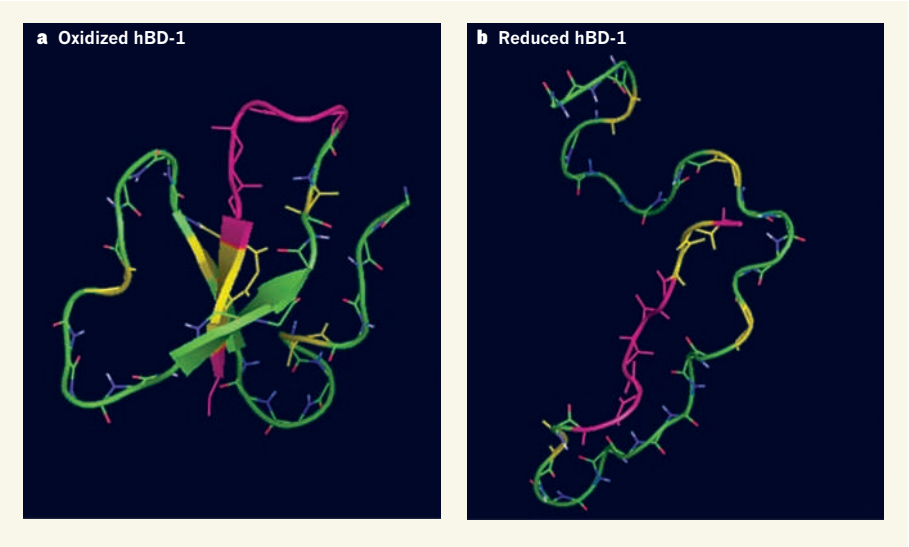

Figure 1 | Computer-generated structures of human β-defensin 1. **a**, In its oxidized form, the peptide human β-defensin 1 (hBD-1) contains three disulphide bonds. The peptide backbone is shown in green except for cysteine residues (yellow) and the six non-cysteine carboxy-terminal residues (pink). The broad arrows represent β-sheet components found only in the oxidized form. **b**, The reduced hBD-1 structure was generated from the lowest-energy conformation of oxidized hBD-1, after breaking its disulphide bonds and causing it to assume a random conformation. (Structure generated by Alan J. Waring, Univ. California, Los Angeles.)

in the colon cannot be grown in culture, and their presence can only be disclosed using various 'gene-sniffing' techniques. It could be, therefore, that the effects of reduced hBD-1 on probiotic bacteria are simply collateral damage on these harmless bystanders by a defence system that also targets less-well-intentioned intestinal residents or transients. Alternatively, even probiotics may require surveillance to keep them from overstepping their boundaries.

Ideally, the activity of an antibiotic should be examined in a defined medium, the composition of which closely resembles, or precisely replicates, the *in vivo* environment. With precise simulation of the colonic content being too challenging to contemplate, it would be informative to learn how defined factors — such as pH and, in particular, salinity — affect the activity of reduced hBD-1 *in vitro*. Another interesting experiment would be to test the antibacterial activity of mixtures of reduced and oxidized hBD-1, because clearly such mixtures occur *in vivo*.

It remains unknown whether Schroeder and colleagues' results are unique to hBD-1 or whether they are also true for other defensin peptides. Defensins and defensin-like peptides are fairly universal participants in host defence against infection[7]: they occur in plants, fungi, invertebrates and vertebrates. Vertebrates have three subfamilies of defensins (designated α, β and θ)[8], the members of which consist exclusively of cationic peptides with six cysteines and three disulphide bonds, which also provide resistance to premature proteolytic digestion.

Although more than 20 genes have been identified[9] that encode hBDs, only hBDs 1–4 have received extensive attention. The net positive charge of these four peptides varies from +4 for hBD-1 to an astounding +11 for hBD-3, whose eight carboxy-terminal residues alone carry a net charge of +6. From previous work[10] on hBD-3, its high net positive charge contributes substantially to the peptide's ability to kill bacteria or fungi such as *Candida albicans*. This is especially true when the assays are performed in media of low ionic strength.

Given the extreme cationicity and high intrinsic activity of oxidized hBD-3, it is not surprising that when Schroeder *et al.*[3] removed its disulphide bonds, they did not detect improved activity of this peptide against bifidobacteria. After all, a bacterium can only be killed once. For thioredoxin reductase to empower hBD-3 to do so twice would be a *reductio ad absurdum*. ■

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CHEMICAL BIOLOGY

Catalytic detoxification

Protein engineering of an enzyme that catalytically detoxifies organophosphate compounds in the body opens up fresh opportunities in the search for therapeutic protection against nerve agents used in chemical warfare.

FRANK M. RAUSHEL

Organophosphates are among the most toxic compounds that have been chemically synthesized. Since the discovery of their biological activity in the 1930s, these compounds have found use as broad-spectrum insecticides for agricultural and domestic applications. But organophosphates have also been developed as chemical-warfare agents, including VX and the 'G-agents' (such as sarin, soman and cyclosarin). Because these compounds are relatively easy to synthesize, their use by international terrorist groups is a serious threat. Current protocols for the prevention and treatment of organophosphate poisoning are largely ineffective, and so new strategies are desperately needed. Reporting in *Nature Chemical Biology*, Gupta *et al.*[1] describe an approach that might one day find use in preventing organophosphate poisoning.

Organophosphates are highly toxic because they rapidly inactivate acetylcholinesterase (AChE), an enzyme required for nerve function (Fig. 1). AChE breaks down (hydrolyses) acetylcholine, a neurotransmitter that relays nerve impulses to muscles and other organs. Organophosphates form a covalent bond to a serine amino-acid residue in the active site of AChE, stopping the enzyme from functioning. The subsequent build-up of acetylcholine blocks cholinergic nerve impulses, leading to paralysis, suffocation and death.

Various prophylactic approaches have been developed to diminish the toxic effect of organophosphates. Atropine, for example, is a competitive antagonist for muscarinic acetylcholine receptors — it blocks the action

of acetylcholine, thereby reducing the effective concentration of the neurotransmitter. Alternatively, chemicals such as pralidoxime react with AChE–organophosphate adducts to regenerate catalytically active AChE.

A relatively new approach for reducing the concentration of organophosphates in the blood is to inject human butyrylcholinesterase (BChE) directly into the bloodstream of a poisoned individual[2]. This enzyme reacts with organophosphates in the same way as AChE, and thus acts as a selective scavenger for the nerve agents. But a problem with this approach is that the scavenging reaction is stoichiometric — one BChE molecule is required to scavenge one molecule of organophosphate. This means that a substantial amount of the enzyme must be injected into the body to reduce a lethal dose to non-toxic levels: approximately 350 milligrams of BChE are required to detoxify every milligram of cyclosarin, for example, because the molecular mass of the enzyme is much greater than that of the nerve agent.

Gupta *et al.*[1] now report genetically modified enzymes that hydrolyse organophosphates. Notably, the nerve agents bind to these enzymes as substrates (non-covalently and reversibly), rather than as potent inactivators (which bind covalently and irreversibly). The enzymes therefore behave as catalysts for organophosphate clearance — each enzyme molecule destroys thousands of molecules of a nerve agent, thus reducing the amount of enzyme required to detoxify a lethal dose.

The authors' work builds on an earlier study[3] in which the active site of a bacterial phosphotriesterase enzyme was optimized by protein engineering to effectively catalyse the hydrolysis of a wide range of organophosphates, including sarin, soman and cyclosarin. Expression of this enzyme in caterpillars reduced the lethal effects of paraoxon[4], an insecticide whose active form is an organophosphate. It is unlikely, however, that a protein of bacterial origin could be used as an effective therapeutic agent for organophosphate toxicity in humans.

Gupta *et al.*[1] have made great strides towards solving this problem by using an enzyme largely of human origin. They worked with a serum paraoxonase enzyme, PON1, which catalyses the hydrolysis of lactones (cyclic molecules often found in nature), but which also catalyses the slow hydrolysis of a range of organophosphate nerve agents. Unfortunately, PON1 isn't stable enough to be expressed and manipulated in bacterial cells, as is required for engineering its catalytic properties. The authors partly overcame this challenge by using a hybrid enzyme[5] that combined parts of human and rabbit PON1 enzymes.

If an enzyme is to be an effective catalyst for the hydrolysis of organophosphate nerve agents, the rate constant (k_{cat}/K_m, a measure of the speed of a chemical reaction) for the process must exceed 10^7 M^{-1} min^{-1}. Another consideration to take into account is that the G-agents are chiral molecules — they form isomers known as enantiomers that are mirror images of each other. Only one of the two enantiomers (the S_P enantiomer) is toxic. Frustratingly, Gupta and colleagues found that their hybrid PON1 enzyme primarily hydrolysed the non-toxic isomer (the R_P enantiomer) of an analogue of cyclosarin, and that k_{cat}/K_m for the hydrolysis of the S_P enantiomer was less than 200 M^{-1} min^{-1}.

The authors therefore subjected the hybrid PON1 to a series of directed-evolution and rational-design experiments, in which alterations were made to the amino-acid sequence of the protein and the resulting mutants were screened to assess their effectiveness in hydrolysing organophosphates. To identify the most effective mutants, the researchers developed a sorting procedure that compartmentalized individual bacteria expressing the mutant enzymes in emulsion droplets. By adding a substrate to the droplets that produces a fluorescent compound when hydrolysed by the enzymes, those bacteria expressing active mutants were easily detected and isolated. The most active mutants were then subjected to further rounds of alterations and screening. After several generations of enzymes had been produced, the authors identified a mutant for which k_{cat}/K_m for the hydrolysis of the toxic isomer of a cyclosarin analogue exceeded 10^7 M^{-1} min^{-1}. This represents an enhancement of 100,000 over the activity of the starting enzyme.

The authors went on to show that the newly evolved enzyme acts as a prophylactic against G-agent exposure in mice. When they injected the animals with the recombinant enzyme at a dose of 2.2 milligrams per kilogram of animal body weight one hour before giving them a lethal dose of a cyclosarin analogue, 75% of the mice were still alive after 24 hours. By contrast, none of the control mice survived. However, when mice were challenged with a lethal dose of the organophosphate six hours after injection of the recombinant enzyme, the survival rate dropped to 50%. No protection was afforded when the mice were challenged after 24 hours.

Gupta and colleagues' study nicely demonstrates that a predominantly human enzyme can be engineered to provide significant therapeutic protection against lethal exposure to nerve agents. The catalytic detoxification of organophosphate nerve agents is clearly superior to the stoichiometric detoxification currently afforded by human BChE, because much lower doses of the modified PON1 enzyme are needed for a therapeutic effect. But for the authors' approach to be truly practical, the duration of the catalytic scavenger's activity in humans must be increased beyond the few hours that the current experiments indicate. In addition, the substrate specificity and catalytic efficiencies of scavengers must be expanded and enhanced for activity against other G-agents and against the even more lethal VX. One might also envisage the development of prophylactics to protect people from exposure to agricultural organophosphate pesticides, which have caused far more medical problems over time than the use of military nerve agents. ■

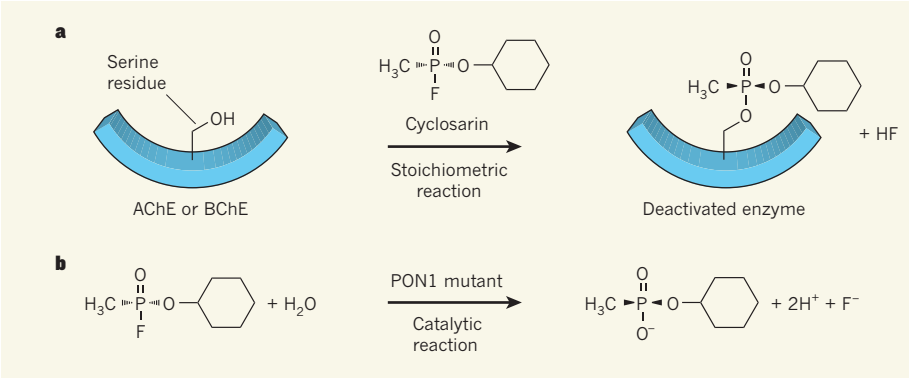

Figure 1 | Enzyme reactions of organophosphate nerve agents. **a**, *In vivo*, organophosphate nerve agents such as cyclosarin react to form a covalent bond with a serine amino-acid residue in the active site of acetylcholinesterase (AChE). This inactivates AChE, preventing it from hydrolysing the neurotransmitter acetylcholine, leading to suffocation and ultimately death. Butyrylcholinesterase (BChE) reacts with organophosphates in the same way, and can be injected into the bloodstream to scavenge the nerve agents, reducing their concentration to non-toxic levels. Because BChE is a stoichiometric scavenger, large concentrations are required for it to be therapeutically effective. **b**, Gupta *et al.*[1] have engineered serum paraoxonase (PON1) to catalyse the rapid hydrolysis of organophosphates. One PON1 molecule catalyses thousands of hydrolysis reactions, and so the amount of enzyme required to reduce the concentration of organophosphates to non-toxic levels is much lower than that needed for BChE.

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