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Title: Squid DFPase and PON1 â€“ Twins or just siblings? Comparison of structure and mechanism of two similar phosphotriesterases

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Blum, Marc-Michael

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Squid DFPase and PON1 – Twins or just siblings?

Comparison of structure and mechanism of two similar phosphotriesterases

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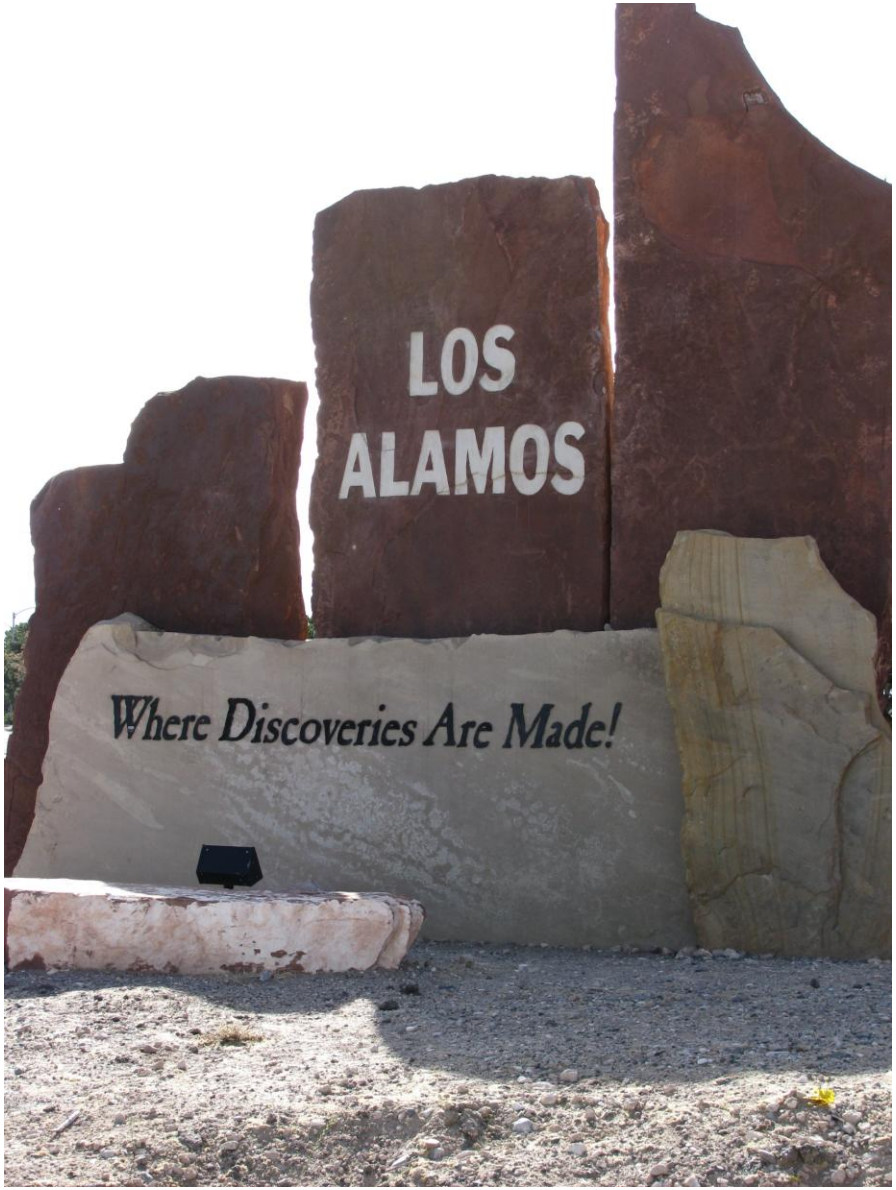
LA-UR-xx-xxxxx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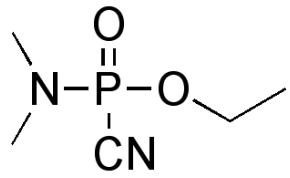
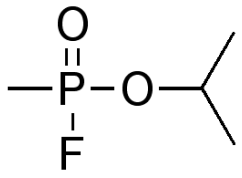
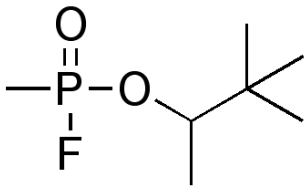
“Classic” Organophosphorus nerve agents



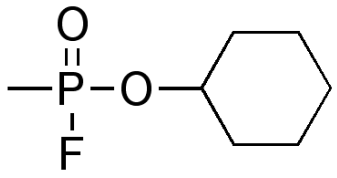
Tabun (GA)



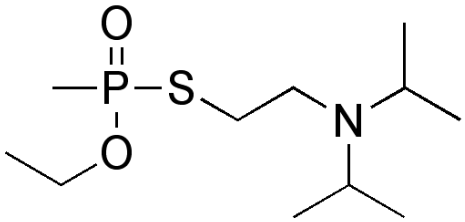
Sarin (GB)



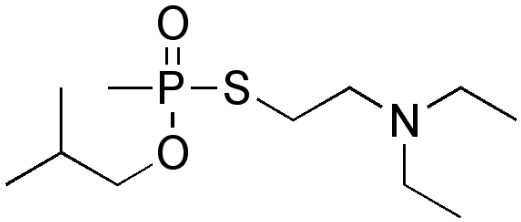
Soman (GD)



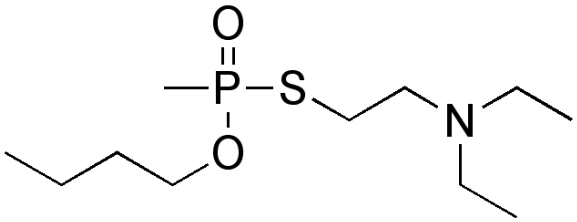
Cyclosarin (GF)



VX

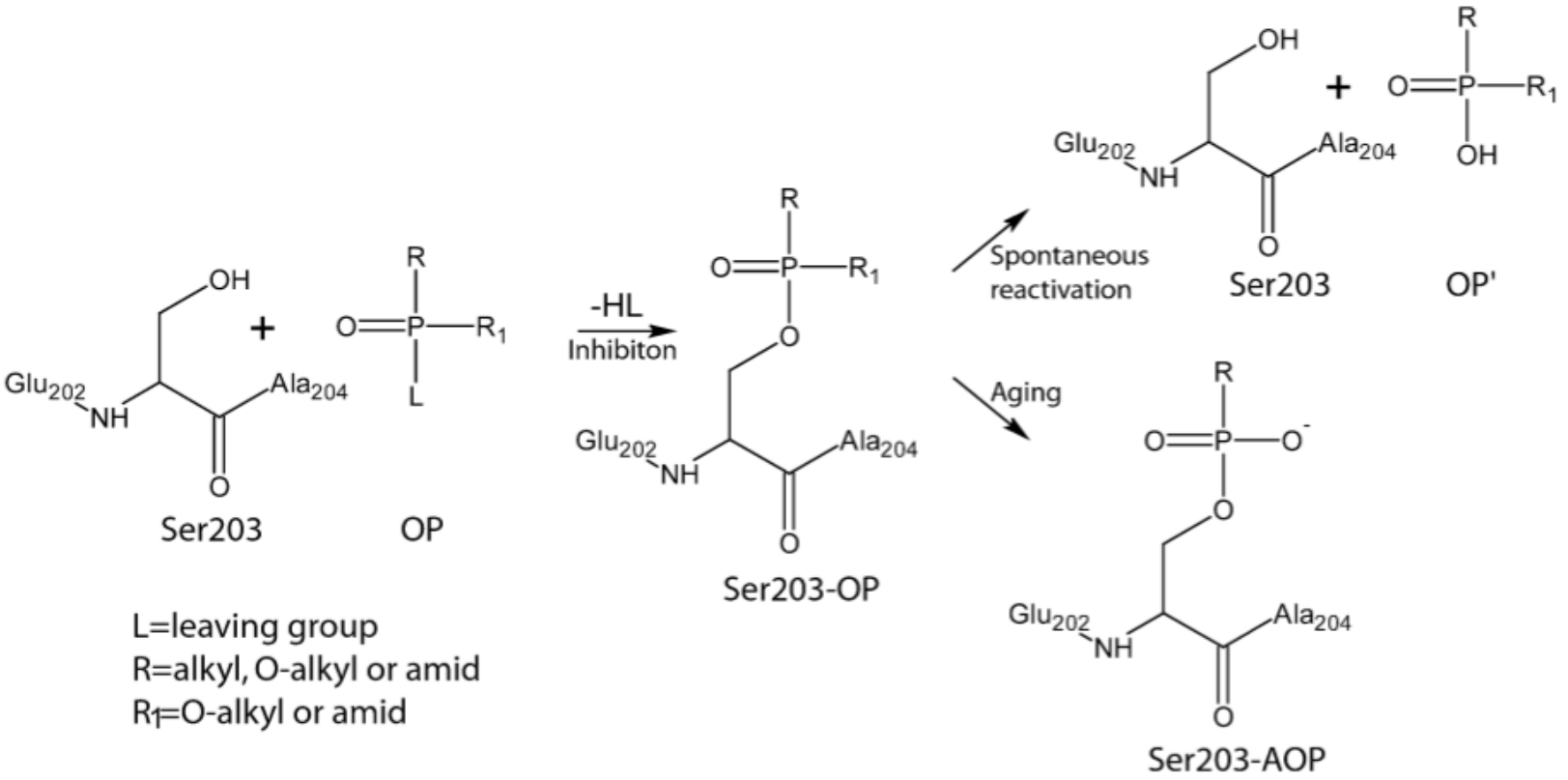


R-VX



C-VX

Nerve agents mode of action



L=leaving group
R=alkyl, O-alkyl or amid
R_1=O-alkyl or amid

Problems of currently fielded countermeasures

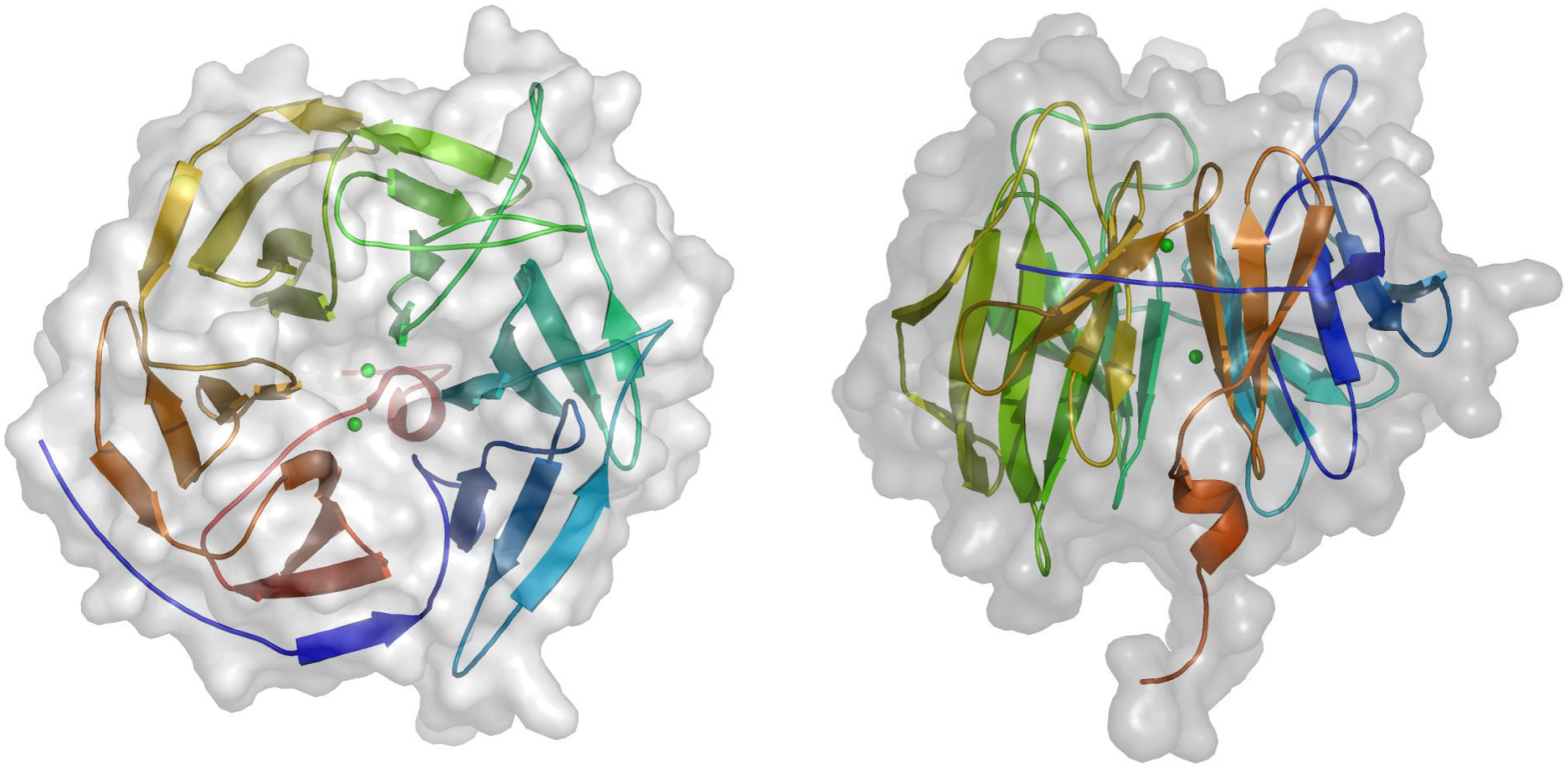
- **Decon**
  - Decon solutions like DS2 are highly corrosive, hazardous and flammable and incompatible with modern sensitive equipment like advanced optics or electronics
  - They pose a high logistical burden in the field
  - Difficult to use in operational theaters with high numbers of unprotected civilian populations
- **Medical**
  - Current treatment regimes based on atropine, oximes (2-PAM in the US) and potentially anticonvulsants
  - Oximes reactivate inhibited AChE but can not reactivate “aged” AChE
  - Some agents lead to rapid aging (e.g. Soman inhibited AChE ages with a half-life of about 2 min)
  - Oxime treatment might be required over several days due to body depots

Enzymes can be a solution to the problem

DFPase from *Loligo vulgaris*

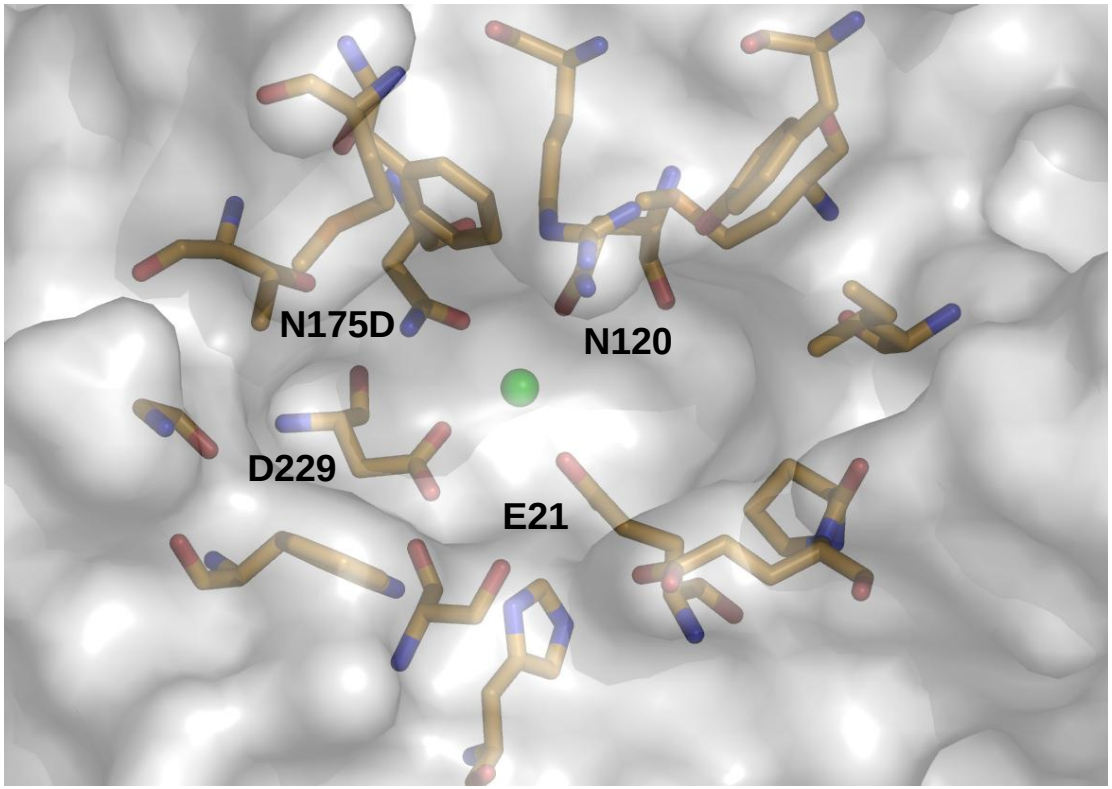
- 314 amino acids, 35kDa, 2 Calcium Ions (one required for catalytic activity and one for structural integrity)
- Six-fold β-propeller
- WT active against DFP and G-type nerve agents (GA, GB, GD, GF)
- WT is inactive against V-type nerve agents
- WT is selective for the less toxic stereoisomers of the G-agents
- A wealth of structural data is available including a 0.85 Å X-ray structure and a neutron structure as well as X-ray structures of a number of mutants.
- A reaction mechanism was proposed in 2006 based and structural and kinetic data as well as isotope labeling experiments.

DFPase from *Loligo vulgaris*

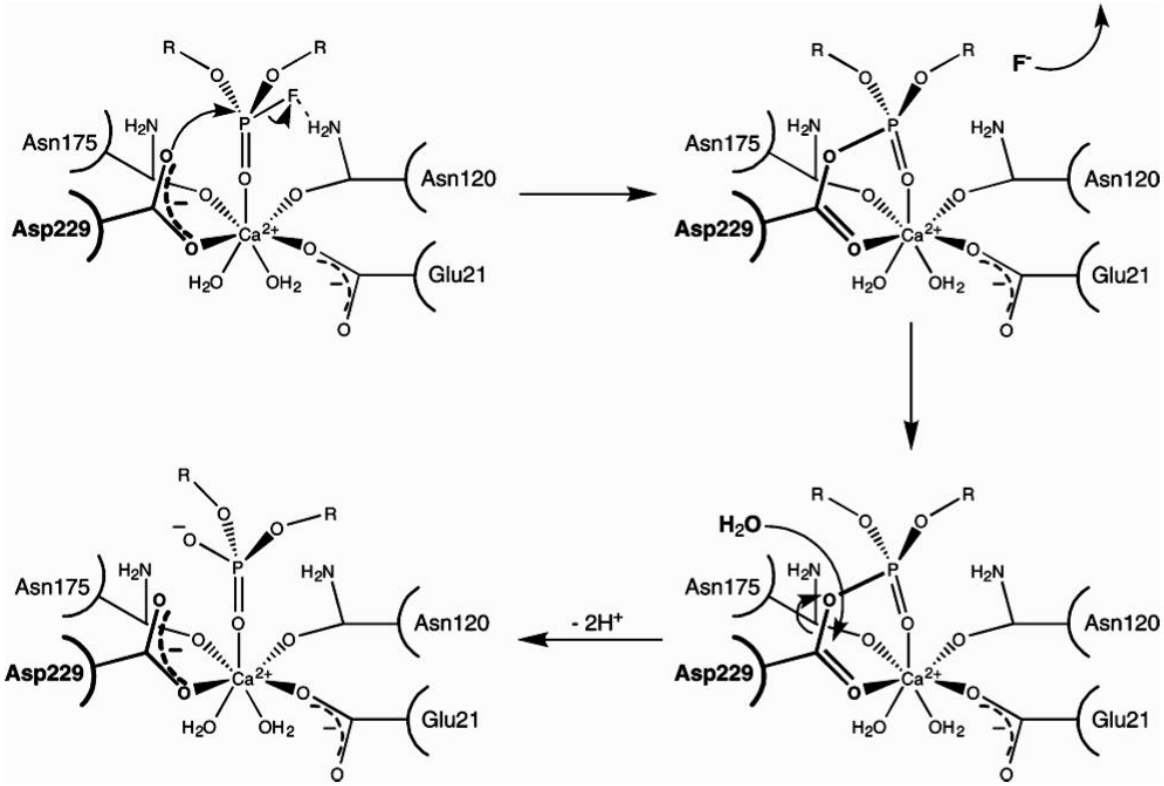


DFPase from *Loligo vulgaris*

- **Active site is an elongated cleft with the the catalytic calcium ion centered on the bottom of binding pocket.**



Reaction mechanism



Blum et al. JACS 2006, 128, 12750

Why this mechanism?

- **Old DFPase mechanism proposed water activation by H287 but the residue can be mutated with retaining activity**
- **Crystal structure of DFPase in complex with a phosphoroamidate inhibitor showes coordination of the phosphoryl oxygen to the calcium ion. A similar coordination is observed in PON1 with inorganic phosphate. Activation of the substrate by coordination to the calcium is now generally accepted as being part of DFPase and PONs reaction mechanism.**
- **Direct water activation by the metal is ruled out because DFPase with Mg instead of Ca in the active site is fully active but the max. coordination numer of Mg is restricted to 6, making it impossible to coordinate substrate and a water molecule for activation at the same time**

Why this mechanism?

- **Mutating D229 completely inactivates DFPase. Double mutants like D229N/N120D that keep the charge balance intact are also inactive.**
- **There might be a significant difference to PON1 as mutating D269 in PON seems to retain some activity (as reported by the Magliery lab). Previous studies with huPON1 also showed complete inactivation upon mutagenesis (Josse et al., Biochemistry, 1999)**
- **Docking studies in agreement with the observed enantioselectivity of DFPase show a substrate orientation with D229 positioned for an in-line attack on the substrate phosphorus. This reaction step was shown to be feasible by DFT calculations and is similar to other enzymes where a phosphoaspartate is formed with inorganic phosphate. D229 is deprotonated as required for activity (shown by neutron diffraction).**
- **^{18}O labeling experiment under single and multiple turnover conditions reveal that ^{16}O is transferred from an enzyme residue to the product.**

Why this mechanism?

- **^{18}O labeling**

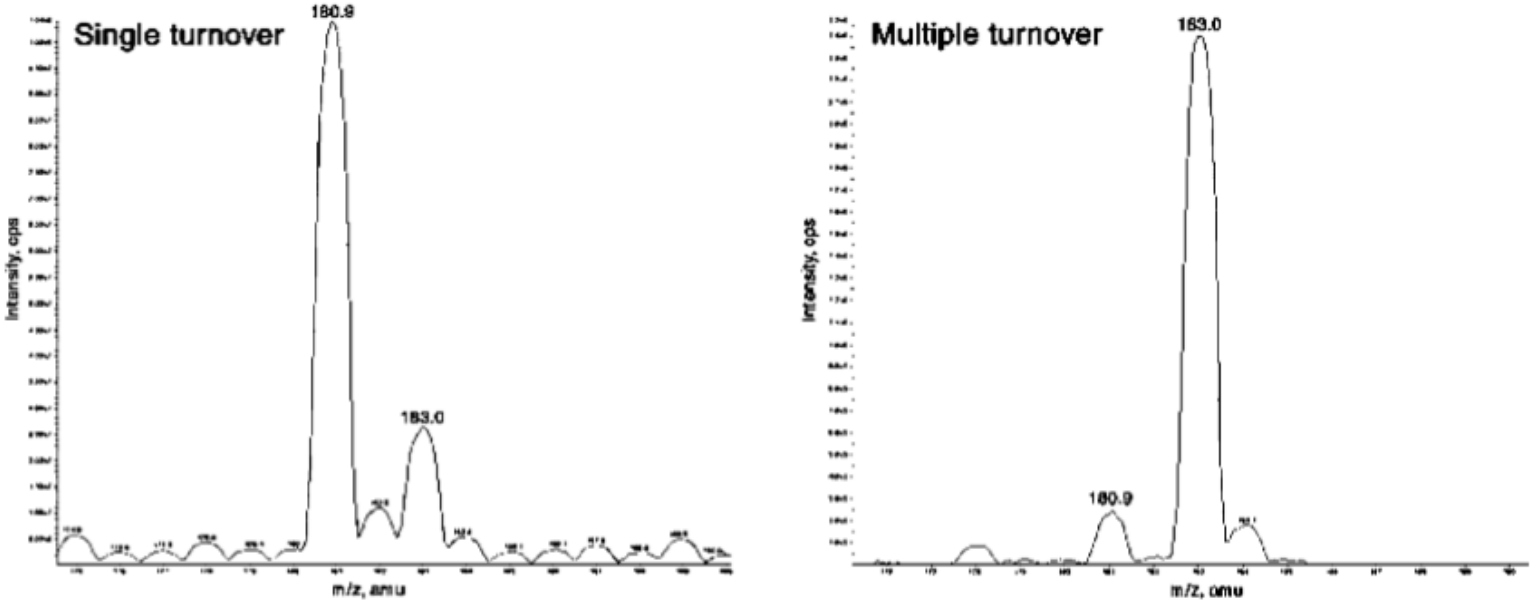
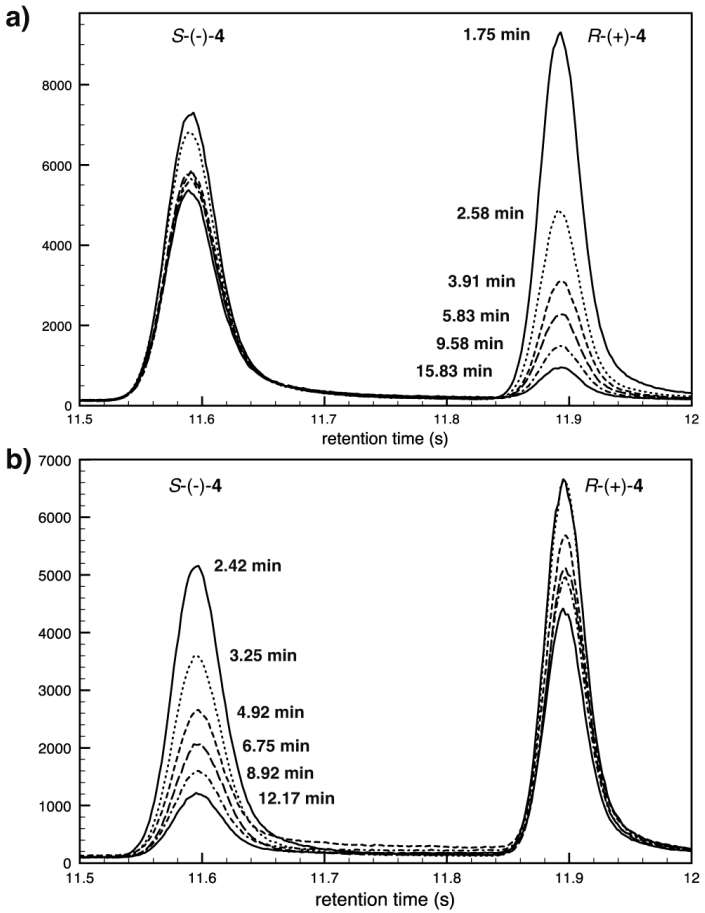


Figure 3. Ion spray mass spectra of diisopropylphosphate produced with DFPase in $H_2{}^{18}O$. The spectra were obtained between 175 and 195 atomic mass units. A single turnover cycle of DFPase yields ^{16}O-diisopropylphosphate $M_r = 181$ amu (left) while multiple turnovers result in ^{18}O incorporation $M_r = 183$ amu (right).

Blum et al. JACS 2006, 128, 12750

Why this mechanism?

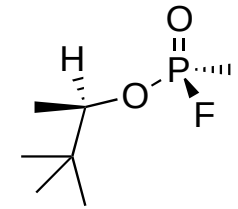
- **Based on the proposed mechanism enzyme variants with reserved enantioselectivity and to accommodate substrates with larger leaving groups were successfully created**
- **Mutant E37A/Y144A/R146A/T195M displayed higher activity and a preference for the more toxic stereoisomers of GB, GD and GF.**



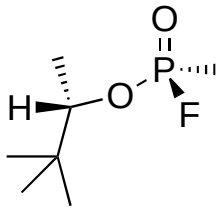
Melzer et al. JACS 2009, 131, 17226

Stereoselectivity is important for *in-vivo* applications

- **Selectivity for the more toxic stereosiomers of the nerve agents is important in applications where rapid reduction in toxicity is required such as for topical or *in-vivo* use (bioscavengers).**
- **Soman (GD) is a prime example**



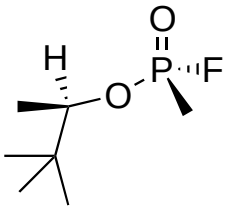
(R_PR_C)-Soman



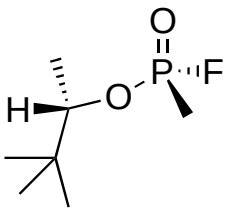
(R_PS_C)-Soman

Non-Toxic

Rate constant of inhibition of AChE: < 5 x 10^3 M^{-1} min^{-1}



(S_PR_C)-Soman



(S_PS_C)-Soman

Toxic

Rate constant of inhibition of AChE: 2 x 10^8 M^{-1} min^{-1}

Shown in a small animal study

Research article

Drug Testing and Analysis

Received: 16 July 2011 Revised: 19 August 2011 Accepted: 22 August 2011 Published online in Wiley Online Library: 15 December 2011

(wileyonlinelibrary.com) DOI 10.1002/dta.363

In vitro and *in vivo* efficacy of PEGylated diisopropyl fluorophosphatase (DFPase)

Marco Melzer,[a,b] Anne Heidenreich,[a] Frederic Dorandeu,[c,d] Jürgen Gäb,[a,e] Kai Kehe,[f,g] Horst Thiermann,[f] Thomas Letzel[h] and Marc-Michael Blum[a,i]*

Drug Test. Analysis **2012**, *4*, 262–270

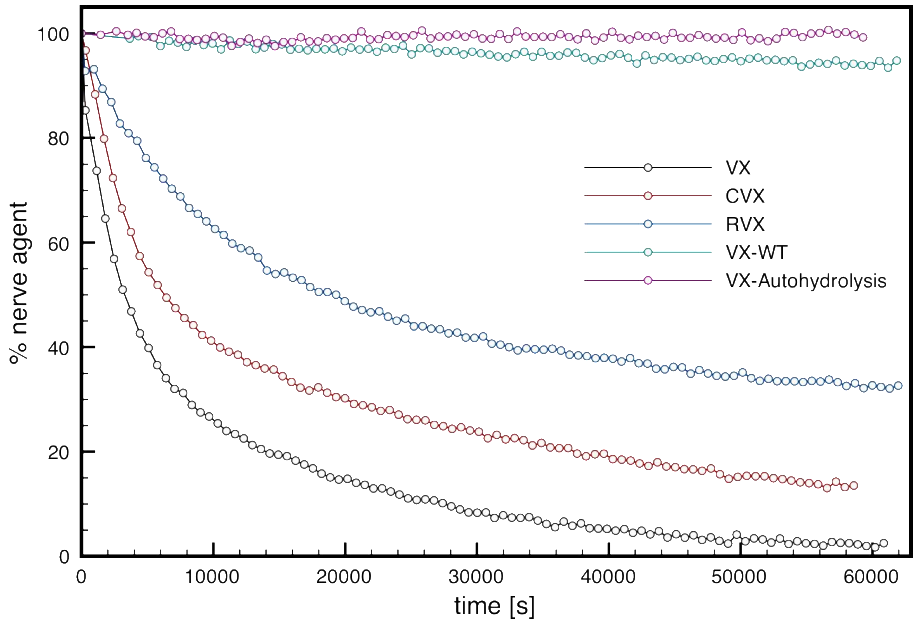
- **Using PEGylated DFPase**
- **Showing protection against 3x LD_{50} of GD in rats (s.c.)**
- **Required enzyme dose still too large**
- **Proof of principle study**

V-agents are problematic

- Have low vapor pressure (persistent agents)
- Hydrolyze slowly in the environment
- Hydrolysis partly results in a highly toxic reaction product
- Are more toxic than G-agents (Lethal dose for VX ca. 10mg)
- Penetrate easily through skin (contact hazard)
- Show complex degradation products, some of them toxic
- Show limited solubility in aqueous solutions
- Can build up depots in body fat / lipophilic body tissues leading to delayed release (problematic medical treatment)

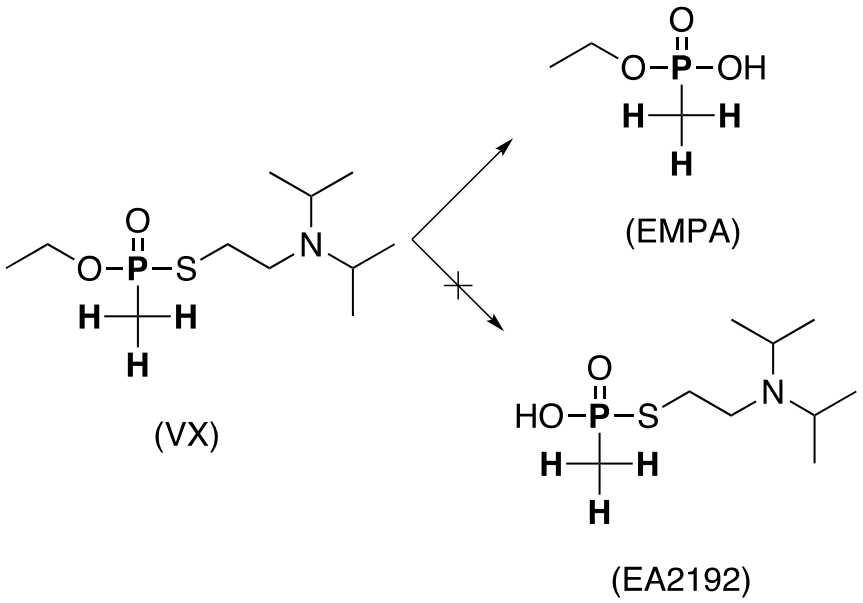
Activity against V-type nerve agents

- **Mutant E37A/Y144A/R146A/T195M displays activity against VX, RVX and CVX.**
- **But activity is rather low with a k_{cat}/K_M in the 10^2 M^{-1} min^{-1} range.**
- **New mutants with further enhances activity (ca. 10 fold)**



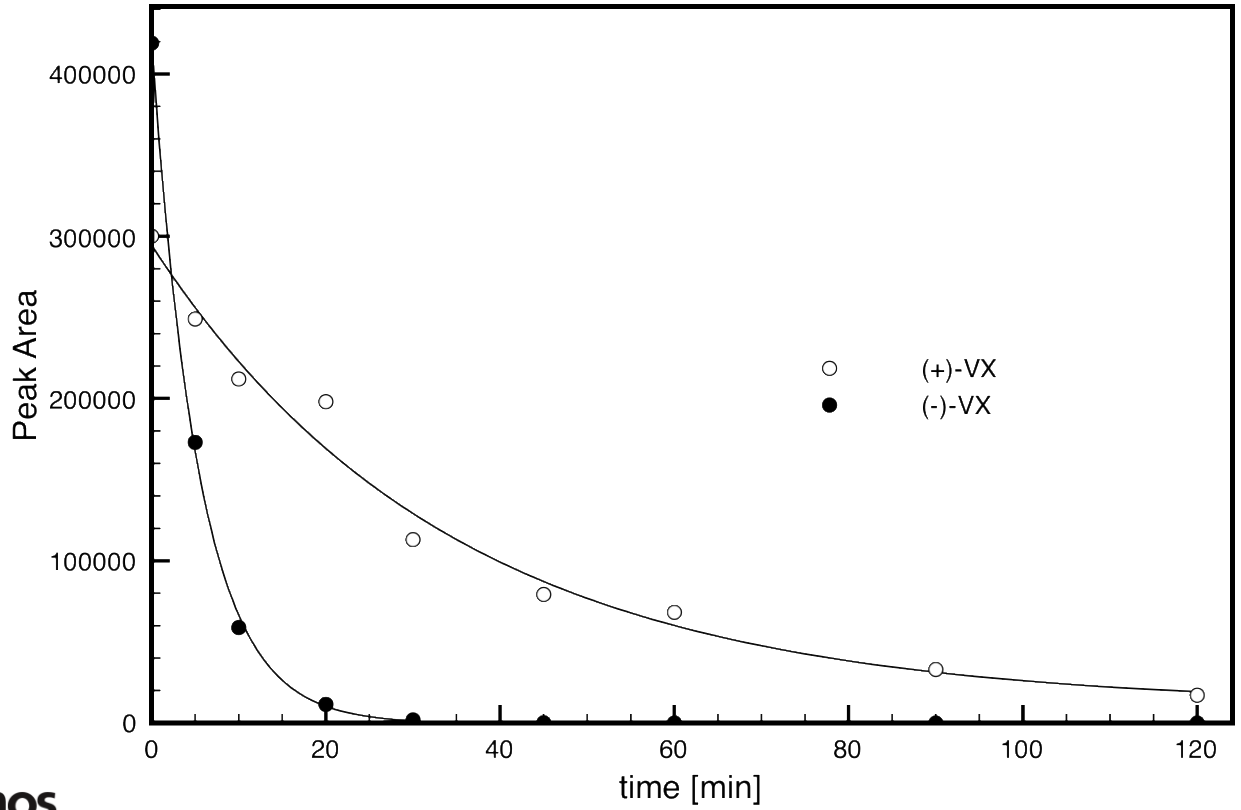
No EA2192 is formed

- During the degradation of VX no toxic EA2192 was formed.
- Reaction was monitored by 1D ^{1}H-^{31}P HSQC NMR spectroscopy employing the phosphorus bound methyl group protons as a probe.
- Only signals for the reaction product EMPA were detected.



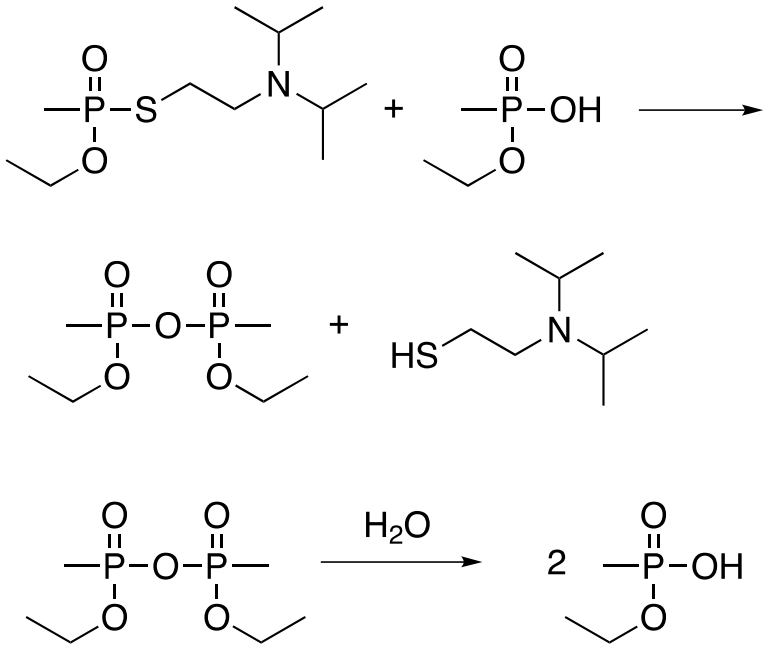
Enantioselectivity with VX

- **The enzyme mutants maintains is preference for the more toxic enantiomer also with VX (determined by ESI-LC-MS employing VX separation on a chiral HPLC column)**



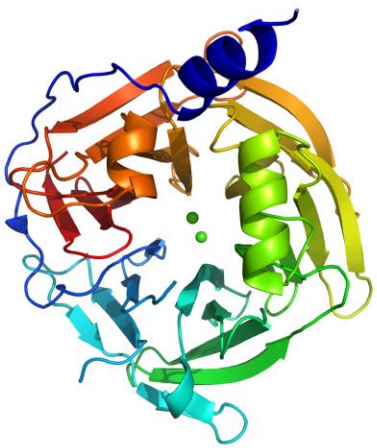
VX-Pyro and why it matters

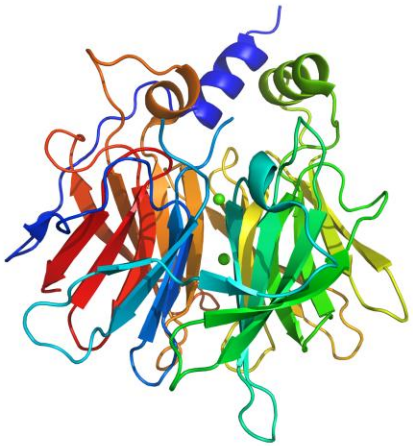
- ***O,O*-Diethyl dimethylpyrophosphonat (VX-pyro) is a byproduct of VX and is formed as a degradation product when VX is exposed to ammounts of moisture that are insufficient for total hydrolysis.**
- **The compound is toxic and can reform VX in a reaction with VX-thiol in appropriate matrices.**
- **VX-pyro was found in an older VX sample in concentrations of ca. 5-8% (v/v).**
- **The DFPase variant that acted on VX showed rapid degradation of VX-pyro.**



Similarities of DFPase and PON1

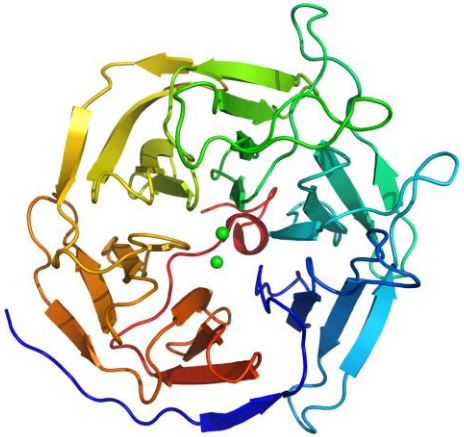
- Similar β-propeller structure

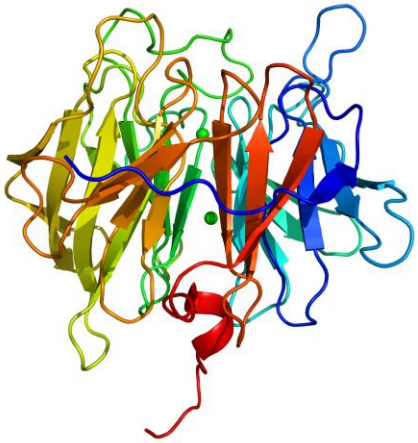




PON1

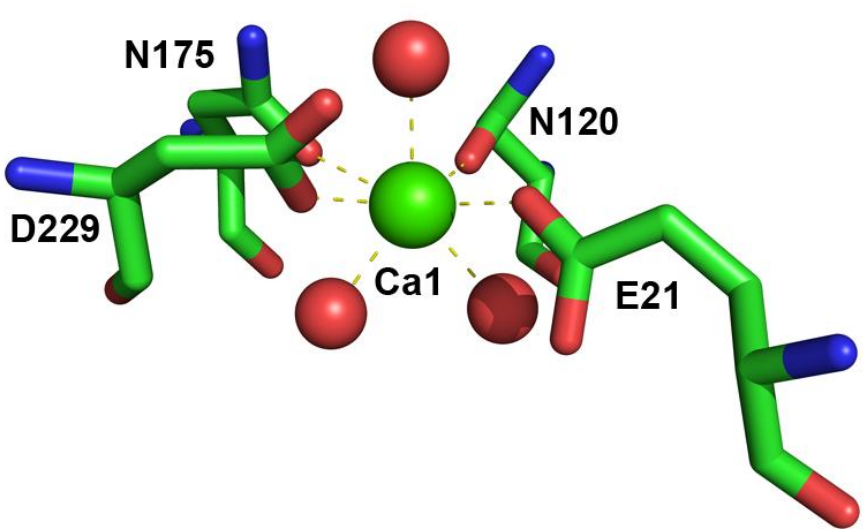
DFPase

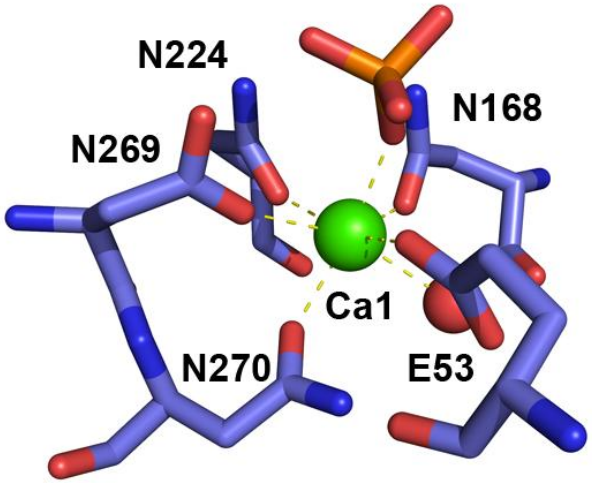




Similarities of DFPase and PON1

- Similar catalytic metal binding site
- Same preference for calcium
- Both enzymes are phosphotriesterases





But...

- PON1 is a lactonase/esterase
- (Glucono)lactonase activity is present in DFPase but is VERY low
- Active site topology is significantly different
- DFPase active site is more open and solvent exposed and also more symetrically shaped (with the calcium ion in the center of the binding pocket)
- Lack of extra helices in DFPase that are found in PON1
- No large flexible loops in DFPase that interact with substrates

Siblings or twins?

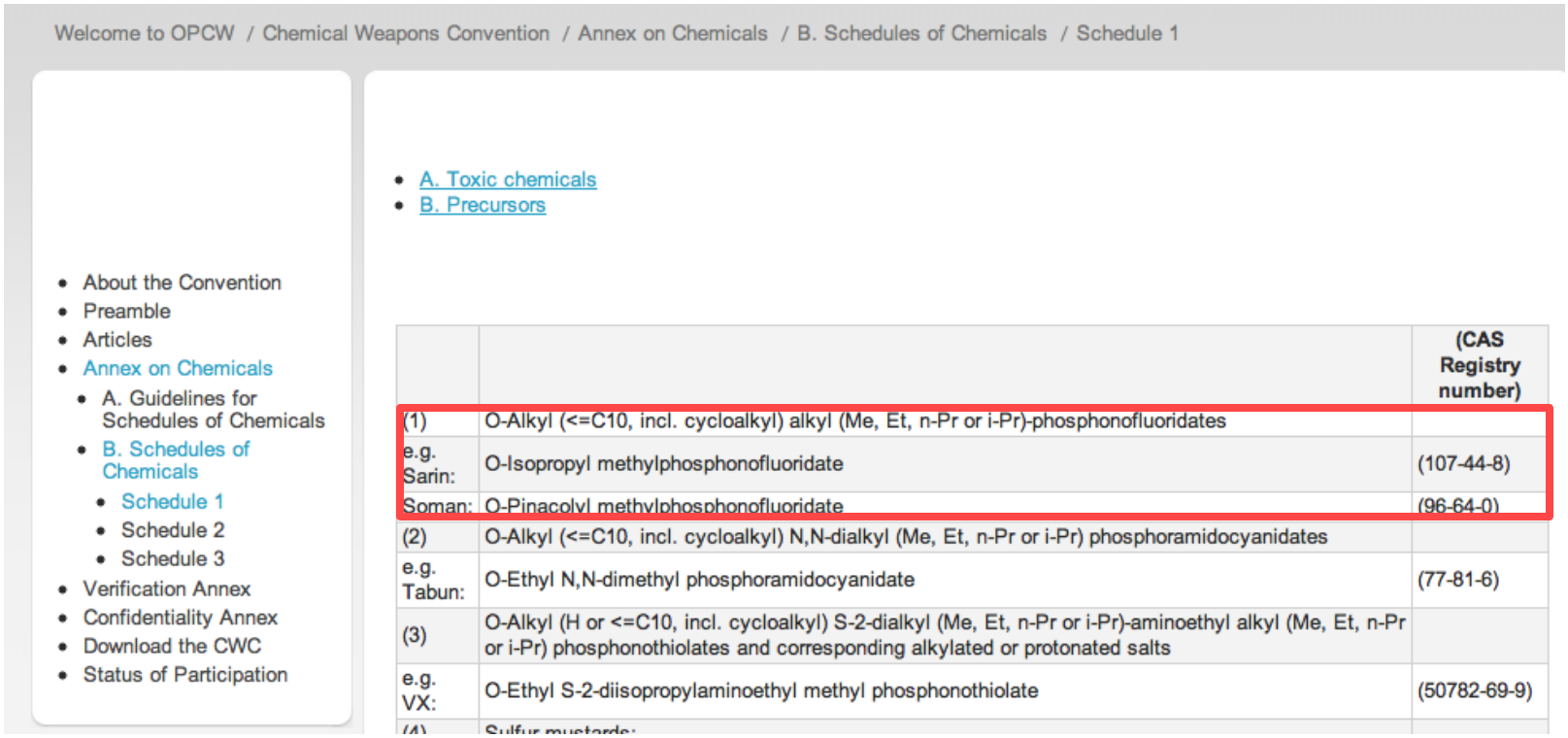
- **Even though a common phosphotriesterase mechanism for DFPase and PON1 appears likely due to the similar metal coordination sites it cannot be ruled out that (slightly?) different mechanisms or even mixed mechanisms exists because of significant differences that exist between the two enzymes.**
- **Therefore there is currently no answer to the question whether the two enzymes are real twins or just distant siblings.**
- **Further work to find supporting evidence for reaction mechanisms (or to effectively rule out options) remains crucial and should be pursued further.**
- **Alternative and complementary methods in structural biology such as neutron diffraction or NMR spectroscopy in additon to kinetic experiments and isotope labeling can help to gain a deeper understanding of the relationship betwwen DFPase and PON1.**

Specificity of enzymes – Advantage and disadvantage

- **While decontamination with chemical formulations like DS2 allows to deal with the whole spectrum of chemical agents their highly reactive and unspecific nature leads to material destruction and environmental issues.**
- **Enzymes target specific substrates at mild conditions making them environmentally benign but restricting the number of agents that can be detoxified.**
- **Engineering enzymes for higher activity and stereoselectivity against single agents or a small subclass currently takes months if not years.**
- **But even in the class of organophosphorus anticholinesterase agents the structural variety goes way beyond the the small number of nerve agents (G- and V-type agents) and pesticides normally targeted.**

Structural variety of Schedule 1 compounds in the CWC

- **Schedule 1 in the Annex of the CWC gives are list of banned toxic chemicals respresenting a large structural variety.**



Welcome to OPCW / Chemical Weapons Convention / Annex on Chemicals / B. Schedules of Chemicals / Schedule 1

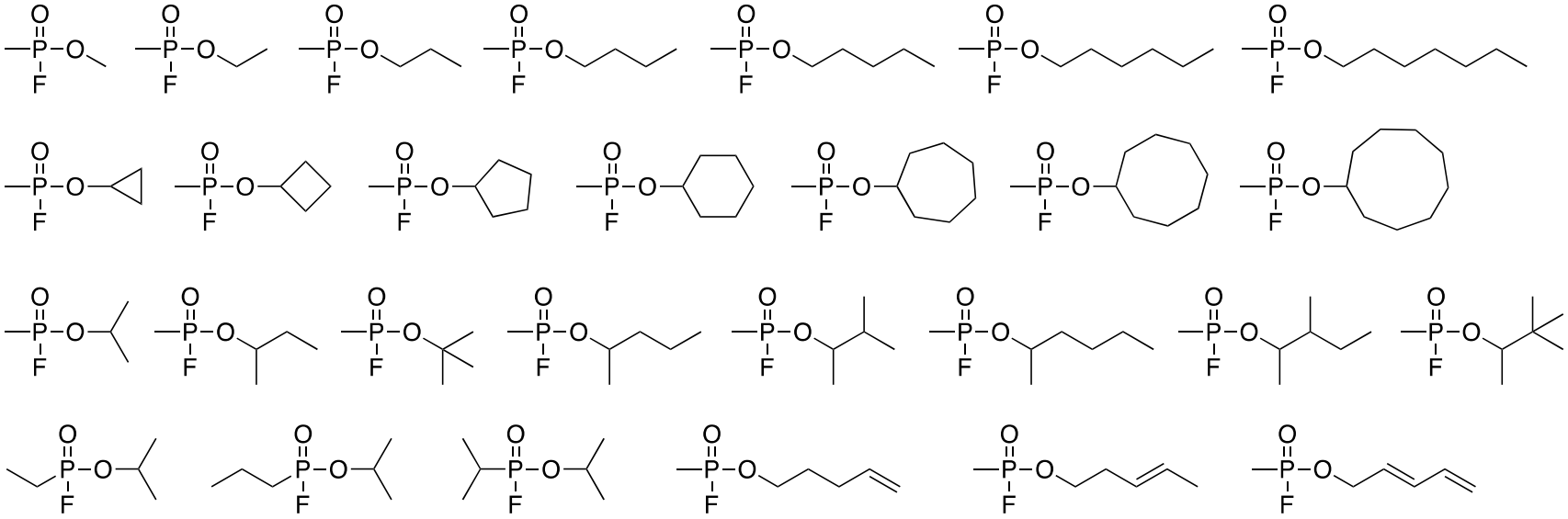
- About the Convention
- Preamble
- Articles
- Annex on Chemicals
  - A. Guidelines for Schedules of Chemicals
  - B. Schedules of Chemicals
    - Schedule 1
    - Schedule 2
    - Schedule 3
- Verification Annex
- Confidentiality Annex
- Download the CWC
- Status of Participation

- A. Toxic chemicals
- B. Precursors

		(CAS Registry number)
(1)	O-Alkyl (<=C10, incl. cycloalkyl) alkyl (Me, Et, n-Pr or i-Pr)-phosphonofluoridates	
e.g. Sarin:	O-Isopropyl methylphosphonofluoridate	(107-44-8)
Soman:	O-Pinacolyl methylphosphonofluoridate	(96-64-0)
(2)	O-Alkyl (<=C10, incl. cycloalkyl) N,N-dialkyl (Me, Et, n-Pr or i-Pr) phosphoramidocyanidates	
e.g. Tabun:	O-Ethyl N,N-dimethyl phosphoramidocyanidate	(77-81-6)
(3)	O-Alkyl (H or <=C10, incl. cycloalkyl) S-2-dialkyl (Me, Et, n-Pr or i-Pr)-aminoethyl alkyl (Me, Et, n-Pr or i-Pr) phosphonothiolates and corresponding alkylated or protonated salts	
e.g. VX:	O-Ethyl S-2-diisopropylaminoethyl methyl phosphonothiolate	(50782-69-9)
(4)	Sulfur mustards:	

Structural variety of Schedule 1 compounds in the CWC

- **Examples for O-Alkyl alkyl phosphonfluoridates listed in Schedule 1**



An engineering challenge

- We must be able to engineer enzymes for existing and emerging threat agents.
- We must be able to either generate libraries of enzyme variants that can cover a wide space in terms of sterical and electronical properties of the agents in advance.
- Or we must be able to rapidly (weeks rather than months) generate such variants that can be produced in bulk rapidly when threats emerge.
- This is hard to achieve by simple point mutagenesis and even for evolutionary approaches.
- Another challenge is the availability of substrates for testing.
- A solution will combine computational approaches im combination with techniques for the rapid generation and testing of enzyme variants.

DFPase as an engineering template

- **Siegel *et al.* used DFPase as a template for a computationally designed Diels-Alderase employing the Rosetta software suite.**

Computational Design of an Enzyme Catalyst for a Stereoselective Bimolecular Diels-Alder Reaction

Justin B. Siegel,[1,2*] Alexandre Zanghellini,[1,2*†] Helena M. Lovick,[3] Gert Kiss,[4] Abigail R. Lambert,[5] Jennifer L. St.Clair,[1] Jasmine L. Gallaher,[1] Donald Hilvert,[6] Michael H. Gelb,[3] Barry L. Stoddard,[5] Kendall N. Houk,[4] Forrest E. Michael,[3] David Baker[1,2,7‡]

The Diels-Alder reaction is a cornerstone in organic synthesis, forming two carbon-carbon bonds and up to four new stereogenic centers in one step. No naturally occurring enzymes have been shown to catalyze bimolecular Diels-Alder reactions. We describe the de novo computational design and experimental characterization of enzymes catalyzing a bimolecular Diels-Alder reaction with high stereoselectivity and substrate specificity. X-ray crystallography confirms that the structure matches the design for the most active of the enzymes, and binding site substitutions reprogram the substrate specificity. Designed stereoselective catalysts for carbon-carbon bond-forming reactions should be broadly useful in synthetic chemistry.

www.sciencemag.org SCIENCE VOL 329 16 JULY 2010 309

DFPase as an engineering template

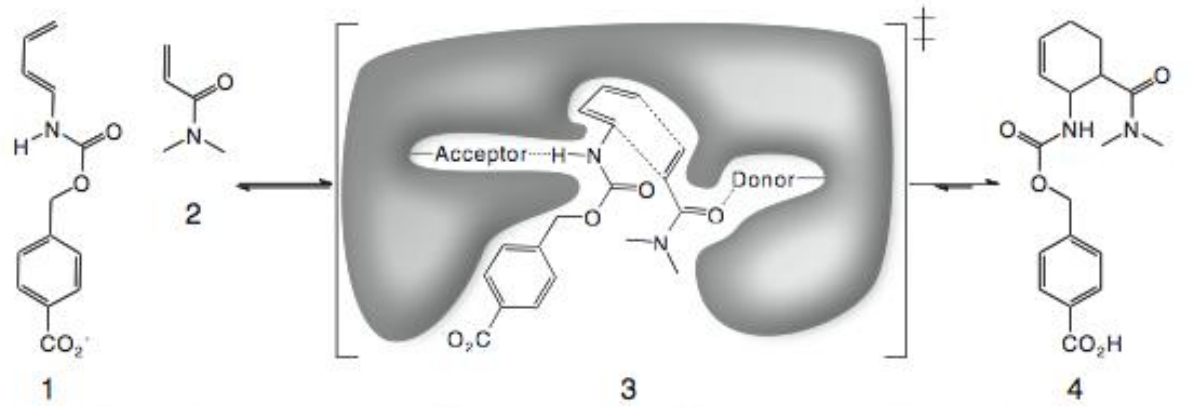
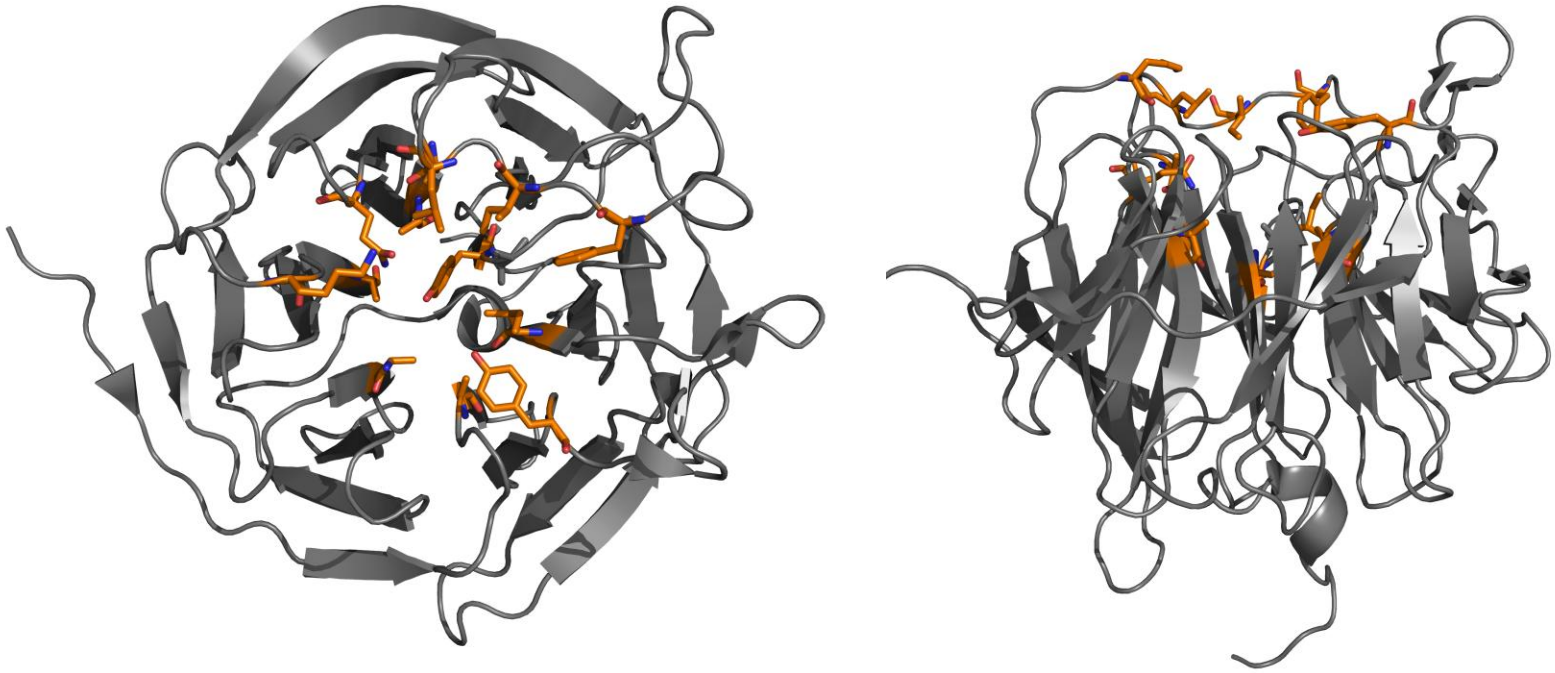


Fig. 1. The Diels-Alder reaction. Diene (1) and dienophile (2) undergo a pericyclic [4 + 2] cycloaddition (3) to form a chiral cyclohexene ring (4). Also shown in (3) is a schematic of the design target active site, with hydrogen bond acceptor and donor groups activating the diene and dienophile and a complementary binding pocket holding the two substrates in an orientation optimal for catalysis.

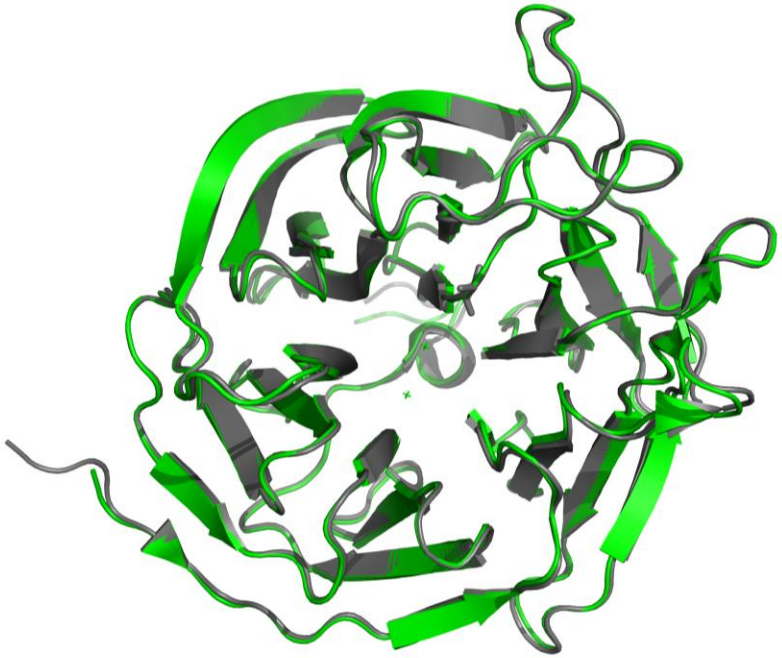
DFPase as an engineering template

- **WT DFPase was used as the template and the designed mutant contained 14 (!) point mutations (shown in orange).**
- **Both metal binding sites of DFPase were removed.**



DFPase as an engineering template

- **Despite the number of mutations and the removal of the metal binding sites, the backbone of the mutant (grey) and of WT DFPase (green) align with a RMSD of 0.36.**
- **The position of the β-sheets is also unaltered.**



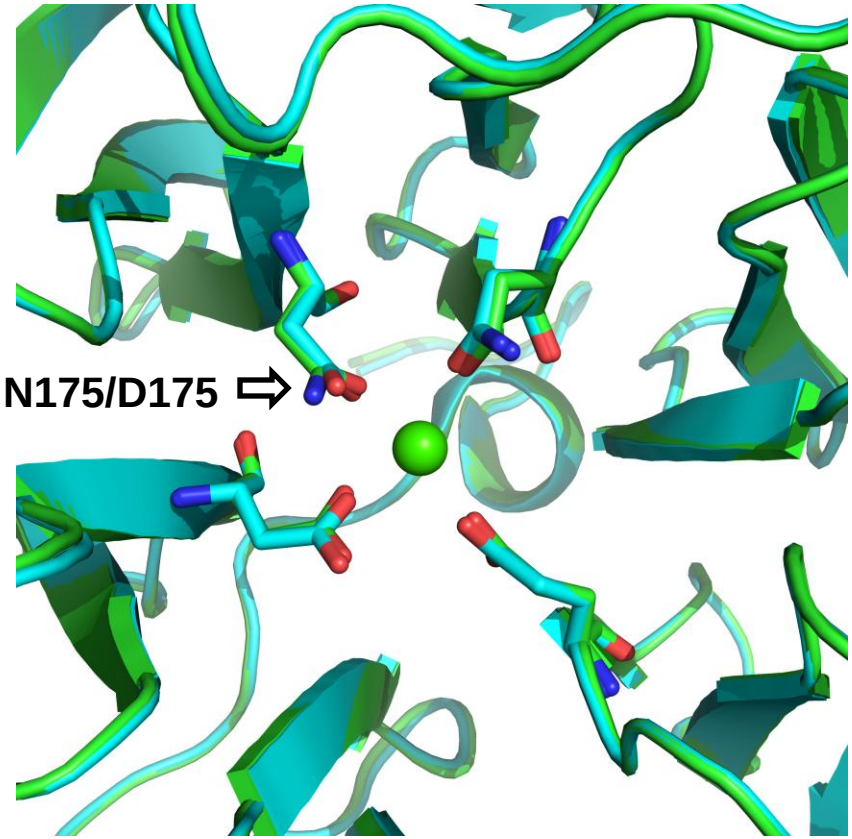
DFPase as an engineering template

- **This demonstrates that the DFPase propeller fold is a highly robust template that tolerates a high number of mutations and even allows complete remodeling of the binding pocket.**
- **Understanding of the enzyme reaction mechanism combined with capabilities of software like Rosetta allows for the re-construction of the enzymes binding pocket potentially allowing to generate a large variety of enzyme variants without significant perturbation of the enzyme fold and backbone. This should lead to maintained beneficial properties such as thermal stability and solvent tolerance.**
- **Therefore we believe that DFPase can serve as a valuable template for the generation of enzyme libraries required to respond to the challenges posed by the structural variety of OP compounds that exists or can emerge as chemical threats.**

Neutron work

- The neutron structure of DFPase revealed that residue D229, which is proposed to act as the nucleophile in the reaction mechanism, is deprotonated as required.
- Mutant N175D only shows 2% of the wildtype activity against DFP
- X-ray structure virtually identical with the WT structure
- Computational modeling and calculations suggest that pK_a of D229 is significantly perturbed by the mutation so it might be protonated.
- This can be shown by a neutron structure of the mutant
- Such a structure might also reveal differences in the orientation of structural water molecules in the active site
- Beam-time for data acquisition at the Protein Crystallography Station (PCS) at the Los Alamos Neutron Science Center (LANSCE) has been allocated for August/September.

Neutron work



- Alignment of the X-ray structures of DFPase WT and mutant N175D
- Structures are virtually identical
- Even the positions of water in the active site remain identical but…
- … orientations of water molecules can not be determined by X-ray diffraction.

Adressing immunogenicity and plasma half-life

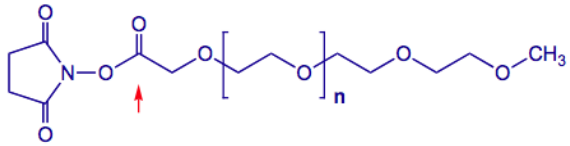
- **In order to be suitable for *in-vivo* use in humans three aspects have to be considered:**
  - Catalytic efficiency (discussed above)
  - Suppressed immunogenicity (as DFPase is of non-human origin)
  - Prolonged plasma half-lives (days to weeks)
- **Immunogenic surface epitopes have to be either removed or masked**
- **Renal clearance has to be minimized**
- **Other clearance pathways (like billary excretion) have to be checked for relevance**

Loss of small proteins from plasma via kidney filtration

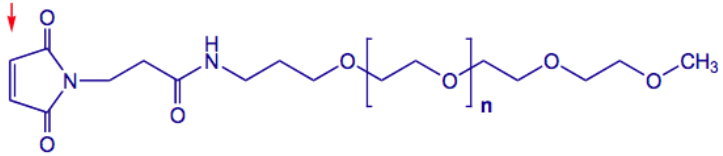
- Plasma proteins with a diameter < 88 A are lost more or less quickly via blood filtration in the kidney glomeruli
- Filtration efficiency increases with decreasing size, down to 30-36 A, below that the sieving coefficient is = 1
- This explains the very short circulation of small proteins and peptides, which are in the range of min to h
- Negative charge hampers filtration due to the anionic basal membrane
- <u>Example:</u> human serum albumin (HSA)
  585 residues, molecular mass 68 kDa, diameter ca. 90x50 A, pI ≈ 5
  extremely small sieving coefficient < 0.001 => plasma half-life: 19 days

PEGylation as a strategy to increase molecular size

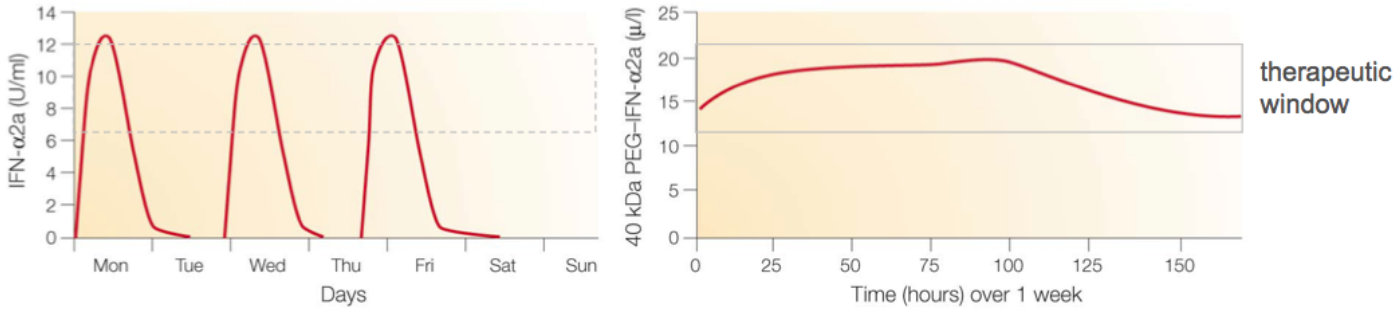
- **Chemical coupling of the recombinant biopharmaceutical protein with synthetic polyethylene glycol (PEG)**
  - Via Lys side chains (randomly, NHS/succinimidyl PEG)



  - Via Cys side chains (site specific, maleimidyl PEG)



Effect of PEGylation on the pharmacokinetic (PK) profile of IFNα2a after s.c. injection into humans:



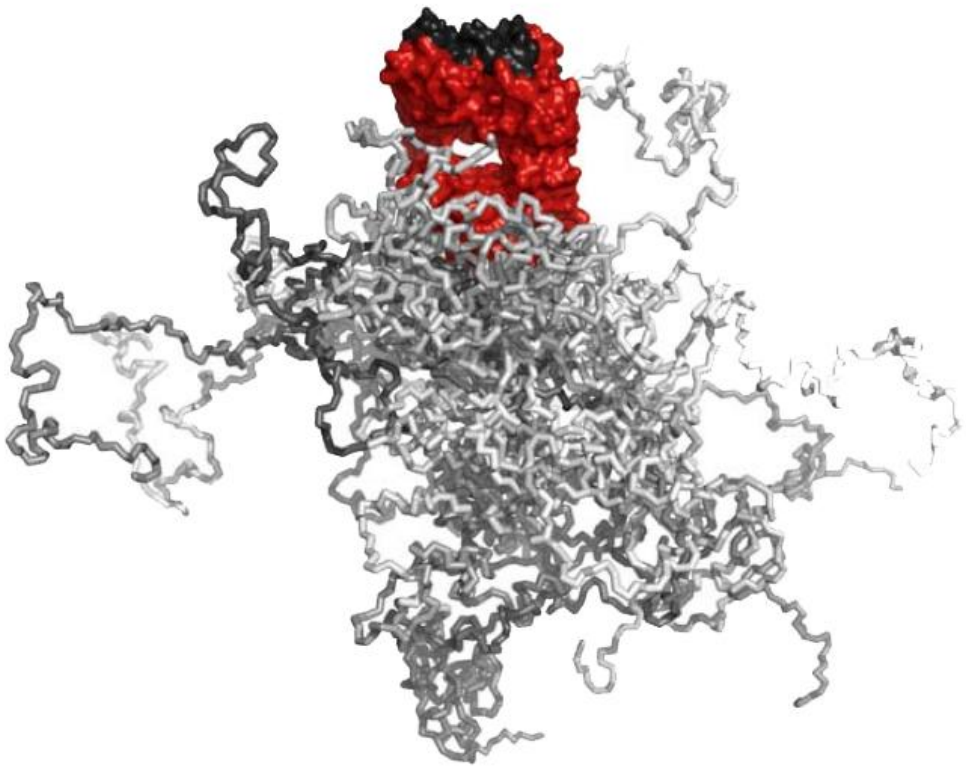
Drawbacks of PEGylation

- Potential negative influence on protein function
- Potential reduction of enzymatic activity
- High cost of clinical grade monodisperse PEG raw materials
- Additonal expensive coupling and purifications steps during GMP production, recution in yield on a molar basis
- Chemical instability due to formation of oxidation products
- Poor bioavailability from concentrated solution due to waxy nature
- PEG can still be immunogenic
- PEG is not biodegradable, can lead to vacuolation of kidney epithelium

Alternatives to PEGylation: 1. PASylation

- **Sequences of the three small amino acids Proline, Alanine, and Serine form a highly soluble biological polymer that can be directly produced as fusion protein with the biopharmaceutical using standard expression systems**
- **Similarly as PEG, PAS sequences adopt a stable random structure with large apparent volume under physiological conditions, which effectively increases the size of the conjugated drug beyond the pore size of the kidney glomeruli and thus slows down filtration**
- **PAS sequences are stable in the blood plasma but can be degraded by kidney enzymes**
- **PAS sequences only marginally influence the biological activity of the drug and they are not immunogenic in animals**
- **BUT: PASylation does not effectively mask immunogenic surface epitopes of the biopharmaceutical**

Alternatives to PEGylation: 1. PASylation

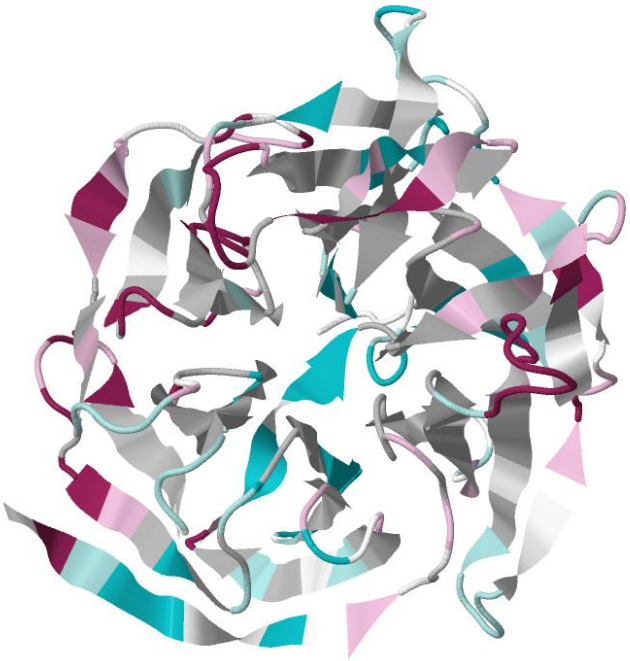


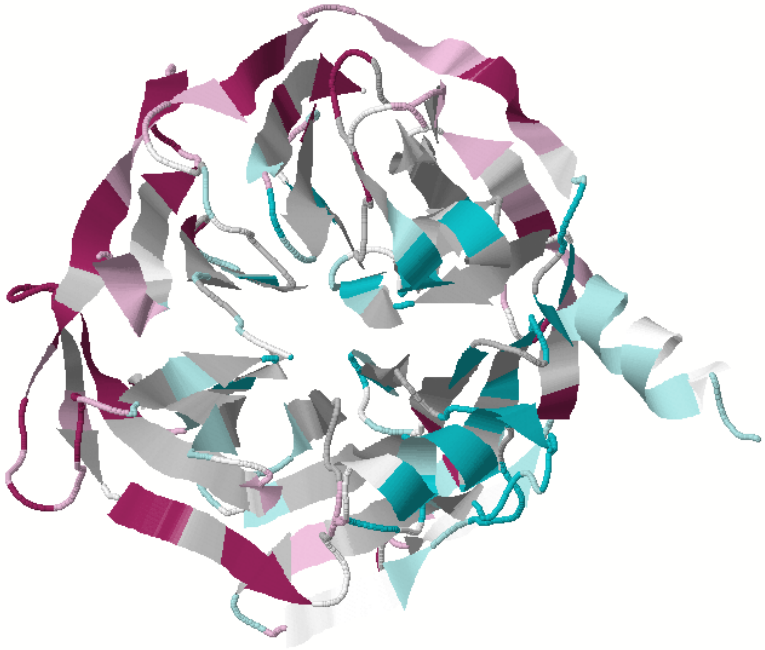
Computer model of a PASylated Fab fragment

Alternatives to PEGylation: 2. Surface engineering

- **While surface epitope engineering does not address the issue of plasma half-life it can be an effective technique to address immunogenicity.**
- **Prediction of B-cell epitopes using Epitopia (epitopia.tau.ac.il)**
- **Mutagenesis of surface residues to eliminate epitopes**
- **Can result in a enzyme variant with significantly reduced immunogenic potential**
- **Potential drawback: Might affect catalytic efficiency**

Alternatives to PEGylation: 2. Surface engineering





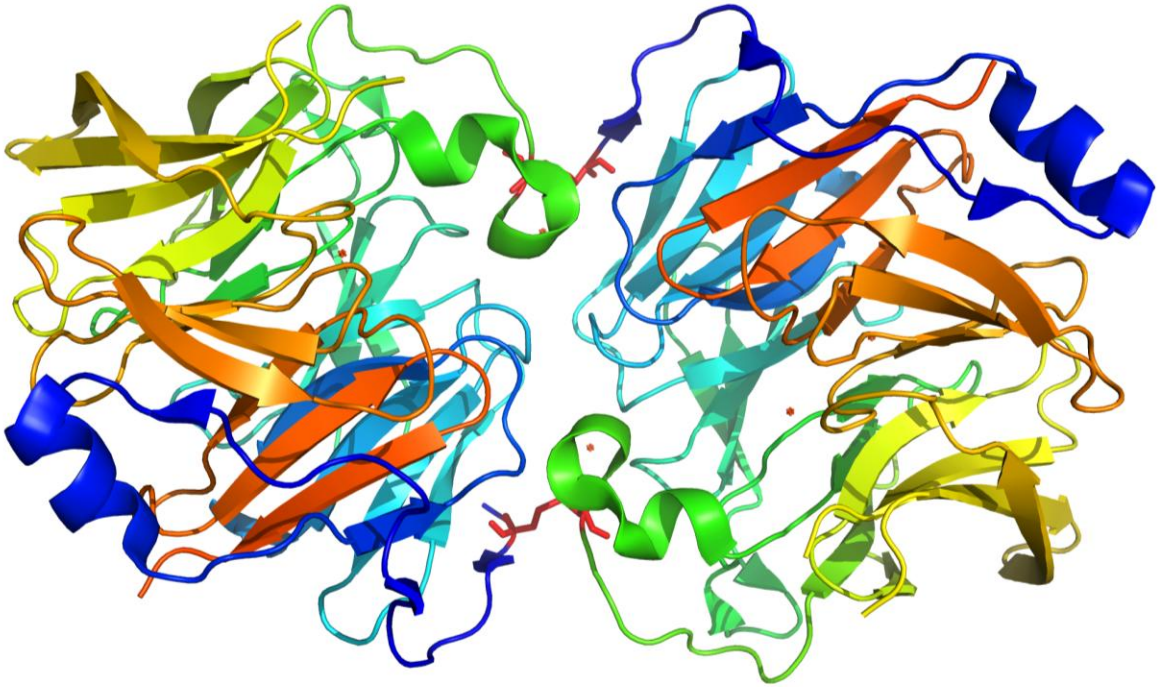
Epitopia results:
DFPase (1E1A)

rePON1 (1V04)

Alternatives to PEGylation: 3. Crosslinking

- **Covalent crosslinking of individual DFPase proteins to form multimeric complexes**
- **Addresses the requirement for enlarging the size of the protein to avoid removal by renal filtration**
- **Major advantage in that it avoids the addition of "dead weight" like PEGylation or PASylation since each subunit of the multimer will be catalytically active**
- **It is however crucial that the binding pockets of the individual subunits are not obstructed by this approach**
- **An example for such an active multimeric complex of a β-propeller structure is the bacterial gluconolactonase from *Xantomonas campestris* that exists as a dimmer covalently linked by disulfide bridges**

Alternatives to PEGylation: 3. Cross-linking



Covalently linked dimer of the gluconolactonase XC5397

Alternatives to PEGylation: 4. Glycoengineering

- …is the addition of glycosyl groups to reduce immunogenicity
- it has been shown that specifically glycosylated proteins display much lower immunogenicity than their non-glycosylated counterparts providing that the correct sugars and linkages are employed
- Since wild type DFPase is not glycosylated, and the β-propeller fold is so robust, there may be significant benefits from glycoengineering
- Requires identification of sites to introduce required consensus sequences by mutation
- Possible secondary structures required for carbohydrate addition within functional glycosylation sites are β or Asn-X turns
- Problems: Carbohydrate addition precedes protein folding. Thus, it might affect correct folding and biological/catalytic activity. Requires eucaryotic expression systems (*P. pastoris* established for DFPase)

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  LANL/DOE LDRD program: Director's Fellowship for Marc-Michael Blum

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- **Live agent work and animal studies**

Bundeswehr Institute for Pharmacology and Toxicology, Munich, Germany

Institut de Recherche Biomédicale des Armées - CRSSA, Département de Toxicologie et Risques Chimiques, La Tronche, France

Backup Slides

Active Site Environment of WT DFPase

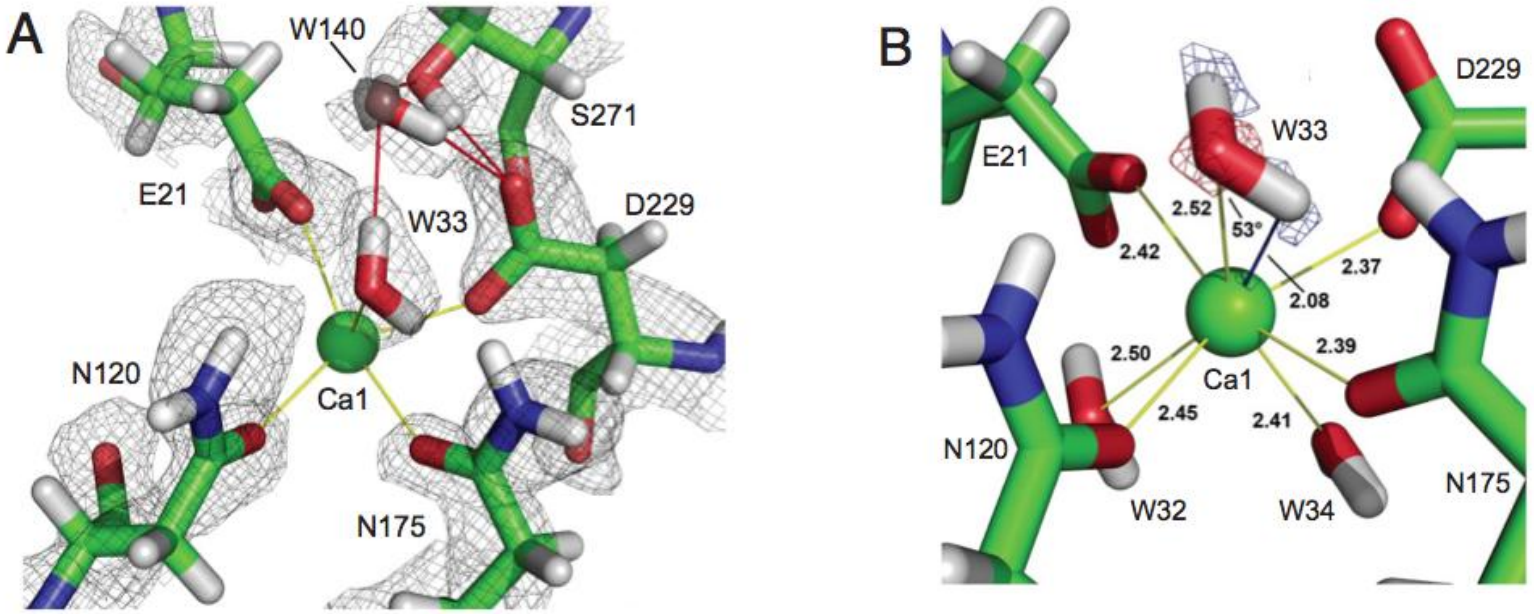
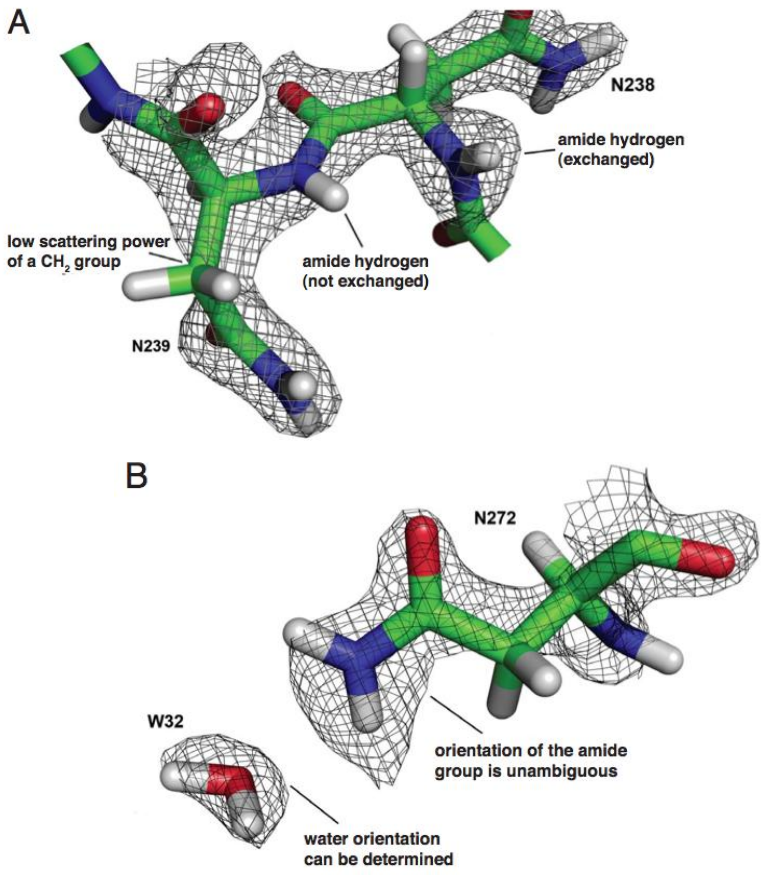


Fig. 5. Active site environment. (*A*) Catalytic Ca1 with surrounding residues and positive nuclear density. Yellow lines indicate direct calcium coordination. Hydrogen bonding interactions are indicated in red. For the poorly resolved water W140 electron density is displayed (transparent gray sphere) showing the position of the oxygen atom. Nucleophilic Asp-229 is deprotonated. (*B*) A F_o-F_c nuclear density omit map (blue), with the 2 deuterium atoms of W33 omitted, contoured at 4.0 σ, showing identity of W33 as a water, and not a hydroxide ion. The electron density $2F_o$-F_c map at 2.5 σ is superimposed (red), showing the location of the oxygen atom. Distances are shown in Å. The W33 hydrogen–Ca1 distance is indicated as a blue line. The Ca–O–H angle is 53°.

Signature Features of Nuclear Density in Proteins



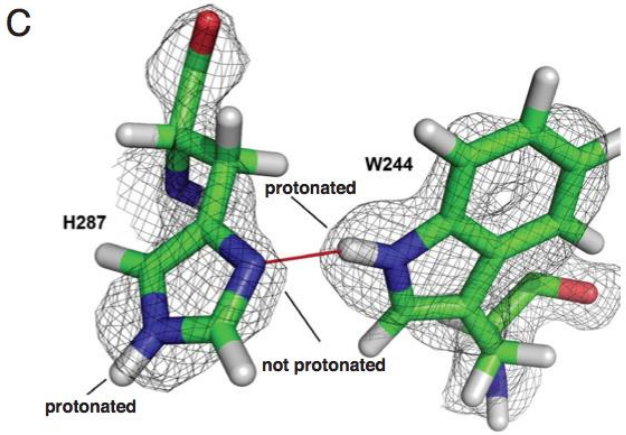
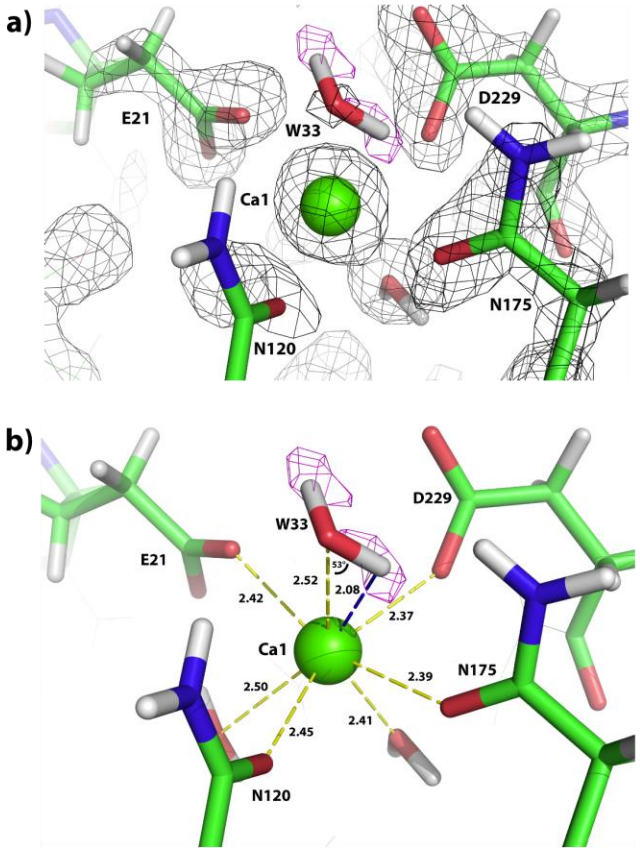
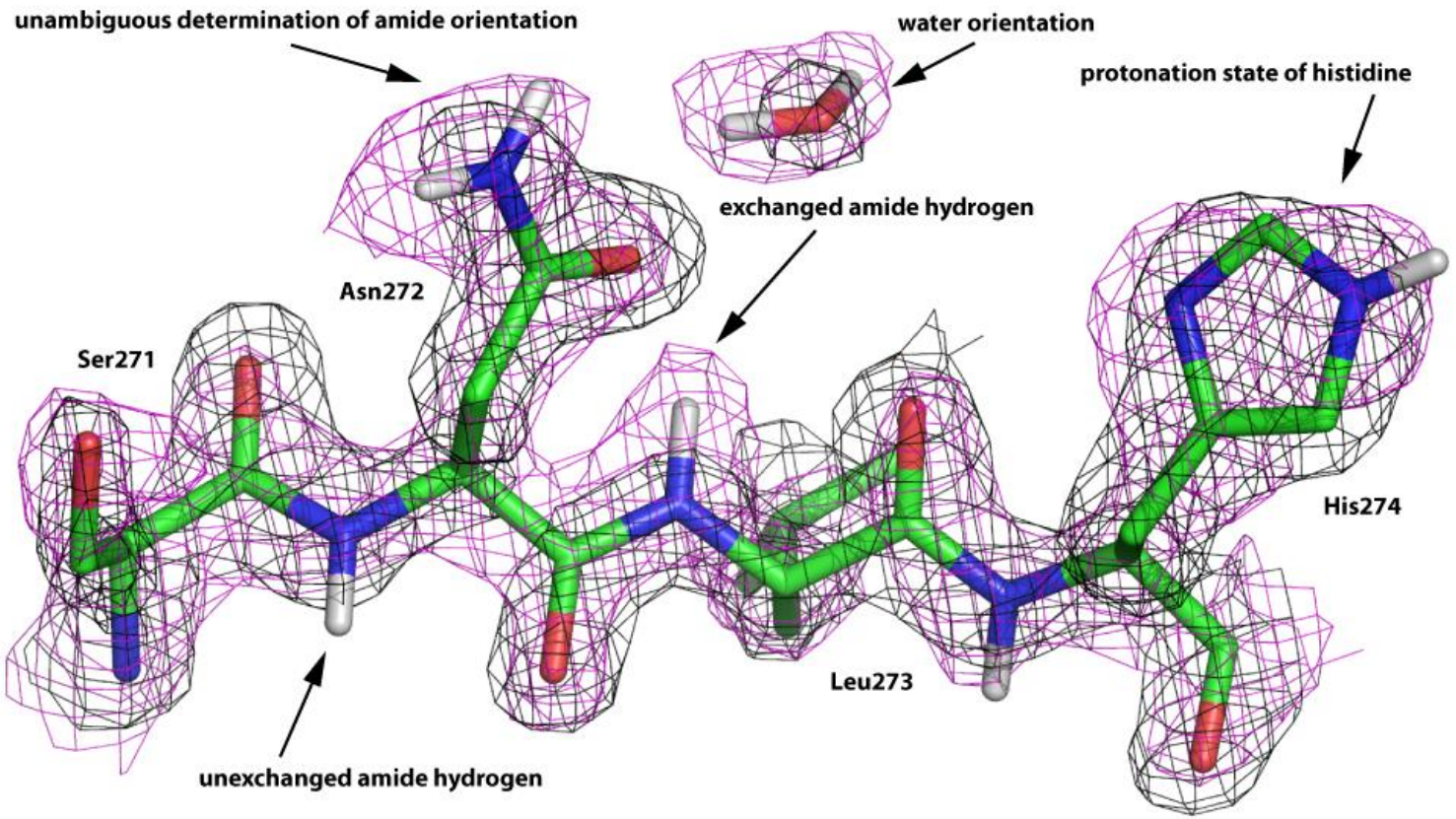


Fig. 3. Signature features of nuclear density in proteins. (*A*) Unexchanged backbone amide hydrogens can be identified by the lack of positive nuclear density for these atoms as shown for Asn-238 (exchanged) and Asn-239 (not exchanged). The sidechain of Asn-239 also shows the weak neutron scattering power of a CH_2 group. Additional information from electron density in joint refinement helps to correctly position the carbon atom. (*B*) Density for the side-chain amide of Asn-272 allows for unambiguous assignment of the oxygen and nitrogen due to the greater neutron scattering of the NH_2 group and the bean shaped density of W32 allows determining the orientation of that water molecule. Also in this case additional information from electron density in joint refinement helps to correctly position the oxygen atom. (*C*) Nuclear density for active site residues His-287 and Trp-244 clearly indicating the protonation states of the imidazole and indole ring nitrogen atoms. Hydrogen bonding is indicated by a red line.

Active Site of DFPase by Neutron Scattering



Detailed Neutron Structure



Maybe worth a look…