Supporting Information

Reversed Enantioselectivity of Diisopropyl Fluorophosphatase Against Organophosphorus Nerve Agents by Rational Design

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Contents

1. Experimental procedures

1.1 Expression and purification of DFPase

The expression and purification of His-tagged wild-type (WT) DFPase and mutants followed the procedure of Hartleib and Rüterjans (Ref. S1). The protein was expressed using the pKKHisND plasmid based on the *E. coli trc*-promotor under the control of the *lac*-repressor. Cells were grown in TB-media containing 100 μg/ml of ampicillin and incubated at 37°C. Cells were induced by addition of IPTG at OD_{600} = 0.7 to a final concentration of 1 mM. At the same time another batch of ampicillin (100 μg/ml) was added and the temperature lowered to 30°C. After overnight incubation (12-16 h) the cells were harvested by centrifugation (30 min, 4°C, 12000 g) and the cell pellet stored at -20°C.

DFPase was purified by Ni-NTA affinity chromatography and Q-Sepharose anion-exchange chromatography. The frozen cell pellet was thawed on ice and resuspended in 30 ml MCAC1 buffer (10 mM Tris, 2 mM $CaCl_2$, 500 mM NaCl, 5 mM Imidazole, pH 7.5). After addition of 15 mg Pefabloc proteinase inhibitor the cells were disrupted using sonication (Branson W-250D sonifier, 70% output power, 0.7s pulses, 5 min, max. 25°C) and cell debris removed by centrifugation (30 min, 4°C, 40000 g). The supernatant was applied to an equilibrated metal chelate affinity column (Ni-NTA Superflow,15 ml column volume, flow rate 2 ml/min). The column was washed with 7% MCAC2 buffer (10 mM Tris, 2 mM $CaCl_2$, 500 mM NaCl, 300 mM Imidazole, pH 7.5), and the target protein eluted with 100% MCAC2 buffer. The protein solution was dialyzed overnight against 5 l IEC-A buffer (10 mM Tris, 2 mM $CaCl_2$, pH 7.5) at 4°C. The N-terminal polyhistidine tag was cleaved by thrombin digestion (3-5 U per mg of DFPase, pH 8.4) for 6 h. Afterwards thrombin was deactivated by lowering the pH to 7.3, the solution was centrifuged (10 min, 4°C, 10000 g), and reapplied to an equilibrated Ni-NTA column. The flow-through was collected and dialyzed overnight using the same buffer and conditions as above. Finally the protein solution was applied to an equilibrated Q-Sepharose ion exchange column (Q-Sepharose High Performance, 15 ml column volume, flow rate 2 ml/min)

using buffer IEC-A. DFPase was eluted by application of a linear gradient from 0 to 500 mM NaCl using buffer IEC-B (10 mM Tris, 2 mM $CaCl_2$, 1 M NaCl, pH 7.5) with the protein eluting from the column at approx. 50 mM NaCl. The purified DFPase was concentrated and buffer was exchanged to IEC-A using a Vivaspin concentrator (10 kDa cutoff).

1.2 Site-directed mutagenesis of the DFPase gene

Site-directed mutagenesis was carried out using the Quik-Change II Site-Directed Mutagenesis Kit (Stratagene) employing PfuUltra high-fidelity DNA polymerase and the oligonucleotide primers required for the introduction of a specific mutation. The cycling parameters consisted of an initial heating phase to 95°C for 30 s followed by 16 cycles consisting of heating to 95°C for 30 s followed by a 1 min annealing phase at 55°C and a 10 min extension phase at 68°C. The PCR product was checked by electrophoresis on a 1% agarose gel. Finally the reaction mix was digested for 1 h at 37°C using the DpnI restriction enzyme. The mutants were verified by sequencing the plasmids.

1.3 Measurement the stereoselective degradation of nerve agents by DFPase

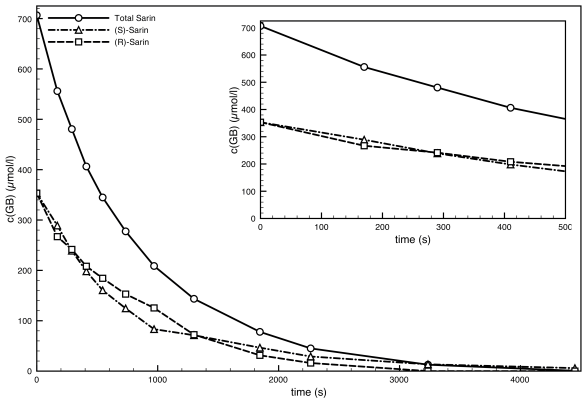
To determine the hydrolysis rates of the two enantiomers of sarin and Cyclosarin and those stereoisomers of Soman with a different configuration on the phosphorus atom (that is mainly responsible for the difference in toxicity) a combination of two methods was employed. The overall hydrolysis of the substrate was determined by a titrimetric method. The hydrolysis of the substrates leads to the release of two protons per substrate molecule, monitored by a pH-stat assay employing a Methrom 799 GPT Titrino automatic titrator, which adds NaOH solution to keep the pH of the reaction solution constant at pH 7.0. The second method is based on the different ability of the nerve agent stereoisomers to inhibit acetylcholinesterase (AChE). For the substrates Sarin, Soman and Cylosarin differences in the configuration on the phosphorus atom renders one form nearly non-toxic with the other form almost completely responsible for the observed toxicity (Ref. S6). AChE inhibition was determined using human hemoglobin-free erythrocyte ghosts prepared according to the method of Worek *et al.* (Ref. S7) as

the AChE source. Inhibition was measured spectrophotometrically using a modified Ellman assay.

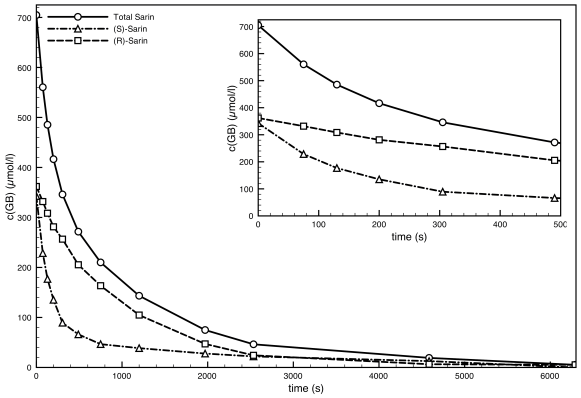
The reaction solution contained 50 µl of substrate stock solution in dry acetonitrile (ca. 2%) in a total volume of 3 ml of NaCl solution (0.1 M) adjusted to a pH of 7.0. The amount of enzyme added was adjusted to assure complete degradation of the substrate in 5000 s to 10000 s. Samples were taken at intervals and quenched by addition of 50 mM sodium formate buffer (pH 3.5) before the samples were stored on ice. The amount of formate buffer was adjusted so that the right concentration range for the AChE inhibition assay was maintained. 50 µl of the solution containing acetylthiocholine (ATCh) (28.4 mM) and 10 µl of the sample were added to a cuvette containing 3000 µl 0.1 M phosphate buffer (pH 7.4) and 100 µl DTNB solution (4 mM in 0.1 M phosphate buffer). The reaction was started by adding 10 µl erythrocyte ghosts solution. The final volume was 3170 µl. ATCh hydrolysis was continuously monitored over a 30 min period. The concentration of the toxic OP stereoisomer(s) was determined by the double-reciprocal method of Hart and O'Brien (Ref. S8) yielding a relationship between measured slopes and substrate concentrations used. The calibration is shown for Cyclosarin as an example in S2.5. To demonstrate that both the titrimetric and the biological assay yield similar results the reaction was carried out using achiral DFP. As can be seen in S2.4 both methods yield curves that are superimposable.

2. Supplementary Results

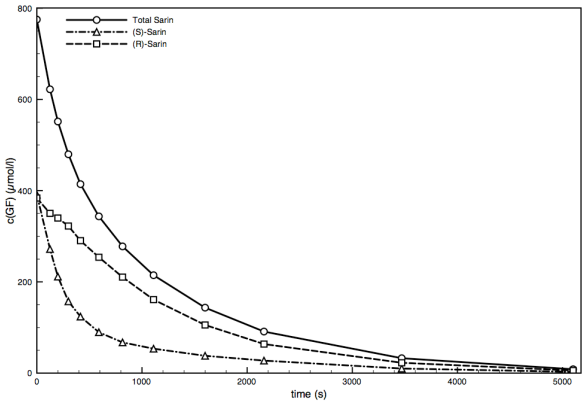
2.1 Hydrolysis of Sarin by DFPase



DFPase WT



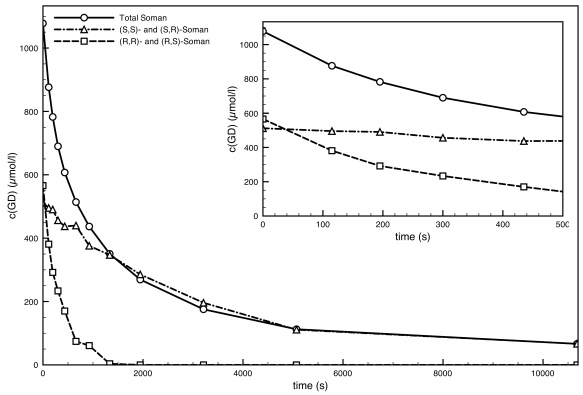
Mut1



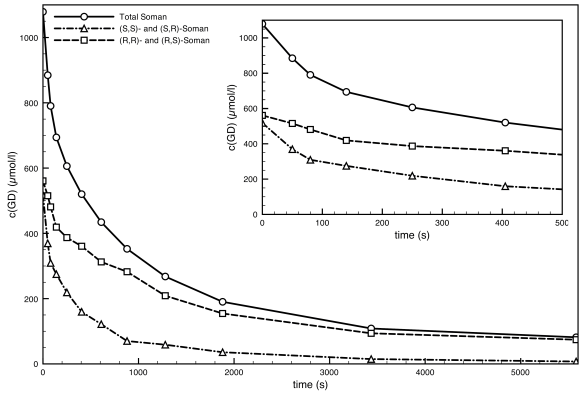
Mut2

Hydrolysis of enantiomers of Sarin (GB) by WT DFPase, Mut1 and Mut2. WT DFPase shows virtually no enantioselectivity with GB as a substrate while Mut1 and Mut2 show a preference for the toxic enantiomer (*S*)-GB.

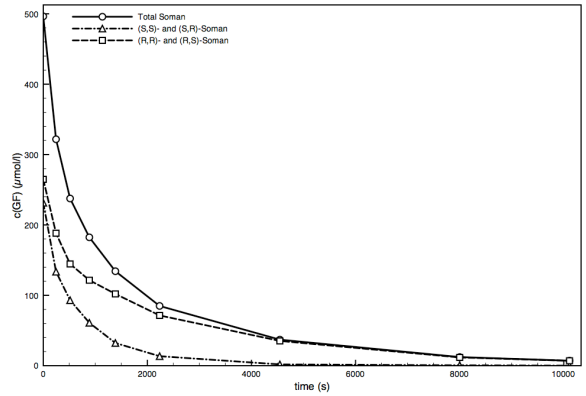
2.2 Hydrolysis of Soman by DFPase



DFPase WT



Mut1

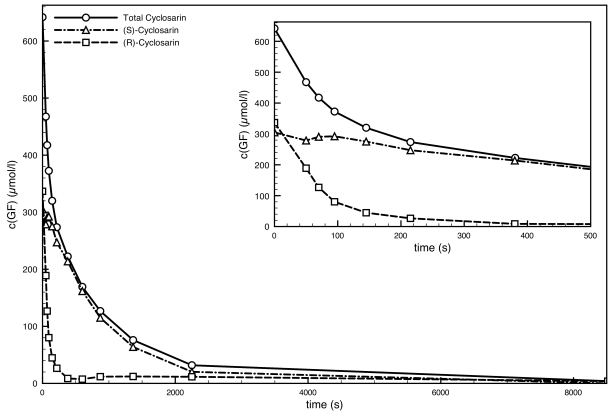


Mut2

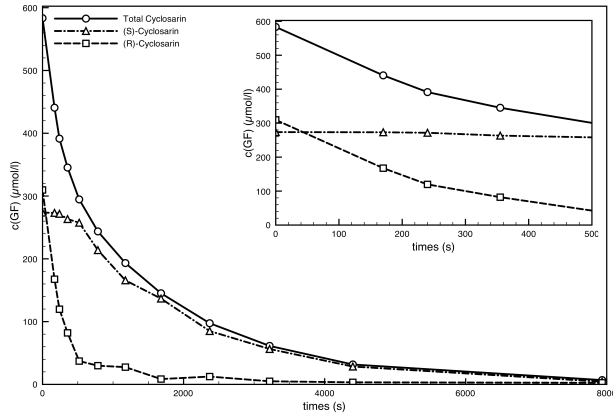
Hydrolysis of stereoisomers of Soman (GD) by WT DFPase, Mut1 and Mut2. The toxicity of GD depends almost exclusively on the configuration at the phosphorus atom. (S_PS_C)-GD and (S_PR_C)-GD are toxic while (R_PR_C)-GD and (R_PS_C)-GD are non-toxic. WT DFPase shows a preference for the two non-toxic stereoisomers of GD while Mut1 and Mut2 hydrolyze the toxic stereoisomers faster than the non-toxic ones.

2.3 Hydrolysis of Cyclosarin by mutants E37A, Y144A/R146A and T195M

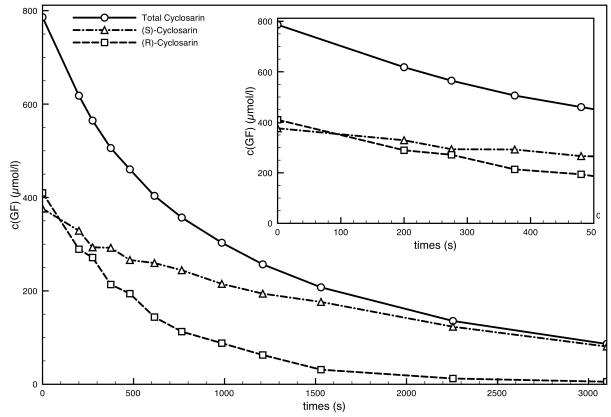
E37A:



Y144A/R146A:

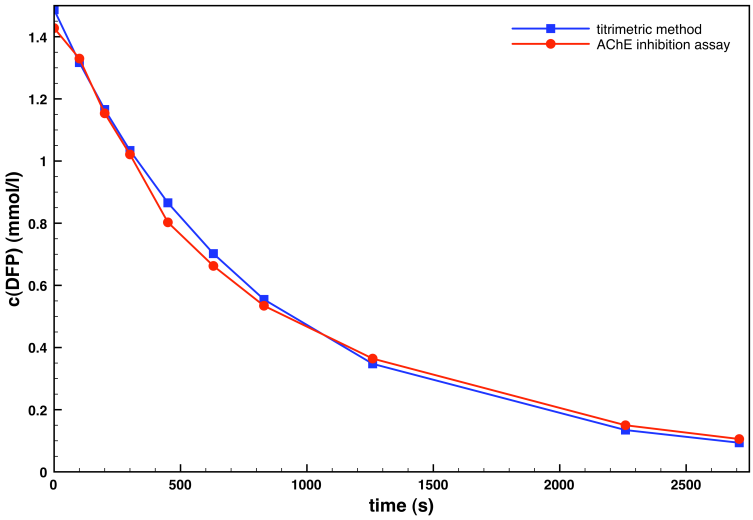


T195M:



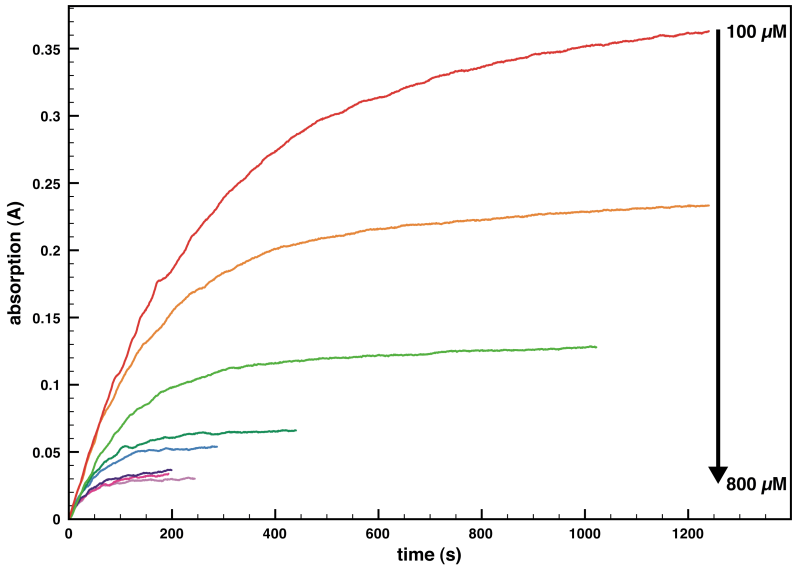
Both E37A and Y144A/R146A are similar in enantioselectivity to the wild type. Mutant T195M exhibits a relaxed selectivity, although the enantioselectivity is not reversed.

2.4 Comparison of titrimetric and inhibition assays for monitoring DFP hydrolysis

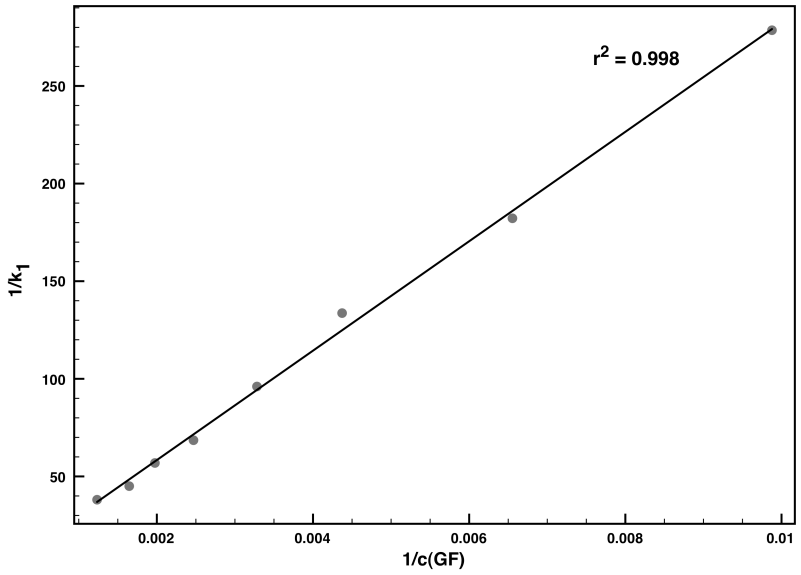


Results obtained with the titrimetric pH-stat method and with the biological AChE inhibition assay yield identical results with the achiral substrate.

2.5 Calibration of the AChE inhibition assay with WT DFPase for cyclosarin



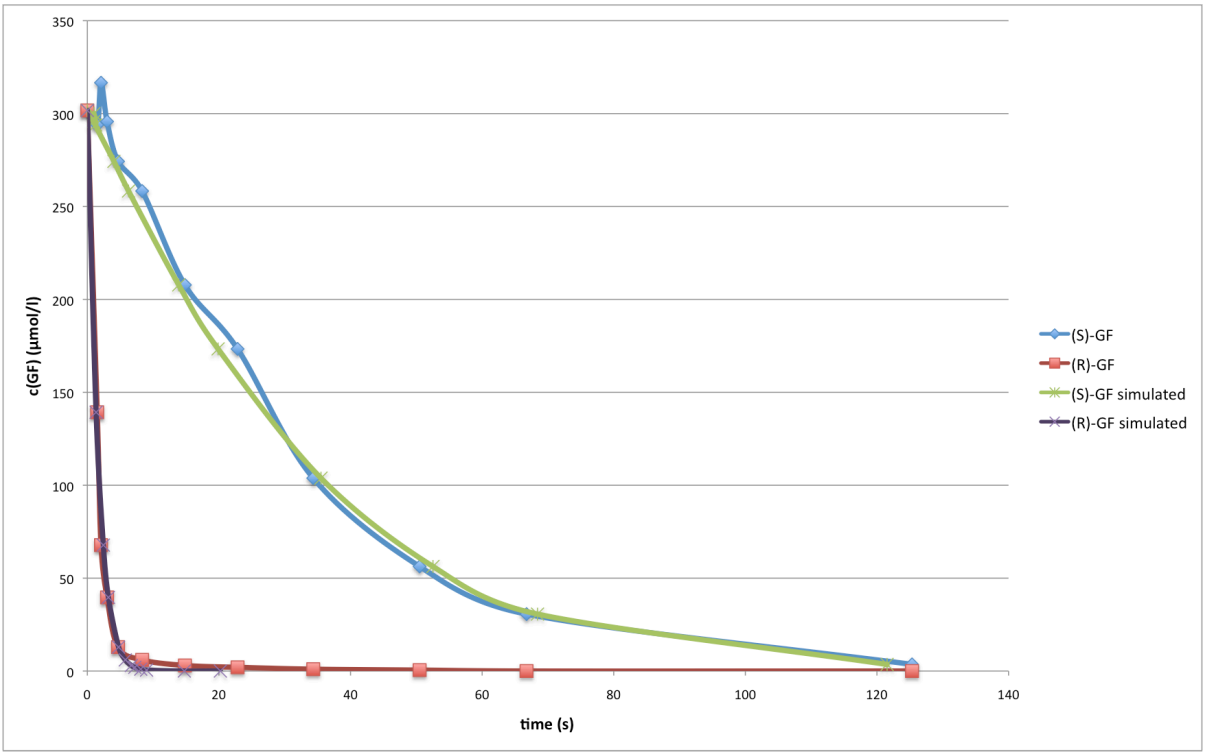
Turnover of acetylthiocholine is monitored over time using an Ellman assay at different concentrations of cyclosarin (GF). The recorded curves were analyzed by nonlinear regression using GraphPadPrism 4.0 assuming a one phase exponential decay to determine the first-order rate constant k_1 (min^{-1}).



The concentration of the toxic OP stereoisomer(s) is determined by plotting $1/k_1$ against 1/c(GF) yielding a linear relationship.

2.6 Comparison of real and simulated data (GF with WT DFPase)

Using the mathematical model published by Yeung *et al.* (S10) in a version for two instead of four different stereoisomers present in the reaction mix and fitting for k_{cat}/K_M instead of individual k_{cat} and K_M values one can obtain simulated reaction curves. Fitting is carried out in MATHEMATICA and the simulated curves are in very good agreement with the experimental data. As an example the experimental and the theoretical reaction curves for WT DFPase with the substrate GF are shown below.



3. Abbreviations

AChE	acetylcholinesterase
ATCh	acetylthiocholine
DFP	diisopropyl fluorophosphate
DTNB	5,5′-dithiobis(2-nitrobenzoic acid)
GB	Sarin
GD	Soman
GF	Cyclosarin
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
Ni-NTA	Ni^{2+}-nitrilotriacetate
TB	terrific broth
TRIS	tris(hydroxymethyl)aminomethane
WT	wild type

4. References

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(S10) D.T. Yeung, J.R. Smith, R.E. Sweeney, D.E. Lenz, D.M. Cerasoli, *FEBS J.* **2007**, 274, 1183-1191.