available at www.sciencedirect.com

ScienceDirect

journal homepage: www.elsevier.com/locate/biochempharm

Preparation and characterization of dialkylphosphoryl-obidoxime conjugates, potent anticholinesterase derivatives that are quickly hydrolyzed by human paraoxonase ($PON1_{192Q}$)

J. Stenzel [a], *F. Worek* [b], *P. Eyer* [a,*]

[a] *Walther-Straub-Institute of Pharmacology and Toxicology, Ludwig-Maximilians-University, Goethestrasse 33, D-80336 Munich, Germany*
[b] *Bundeswehr Institute of Pharmacology and Toxicology, Neuherbergstrasse 11, 80937 Munich, Germany*

ARTICLE INFO

Article history:
Received 1 June 2007
Accepted 10 July 2007

Keywords:
Acetylcholinesterase
Paraoxonase
Phenotypes
Phosphoryl oximes
Obidoxime
Kinetic constants

ABSTRACT

The potential of the most active pyridinium-4-aldoximes, such as obidoxime and trimedoxime, to reactivate phosphorylated acetylcholinesterase is not fully exploited because of inevitable formation of phosphoryloximes (POXs) with extremely high anticholinesterase activity. Hence, a topochemical equilibrium is expected at the active site, with the freshly reactivated enzyme being rapidly re-inhibited by POX produced during reactivation. In the present study, dimethylphosphoryl-, diethylphosphoryl-, and diisopropyl-obidoxime conjugates were generated and isolated in substance. Their inhibition rate of acetylcholinesterase from human red cell membranes was by a factor of 2250, 480 and 600 higher than that observed with paraoxon-methyl, paraoxon-ethyl, and diisopropyl phosphorofluoridate, respectively. All three POXs were hydrolyzed by human paraoxonase (PON1), with the alloenzyme $PON1_{192Q}$ being about 50-fold more active than $PON1_{192R}$. The rate of hydrolysis, yielding obidoxime, was 1:6:0.03 for the three POXs, respectively. The rate of non-enzymic degradation, yielding obidoxime mononitrile, was similar with the three POXs and showed a high dependency on the reaction temperature (activation energy 83 kJ/mol), while enzymic hydrolysis required less energy (16 kJ/mol). To determine POX-hydrolase activity, we preferred a reaction temperature of 20 °C to reduce the noise of spontaneous degradation. A plot of POX-hydrolase versus salt-stimulated paraoxonase activity showed a highly discriminating power towards the $PON1_{Q192R}$ alloenzymes, which may be based on repulsive forces of the quaternary nitrogen atoms of the protonated arginine subtype and the bisquaternary POXs. It is concluded that the pharmacogenetic $PON1_{Q192R}$ polymorphism may be another contributor to the large variability of susceptible subjects seen in obidoxime-treated patients.

* *Corresponding author*. Tel.: +49 89 2180 75702; fax: +49 89 2180 75701.
E-mail address: peter.eyer@lrz.uni-muenchen.de (P. Eyer).

Abbreviations: AChE, acetylcholinesterase; DEP-obidoxime, *O*,*O*′-diethylphosphoryl-obidoxime; DMP-obidoxime, *O*,*O*′-dimethylphosphoryl-obidoxime; DIP-obidoxime, *O*,*O*′-diisopropylphosphoryl-obidoxime; DFP, diisopropyl phosphorofluoridate; MOPS, 3-(*N*-morpholino)propanesulfonate; OP, organophosphorus compounds; 2-PAM, pyridinium-2-aldoxime; PON, paraoxonase; POX, phosphoryloxime; TMB-4, trimedoxime

0006-2952/$ – see front matter © 2007 Elsevier Inc. All rights reserved.
doi:10.1016/j.bcp.2007.07.013

1. Introduction

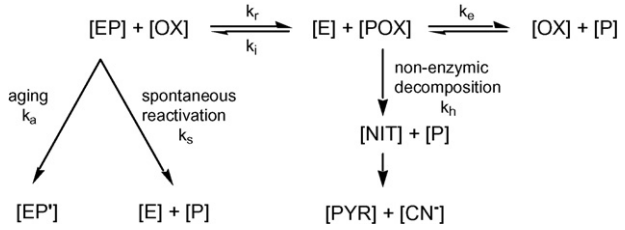
Shortly after the introduction of pyridinium oximes as causal antidotes in organophosphate poisoning by Wilson and Ginsburg, it was noticed that the reactivation of inhibited acetylcholinesterase (AChE) was more complete when diluted AChE preparations were used. More detailed analysis of this phenomenon led to the suggestion that a reaction product of the reactivation process, a phosphylated oxime (POX), could be an inhibitor of AChE [1]. Subsequently, it was observed that POXs were also formed in a direct reaction of oximes with the more reactive anticholinesterase agents such as sarin [2]. POX conjugates between sarin and 4-pralidoxime (4-PAM), 2-PAM and trimedoxime (TMB-4) were prepared and it was noted that the AChE inhibiting potency increased in the order of the conjugate formed with 2-PAM, 4-PAM and TMB-4 [3]. Subsequent studies with a variety of pyridinium oximes and organophosphorus compounds (OP) revealed the general rule that four-substituted pyridinium oximes yield rather stable OP conjugates, while two-substituted pyridinium oxime conjugates appeared to be highly unstable [4,5].

The decomposition of the pyridinium oximes in aqueous solutions near neutrality, with loss of anticholinesterase activity, gave mainly the corresponding nitrile, rather than the parent oxime [6,7]. The mechanism underlying nitrile formation was postulated as a Beckmann elimination reaction [6,7], depending on the acidity of the methine proton [8,9].

The high inhibitory potency of the POXs towards AChE has been demonstrated *in vitro* and often exceeds that of the parent OP by one or two orders in magnitude [5,10–14]. This holds particularly true for the rather stable 4-pyridinium-aldoximes and 4-bispyridinium-aldoximes, for review see [15], and it has been suggested that formation of rather stable POXs *in vivo* may thwart effective reactivation of phosphylated AChE be oximes [16]. In fact, the relative inefficacy of TMB-4 against soman in isolated rat phrenic nerve diaphragm preparations was related to formation of a TMB-4-soman complex which itself is a strong inhibitor of AChE [17]. Definite proof of the *in vivo* toxicity of phosphylated oximes was obtained in rats that were dosed with the isopropoxy ethylphosphonyl oxime derived from 2- and 4-PAM [18].

The particularly dramatic increase in anticholinesterase activity of paraoxon by more than two orders of magnitude upon reaction with obidoxime has been already supposed earlier [11,13] when the compound was generated *in situ* upon reactivation of erythrocyte AChE and was confirmed when (mono)diethylphosphoryl-obidoxime (DEP-obidoxime) was isolated and fully characterized [19]. Formation and the high inhibitory potency of DEP-obidoxime contrasted with the well-known reactivating efficacy of obidoxime in parathion poisoning and prompted Schoene to argue that accumulating acetylcholine may compete for re-inhibition of AChE by POX at the active site of the enzyme [11]. While this effect may contribute to a more effective reactivation than expected from POX formation, another mechanism may be more important.

It was detected fortuitously that human plasma was able to quickly destroy DEP-obidoxime. It turned out that paraoxonase (PON1) was responsible for the hydrolysis of POX [20], but that some human plasma samples had rather low POX-hydrolase activity while the capacity to hydrolyze paraoxon was high. This phenomenon was attributed to a substrate-dependent activity polymorphism of $PON1_{Q192R}$ [21–24]. Substitution at position 192 by arginine (R) for glutamine (Q) largely abolished the hydrolytic capacity of PON1 towards DEP-obidoxime and DEP-TMB-4 [19]. There were experimental hints that $PON1_{Q192}$ was not only able to hydrolyze DEP-obidoxime, but also dimethylphosphoryl-obidoxime [20]. A similar behavior was shown for rabbit serum paraoxonase which accelerated the obidoxime and TMB-4 induced reactivation of diethylphosphoryl mouse AChE. The effect was less pronounced in the case of ethoxyl methylphosphonyl AChE [25]. In contrast, isopropoxy methylphosphonyl obidoxime, the reaction product with the organophosphonate sarin, was not amenable to hydrolysis by PON1 [26]. When it was shown that the PON1 polymorphism was a modulating factor that influences the obidoxime effectiveness in OP poisoned patients [27,28], it became of major interest to characterize the substrate specificity in more detail.



Scheme 1 – Reactivation of phosphorylated enzyme [EP] by an oxime [OX] with formation of free enzyme [E] and the phosphoryl-oxime [POX]. [EP] is capable to reactivate spontaneously or undergo the process of aging by liberating an alkyl moiety, leading to an irreversible inhibition state [EP']. [POX] is hydrolyzed by PON1 to give the parent oxime and alkoxyphosphoric acid [P] or decomposes to a nitrile [NIT] and alkoxyphosphoric acid to give eventually the pyridone [PYR] and cyanide [CN^-].

In the present study, two new POX compounds, dimethylphosphoryl-obidoxime (DMP-obidoxime) and diisopropylphosphoryl-obidoxime (DIP-obidoxime), were isolated and characterized. These compounds arise upon reactivation of AChE inhibited by dimethylphosphoryl OP insecticides and by diisopropyl phosphorofluoridate (DFP). Both POXs were highly reactive inhibitors of AChE and are prone to hydrolysis mainly by $PON1_{Q192}$. In addition, a simple HPLC method was elaborated to assess POX-hydrolase activity of human plasma at low substrate concentration, which allows precise discrimination of the $PON1_{Q192R}$ phenotype. Scheme 1 gives an overview on the most important reactions that are dealt with in this article.

2. Materials and methods

2.1. Chemicals

Ion-pairing reagents (PIC B7® and PIC A® low UV) for HPLC analysis were obtained from Waters (Milford, USA). Diazoxon was obtained from Chem Service Inc. (West Chester, USA), alcohol dehydrogenase from horse liver, sodium pyrophosphate decahydrate, semicarbazide hydrochloride

Table 1 – Rate constants for the inhibition of AChE by POXs and OP and for non-enzymic decomposition of POXs

Inhibitor (concentration)	k_{obs} [a] (min^{-1})	k_i [a] ($M^{-1}\,min^{-1}$)	k_h (min^{-1})	k_i ($M^{-1}\,min^{-1}$) (reference)
DMP-obidoxime (2 nM)	1.26	6.3×10^8	0.023[a]; 0.11[b]; 0.12 ($n = 2$)	
DEP-obidoxime (2 nM)	0.53	2.65×10^8	0.013[a]; 0.088 ± 0.01[b] ($n = 3$)	1.2×10^8, 10 °C [19]
DIP-obidoxime (30 nM)	0.37	0.12×10^8	n.d.; 0.070 ± 0.004[b] ($n = 3$)	
Paraoxon-methyl (1 μM)	0.53	27×10^4	n.d.	120×10^4, 37 °C [48]
Paraoxon-ethyl (1 μM)	0.28	52.5×10^4	n.d.	35×10^4, 10 °C [19]; 220×10^4, 37 °C [64]
DFP (6 μM)	0.12	2×10^4	n.d.	14×10^4, 27 °C [65]; 14×10^3, 15 °C [66]

n.d.: not determined.
[a] Rate constants were determined in 0.1 M phosphate buffer, pH 7.4, at 10 °C.
[b] Rate constants were determined in 50 mM MOPS, 0.5 mM Ca^{2+}, pH 7.4 at 20 °C.

from Sigma–Aldrich (Deisenhofen, Germany), glycine from Merck (Darmstadt, Germany), NAD from Boehringer Mannheim (Mannheim, Germany). All other chemicals were obtained from suppliers as mentioned previously [20].

2.2. Generation and isolation of DMP-, DEP-, and DIP-obidoxime

Caution: Generation of POXs produces extremely toxic anticholinesterase agents!

Obidoxime (10 mM) was incubated with equimolar amounts of paraoxon-methyl, paraoxon-ethyl or diisopropyl phosphorofluoridate (DFP) in 10 mM sodium veronal buffer adjusted to pH 7.5 with NaOH. The incubation mixture was allowed to react on ice over night. Aliquots of 0.1 ml were separated by HPLC (method A) and the peak fractions of DMP-obidoxime (r.t. 9 min, method A1), DEP-obidoxime and DIP-obidoxime (r.t. 18 min and 23 min, respectively, method A2) were collected on ice. To reduce the MeOH concentration, the fractions were pooled and evaporated at −1 °C shelf temperature at 0.014 mbar (freeze-dryer Alpha 1-4 LSC; M. Christ Osterode, Germany) to near-dryness and subsequently constituted in water, adjusted to pH 4.5 by a few drops of 0.1 M acetic acid. In this way the MeOH concentration was reduced below 0.5%. By completely drying and reconstitution, a large amount of POX degraded to obidoxime mononitrile. The POX solutions were stored at −80 °C and were found to be stable over 3 years. The concentrations of the POX derivatives of obidoxime were quantified as obidoxime after complete hydrolysis with $PON1_{192QQ}$. It turned out that the absorbance coefficient of DEP-obidoxime solutions (31.6 $mM^{-1}\,cm^{-1}$, pH 4.5 [19]) was also applicable to DMP- and DIP-obidoxime.

2.3. AChE preparation

Hemoglobin-free human erythrocyte membrane suspensions (ghost suspensions) were prepared according to Dodge et al. [29] with minor modifications [30]. Finally, the suspension was adjusted to an appropriate AChE activity of 4–5 U/ml with sodium phosphate buffer (0.1 M, pH 7.4). Aliquots of the AChE suspension were stored at −20 °C until use.

2.4. Titration of AChE active sites

In order to estimate the concentration of AChE active sites, suspensions of erythrocyte membranes were titrated with DEP-obidoxime. To reduce errors due to spontaneous decomposition of DEP-obidoxime concentrated AChE (6 U/ml) was incubated with nine different DEP-obidoxime concentrations (0.5–12 nM) at 10 °C until activity reached a stable level after about 5–10 min. (Under these conditions DEP-obidoxime decomposed spontaneously with $t_{1/2} = 53$ min; Table 1.) The residual activity was plotted versus the DEP-obidoxime concentration. Linear regression analysis ($r^2 = 0.97$) gave an X-axis intercept at 16 nM active sites. Assuming a normal activity of 5.6 U/ml in whole blood [31] gives an AChE concentration of about 15 nM in whole blood. These findings agree with data of Worek et al. [32] stating 13.7 nM active sites for an AChE suspension of 5 U/ml, but is five times higher than reported by Lockridge and Masson [33].

2.5. Inhibition kinetics of AChE by OPs and POXs

Inhibition kinetics of human AChE in the absence of substrate were followed under pseudo-first-order conditions. Stock solutions of POX (11–16 μM) were thawed immediately before use and diluted appropriately with water (pH 4.5). AChE suspensions were diluted in 0.1 M phosphate buffer (pH 7.4) to a concentration of approximately 0.2–0.3 nM active sites and incubated with 2 nM DMP- or DEP-obidoxime, 30 nM DIP-obidoxime, and 1 μM paraoxon-methyl or paraoxon-ethyl and 10 μM DFP. To reduce POX decomposition the inhibition kinetics, and for purpose of comparison also the OP-inhibition kinetics, were followed at 10 °C, while AChE activity was determined at 37 °C. At selected time intervals, aliquots were diluted into Ellman's assay mixture for measurement of residual activity (see below). The following function was fitted to the data:

$$E_t = E_0\, e^{-k_{obs} t} + \text{plateau}$$

E_t and E_0 denote enzyme activities at time t and zero time. The second-order rate constant of the inhibition k_i was obtained by dividing k_{obs} through the POX concentration. In order to take the non-enzymic decomposition of POXs, spontaneous reactivation and aging into account, a computer simulation for the inhibition kinetics of AChE by DMP-obidoxime was performed with Maple 9.00 (Maplesoft, Waterloo, Canada) using the following differential equations:

$$\frac{d[E]}{dt} = -k_i[POX][E](t) + k_s[EP](t),$$
$$\frac{d[EP]}{dt} = k_i[POX][E](t) - k_s[EP](t) - k_a[EP](t)$$

The actual POX concentration $[POX]_t$ due to non-enzymic decomposition was calculated by the equation $[POX]_t = [POX]_0 e^{-k_h t}$ and integrated in the equations above (see Scheme 1).

2.6. Spectrophotometric determination of enzyme activities

2.6.1. AChE activity assay

The activity of AChE from erythrocyte membranes was determined by a modified Ellman method [31]. The assay contained 0.45 mM acetylthiocholine, 0.28 mM 5,5′-dithio-bis-2-nitrobenzoic acid in 0.1 M sodium phosphate buffer, pH 7.4 at 37 °C. The reaction was followed at 436 nm.

2.6.2. Diazoxonase and paraoxonase activity assays

Rates of diazoxon and paraoxon hydrolysis were determined in 0.1 M Tris–HCl buffer, pH 8.5, 2 M NaCl, 2 mM $CaCl_2$ at 37 °C and 270 nm (diazoxonase) or 405 nm (paraoxonase), substrate concentrations were 0.5 mM diazoxon for diazoxonase and 1.2 mM PX for paraoxonase, respectively, for details see [24,34]. It should be noted that the final acetonitrile concentration in the diazoxonase test (0.7%) inhibited PON1 by about 10%. For comparability with published results the data were not corrected.

2.6.3. Assay for MeOH determination

Since MeOH is a very poor substrate for liver alcohol dehydrogenase and the Michaelis constant lies far above the MeOH concentrations of interest, the initial velocity of the conversion was used as a measure of the MeOH concentration. An appropriate calibration curve with MeOH standards followed first-order kinetics. The reaction was carried out at pH 8.7 in 68 mM sodium pyrophosphate/19 mM glycine buffer, containing 68 mM semicarbazide and 0.65 mM NAD at 37 °C and recorded at 340 nm [35]. The reaction was started with 20 mU of alcohol dehydrogenase/ml.

2.7. Determination of POX-hydrolase activity (k_e) and POX decomposition rate constant (k_h)

Enzymic hydrolysis (k_e) and spontaneous decomposition (k_h) of POX were determined at 20 °C in 50 mM MOPS buffer, 0.5 mM Ca^{2+}, pH 7.4. An aliquot of POX-solution on ice was brought to reaction temperature and mixed with plasma (1:1) at 20 °C. Plasma showing high activity was diluted in 0.1 M MOPS buffer, 1 mM Ca^{2+}, pH 7.4; plasma with low activity was dialyzed against the same buffer and used more concentrated. For determination of the spontaneous decomposition rate of POX, dilution buffer only without plasma was used.

At appropriate time intervals, an aliquot was mixed with trichloroacetic acid to yield a final concentration of 0.23 M. After centrifugation, one part of the supernatant was mixed with two parts of a PIC B7®/NH_3 solution (1 ml PIC B7® was mixed with 120 μl of concentrated NH_3 solution) and maintained on ice. Fifty microlitres was injected on the HPLC system and analyzed by method B or C. The decrease of the POX concentration versus time was used as a measure of POX-hydrolase activity. As the substrate concentration was very low ($[S] \ll K_m$) first-order kinetics were assumed:

$$[POX]_t = [POX]_0 e^{-k_{el} t}$$

$[POX]_t$ and $[POX]_0$ are POX concentrations at time t and zero time and k_{el} expresses the first-order reaction constant [36] of POX disappearance. When POX was hydrolyzed enzymically, the rate of POX disappearance consisted of the spontaneous decomposition rate k_h, and the rate of enzymic cleavage, denoted as k_e, with $k_e = k_{el} - k_h$ (Scheme 1). Consistently, k_e was proportional to the plasma concentration.

2.8. HPLC methods

DEP-obidoxime and the degradation products were analyzed by HPLC using a Hitachi L-6200 pump (Merck). For all methods, a LiChroCart Superspher 60, RP-select B (3 mm i.d. × 125 mm; 4 μm (Merck)) column was used. Peaks were quantified by peak integration with an SPD M6A UV–vis diode-array detector (Shimadzu) coupled with a personal computer Compaq ProLinea 4/66; software Shimadzu Class LC-10 1992. Identification was achieved by comparison of retention times and UV spectra with authentic standards or compounds isolated by HPLC. For chromatography with ion pairing, PIC B7®/NH_3 was added to the sample to prevent peak broadening.

2.8.1. Method A

For preparation of POX, chromatography at a flow rate of 0.6 ml/min without ion-pairing reagents was applied. The mobile phase consisted of 10 mM ammonium acetate, pH 4.5 (eluent A) and the same buffer containing 50% MeOH (eluent B). The detection wavelengths were set at 220 and 285 nm. Method A1: samples of DMP-obidoxime were eluted with a linearly increasing gradient from 0% B to 24% B in 12 min, linear increase to 100% B in 9 min, 100% B for 12 min, return to 0% B in 3 min and 0% B for 9 min; the retention time was 9 min. Method A2: samples of DEP- and DIP-obidoxime were eluted with a linearly increasing gradient from 0% B to 40% B in 20 min, linear increase to 100% B in 10 min, 100% B held for 12 min, return to 0% B in 3 min and 0% B for 5 min; the retention times were 18 min for DEP-obidoxime and 23 min for DIP-obidoxime.

2.8.2. Method B

For the simultaneous detection and quantification of POX, obidoxime and obidoxime mononitrile, a flow rate of 0.8 ml/min was used. The mobile phase consisted of aqueous 4% PIC B7®, 0.35% PIC A® low UV. For samples of DMP- and DEP-obidoxime gradient elution was started with 2% acetonitrile, linearly increased to 5% in 12 min, linearly increased to 12% in 3 min, 12% held for 5 min, return to 2% in 2 min and held for 10 min. The separation of obidoxime and obidoxime mononitrile was improved when applying a column oven temperature of 40 °C.

2.8.3. Method C

For quantification of POXs only in the isocratic mode, the same aqueous mobile phase as described in method B was used, containing 6% acetonitrile for DMP-obidoxime, 12% acetoni-

trile for DEP-obidoxime and 16% for DIP-obidoxime. The column oven temperature was adjusted to 30 °C.

2.9. Calculations

Data analysis was performed by curve fitting programs provided by Prism™ Version 3.0 (GraphPad Software, San Diego, CA). Computer calculations for simulation of dynamic changes of AChE activities were performed with the software Maple 9.0 (Maplesoft, Waterloo, Canada) by solving numerically multiple differential equations (basics are detailed in [37]). Arithmetic means are given with standard deviations for $n \geq 3$.

3. Results

3.1. Determination of PON1 status

Fig. 1 shows a plot of diazoxonase versus paraoxonase activities resolving $PON1_{Q192R}$ phenotypes. All samples showed sufficient high activities to provide a clear differentiation. Even the sample with very low paraoxonase activity would have been clearly assigned to the $PON1_{192QQ}$ phenotype; in fact it was purified $PON1_{192QQ}$ that was generously provided by the lab of Prof. P. Masson.

3.2. Long-term stability of PON1 in frozen plasma

The stability of paraoxonase, diazoxonase and arylesterase was explored using samples from one individual of $PON1_{192QQ}$ or $PON1_{192RR}$ phenotype each, stored at −20 °C up to 6 years. Activities with all substrates decreased slowly upon storage. By fitting an exponential function to the data, a half-life of 9.5 years was found (11.7–8.1, 95% CI). For comparison, paraoxonase activities obtained by Eckerson et al. [38] were included (Fig. 2). In general, the ratios between the three substrates remained constant during storage, with the tendency of a slight increase in the ratio diazoxonase/paraoxonase of the $PON1_{192RR}$ sample.

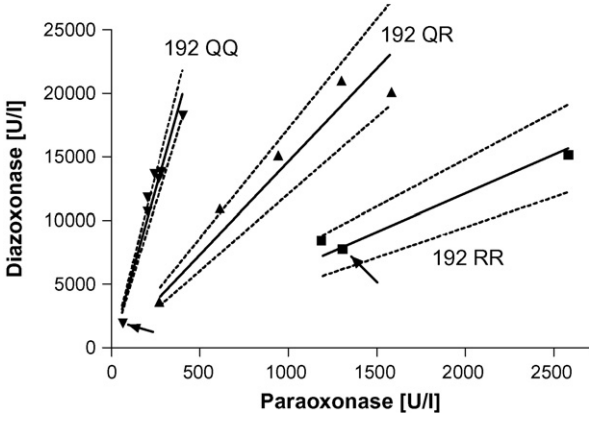


Fig. 1 – Assignment of plasma samples to the supposed $PON1_{Q192R}$ phenotype by the plot of diazoxonase vs. paraoxonase activity [24]. Arrows point to samples provided by the laboratory of P. Masson with confirmed $PON1_{Q192R}$ phenotype. Linear regression lines with 95% CI; lines forced through the origin.

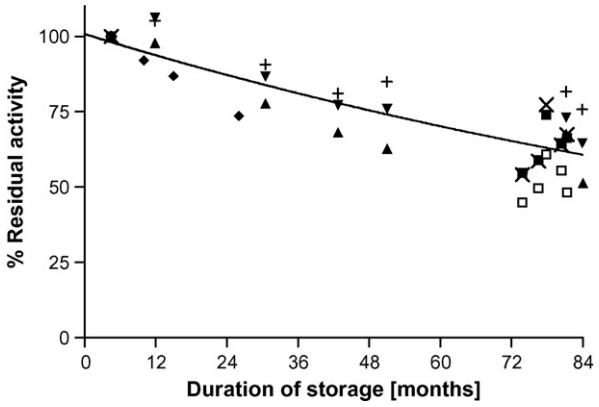


Fig. 2 – Stability of paraoxonase, diazoxonase and arylesterase activity of $PON1_{192QQ}$ or $PON1_{192RR}$, respectively, stored at −20 °C. Paraoxonase activities (◆) from Eckerson [38] were included for calculation of the half-life (9.5 years) for PON1 activity. Enzyme activities were related to protein content of plasma; paraoxonase: (▼) 192RR, (■) 192QQ; arylesterase: (▲) 192RR, (□) 192QQ; diazoxonase: (+) 192RR, (×) 192QQ.

3.3. Generation and isolation of DMP-, DEP-, and DIP-obidoxime

DMP- and DIP-obidoxime were synthesized in analogy to DEP-obidoxime [19]. Paraoxon-methyl or DFP were allowed to react directly with equimolar (10 mM) obidoxime on ice. The new POXs were isolated by HPLC and exhibited nearly identical UV spectra as DEP-obidoxime, characterizing them as mono-dialkylphosphoryl-obidoximes. Another evidence for their identity was their high inhibitory potency towards AChE. Hydrolysis of the new POX compounds by $PON1_{192Q}$ yielded obidoxime as reaction product, while non-enzymic decomposition resulted in obidoxime mononitrile, which was already characterized by mass spectroscopy [19].

3.4. Inhibition rate constants of AChE by POX compounds and OPs

Inhibition rate constants were determined under pseudo-first-order conditions at 10 °C in the absence of substrate. The low temperature was chosen to keep the spontaneous decomposition rate of POXs low. An AChE concentration of approximately 0.2–0.3 nM was incubated with POXs or OPs in excess. Pseudo-first-order rate constants were obtained by fitting a one-phase exponential decay function to the data points (Fig. 3). Second-order rate constants k_i were derived from the inhibitor concentrations. It should be kept in mind that the data are approximate values, since the K_d values describing the affinity of the OPs and POXs to AChE have not been determined. We roughly estimated the value for paraoxon-ethyl at 10 °C in the range of about 90 μM [39–41]. Hence, pseudo-first-order conditions are probably fulfilled. DMP-obidoxime turned out to be more potent than DEP-

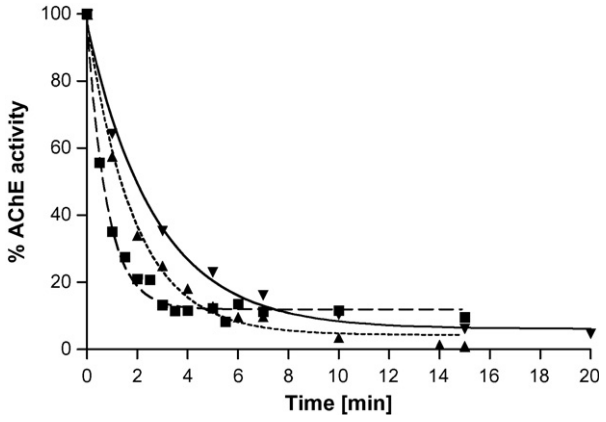


Fig. 3 – Inhibition of AChE by dialkylphosphoryl-obidoxime derivatives. Dimethylphosphoryl-obidoxime (■) (2 nM), diethylphosphoryl-obidoxime (▲) (2 nM), and diisopropyl-obidoxime (▼) (30 nM) were incubated with 0.2–0.3 nM human ghost membrane AChE in 0.1 M phosphate buffer, pH 7.4, at 10 °C. After various time intervals aliquots were taken and the residual AChE activity determined at 37 °C by a modified Ellman method. A monoexponential function was fitted to the data points allowing variable plateau calculation.

obidoxime, with DIP-obidoxime being the weakest AChE inhibitor (Table 1). Surprisingly, AChE was not completely inhibited by the above POXs. A plateau of 10% residual activity remained with DMP-obidoxime, 4.3% with DEP-obidoxime and 6.2% with DIP-obidoxime (Fig. 3).

3.5. *Characterization of POX-hydrolase*

3.5.1. *Establishment of a new determination method*

Handling of large amounts of highly toxic POX compounds requires particular precautions and demands special laboratory facilities. Because of this situation, we pursued a way to establish a method for quantifying POX-hydrolase activity at low POX concentrations, which were presumed to be far below K_m. Under these conditions, determination of product formation or substrate reduction are both possible. Following decreasing POX concentrations was favored, as it was a problem to precisely quantify obidoxime and obidoxime mononitrile by HPLC (Fig. 4). Nevertheless, useful kinetics of

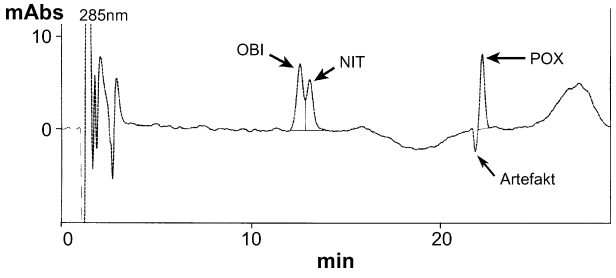


Fig. 4 – HPLC chromatogram of an incubate of POX and human plasma (method B) showing the separation of POX, obidoxime (OBI), and obidoxime mononitrile (NIT).

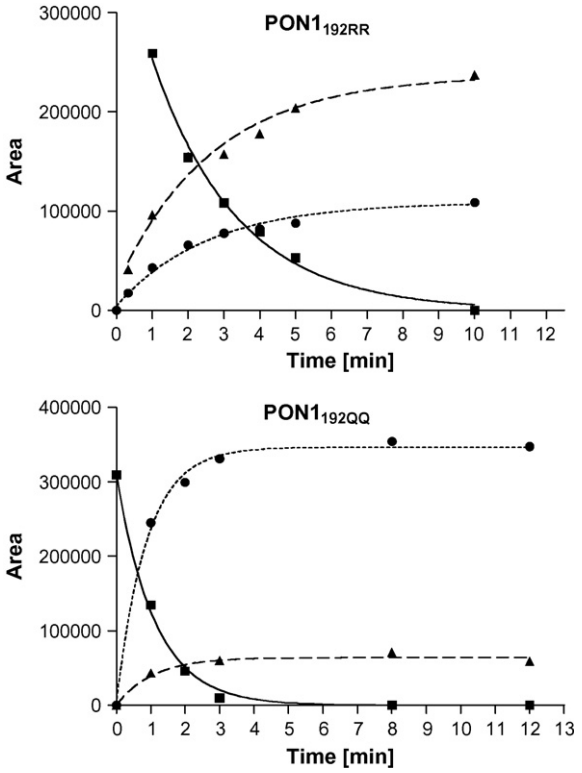


Fig. 5 – Degradation and product formation of diethylphosphoryl-obidoxime in the presence of different PON1 phenotypes in human plasma. Diluted plasma (1:1 with $PON1_{192RR}$ and 1:20 with $PON1_{192QQ}$) was incubated with diethylphosphoryl-obidoxime (8 μM) in 50 mM MOPS buffer, 0.5 mM Ca^{2+}, pH 7.4, at 37 °C. Diethylphosphoryl-obidoxime (■), obidoxime (●), and obidoxime mononitrile (▲) were determined by HPLC, method B with peak area recorded at 285 nm wavelength.

the formation of obidoxime and obidoxime mononitrile were obtained (Fig. 5) and allowed unambiguous proof of obidoxime liberation upon enzymic hydrolysis. Since it was not necessary to quantify the hydrolysis product of POX, isocratic HPLC method C was preferred to accelerate POX determination.

3.5.2. *Influence of EDTA, temperature and pH on POX-hydrolase*

Obidoxime as a product of the enzymic reaction with samples phenotyped as $PON1_{192QQ/QR}$ was found for all POX compounds. However, only DMP- and DEP-obidoxime were substrates for the $PON1_{192R}$ phenotype, while DIP-obidoxime appeared to be no substrate for the $PON1_{192R}$ alloenzyme (Table 2). The effect of 2 mM EDTA was tested with a $PON1_{192QR}$ sample. Assay conditions were as described except calcium was substituted by EDTA. The POX-hydrolase activity disappeared under these conditions.

Since it was our intention to comply with physiological conditions, we tried to work at 37 °C and pH 7.4. When we examined the dependence of POX-hydrolase activity and spontaneous decomposition of POX from temperature (Fig. 6), POX turned out to be too unstable at 37 °C for reliable

Table 2 – Dependence of POX-hydrolase activities (k_e) in human plasma on the $PON1_{Q192R}$ phenotype

Sample	$PON1_{Q192R}$ phenotype	Substrate	DF	$k_e \times DF$ (min^{-1})	Paraoxonase (U/l)/$k_e \times DF$
1	192RR	DMP-obidoxime	2	0.12	21667
7	192QR		40	6.8	192
11	192QQ		40	9.6	30
1	192RR	DEP-obidoxime	4	1.7	1530
7	192QR		60	41	32
11	192QQ		60	55	6
1	192RR	DIP-obidoxime	2	0	Not applicable
7	192QR		2	0.32	4063
11	192QQ		2	0.13	2154

Activities of human plasma were determined with approximately 8 μM of the respective dialkylphosphoryl-obidoxime derivatives in 50 mM MOPS, 0.5 mM Ca^{2+}, pH 7.4, MeOH < 0.5%, at 20 °C and related to undiluted plasma ($k_e \times DF$: dilution factor).

determination of low POX-hydrolase activities. In contrast, the enzymic hydrolysis of POX was less influenced by temperature. Due to these findings 20 °C were adopted as reaction temperature. Enzymic hydrolysis of POX was more influenced by changes in pH than the non-enzymic decomposition (Fig. 7).

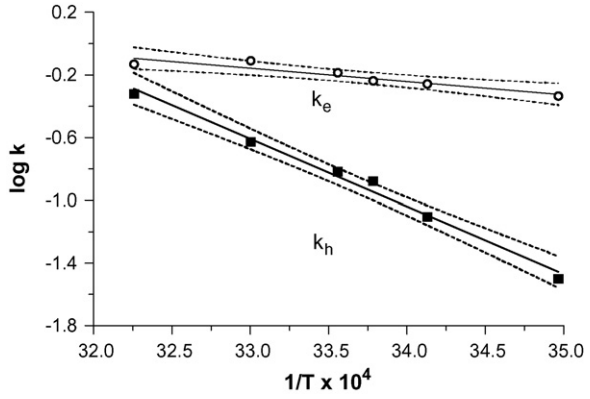


Fig. 6 – Arrhenius plot of the non-enzymic decomposition rate constants k_h (min^{-1}) and of POX-hydrolase activity k_e (min^{-1}) vs. the reciprocal of absolute temperature ($PON1_{192QR}$, DEP-obidoxime, pH 7.4; linear regression analysis and 95% CI).

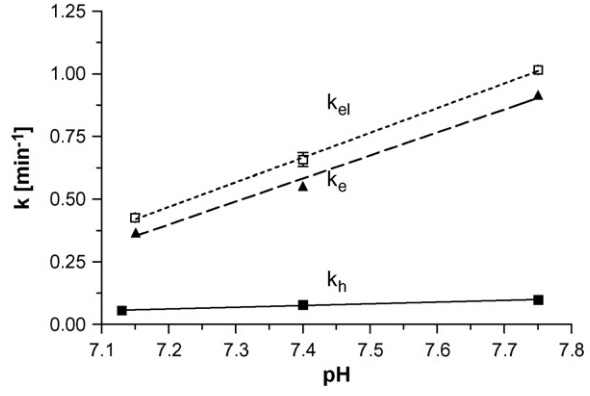


Fig. 7 – Dependence of k_{el}, k_e, and k_h of the degradation of diethylphosphoryl-obidoxime on pH (50 mM MOPS buffer, 0.5 mM Ca^{2+}, 20 °C).

3.5.3. *Dependence of POX-hydrolase activity on $PON1_{Q192R}$ phenotype*

The enzyme-catalyzed decomposition of DEP-obidoxime by PON1 was tested with 15 samples ($PON1_{192QQ}$, $n = 7$; $PON1_{192QR}$, $n = 5$; $PON1_{192RR}$, $n = 3$, phenotyped by the diazoxonase/paraoxonase ratio [24]). Groups of $PON1_{Q192R}$ phenotypes were clearly differentiated (Fig. 8). Interestingly, DEP-obidoxime was hardly split by the $PON1_{192RR}$ phenotype. When using DMP-obidoxime as substrate, enzyme activity was markedly less while the same rank order was observed. DIP-obidoxime was a poor substrate, which apparently was not split at all by the $PON1_{192RR}$ phenotype. Since HPLC-isolated DIP-obidoxime contained significant amounts of MeOH, we tested the influence of MeOH on PON1 activity.

3.5.4. *Effect of MeOH on PON1 activities*

With paraoxon as substrate, PON1 activity was markedly depressed by MeOH concentrations exceeding 0.5% (Fig. 9). A hyperbola was applied to the data points indicating an IC_{50} value of 2.7% MeOH. There was no significant difference between the $PON1_{Q192R}$ phenotypes. In contrast, MeOH was a stronger inhibitor for $PON1_{192RR}$ phenotype when using DEP-obidoxime as substrate with an IC_{50} value of 1% MeOH. The sensitivity of the $PON1_{192QQ}$ phenotype towards MeOH was independent of the substrate used.

Based on these observations, POX samples were evaporated to near-dryness at −1 °C. The reconstituted POX samples contained less than 0.5% MeOH. With these samples representative $PON1_{Q192R}$ phenotypes were tested for POX-hydrolase activity (Table 2). These data indicate that the POX-hydrolase turnover of DEP-, DMP-, and DIP-obidoxime are 6:1:0.03, respectively, with the $PON1_{192QQ}$ phenotype being about 50 times more active than the $PON1_{192RR}$ phenotype.

4. Discussion

4.1. *Long-term stability of PON1*

Information about the stability of stored PON1 is rare in the accessible literature. Only some hints, but no systematic investigations were found [42–44]. Intraindividual PON1 activity reaches its normal level at an age of 2 years and remains stable in adults [45]. Activity is modified by drugs, diseases or changes in life-style [46]. Our results comprising

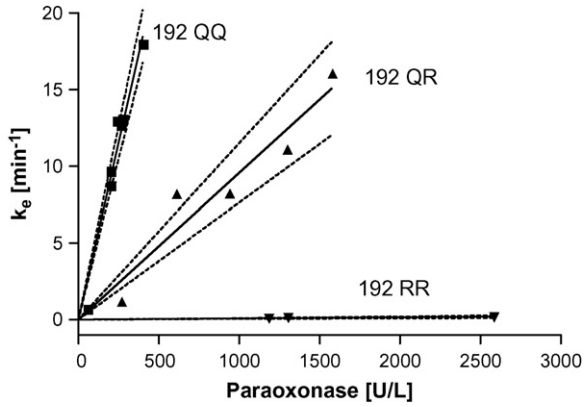


Fig. 8 – Two-dimensional plot of POX-hydrolase k_e vs. paraoxonase activity in human plasma of different phenotypes. Plasma samples were phenotyped as shown in Fig. 1. Plasma samples were incubated with 8 μM DEP-obidoxime at pH 7.4 and 20 °C (see Section 2). Linear regression analysis with 95% CI; lines forced through the origin.

plasma samples of two individuals with up to 84 months storage at −20 °C may have been altered by inconsistent treatment (different numbers of thawings, dialysis conditions, some were treated with soman to inhibit butyrylcholinesterase). Hence the calculated half-life of some 10 years should be regarded as a hint only. Importantly, the two different homozygous $PON1_{Q192R}$ phenotypes did not show major differences, regardless of the substrate used.

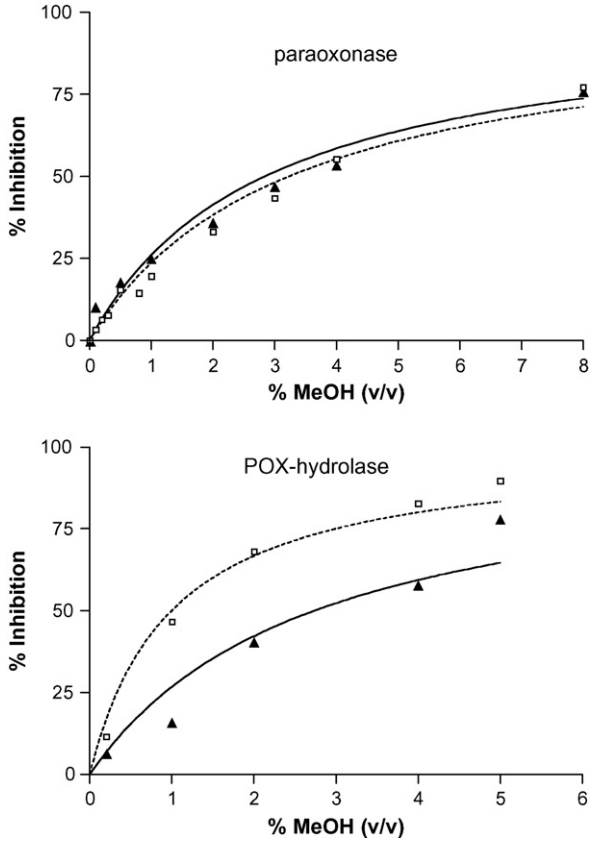


Fig. 9 – Influence of MeOH on paraoxonase and POX-hydrolase activities of PON1 in human plasma. A hyperbola was fitted to the data ((□) $PON1_{192RR}$; (▲) $PON1_{192QQ}$).

4.2. *Synthesis and inhibitory potency of POX compounds*

Kiderlen et al. [19] succeeded in generation and characterization of DEP-obidoxime in a direct reaction between paraoxon-ethyl and obidoxime and also showed the pattern of degradation. Under identical conditions paraoxon-methyl and DFP were allowed to react with obidoxime assuming the formation of DMP- and DIP-obidoxime, respectively. The novel compounds emerged as newly occurring HPLC peaks with characteristic retention times. The tentatively assigned POX compounds exhibited largely similar UV spectra. The different alkyl residues were not supposed to cause significant changes to the absorption of the major chromophor, the obidoxime moiety. Absorption maxima, 275 nm, were indistinguishable from DEP-obidoxime, so that the absorption coefficient of DEP-obidoxime was applied (31.6 mM^{-1} cm^{-1}) [19]. In fact, non-enzymic degradation yielded obidoxime mononitrile which had been fully characterized [19], while enzymic hydrolysis liberated obidoxime. Non-enzymic decomposition of the three POX compounds at pH 7.4 and 20 °C proceeded at similar rates (Table 2). Finally, the high AChE inhibitory activity underlined the identity of the compounds as POXs.

Testing the inhibitory potency of POX against AChE gave bimolecular rate constants k_i of about 10^8 M^{-1} min^{-1} at 10 °C. The ability to inhibit AChE decreased in the following order DMP-obidoxime > DEP-obidoxime ≫ DIP-obidoxime, which was an unexpected result considering the potency of the parent compounds paraoxon-methyl and paraoxon-ethyl, with paraoxon-ethyl being the stronger inhibitor. Thus, conjugation with obidoxime increased the toxicity of the parent dialkylphosphates by a factor of 2250, 480, and 600, respectively. The comparably lower toxicity of DFP and DIP-obidoxime might be explained by steric interactions of the bulky isopropyl moieties [47].

It should be noted that even 6–100-fold excess POX did not completely inhibit AChE. Fitting an exponential equation to the residual activities of inhibition experiments with POX did not provide satisfying results after all. Especially the inhibition kinetics with DMP-obidoxime levelled of at a plateau of 10% residual activity. Spontaneous reactivation of DMP-AChE ($t_{1/2}$ = 41 min at 37 °C) or reactivation by the liberated obidoxime do not explain these observations under quantitative aspects. These considerations were fostered by a computer simulation of AChE inhibition by DMP-obidoxime (see Section 2). With the rate constants applied for aging and spontaneous reactivation at 37 °C [48] and POX decomposition (k_h = 0.023 min^{-1}, 10 °C) a residual activity approaching 1.5% only was calculated. This plateau would be even lower if the pertinent data at 10 °C were at hand. Conceivably, the rapid liberation of obidoxime, which is connected with rapid inhibition at rates approaching diffusion controlled reactions, leads to higher local concentrations of the oxime near the active site than calculated for homogenous distribution.

4.3. POX-hydrolase activity and PON1$_{Q192R}$ polymorphism

Kiderlen et al. introduced two methods for measuring POX-hydrolase activity [19]. The first one was based on the reactivation ratio of inhibited AChE in absence/presence of plasma, while the second one exploited the liberation of obidoxime as analyzed by HPLC. We preferred to follow POX disappearance under first-order conditions, which was not disturbed by product formation and allowed a fast isocratic HPLC analysis. When working with 6–8 μM POX we consistently observed a monoexponential decay, indicating a much higher K_m value $\gg$ 8 μM. A K_m value of 0.78 mM was reported for DEP-4-pralidoxime in the case of phosphotriesterase from *Pseudomonas* sp. [49], and K_m values for human PON1 were in the range of 0.5 mM with organophosphorus compounds such as paraoxon and diazoxon [24,50]. Hence, the first-order rate constant for elimination was directly proportional to the enzyme activity and had to be corrected only for the spontaneous decomposition of POX.

The spontaneous decomposition of DEP-obidoxime exhibited a high activation energy (82.5 kJ/mol) while the enzymic hydrolysis required less energy (16.2 kJ/mol). It should be noted, however, that two different chemical reactions underlie the degradation of POX. In the first case, POX is presumably eliminated by a cyclic β-*cis* elimination with formation of the nitrile, which is markedly influenced by the acidity of the methine proton [8,9]. The rate of this reaction was not markedly affected by pH in the range between 7.1 and 7.8. Conversely, the enzymic hydrolysis of DEP-obidoxime showed a distinct dependence on pH: in the range observed the increase in hydrolysis was directly proportional to the OH^- concentration (slope 0.92 ± 0.08).

It has been reported that oximate ions may catalyze the decomposition of POXs [4,7]. Two possible reaction pathways were considered: (i) a general base catalysis of the elimination reaction of the acidic methine proton yielding the nitrile or (ii) nucleophilic attack on the phosphorus atom. Since the latter pathway would lead only to re-synthesis of the phosphylated oxime, this way was previously ruled out [6]. While these considerations may apply for the synthesis of the POXs, when rather high concentrations are used, we could not detect such an oxime interference when reacting DEP-obidoxime (5 μM) with obidoxime (10 μM) at pH 7.1 and 20 °C [19]. We re-investigated this matter reacting 10 μM DEP-obidoxime with 100 μM obidoxime or TMB-4 at pH 7.4 and 20 °C (data not shown) and did not detect any increase in disappearence of the POX. Also transoximation (pathway (ii), see above) was excluded, because no new compound could be detected by HPLC and spectroscopic analysis during degradation of DEP-obidoxime in the presence of TMB-4. We hence conclude that DEP-obidoxime decomposition will be probably not accelerated by oximes at therapeutic concentrations.

PON1 is quite sensitive towards organic solvents, an effect which was detected quite late when many of our experiments had already been finished. Even 1% (v/v) MeOH or acetonitrile inhibited paraoxon hydrolysis by around 20%, hence POX samples isolated by HPLC had to be treated to reduce the MeOH content to a minimum. Unfortunately, lyophilization to dryness resulted in marked nitrile formation and has to be avoided. Data on the sensitivity of PON1 towards solvents are rare in literature, but inhibition by MeOH of chlorpyrifos oxon hydrolysis was reported by Furlong et al. [51] with an IC_{50} of about 5%. The inhibition of both PON1$_{Q192R}$ alloenzymes by MeOH, using phenylacetate as substrate revealed an IC_{50} of about 4% [52]. Since we did not notice a difference between PON1$_{Q192R}$ phenotypes for inhibition of paraoxon hydrolysis (IC_{50} about 3%), it was surprising that POX-hydrolase activity of PON1$_{192RR}$ seemed to be more influenced by MeOH than the activity of PON1$_{192QQ}$. This observation could originate from the application of different plasma concentrations, rather than from different sensitivities of the alloenzymes towards MeOH. Nevertheless, phenotyping of the PON1$_{Q192R}$ alloenzymes by the paraoxonase/POX-hydrolase ratio was not challenged.

A plasma sample of the 192RR phenotype showed a 32- and 80-fold lower activity of DMP- and DEP-obidoxime hydrolase, respectively, than the 192QQ type (Table 2). Thus, the ratio of paraoxonase/POX-hydrolase discriminates very selectively the PON1$_{Q192R}$ phenotypes comparable with the paraoxonase/sarinase ratio [21].

The catalytic mechanism of PON1 is still not fully elucidated. Various substrates were described so far including aromatic carboxyl esters, lactones, OPs and also some widely used drugs. The rate of hydrolysis for several substrates depends on the Q192R polymorphism [21,51,53,54]. Recent findings pointed to lactonase activity as the native role of PON1 [55], whereas its further catalytic capabilities are considered as ''promiscuous'' functions [56]. Harel et al. [57] introduced the 3D structure of a recombinant PON1 showing the location of the Q192R polymorphism near the catalytic center. Conceivably the protonated nitrogen atom in arginine interacts with the positive charged pyridinium ring(s) of POXs. In contrary the glutamine residue is not charged at pH 7.4.

4.4. Conclusion

POX compounds are inevitably produced during reactivation of OP inhibited ChEs by oximes [2,3,12–14]. The enhancement of reactivation by 4-oximes (4-PAM, obidoxime, TMB-4) in the presence of POX decomposing enzymes like phosphotriesterase from *Pseudomonas* sp. or human PON1 could be confirmed [19,20,49]. 2-POXs formed by reactivation with pyridinium-2-aldoximes (2-PAM, Hagedorn oximes) appear to be too unstable, thus re-inhibition by 2-POXs is of no importance [5]. PON1 is thought to be mainly expressed in the liver and released into plasma [58–60]. It is probably absent in the immediate vicinity of the neuromuscular junction. However, the presence of the patrolling PON1$_{192Q}$ alloenzyme in the capillary tree could augment the effectiveness of 4-oximes. In addition removing of POXs during reactivation of inhibited ChE by 4-oximes in the blood, may help lowering acetylcholine concentration in blood. Thus dangerous states of hypotension could possibly be prevented, because endothelial muscarine receptors are less stimulated [61,62]. It is unclear at present whether an OP poisoned patient homozygous for PON1$_{192RR}$ may gain more profit from pralidoxime than from obidoxime that has a higher potency and efficacy in reactivating AChE inhibited by OP insecticides [60]. While the frequency of the homozygous PON1$_{192RR}$ phenotype in the Caucasian population is only around 6%, its frequency is considerably higher in

several regions of East Asia [63]. Hence, the pharmacogenetics of paraoxonase polymorphism may have decisive effects on the reactivating potential of pyridinium-4-aldoximes and is another contributor to the large variability of susceptible subjects seen in obidoxime-treated patients [28].

Acknowledgement

The authors are indebted to the laboratory of Prof. Patrick Masson (La Tronche, France) for providing us with purified human $PON1_{192QQ}$ and $PON1_{192RR}$.

REFERENCES

[1] Wilson IB, Ginsburg S. A powerful reactivator of alkylphosphate-inhibited acetylcholinesterase. Biochim Biophys Acta 1955;18:168–70.
[2] Hackley Jr BE, Steinberg GM, Lamb JC. Formation of potent inhibitors of AChE by reaction of pyridinaldoximes with isopropyl methylphosphonofluoridate (GB). Arch Biochem Biophys 1959;80:211–4.
[3] Lamb JC, Steinberg GM, Hackley JBE. Isopropyl methylphosphonylated bisquaternary oximes; powerful inhibitors of cholinesterase. Biochim Biophys Acta 1964;89:174–6.
[4] Lamb JC, Steinberg GM, Solomon S, Hackley Jr BE. Reaction of 4-formyl-1-methylpyridinium iodide oxime with isopropyl methylphosphonofluoridate. Biochemistry 1965;4:2475–84.
[5] Ashani Y, Bhattacharjee AK, Leader H, Saxena A, Doctor BP. Inhibition of cholinesterases with cationic phosphonyl oximes highlights distinctive properties of the charged pyridine groups of quaternary oxime reactivators. Biochem Pharmacol 2003;66:191–202.
[6] Steinberg GM, Solomon S. Decomposition of a phosphonylated pyridinium aldoxime in aqueous solution. Biochemistry 1966;5:3142–50.
[7] Blanch JH. Stability of *N*-heterocyclic oxime derivatives. Part V. Kinetics of the reaction of 2-hydroxyiminomethyl-1-methylpyridinium iodide and isopropyl methylphosphonofluoridate in water at 15, 25 and 35 °C and at pH 5.7–7.7. J Chem Soc B 1969;1172–8.
[8] Hagedorn I, Gündel WH, Schoene K. Reaktivierung phosphorylierter Acetylcholin-Esterase mit Oximen: Beitrag zum Studium des Reaktionsablaufes. Arzneim Forsch 1969;19:603–6.
[9] Hagedorn I, Stark I, Lorenz HP. Reaktivierung phosphorylierter Acetylcholin-Esterase—Abhängigkeit von der Aktivator-Acidität. Angew Chem 1972;84:354–6.
[10] Aldridge WN, Reiner E. Enzyme inhibitors as substrates Interactions of esterases with esters of organophosphorus and carbamic acids, vol. 26. Amsterdam: North-Holland Publ. Co.; 1972.
[11] Schoene K. Reaktivierung von *O*,*O*-Diäthylphosphoryl-Acetylcholinesterase. Reaktivierungs-Rephosphorylierungs-Gleichgewicht. Biochem Pharmacol 1972;21:163–70.
[12] Nenner M. Phosphonylierte Aldoxime. Hemmwirkung auf Acetylcholinesterase und hydrolytischer Abbau. Biochem Pharmacol 1974;23:1255–62.
[13] De Jong LPA, Ceulen DI. Anticholinesterase activity and rate of decomposition of some phosphylated oximes. Biochem Pharmacol 1978;27:857–63.
[14] Harvey B, Scott RP, Sellers DJ, Watts P. In vitro studies on the reactivation by oximes of phosphylated acetylcholinesterase. I. On the reactions of P2S with various organophosphates and the properties of the resultant phosphylated oximes. Biochem Pharmacol 1986;35:737–44.
[15] Eyer P, Worek F. Oximes. In: Sidell FR, editor. Chemical warfare agents. Chichester: John Wiley & Sons; 2007. p. 305–29.
[16] Schoene K. Kinetic studies on chemical reactions between acetylcholinesterase, toxic organophosphates and pyridinium oximes. In: SIPRI, editor. Medical protection against chemical-warfare agents. Stockholm, Sweden: Almqvist & Wiksell; 1976. p. 88–100.
[17] Wolthuis OL, Cohen EM. The effects of P2S, TMB4 and LüH6 on the rat phrenic nerve diaphragm preparation treated with soman or tabun. Biochem Pharmacol 1967;16:361–7.
[18] Barstad JAB, Lilleheil G, Skobba TJ. Phosphylated oximes. Some pharmaco-toxicological and biochemical features. Arch Int Pharmacodyn 1969;179:352–63.
[19] Kiderlen D, Eyer P, Worek F. Formation and disposition of diethylphosphoryl-obidoxime, a potent anticholinesterase that is hydrolysed by human paraoxonase (PON1). Biochem Pharmacol 2005;69:1853–67.
[20] Kiderlen D, Worek F, Klimmek R, Eyer P. The phosphoryl oxime-destroying activity of human plasma. Arch Toxicol 2000;74:27–32.
[21] Davies HG, Richter RJ, Keifer M, Broomfield CA, Sowalla J, Furlong CE. The effect of human serum paraoxonase polymorphism is reversed with diazoxon, soman and sarin. Nat Genet 1996;14:334–6.
[22] Humbert R, Adler DA, Disteche CM, Hassett C, Omiecinski CJ, Furlong CE. The molecular basis of the human serum paraoxonase activity polymorphism. Nat Genet 1993;3:73–6.
[23] Adkins S, Gan KN, Mody M, La Du BN. Molecular basis for the polymorphic forms of human serum paraoxonase/arylesterase: glutamine or arginine at position 191, for the respective A or B allozymes. Am J Hum Genet 1993;52:598–608.
[24] Richter RJ, Furlong CE. Determination of paraoxonase (PON 1) status requires more than phenotyping. Pharmacogenetics 1999;9:745–53.
[25] Luo C, Saxena A, Smith M, Garcia G, Radic Z, Taylor P, et al. Phosphoryl oxime inhibition of acetylcholinesterase during oxime reactivation is prevented by edrophonium. Biochemistry 1999;38:9937–47.
[26] Worek F, Eyer P, Kiderlen D, Thiermann H, Szinicz L. Effect of human plasma on the reactivation of sarin-inhibited human erythrocyte acetylcholinesterase. Arch Toxicol 2000;74:21–6.
[27] Kiderlen D, Meischner V, Worek F, Eyer P. Phosphoryl oxime-hydrolase in human serum influences oxime effectiveness in organophosphate poisoning. Drug Metab Rev 2001;33(Suppl 1):110.
[28] Eyer P, Kiderlen D, Stenzel J, Thiermann H, Worek F. Paraoxonase polymorphism. A modifying factor that influences oxime effectiveness. Clin Toxicol 2006;44:440–1.
[29] Dodge JT, Mitchell C, Hanahan DJ. The preparation and chemical characteristics of hemoglobin-free ghosts of human erythrocytes. Arch Biochem Biophys 1963;100:119–30.
[30] Worek F, Thiermann H, Szinicz L, Eyer P. Kinetic analysis of interactions between human acetylcholinesterase, structurally different organophosphorus compounds and oximes. Biochem Pharmacol 2004;68:2237–48.
[31] Worek F, Mast U, Kiderlen D, Diepold C, Eyer P. Improved determination of acetylcholinesterase activity in human whole blood. Clin Chim Acta 1999;288:73–90.

[32] Worek F, Eyer P, Szinicz L. Inhibition, reactivation, and aging kinetics of cyclohexylmethylphosphonofluoridate-inhibited human cholinesterases. Arch Toxicol 1998;72:580–7.
[33] Lockridge O, Masson P. Pesticides and susceptible populations: people with butyrylcholinesterase genetic variants may be at risk. Neurotoxicology 2000;21:113–26.
[34] Furlong CE, Richter RJ, Seidel SL, Motulsky AG. Role of genetic polymorphism of human plasma paraoxonase/arylesterase in hydrolysis of the insecticide metabolites chlorpyrifos oxon and paraoxon. Am J Hum Genet 1988;43:230–8.
[35] Bernt E, Gutmann I. Äthanol. Bestimmung mit Alkohol-Dehydrogenase und NAD. In: Bergmeyer HU, editor. Methoden der enzymatischen Analyse, vol. II. Weinheim: Verlag Chemie; 1970. p. 1457–60.
[36] Segel IH. Enzym kinetics. New York: Wiley & Sons; 1975.
[37] Worek F, Szinicz L, Eyer P, Thiermann H. Evaluation of oxime efficacy in nerve agent poisoning: development of a kinetic-based dynamic model. Toxicol Appl Pharmacol 2005;209:193–202.
[38] Eckerson HW, Romson J, Wyte C, La Du BN. The human serum paraoxonase polymorphism: identification of phenotypes by their response to salts. Am J Hum Genet 1983;35:214–27.
[39] Chiu YC, Main AR, Dauterman WC. Affinity and phosphorylation constants of a series of *O*,*O*-dialkyl malaoxons and paraoxons with acetylcholinesterase. Biochem Pharmacol 1969;18:2171–7.
[40] Barak D, Ordentlich A, Bromberg A, Kronman C, Marcus D, Lazar A, et al. Allosteric modulation of acetylcholinesterase activity by peripheral ligands involves a conformational transition of the anionic subsite. Biochemistry 1995;34:15444–52.
[41] Rosenfeld CA, Sultatos LG. Concentration-dependent kinetics of acetylcholinesterase inhibition by the organophosphate paraoxon. Toxicol Sci 2006;90:460–9.
[42] Mueller RF, Hornung S, Furlong CE, Anderson J, Giblett ER, Motulsky AG. Plasma paraoxonase polymorphism: a new enzyme assay, population, family, biochemical, and linkage studies. Am J Hum Genet 1983;35:393–408.
[43] Brackley M, Carro-Ciampi G, Stewart DJ, Lowden JA, Ray AK, Kalow W. Stability of the paraoxonase phenotyping ratio in collections of human sera with differing storage times. Res Commun Chem Pathol Pharmacol 1983;41:65–78.
[44] Eckerson HW, Wyte CM, La Du BN. The human serum paraoxonase/arylesterase polymorphism. Am J Hum Genet 1983;35:1126–38.
[45] Geldmacher-von Mallinckrodt M, Diepgen TL. The human serum paraoxonase-polymorphism and specificity. Toxicol Environ Chem 1988;18:79–196.
[46] Costa LG, Vitalone A, Cole TB, Furlong CE. Modulation of paraoxonase (PON1) activity. Biochem Pharmacol 2005;69:541–50.
[47] Briseno-Roa L, Hill J, Notman S, Sellers D, Smith AP, Timperley CM, et al. Analogues with fluorescent leaving groups for screening and selection of enzymes that efficiently hydrolyze organophosphorus nerve agents. J Med Chem 2006;49:246–55.
[48] Worek F, Diepold C, Eyer P. Dimethylphosphoryl-inhibited human cholinesterases: inhibition, reactivation, and aging kinetics. Arch Toxicol 1999;73:7–14.
[49] Leader H, Vincze A, Manisterski B, Rothschild N, Dosoretz C, Ashani Y. Characterization of *O*,*O*-diethylphosphoryl oximes as inhibitors of cholinesterases and substrates of phosphotriesterases. Biochem Pharmacol 1999;58:503–15.
[50] Smolen A, Eckerson HW, Gan KN, Hailat N, La Du BN. Characteristics of the genetically determined allozymic forms of human serum paraoxonase/arylesterase. Drug Metab Dispos 1991;12:107–12.
[51] Furlong CE, Richter RJ, Seidel SL, Costa LG, Motulsky AG. Spectrophotometric assays for the enzymatic hydrolysis of the active metabolites of chlorpyrifos and parathion by plasma paraoxonase/arylesterase. Anal Biochem 1989;180:242–7.
[52] Debord J, Dantoine T, Bollinger J-C, Abraham MH, Verneuil B, Merle L. Inhibition of arylesterase by aliphatic alcohols. Chem Biol Interact 1998;113:105–15.
[53] Furlong CE, Li W-F, Shih DM, Lusis AJ, Richter RJ, Costa LG. Genetic factors in susceptibility: serum PON1 variation between individuals and species. Hum Ecol Risk Assess 2002;8:31–43.
[54] Draganov DI, La Du BN. Pharmacogenetics of paraoxonases: a brief review. Naunyn-Schmiedeberg's Arch Pharmacol 2004;369:78–88.
[55] Khersonsky O, Tawfik DS. Structure–reactivity studies of serum paraoxonase PON1 suggest that its native activity is lactonase. Biochemistry 2005;44:6371–82.
[56] Aharoni A, Gaidukov L, Khersonsky O, McQ Gould S, Roodveldt C, Tawfik DS. The 'evolvability' of promiscuous protein functions. Nat Genet 2005;37:73–6.
[57] Harel M, Aharoni A, Gaidukov L, Brumshtein B, Khersonsky O, Meged R, et al. Structure and evolution of the serum paraoxonase family of detoxifying and anti-atherosclerotic enzymes. Nat Struct Mol Biol 2004;11:412–9.
[58] Hassett C, Richter RJ, Humbert R, Chapline C, Crabb JW, Omiecinski CJ, et al. Characterization of cDNA clones encoding rabbit and human serum paraoxonase: the mature protein retains its signal sequence. Biochemistry 1991;30:10141–9.
[59] Reddy ST, Wadleigh DJ, Grijalva V, Ng C, Hama S, Gangopadhyay A, et al. Human paraoxonase-3 is an HDL-associated enzyme with biological activity similar to paraoxonase-1 protein but is not regulated by oxidized lipids. Arterioscler Thromb Vasc Biol 2001;21:542–7.
[60] Zech R, Chemnitius JM. PON1 in different species. In: Furlong CE, editor. Paraoxonase (PON1) in health disease. Boston: Kluwer Academic Publishers; 2002. p. 137–63.
[61] Buckley NA, Dawson AH, Whyte IM. Organophosphate poisoning: peripheral vascular resistance—a measure of adequate atropinization. Clin Toxicol 1994;32:61–8.
[62] Lamping KG, Wess J, Cui Y, Nuno DW, Faraci FM. Muscarinic (M) receptors in coronary circulation: gene-targeted mice define the role of M2 and M3 receptors in response to acetylcholine. Arterioscler Thromb Vasc Biol 2004;24:1253–8.
[63] Brophy VH, Jarvik GP, Furlong CE. PON1 polymorphism. In: Furlong CE, editor. Paraoxonase (PON1) in health and disease: basic and clinical aspects. Boston: Kluwer Academic Publications; 2003. p. 52–77.
[64] Worek F, Bäcker M, Thiermann H, Szinicz L, Mast U, Klimmek R, et al. Reappraisal of indications and limitations of oxime therapy in organophosphate poisoning. Hum Exp Toxicol 1997;16:466–72.
[65] Ordentlich A, Barak D, Kronman C, Ariel N, Segall Y, Velan B, et al. The architecture of human acetylcholinesterase active center probed by interactions with selected organophosphate inhibitors. J Biol Chem 1996;271:11953–62.
[66] Main AR, Iverson F. Measurement of the affinity and phosphorylation constants governing irreversible inhibition of cholinesterases by di-isopropyl phosphorofluoridate. Biochem J 1966;100:525–31.