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In search of a catalytic bioscavenger for the prophylaxis of nerve agent toxicity☆

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ABSTRACT

A novel approach for treating organophosphorus (OP) poisoning is the use of enzymes, both stoichiometric and catalytic, as bioscavengers to sequester these compounds in circulation before they reach their physiological targets. Human serum butyrylcholinesterase and a recombinant form of this enzyme produced in the milk of transgenic goats have completed Phase I clinical trials as stoichiometric bioscavengers for the protection of humans against OP nerve agents. However, a major limitation of the first generation bioscavenger is the 1:1 stoichiometry between the enzyme and the OP. Therefore, efforts are underway to develop the second generation catalytic bioscavenger, which will neutralize/hydrolyze multiple OP molecules. To avoid any complications related to adverse immune reactions, three enzymes from human (Hu) sources are being considered for development as catalytic bioscavengers: (1) prolidase; (2) paraoxonase 1 (PON1); (3) senescence marker protein-30 (SMP-30). Towards this effort, native or recombinant (r) forms of candidate catalytic bioscavengers were isolated and characterized for their ability to hydrolyze G-type nerve agents at concentrations of 10 μM and 1 mM. Results show that mammalian enzymes were significantly less efficient at hydrolyzing nerve agents as compared to bacterial organophosphorus hydrolase (OPH) and organophosphorus acid anhydrolase (OPAA). Recombinant Hu prolidase was the most efficient and the only mammalian enzyme that hydrolyzed all four G-type nerve agents. On the other hand, both rHu PON1 and Mo SMP-30 showed 10-fold lower activity towards sarin compared to rHu prolidase and did not hydrolyze tabun. Based on these results, Hu prolidase appears to be the most promising candidate for further development: (1) it can be easily expressed in *E. coli*; (2) of the three candidate enzymes, it is the only enzyme that hydrolyzes all four G-type agents. Efforts to improve the catalytic efficiency of this enzyme towards OP nerve agents are underway.

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1. Introduction

Organophosphorus (OP) nerve agents, such as sarin, soman, cyclosarin, tabun, VX, and VR, represent a risk to both military and civilian populations. Current antidotal regimen for OP poisoning includes a combination of pretreatment with a spontaneously reactivating AChE inhibitor such as pyridostigmine bromide, and post-exposure therapy with anticholinergic drugs such as atropine sulfate and oximes such as 2-PAM chloride [1]. Unfortunately, there are several problems with these treatments: (1) they produce serious side effects when administered in the absence of OP exposure [2]; (2) they must be administered within a fairly short time frame following OP exposure [2,3]; (3) they are successful in preventing lethality of animals to OP poisoning but are unable to prevent post-exposure incapacitation, convulsions, performance deficits, or in many cases, permanent brain damage [4].

As alternative approaches of protection against OP poisoning, the focus has shifted towards identifying human proteins that can remain stable in circulation for prolonged periods of time [5] thus acting as biological scavengers. A biological scavenger should be fast acting, specific, ideally have an extended circulation time, and not present an antigenic challenge to the immune system. As a prophylactic, human proteins can provide protection by inactivating OP compounds through sequestration or hydrolysis. This inactivation must occur before endogenous AChE is affected, which is within 2 min following OP exposure [6]. Human serum butyrylcholinesterase (Hu BChE) and a recombinant form of this enzyme produced in the milk of transgenic goats have completed Phase I clinical trials as stoichiometric bioscavengers for the protection of humans against OP nerve agents. However, a major limitation of the first generation bioscavenger is the 1:1 stoichiometry between the enzyme and the OP. Therefore, efforts are underway to develop the second generation catalytic bioscavenger, which will neutralize/hydrolyze multiple OP molecules. To avoid any complications related to adverse immune reactions, three enzymes from human

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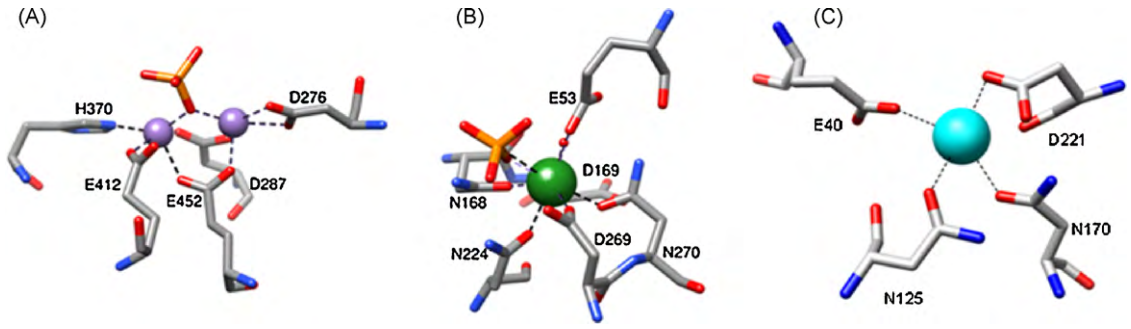

Fig. 1. Structures of active-sites of candidate catalytic bioscavengers. Active-site structures obtained from reported X-ray crystallography or homology modeling: (A) Hu prolidase containing two Mn^{2+} ions (PDB: 2OKN); (B) mammalian PON1 showing the catalytic Ca^{2+} ion (PDB: 1V04); (C) homology built model of SMP-30 containing one Ca^{2+} ion using the crystal structure of *Agrobacterium tumefaciens* regucalcin (PBD: 2GHS).

sources are being considered for development as catalytic bioscavengers: (1) Hu prolidase; (2) Hu paraoxonase 1 (PON1); (3) Hu senescence marker protein-30 (SMP-30).

All three candidate enzymes are metalloproteins whose active sites contain one or two metal ions, which are used to hydrolyze a wide variety of substrates (Fig. 1). The first candidate, Hu prolidase (EC 3.4.13.9) is a 54 kDa binuclear Mn^{2+}-dependent enzyme, which breaks the amide bond in dipeptides containing proline or hydroxyproline as the C-terminal amino acid ([7] and references within). Due to the association of Hu prolidase activity with prolidase deficiency, it has been cloned, sequenced and expressed. Native prolidase has been purified from human erythrocytes, liver, kidney and fibroblast cells and has been extensively characterized with regard to prolidase activity. A comparison of protein sequences and biochemical properties suggest that Hu prolidase maybe similar to bacterial OP acid anhydrolase (OPAA) in catalyzing the hydrolysis of OP nerve agents [8]. The X-ray crystal structure of Hu prolidase shows that it belongs to the 'pita-bread' family of enzymes, with a binuclear catalytic site located within the fold (Fig. 1A) [9].

Hu PON1 (EC 3.1.8.1) is a 43 kDa glycoprotein that is expressed predominantly in the liver and is present in circulation associated with high-density lipoprotein [10]. Although the physiological function of Hu PON1 is not known, it seems to be involved in drug metabolism and in preventing atherosclerosis. PON1s are Ca^{2+}-dependent esterases that catalyze the hydrolysis of a wide variety of esters and lactones [11]. A crystal structure of a mammalian PON1 variant revealed a six-bladed β-propeller with two Ca^{2+} ions in its central tunnel, one having a catalytic function and the other appears to be structural (Fig. 1B) [12].

SMP-30 (EC 3.1.8.2) is a 34 kDa metalloprotein that is expressed predominantly in the liver and kidney of mammals. Although the identity of the metal ion is not clearly established, it was shown that the activity of purified SMP-30 from rat liver was stimulated by $MgCl_2$, $MnCl_2$, $CoCl_2$, and $CdCl_2$ but not by $CaCl_2$ [13]. It has been linked with the regulation of calcium homeostasis and the protection of cells from apoptosis [14]. Consistent with these observations, it was found that the sequence of Mo SMP-30 was identical to that of a Ca^{2+}-binding protein, regucalcin [15]. A homology model built using an unpublished crystal structure of regucalcin from *Agrobacterium tumefaciens*, suggests that the mammalian protein may adopt a six-fold β-propeller structure (Fig. 1C). Rat liver SMP-30 was shown to hydrolyze DFP, phenyl acetate, and more recently glucono-lactones [14,16]. *In vivo* studies with SMP-30 knockout mice confirmed its role in the hydrolysis of DFP in the liver of these animals [16].

Although, the catalytic activity of these enzymes is well characterized, limited information is available on their ability to hydrolyze OP nerve agents. It was reported that recombinant Hu prolidase catalyzed the hydrolysis of soman [17,18], and it is possible that this enzyme is also capable of hydrolyzing other G-type nerve agents such as sarin, cyclosarin, and tabun. Partially purified rat liver SMP-30 was shown to hydrolyze OP compounds in the following order: sarin > soman > tabun > DFP (2694, 718, 586, and 54 nmol/min/mg protein, respectively) [13]. Similarly, PON1s were also shown to catalyze the hydrolysis of OPs including nerve agents sarin and soman [19].

In this study, we obtained purified native or recombinant (r) Hu prolidase, rHu PON1, and mouse (Mo) SMP-30, and characterized their ability to hydrolyze G-type nerve agents. Furthermore, their OP hydrolyzing properties were confirmed by comparing them with those of bacterial OP hydrolase (OPH) and OPAA, two enzymes with well characterized OP hydrolyzing activities [20,21]. Results show that mammalian enzymes are significantly less efficient at hydrolyzing G-type nerve agents as compared to bacterial OPH and OPAA. These results suggest that wild-type mammalian prolidase, SMP-30, and PON1, are not suitable as catalytic bioscavengers of OP nerve agents. However, the bioscavenging capability of these enzymes can be improved using site-directed mutagenesis and/or directed evolution techniques, as shown with mammalian PON1 [22].

2. Materials and methods

2.1. Materials

Acetylthiocholine (ATC), 5,5-dithiobisnitrobenzoic acid (DTNB), sodium phosphate, DFP, $CaCl_2$, $MgCl_2$, $MnCl_2$, (N-[2-hydroxyethyl]-1-piperazine-N′-[ethane-sulfonic acid]) (HEPES) and all other analytical grade chemicals were purchased from Sigma Chemical Co. (St. Louis, MO). BSA solution was purchased from KPL (Gaithersburg, MD). SDS-Polyacrylamide gels were from ThermoFisher (Rockford, IL). Purified FBS AChE was obtained as described [23].

2.2. Candidate catalytic bioscavengers

Recombinant Hu prolidase was expressed in *E. coli* and purified as described [24]. Purified rHu PON1 was purchased from Chesapeake PERL (Savage, MD). SMP-30 was purified from mouse liver as described [25]. Purified OPAA and OPH were purchased from the Edgewood Chemical Biological Center (APG, MD).

2.3. Assay for prolidase activity

Prolidase enzyme activity was determined colorimetrically [26]. Briefly, different dilutions of the enzyme solution in a total of 100 μl of 50 mM Tris–HCl, pH 7.8 containing 1 mM $MnCl_2$ were allowed to react with 100 μl of 94 mM solution of the substrate Gly-Pro for 30 min. The enzyme was then precipitated by the addition of