In summary, we prepared five α-ketothiohydroximates given by the general formula 4-BrC$_6$H$_4$C(:O)C(:NOH)SR where R = $-(CH_2)_2N(CH_3)_2 \cdot HCl$, $-(CH_2)_3N(CH_3)_2 \cdot H_2C_2O_4$, $-(CH_2)_2N(C_2H_5)_2 \cdot HCL$, $-(CH_2)_2N(CH_3)_3 \cdot I$, and $-(CH_2)_2CH_3$. All five compounds were tested as reactivators of eel acetylcholinesterase inhibited *in vitro* by ethyl *p*-nitrophenyl methylphosphonate. For comparison we also determined the activities of two well-known pyridinium oximes, 2PAM and HI6. The α-ketothiohydroximates, as a class, demonstrated modest activity relative to the pyridinium oximes. Thus, the most reactive α-ketothiohydroximate [$R = -(CH_2)_2N(CH_3)_3 \cdot I$] was less reactive than 2PAM and HI6 by factors of 64 and 4 respectively. The α-ketothiohydroximates reversibly bind to hydrophobic regions of the inhibited enzyme, and the ethyl methylphosphonyl moiety shields the inhibited enzyme anionic region from interaction with the protonated alkylaminoalkyl groups featured by the α-ketothiohydroximates.

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