Kinetic and physicochemical analysis of structurally different bis-pyridinium oximes against pesticide inhibited AChE

Arvind Kumar Sahu a, Bhanushree Gupta a, Rahul Sharma a, Yama Singh a, Kamil Musilek b, Kamil Kuca a & Kallol K Ghosh a *

aSchool of Studies in Chemistry, Pt. Ravishankar Shukla University, Raipur (CG) 492 010, India
Email: kallolkghosh@yahoo.com
bUniversity of Hradec Kralove, Faculty of Science, Department of Chemistry, Rokitanskeho 62, Hradec Kralove, Czech Republic
cUniversity Hospital, Biomedical Research Center, Sokolska 581, 50005 Hradec Kralove, Czech Republic

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Physicochemical properties such as acid dissociation constant, \( pK_a \), and lipophilicity, \( \log P \), of some bis-pyridinium mono- and bis-pyridinium oximes with varying functionalities in the second pyridinium ring of the reactivator have been evaluated spectrophotometrically. *In vitro* reactivation kinetics of the investigated oximes against paraoxon inhibited electric eel AChE has been carried out. The studied oximes have \( pK_a \) values within the range of 7.85–8.38 and negative \( \log P \) values, exhibiting their low lipophilic character. Further, on the basis of reactivation parameters (\( k_r, K_D, k_2 \)) the efficacy of bis-pyridinium mono-oximes has been explained.

Keywords: Reactivators, Acetylcholinesterase, Paraoxon, Acid dissociation constant, Lipophilicity, Bis-pyridinium oximes

The extensive use of organophosphorus (OPs) based pesticides are of major concern due to their highly deleterious effects in humans, animals and wildlife 1,2. The high toxicity of organophosphorus nerve agents and pesticides is ascribed to their ability to irreversibly inhibit the activity of enzyme acetylcholinesterase (AChE) 3,4. OPs exert their toxic effects via the formation of O-P covalent bond at the serine residue of the active site of the enzyme 5,6. Hence, the resulting inhibited enzyme is unable to perform its normal function, i.e. hydrolysis of neurotransmitter acetylcholine (ACh) into choline and acetic acid in synaptic cleft. This leads to over stimulation of cholinergic receptors and causes system wide cholinergic crises 7. Therefore, it is important to design and develop efficient oximes reactivators as antidotes which can reactivate the inhibited AChE enzyme.

Customary treatment of OP poisoning underlines the use of an antimuscarinic agent like atropine along with oxime based reactivators. Reactivators work as nucleophiles to displace the organophosphorus moieties from OP-AChE adduct and restore activity of the enzyme 8,9. Considerable attention has been paid to the design and synthesis of several mono- and bis-pyridinium oximes as antidotes for reactivation of OP-inhibited AChE10-14. 2-PAM, obidoxime and TMB-4 are commercially available antidotes15. Most of the reactivators are \( \alpha \)-nucleophiles but their efficiency remains to be improved16. In order to develop more potent reactivators of OP-inhibited AChE, study of physicochemical properties such as acid dissociation constant (\( pK_a \)) and lipophilicity (\( \log P \)) including their reactivation kinetics analysis, is essential.

Since the oximate anion is responsible for the nucleophilic attack during the process of reactivation (*in vitro* and *in vivo*) and its concentration is \( \text{pH} \) dependent, it is essential to determine \( pK_a \) values of newly developed oximes. Lipophilicity (\( \log P \)) is the first preferred physicochemical property which describes the participation of oxime molecule in lipid phase with respect to hydrophilic phase and their ability to penetrate the lipid bilayer membrane to reach the target site19. The acid dissociation constant (\( pK_a \)) characterizes the charged state of an oxime at particular \( \text{pH} \) of its environment whereas lipophilicity (\( \log P \)) is chemical equilibrium of partitioning of all the charged states of oxime between two immiscible liquids like water and 1-octanol. The properties of 1-octanol resemble those of lipid bi-layer membranes. This is due to its structure, which has a polar head group and a hydrophobic chain that leads to moderate water saturation14. Hence, the octanol-water system is widely accepted as a reference system to determine the lipophilicity of various drug molecules. The \( pK_a \) of various carbonyl bis-pyridinium mono-oximes and bis-pyridinium dioximes with different connecting linkers, have been determined by Acharya *et al.*20,21. The \( \log P \) values of various mono- and bis-pyridinium oximes with different linking chains have been reported by Sepsova *et al.*22. Although the evaluation of physicochemical properties gives a preliminary
overview of oximes as reactivators, their actual ability as reactivators can be characterized with the help of reactivation kinetics. Kinetic investigations as a tool allow us to understand the mechanism of reactivation. Worek et al. 23 significantly provided the kinetic basis for the evaluation of reactivation potency of structurally different oximes against nerve agents and pesticides inhibited AChE. Ghosh and co-workers 24-28 also studied in vitro reactivation kinetics of mono and bis-pyridinium oximes against structurally different organophosphorus compounds (paraoxon, DFP, VX, sarin).

In the present investigation we have studied the effect of functional group present on the second pyridinium ring of bis-pyridinium oximes for the reactivation of paraoxon inhibited AChE. The functional groups of the investigated oximes (Table 1) vary from carbamoyl (-CONH$_2$), carboxylic (-COOH) to esteric (-COOEt) groups. Initially, acid dissociation constants and lipophilicity were determined for each reactivator, which was followed by in vitro screening of reactivation potency for paraoxon poisoned AChE. The reactivation results have been compared with standard 2-PAM commercial.

**Experimental**

2-PAM and obidoxime were synthesized by reported methods 29-30. Electric eel AChE (500 UN), paraoxon-ethyl (paraoxon), acetylthiocholine iodide (ATChI) and 5, 5-dithiobis(2-nitrobenzoic acid) (DTNB) were obtained from Sigma Aldrich. pH measurements were made by using a Systronics (Type- 362) pH-meter containing a glass electrode. The pH meter was calibrated at 25 ºC using two point calibration method with commercially available standard buffer solutions pH 4.00 and 9.00. Stock solution of paraoxon (1.5×10$^{-4}$) was prepared in 2-propanol (1%, v/v), stored at −80 ºC and appropriately diluted in distilled water immediately before use. Stock solutions of oximes (0.01 M) were prepared in distilled water and stored at −60 ºC and diluted accordingly. All the inhibition and reactivation

<table>
<thead>
<tr>
<th>K-Oxime</th>
<th>Chemical Structure</th>
<th>$pK_a$</th>
<th>log $P$</th>
</tr>
</thead>
<tbody>
<tr>
<td>2-PAM (K026)</td>
<td><img src="image1" alt="Structure" /></td>
<td>7.85±0.12</td>
<td>-0.49±0.04</td>
</tr>
<tr>
<td>K203</td>
<td><img src="image2" alt="Structure" /></td>
<td>8.34±0.17</td>
<td>-0.47±0.05</td>
</tr>
<tr>
<td>K250</td>
<td><img src="image3" alt="Structure" /></td>
<td>8.38±0.20</td>
<td>-0.31±0.02</td>
</tr>
<tr>
<td>K252</td>
<td><img src="image4" alt="Structure" /></td>
<td>8.24±0.36</td>
<td>-0.88±0.03</td>
</tr>
<tr>
<td>K255</td>
<td><img src="image5" alt="Structure" /></td>
<td>8.25±0.10</td>
<td>-0.82±0.02</td>
</tr>
</tbody>
</table>
experiments were performed using phosphate buffer solutions pH 7.6, sodium dihydrogen phosphate (0.1 M) and disodium hydrogen phosphate (0.1 M) pH 7.6 at room temperature.

The spectrophotometric measurements were made using a Varian Cary 50 UV-visible spectrophotometer equipped with a Peltier (temperature controller unit). All the spectrophotometric measurements were made at 27±0.5 °C and the spectra were recorded within the range of 200–420 nm.

Stock solutions (5.0×10⁻⁴ M) of all the investigated oximes were prepared in triply distilled water and the pKₐ values were determined by the method of Albert and Sergeant. Further, an aliquot (1-3 mL) of stock of oxime was diluted with (25 mL) phosphate buffer solution. The absorption spectrum were recorded at varying pH adjusted to the desired value by the addition of dilute sodium hydroxide solution (0.1 M). After each pH adjustment, the solution was transferred into the cuvette, and the absorption spectra were recorded. Absorbance at selected wavelengths was obtained from the spectra. The absorption spectra of K252 at different pH values at 27 °C are shown in Fig. 1. The pKₐ calculations were made around half neutralization using Eq. (1),

$$pK_a = pH_{exp} - \log \frac{Abs_\psi - Abs_{H\Delta}}{Abs_{Ox} - Abs_\psi} \ldots (1)$$

where Abs_{HΔ} is the absorbance of molecular form of oxime (270-275 nm), Abs_ψ is the absorbance of partially ionized form of oxime and Abs_{Ox} is the absorbance of completely ionized form (342-348 nm) of oxime.

Lipophilicity of the oximes was determined at room temperature by the shake flask method. The standard solution of 1-octanol saturated with water or buffer (pH 7.6) in the ratio 1:1 v/v was prepared by shaking for 24 h. Stock solution of oxime (10⁻²–10⁻⁵ M) in previously saturated 1-octanol was prepared and absorbance was measured at room temperature at the wavelength of its absorption maximum. The absorbance maxima for tested oximes were found to be within range of 280–290 nm. This absorbance (A₁₀₀) corresponds to 100% of the oxime in the solution. Then, a definite volume of this solution and the same volume of the buffer were intensively mixed together for 15 min and centrifuged for 10 min at 3000 rpm. In the perfectly separated 1-octanol phase the absorbance (A_δ) at the same wavelength was determined. Lipophilicity was calculated by Eq. (2).

$$P = \frac{A_\delta}{A_{100} - A_\delta} \ldots (2)$$

The in vitro inhibition and reactivation experiments were carried out at room temperature in duplicate at pH 7.6 by the modified Ellman’s method. AChE stock solution (stock A) was prepared in phosphate buffer (pH 7.6, 100 mM) (364 units/mL). An aliquot of stock A was then diluted 50 times with phosphate buffer to give stock B. Stock solution of paraoxon (1.5×10⁻⁴ M) was prepared in 1% v/v isopropanol and then diluted appropriately just before use. Stock solution of chromogen DTNB (0.01 M) was prepared in buffer (7.6 pH) and substrate ATChI (0.075 M) was dissolved in triply distilled water.

The activities of AChE (inhibition and reactivation) were monitored spectrophotometrically using 0.48 mM ATChI as substrate and 0.32 mM DTNB as chromogen in 0.1 M phosphate buffer (pH 7.6). Here the concentrations of chromogen and substrate refer to the final concentration in inhibition and reactivation cocktail. Further, the enzyme suspended in phosphate buffer (pH 7.6, 100 mM) was incubated with paraoxon (Final conc.: 1.5×10⁻⁷ M) at room temperature for 10-15 min, resulting in inhibition of >95% of control activity of AChE.

Inhibited-AChE cocktail was then followed by the addition of oximes at a final concentration ranging from 0.01–1 mM. Aliquots of 50 µL of reactivation cocktail were withdrawn at specified time intervals and diluted in cuvettes containing 3000 µL phosphate buffer

Fig. 1—Absorption spectra of K252 in water at 27 °C at different pH values. [Conc. = 2.0×10⁻⁵ M; Run and pH: (1) 7.05; (2) 7.15; (3) 7.29; (4) 7.44; (5) 7.60; (6) 7.99; (7) 8.23; (8) 8.44; (9) 8.68; (10) 9.06; (11) 9.47; (12) 9.69; (13) 9.96].
buffer and 100 µL DTNB to monitor the change in the enzyme activity after addition of 20 µL AChI.

According to Aldridge and Reiner\(^4\), the process of reactivation occurs in two sequential steps (Scheme 1),

\[
[\text{EP} + [\text{OX}]] \xrightleftharpoons[k_r]{K_D} [\text{EPOX}] \xrightarrow[k_r^2]{r^2} [\text{E} + [\text{POX}]}
\]

where [EP] is the phosphorylated enzyme, [OX] the reactivator, [EPOX] is the Michaelis-type OP-AChE-oxime complex, [E] is the reactivated enzyme and [POX] is phosphorylated oxime. \(K_r = [\text{EP}][\text{OX}]/[\text{EPOX}]\) is the dissociation constant, which is inversely proportional to the affinity of oxime to [EP], and \(k_r\) is the rate constant for the removal of OP residue from [EPOX] by oxime specifying its reactivity. In the case of complete reactivation and with \([\text{OX}] \gg [\text{EP}]\), a pseudo-first-order rate can be applied for the reactivation (Eq. 3).

\[
k_{\text{obs}} = \frac{k_r \cdot X[\text{OX}]}{K_D + [\text{OX}]}
\]

\(k_{\text{obs}}\) was calculated by nonlinear regression analysis\(^3^5\) using Eq. 4,

\[
v = v_0 \times (1 - e^{-k_{\text{obs}}x})
\]

The value of \(k_{\text{obs}}\) is not proportional to the concentration of oxime, but indicates a saturated kinetics\(^3^6\). \(k_r\) and \(K_D\) were determined by nonlinear fit to the relationship between \(k_{\text{obs}}\) versus [OX]. The second-order reactivation rate constant \(k_{r^2}\) was derived from ratio of \(k_r\) and \(K_D\) (both follow the Michaelis–Menten kinetics\(^3^7\)).

## Results and discussion

### pKa and log \(P\) of tested reactivators

It has to be mentioned that most of the investigated pyridinium oximes have \(pK_a\) within the range of 7.85–8.38 (Table 1) and may be efficient to reactivate the phosphorylated enzyme at physiological pH. The absorption spectra of the investigated bis-pyridinium oximes connected to butene linker were recorded in different pH solutions ranging from 7.05–9.96. All the oximes show electronic absorption spectra with two pH-dependent absorption maxima, one in the range from 250–300 nm appearing at lower pH and the longer wavelength maximum in the range from 325–380 nm at higher pH (Fig. 1). Both maxima are in accordance with the \(\pi \rightarrow \pi^*\) transitions within the aromatic ring of pyridinium oximes. The change in the absorption spectra with the change of pH indicates that, the dissociation of oxime group into oximate ions occur in the investigated pH range of 7.05–9.96.

Lipophilicity of oximes is an important parameter to predict the extent of their permeability through membranes. log\(P\) values of all mono- and bis-pyridinium oximes were negative due to the permanent positive charge on the pyridinium ring, which indicates their easy distribution within the peripheral nervous system. log\(P\) values were found to be in the range of 0.31-0.88 (Table 1).

### Reactivation of paraoxon-inhibited AChE

In the present investigation we have tested five structurally different pyridinium oxime based reactivators for their in vitro potency to reactivate inhibited AChE. The observed reactivation rate constants of tested oximes (Table 2) against paraoxon inhibited-AChE resulted in marked differences of constants of tested reactivators (Table 2) against paraoxon inhibited AChE. The observed reactivation rate constants were found to be in the range of 0.31-0.88 (Table 1).

### Table 2—Reactivation parameters for oxime-induced reactivation of paraoxon-inhibited AChE\(^a\)

<table>
<thead>
<tr>
<th>Oxime</th>
<th>(k_r) (min(^{-1}))</th>
<th>(K_D) (µM)</th>
<th>(k_{r^2}) (mM(^{-1}) min(^{-1}))</th>
<th>Max. react. (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2-PAM(^b)</td>
<td>0.082±0.006</td>
<td>8.81±0.081</td>
<td>9.350±0.011</td>
<td>68</td>
</tr>
<tr>
<td>K203(^b)</td>
<td>0.185±0.014</td>
<td>220.60±0.012</td>
<td>0.841±0.006</td>
<td>51</td>
</tr>
<tr>
<td>K250</td>
<td>0.115±0.010</td>
<td>48.20±0.024</td>
<td>2.380±0.015</td>
<td>61</td>
</tr>
<tr>
<td>K252</td>
<td>0.102±0.026</td>
<td>97.10±0.024</td>
<td>1.060±0.020</td>
<td>58</td>
</tr>
<tr>
<td>K255</td>
<td>0.145±0.018</td>
<td>173.60±0.014</td>
<td>0.835±0.016</td>
<td>45</td>
</tr>
</tbody>
</table>

\(^a\)The pseudo first order rate constant \(k_{\text{obs}}\) was determined by non-linear regression analysis and these data were used for the calculation of the reactivity rate constant \(k_r\), the dissociation constant \(K_D\) and the hybrid reactivation rate constant \(k_{r^2}\) (from the ratio of \(k_r/K_D\)). Data are given as means ±SD (\(n = 2\)). Maximum reactivation gives the highest AChE activity after 60 min incubation of inhibited AChE by \(2-PAM\) and oxime connected to butene linker were recorded in different pH solutions ranging from 7.05–9.96. All the oximes show electronic absorption spectra with two pH-dependent absorption maxima, one in the range from 250–300 nm appearing at lower pH and the longer wavelength maximum in the range from 325–380 nm at higher pH (Fig. 1). Both maxima are in accordance with the \(\pi \rightarrow \pi^*\) transitions within the aromatic ring of pyridinium oximes. The change in the absorption spectra with the change of pH indicates that, the dissociation of oxime group into oximate ions occur in the investigated pH range of 7.05–9.96.

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Lipophilicity of oximes is an important parameter to predict the extent of their permeability through membranes. log\(P\) values of all mono- and bis-pyridinium oximes were negative due to the permanent positive charge on the pyridinium ring, which indicates their easy distribution within the peripheral nervous system. log\(P\) values were found to be in the range of 0.31-0.88 (Table 1).
reactivators with 4, 4′-oximes. 2-PAM was used as standard \(^{39}\). Reactivation of inhibited-AChE with tested oxime followed first order kinetics \(^{40}\). The representative graph for paraoxon-inhibited AChE is given in Fig. 2. The obtained data revealed that the reactivation constants are greatly influenced by the chemical structures of oximes. The affinity of the oxime towards the phosphorylated AChE (dissociation constant, \(K_D\)) showed a large variability (8.81–220.6 \(\mu M\)), while the reactivity rate constant (\(k_r\)) exhibited very little variation (0.082 –0.185 \(min^{-1}\)). As specific reactivity of oximes (\(k_{r2}\), \(mM^{-1} min^{-1}\)) is dependent on both affinity (\(K_D\)) and reactivity (\(k_r\)), hence \(k_{r2}\) resulted in marked differences for all oximes against the investigated pesticide. Amongst tested oximes, K250 (\(k_{r2}: 2.38 \text{mM}^{-1} \text{min}^{-1}\)) was found to be the most efficient reactivator for paraoxon poisoning whereas K255 was found to be least effective with \(k_{r2}: 0.835 \text{mM}^{-1} \text{min}^{-1}\). Although none of the investigated oximes (Table 1) could exhibit comparable reactivity with standard oxime (2-PAM), they showed moderate to good ability. Figure 3 represents comparative second order rate profile for studied reactivators.

In the search for a powerful reactivator of inhibited-AChE, evaluation of its \(pK_a\) is important. Also, it has been assumed from previous studies \(^{41}\) that reactivators with lower \(pK_a\) may be more effective at physiological pH. The present study reveals that this assumption is followed by only the standard oxime reactivator 2-PAM. However, the trend indicates limited dependence of nucleophilicity of reactivators on the availability of oximate anions. Taylor et al. \(^{42,43}\) have reported that the reactivation poteny of a reactivator is also dependent on AChE-OP-oxime trigonal bipyramidal intermediate which is formed during the reactivation process. Thus for the prediction of complete nucleophilic efficacy of a reactivator, several other factors have to be considered including its acid dissociation constant.

This study of physicochemical properties (acid dissociation constant and lipophilicity) and reactivation kinetics of structurally different oxime reactivators may provide a direction to design efficient reactivators against pesticides. The negative log \(P\) indicates low lipid solubility of the investigated oximes. The \(pK_a\) of tested oximes are found to be in the range of 7.85–8.38. Reactivation studies show that K250 is the most efficient reactivator among all the investigated oximes, although it could not surpass the reactivity of standard oximes against paraoxon. The dephosphorylation of phosphorylated enzyme is dependent on various factors such as \(pK_a\), lipophilicity...
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References


and reactivation rate constants. The detailed study of antidotal efficacy including in vitro reactivation against nerve agents shall be reported in future.