Catalytic biomaterials: engineering organophosphate hydrolase to form self-assembling enzymatic hydrogels

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Received December 31, 2009; revised February 17, 2010; accepted April 1, 2010

Edited by Ashutosh Chilkoti

Organophosphate (OP) neurotoxins have contaminated the environment, contributed to millions of poisoning annually, and have been used as chemical weapons. Biomaterials incorporating the native activity of the OP hydrolase (OPH) enzyme are of interest for applications including OP sensing, environmental bioremediation and prophylactic decontamination. We have engineered and characterized four novel hydrogel-forming OPH variants by genetically fusing the OPH enzyme with α-helical leucine zipper domains (H), unstructured soluble linker domains (S) and polyhistidine purification tags. The appended H domains form physical cross-links between the enzymes and enable self-assembly of the enzymes into hydrogels. The addition of the H and S fusions significantly increased the expression levels of soluble protein. OPH constructs with biterminal H domains form hydrogels at lower protein weight percents and exhibit higher enzymatic activity than those variants modified with a single H domain fusion. Polyhistidine tags were not useful for purification but they were not benign, as the addition of the 6His tags increased the hydrogel-forming abilities of the proteins with a concomitant reduction in both the $k_{cat}$ and $K_M$ values. Active enzymatic hydrogels could be made from concentrated unpurified crude protein lysates, significantly simplifying the processing and utilization of the biomaterials. And, a simple proteinaceous bioactive surface coating exhibiting OPH activity is demonstrated. The hydrogels were stable over long-term storage, as activity was retained after cold storage in buffer after 5 months. These new protein constructs further show the use of rational protein design to create novel, bifunctional, self-assembling units for the formation of catalytic biomaterials.

Keywords: bifunctional protein/leucine zipper/organophosphate hydrolase/protein hydrogel/surface modification

Introduction

Organophosphates (OPs) can deactivate serine proteases and inhibit vital metabolic functions, which can lead to subsequent neurological failure and death upon exposure (Chambers and Oppenheimer, 2004). OP compounds have been developed and exploited for their neurological toxicity. For example, parathion has been widely used as a pesticide and sarin has been used as a chemical nerve agent. The widespread use of OP-based pesticides has resulted in extensive environmental contamination, which contributes to millions of cases of OP poisoning annually (Karalliedde and Senanayake, 1989, Mulbry et al., 1996, Singh and Walker, 2006). The use and possession of OP-based weapons has been banned among party members of the Chemical Weapons Convention (Chauhan et al., 2008). For these reasons, there is a broad interest in the development of methods to both detect and to degrade OP compounds.

OP hydrolase (OPH) from Flavobacterium sp. (Mulbry and Kars, 1989) is a dimeric metalloenzyme that catalyzes the hydrolysis of the P–O, P–F or P–S phosphoric acid ester bonds of OP compounds. OPH consists of two 36 kDa monomers, is active over a broad pH range (pH 6.5–12), and exhibits Michaelis–Menten kinetics (Chen-Goodspeed et al., 2001; Efremenko and Sergeeva, 2001). Many research groups have reported the modification and use of OPH in biosensors and decontamination systems. For example, OPH–polymer complexes and glutaraldehyde cross-linked OPH hydrogels have been applied in electrochemical and spectroscopic OP sensors (Mulchandani et al., 2001; Trojanowicz, 2002). OPH-based sensors have been engineered with OP detection limits as low as parts per trillion (White and Harmon, 2005; Lei et al., 2007; Luckarift et al., 2007). OPH-based materials have also been used in bio-reactors for the industrial-scale destruction of OP weapon stockpiles, and OPH-displaying microorganisms have been engineered for the bioremediation of OP contaminated ecosystems (Richins et al., 2000; Singh and Walker, 2006). OPH has also been added to composite materials, including fire-fighting foams, sponges, paint coatings, cotton and a variety of polymers for use in personal OP decontamination (Russell et al., 2003).

We have been developing a protein engineering approach to make enzymes bifunctional, such that they retain their native activity while gaining the ability to self-assemble into hydrogels. This is accomplished by fusing the enzymes with previously designed α-helical leucine zipper domains (H), which reversibly assemble and form non-covalent cross-links (Shen et al., 2005). We have demonstrated this approach for creating fluorescent protein hydrogels (Wheeldon et al., 2007), bioelectrocatalytic protein hydrogels (Wheeldon et al., 2008) and hydrogels made from a thermostable dehydrogenase enzyme (Wheeldon et al., 2009). In the present work, we have applied this protein engineering strategy to the OPH enzyme to enable it to self-assemble and form a catalytic biomaterial that can degrade OP compounds. Cartoon representations of the newly created bifunctional OPH constructs are shown in Fig. 1. This new technology could be used in the development of new decontaminating or
bioremediating surfaces, as well as surface modifications for OP biosensors.

Materials and methods

Materials

β-D-1-thiogalactopyranoside (IPTG; Promega) and complete protease inhibitor cocktail (Roche) were used without modification. Sodium dodecyl sulfate polyacrylamide electrophoresis gels (SDS-PAGE) and running buffers were purchased from Invitrogen. Restriction endonucleases were purchased from New England Biolabs and used as directed. Amicon centrifugal filter devices (Millipore) and Bradford protein concentration assay kit (Pierce) were also used as directed. Terrific Broth powder was purchased from Sigma-Aldrich. All other chemicals were purchased from Sigma-Aldrich and used without modification.

Plasmid constructs

The plasmid pQE9AC10Acyx, expressing the hydrogel-forming triblock polypeptide AC10Acyx (Shen et al., 2005) here termed HSH, was a kind gift from David Tirrell (California Institute of Technology). The plasmid encoding the OPH gene from Flavobacterium sp. ATCC27 551, pE1OPD (Barnard et al., 2005) was a kind gift from David Wood (Princeton University).

The OPH gene was isolated from the pE1OPD plasmid using the forward primer 5'-ATATATAGGATCCATGTCTATC GTG-3' and the reverse primer 5'-ATATATAAGCTTTTA TGAGCGCCG-3' by overlap-extension PCR. These primers introduced an upstream BamHI restriction site and a downstream HindIII restriction site. The doubly digested product was ligated into pQE9AC10Acyx at the first vector upstream BamHI site and the unique HindIII site, resulting in pQ9AC10Acyx, coding for the OPH protein with an N-terminal hexa-histidine tag (6His-OPH).

The OPH was also isolated from pE1OPD using the forward primer 5'-ATATAAGCATGCGTAGAATGTCTATCG TGAC-3' and the reverse primer 5'-GCTGTTAACCTTTT CATGACCCTCC-3' by overlap extension PCR. These primers introduced an upstream Spel restriction site and a downstream SpeI restriction site. The doubly digested fragment was ligated into pQE9AC10Acyx at the unique Spel and SpeI sites, resulting in pQ9HS-OPH, coding for the protein 6His-OPH. This was repeated with a different reverse primer, 5'-AATATACCTTGCCTAGCCCGCAAG-3', which incorporated a downstream SpeI restriction site. The SpeI-digested fragment was ligated into pQE9AC10Acyx at the unique SpeI site, resulting in pQ9HS-OPH-H, coding for the protein 6His-OPH-H.

Complementary oligonucleotides, 5'-AGATCTGGATCC ATAGTTAATTTTCTCCTTTT TAATTAGATCTACTTTTG-3' and 5'-GAAACTGAATTCATTAAAGAGGAGAAATTAAC TA TGCCATGCGATCT-3' were hybridized, double digested with EcoRI and BamHI and ligated into pQ9AC10Acyx that was double digested with the same enzymes. The resultant vector, no longer containing a 6His tag, was named pQ9-del.

The HS-OPH and HS-OPH-H genes were excised from their respective plasmids using unique EcoRI and BamHI sites, and these were ligated into the doubly digested pQ9-del vector. This resulted in the plasmids pQ9-delHS-OPH and pQ9-delHS-OPH-H, which code for the proteins HS-OPH and HS-OPH-H, respectively.

The five plasmid constructs were propagated into E. coli strain SG13009, which contains the pREP4 repressor plasmid. The fidelity of all constructed plasmids was confirmed by DNA sequencing. The DNA and amino acid sequences of the four new bifunctional OPH constructs can be found in the Supplementary data.

Protein expression and purification

All protein constructs were expressed identically in Terrific Broth growth medium with one-half the prescribed glycerol content (5 g/l glycerol), as per previous OP expression protocols (Omburo et al., 1992). One liter cultures of growth media supplemented with 200 µg/ml ampicillin and 50 µg/ml kanamycin were inoculated with 1 ml of saturated overnight culture. Upon reaching OD600 ≈ 0.5, IPTG was added to a final concentration of 0.5 mM and CoCl₂ was also added to a final concentration of 1 mM. Expression was allowed to continue for 15–16 h at 24°C, and cells were harvested by centrifugation at 15 000 g for 10 min. Cell pellets (from 1/3 l of culture) were resuspended in 20 ml of Buffer A (50 mM HEPES, 100 µM CoCl₂, pH 8.5) and pelleted by centrifugation. The pellets were again resuspended in 35 ml of buffer A containing protease inhibitor, and sonicated (Misonix 3000) on ice for 6 min. The lysate was clarified by centrifugation at 15 000 g for 30 min. The supernatant was subjected to fractionation by ammonium sulfate precipitation.
over ice by slowly adding concentrated aqueous ammonium sulfate in Buffer A to 40% saturation (28 mg ammonium sulfate/100 ml of Buffer A) while mixing. A precipitated pellet was collected by centrifugation at 10 000 g for 10 min. The salt precipitated pellet was spun for an additional minute and the remaining supernatant was aspirated. The pellet was resuspended in 10 ml of Buffer A, and thoroughly desalted by dialfiltration over a 3 kDa centrifugal filter (Amicon Ultra 15). When required, the desalted solution was subsequently loaded onto a gel filtration column (HiLoad 16/60 Superdex 200pg, GE Healthcare) equilibrated with Buffer A and eluted at 0.5 ml/min at 4°C. Fractions containing pure recombinant OPH, as identified by SDS-PAGE, were pooled. Protein was either used directly, or it was further concentrated by ultrafiltration and stored at −80°C.

Concentrations of pure protein samples were determined by measuring $A_{280}$ values and using theoretical extinction coefficients [$\varepsilon_{\text{HS-OPH}} = 0.69; \varepsilon_{\text{T-HS-OPH-H}} = 0.70; \varepsilon_{\text{HS-OP-H}} = 0.61; \varepsilon_{\text{T-HS-OPH-H}} = 0.62$ (mg/ml)$^{-1}$cm$^{-1}$] (Gill and von Hippel, 1989). Protein concentrations were verified using the Bradford method. Expression and purity were monitored throughout the purification process by SDS-PAGE under denaturing conditions using 4–12% Bis-Tris polyacrylamide gels.

Protein activity assays

The activity of freshly purified protein constructs in dilute solution was measured by monitoring the accumulation of $p$-nitrophenolate, a paraoxon, parathion and methyl parathion hydrolysis product, colorimetrically ($A_{405}$ nm) (Votchitseva et al., 2006) at 25°C using a SpectraMax M2 spectrophotometer. Activity at various pH values was measured using 0.50 mM parathion substrate in 50 mM carbonate or 50 mM Tris buffer with an enzyme concentration of approximately $10^{-8}$ M. Activity measurements under saturated conditions were also recorded with 1.0 mM paraoxon, 0.50 mM parathion or 1.0 mM methyl parathion as substrates in 50 mM carbonate buffer (pH 10.5). Other researchers have reported OPH activity on a mass basis of the enzyme. Here we define a unit (U) as moles of substrate hydrolyzed per mol enzyme per second at a specified substrate concentration since our constructs vary significantly in molecular weight.

The kinetic parameters $k_{\text{cat}}$ and $K_M$ were determined by non-linear regressions of reaction rates in 50 mM carbonate buffer (pH 10.5) with varying parathion concentrations. Enzymatic activity of the protein constructs at hydrogel-forming concentrations was verified by visually confirming the production yellow $p$-nitrophenolate after 10 μl of 0.4 M parathion in ethanol was added to $\sim$100 μl of the protein hydrogels.

Hydrogel preparation

Hydrogels were formed by either concentrating protein solutions directly or by dissolving lyophilized protein in buffer. For hydrogel formation by concentration, protein was concentrated by dialfiltration (3 kDa cellulose filter; Amicon Ultra 15). For controlled hydrogel formation, protein aliquots with known concentrations and volumes were prepared in glass vials and frozen at −80°C. The samples were lyophilized and rehydrated with 100 μl distilled water. Mechanical mixing with a pipette tip and low speed centrifugation aided in dissolution and homogenization of the rehydrated protein. Hydrogel formation was determined by observing the rehydrated protein’s extent of adherence to the top of a glass vial container upon inversion at room temperature.

Hydrogel films were prepared by spreading small amounts of hydrogel onto a surface of a glass microscopic slide. More than 20 μl of hydrogel was spotted on the surface of one slide and a second slide was placed firmly on top of the first, sandwiching the hydrogel into a thin film. Excess hydrogel was wiped away and the second slide was removed with a gentle sliding motion.

Statistical analysis

All measurements were performed in at least triplicate, and reported errors are standard deviations. In the cases where two-way ANOVA statistics were performed, statistical significance was achieved for parameters with a $P$-value <0.05.

Results

Protein expression and purification

Four new bifunctional protein constructs based on the OP hydrolase (OPH) enzyme were designed and produced: HS-OPH and HS-OPH-H with and without N-terminal hexahistidine tags (Fig. 1A–D). Physically cross-linked networks of the bifunctional proteins are schematically shown in Fig. 1E and F. 6His-OPH (without helical appendages) was also engineered to serve as a control construct. SDS-PAGE of whole-cell lysate of E.coli expressing 6His-OPH shows a distinct band of the anticipated monomeric molecular weight (~36 kDa), but the majority of this protein is in the insoluble fraction (Fig. 2A), which is similar to what has been observed by other researchers working with overexpressed OPH modified with a polyhistidine tag (Cha et al., 2000; Wu et al., 2000). No further work was done with 6His-OPH since it was mostly expressed in an insoluble form under conditions of proteins HS-OPH (i), HS-OPH-H (ii), 6His-HS-OPH (iii), 6His-HS-OPH-H (iv) and protein standards (V) are shown. Protein samples obtained after the purification process are shown, HS-OPH (vi), 6His-HS-OPH (vii), HS-OPH-H (viii) and 6His-HS-OPH-H (ix).

Fig. 2. SDS-PAGE analysis of OPH and OPH hydrogel-forming proteins. (A) Whole-cell protein extract from E.coli expressing 6His-OPH (i) is compared with the soluble portion of the sample (ii) with protein standards (iii). (B) Protein samples from clarified lysates from E.coli expressing HS-OPH (i), 6His-HS-OPH (ii), HS-OPH-H (iii), 6His-HS-OPH-H (iv) and protein standards (V) are shown. Protein samples obtained after the purification process are shown, HS-OPH (vi), 6His-HS-OPH (vii), HS-OPH-H (viii) and 6His-HS-OPH-H (ix).
these conditions, SDS-PAGE of clarified lysate of *E. coli* expressing the four proteins with the helical appendages shows that the addition of the HS domains to the OPH protein significantly increased the expression of soluble protein. Ammonium sulfate fractionation followed by size exclusion chromatography resulted in samples of greater than >95% purity as judged by SDS-PAGE (Fig. 2B).

Purification of the constructs with both the helical appendages and the hexa-histidine tags by Ni-NTA affinity chromatography unexpectedly resulted in low yields (data not shown). Since the hexa-histidine tags appeared to have an impact on the kinetics and the gel-forming abilities of the constructs, the 6His-containing constructs were characterized in this work, although the 6His tags were not used for purification. All of the new OPH constructs used here were purified in the same way, when necessary, by ammonium salt precipitation and size exclusion chromatography.

**Protein activity**

Activity assays with saturating substrate concentrations were performed at varying pH values (Fig. 3). The four fusion constructs had similar optimal pHs (pH 10.5–11.0), and had similar pH-dependent activity profiles between pH 7.5 and 12.0; however, each construct exhibited a different specific activity. The most active construct, HS-OPH-H, had a maximum activity of 1.3 ± 0.1 U at pH 11.0, and the least active construct, 6His-HS-OPH, had a maximum activity of 0.43 ± 0.03 U at pH 10.5. For all pH values tested, the specific activities at saturating substrate values were ordered according to: HS-OPH-H > 6His-HS-OPH-H > HS-OPH > 6His-HS-OPH.

Activity assays using different OP substrates reveal the same trend, with the activity of HS-OPH-H > 6His-HS-OPH-H > HS-OPH > 6His-HS-OPH for paraoxon, parathion and methyl parathion substrates (Table I). The rate of hydrolysis was highest with the paraoxon substrate and slowest with the methyl parathion substrate for all four variants.

Michaelis–Menten kinetic parameters were determined for the four protein constructs with parathion as a substrate (Fig. 4, Table II). As expected the *k*~cat~ values were consistent with the saturation activity measurements, with HS-OPH-H > 6His-HS-OPH-H > HS-OPH > 6His-HS-OPH; the highest *k*~cat~ was 1.5 ± 0.1 per second and the lowest *k*~cat~ was 0.47 ± 0.03 per second. The addition of a 6His fusion significantly lowered both the *k*~cat~ and *K*~M~ values for the constructs, but when combined, the addition of the 6His tags had no significant impact on the *k*~cat~/*K*~M~ values. In contrast, the addition of the C-terminal H domain caused a significant increase in the *k*~cat~ values for the constructs, which also lead to a significant improvement in the *k*~cat~/*K*~M~ values.

**Hydrogel formation**

The constructs ability to self-assemble into hydrogels was determined by rehydrating the lyophilized protein with small amounts of distilled water. Mechanical mixing and low-speed centrifugation were required to ensure that the hydrogels were homogenous and free of bubbles. Hydrogel formation was judged by inspection as well as by adhesion of the rehydrated protein to the top of a glass vial upon inversion, similar to the methods used by others (Das et al., 2006; Cao and Li, 2008).

All of the protein constructs were capable of forming hydrogels, but the minimum weight percent of protein required to

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**Table I.** Specific activity of OPH constructs with different OP substrates

<table>
<thead>
<tr>
<th>Name</th>
<th>Specific activity (U)</th>
<th>Parathion</th>
<th>Methyl parathion</th>
</tr>
</thead>
<tbody>
<tr>
<td>HS-OPH</td>
<td>2.2 ± 0.1</td>
<td>0.61 ± 0.01</td>
<td>0.015 ± 0.001</td>
</tr>
<tr>
<td>6His-HS-OPH</td>
<td>1.6 ± 0.1</td>
<td>0.43 ± 0.03</td>
<td>0.011 ± 0.003</td>
</tr>
<tr>
<td>HS-OPH-H</td>
<td>5.0 ± 0.1</td>
<td>1.3 ± 0.1</td>
<td>0.036 ± 0.003</td>
</tr>
<tr>
<td>6His-HS-OPH-H</td>
<td>4.1 ± 0.2</td>
<td>0.99 ± 0.07</td>
<td>0.025 ± 0.008</td>
</tr>
</tbody>
</table>

*U* is defined as moles of substrate hydrolyzed per mol enzyme per second at specific substrate concentrations (1 mM paraoxon, 0.5 mM parathion, 1 mM methyl parathion) in 50 mM carbonate buffer, pH 10.5, 25°C. All data were collected in triplicate and error bars represent standard deviations.
form a hydrogel varied among each construct. Protein samples with varying weight percent values were prepared to identify a minimum weight percent range where constructs would form hydrogels. Images of protein hydrogels above and below the critical weight percent are shown (Fig. 5A). HS-OPH formed a hydrogel at 18 wt% (4.3 mM) and not at 14 wt% (3.2 mM). HS-OPH-H formed a hydrogel at 14 wt% (2.8 mM) but not at 11 wt% (2.1 mM), 6His-HS-OPH formed a hydrogel at 11 wt% (2.4 mM) but not 8 wt% (1.7 mM) and 6His-HS-OPH-H formed a hydrogel at 8 wt% (1.5 mM) but not 4 wt% (0.70 mM) (Table II, Supplementary Table S1).

Hydrogels were also formed from protein collected after different steps of the purification process (Fig. 5B). First, to simplify the processing procedure, the samples were concentrated by diafiltration instead of lyophilization. Samples were obtained during the purification of the 6His-HS-OPH-H construct, and hydrogels were made from clarified crude cell lysate, protein purified by ammonium sulfate precipitation and protein purified by size exclusion chromatography (Fig. 5B i, ii and iii, respectively). All of these samples formed hydrogels, and all of the hydrogels retained OPH catalytic activity as they visibly showed the production of p-nitrophenolate from parathion degradation. The kinetic activities of the proteins in the bulk hydrogels were not quantified because the presence of the hydrogel complicates absorbance measurements, and because the OP hydrolysis

Table II. Summary of kinetic parameters and hydrogel-forming properties of protein constructs

<table>
<thead>
<tr>
<th>Name</th>
<th>(k_{cat}) (per s)(^a)</th>
<th>(K_M) (µM)(^b)</th>
<th>(k_{cat}/K_M) (per mM per s)(^c)</th>
<th>Calculated MW (Da)</th>
<th>Lowest hydrogel forming wt%</th>
</tr>
</thead>
<tbody>
<tr>
<td>HS-OPH</td>
<td>0.67 ± 0.02</td>
<td>59 ± 3</td>
<td>11 ± 1</td>
<td>51 437</td>
<td>18</td>
</tr>
<tr>
<td>6His-HS-OPH</td>
<td>0.47 ± 0.03</td>
<td>42 ± 4</td>
<td>11 ± 1</td>
<td>52 561</td>
<td>11</td>
</tr>
<tr>
<td>HS-OPH-H</td>
<td>1.5 ± 0.1</td>
<td>56 ± 1</td>
<td>26 ± 1</td>
<td>58 117</td>
<td>14</td>
</tr>
<tr>
<td>6His-HS-OPH-H</td>
<td>1.1 ± 0.1</td>
<td>50 ± 5</td>
<td>23 ± 2</td>
<td>59 240</td>
<td>8</td>
</tr>
</tbody>
</table>

Parathion substrate was used for kinetics experiments. All data were collected in triplicate. Errors are standard deviations.

\(^a\) The effect of the addition of the 6His tag and the C-terminal H domain on the \(k_{cat}\) value is statistically significant (two-way ANOVA, \(P < 0.05\)).

\(^b\) The effect of the addition of the 6His tag on the \(K_M\) value is statistically significant (two-way ANOVA, \(P < 0.05\)).

\(^c\) The effect of the addition of the C-terminal H domain on the \(k_{cat}/K_M\) value is statistically significant (two-way ANOVA, \(P < 0.05\)).

Fig. 5. Hydrogel formation by OPH hydrogel-forming proteins. (A) Concentrated protein samples were lyophilized and rehydrated with 100 µl of distilled water to various weight percent protein mixtures in glass vials at room temperature. The protein weight percent of each sample is labeled adjacent to the sample. All experiments were done in duplicate. (Left) Rehydration with water only, no reactant added. (Right) Rehydration with water and 10 µl of 0.4 M parathion. All vials were inverted 10 min after rehydration. (B) Two hundred microliters of the 6His-HS-OPH-H hydrogels made from protein collected from clarified cell lysate (i), protein pooled from the salt precipitation purification step (ii), and protein collected from the size exclusion chromatography step (iii) (top). The same hydrogel samples after 10 µl of 0.4 M parathion in ethanol was added to each vial before inversion (bottom). (C) A glass slide covered by a thin film of 6His-HS-OPH-H on the left side of the blue divider is shown. Five microliters of 50 mM carbonate buffer pH 10.5 without parathion (i) and with 0.50 mM parathion (ii) are placed on top of the glass slide covered with protein film, and the same buffer without parathion (iii) and with parathion (iv) is placed on top of the uncoated section of the glass slide. The yellow color indicates the degradation of the parathion substrate to produce p-nitrophenolate. (D) Image of a 6His-HS-OPH-H hydrogel on a spatula that has been stored for over 5 months in 50 mM HEPES buffer pH 8.5 at 4°C.
reaction occurred too rapidly to be accurately measured at the high protein concentrations required for hydrogel formation.

The ability of the 6His-HS-OPH to function as a catalytic film coating was investigated (Fig. 5C). Half of a glass slide was coated with a film of the 6His-HS-OPH hydrogel. Drops of parathion substrate placed on the hydrogel-covered slide turned from clear to yellow, indicating hydrolysis of the substrate. Drops of buffer alone on hydrogel-covered slide remained clear. Moreover, substrate drops on bare glass also remained clear. The results show that the protein film was catalytically active and could readily degrade OP samples.

The long-term stability of hydrogels was also partially investigated. A sample of 6His-HS-OPH-H was formed into hydrogel and was subsequently stored for over 5 months in Buffer A at 4°C. The sample retained its three dimensional shape (Fig. 5D) and was still active with paraoxon as a substrate (Supplementary Fig. S2). A sample of lyophilized 6His-HS-OPH-H protein was stored for over 6 months at −20°C and upon rehydration, the sample was capable of hydrogel formation and paraoxon degradation (not shown).

**Discussion**

Protein engineering provides a powerful tool set to design and produce macromolecules with novel functionalities and new materials with responsive action and catalytic functions (van Hest and Tirrell, 2001; Chockalingam et al., 2007; Banta et al., 2010). We have developed a method for endowing enzymes the ability to self-assemble into enzymatic biomaterials by fusing α-helical leucine zipper domains (H domains) to the protein termini (Wheeldon et al., 2007). In the present work, we created four variants of the OP-hydrolyzing enzyme, OPH, and have demonstrated that hydrogels made from these proteins retain the ability to degrade OP compounds (Fig. 5). These new catalytic materials will be useful in the development of new OP detection, bioremediation and decontamination platforms.

The unmodified OPH protein has previously been shown to be prone to inclusion body formation upon high-yield production in *E. coli* (Cha et al., 2000), which has limited its widespread use. Similar to other reports (Cha et al., 2000), in our hands the expression of the OPH gene with a hexahistidine tag resulted in protein that was predominantly in the insoluble fraction; however, the addition of the H and S domains to OPH significantly increased the soluble overexpression of the proteins (Fig. 2). The increase in soluble expression is likely due to the presence of the S domain (polypeptide (AGAGAGPEG)10), which has previously been shown to improve protein solubility (Richins et al., 2000).

All of the new hydrogel-forming protein constructs retained native OPH activity and were able to hydrolyze various OP compounds including paraoxon, parathion and methyl parathion (Table I, Figs 3 and 4). The activity–pH relationship of all the hydrogel-forming constructs was found to be similar to that of the wild-type OPH (Votchitseva et al., 2006). The wild-type OPH enzyme has previously been characterized by several research groups, and there has been a range of values reported for the specific activity of the enzyme, which depend on the expression systems and constructs used. Omburo et al. (1992) reported one of the highest specific activities (8120 μmol of paraoxon degraded per minute per milligram OPH, or approximately 4870 U) with paraoxon as a substrate, and this was obtained with OPH produced in a low-level, constitutive expression system. Cha et al. (2000) has reported a much lower specific activity (0.014 μmol paraoxon degraded per minute per microgram 6His-OPH, or approximately 8.5 U) with paraoxon substrate, but this was obtained using a hexa-histidine modified OPH overexpressed with a strong promoter system. The constructs made in the present work are more consistent with the latter report, as the activities for the bifunctional constructs with paraoxon were close to 1 U. Leader peptides fused to the N-terminus of OPH have been previously observed to decrease the activity of OPH (Mulbry and Karns, 1989), and this effect may have occurred in our case as well, as all N-terminally modified constructs exhibit lower enzymatic activity than what has been reported for the wild type. But importantly, the activity is retained by the mutant enzymes, and when self-assembled into high-density hydrogels, the high concentration of enzymes will dominate the overall turnover rate of the biomaterial.

Comparisons of the kinetic properties of the four constructs revealed unanticipated trends. Two-way ANOVA shows that the addition of the C-terminal H domain increased the *k*~cat~ values for the constructs while having no significant impact on the *K*~M~ values. The addition of a 6His tag to the constructs resulted in decreased *k*~cat~ and *K*~M~ values, leading to no effect on the *k*~cat~/*K*~M~ values (Table II). Changes in the *K*~M~ values have been previously observed when polyhistidine tags have been fused to other metalloenzymes including OPH, and it has been proposed that the histidine residues may interact with the divalent metal ion in the active site (Lai et al., 1994; Efremenko et al., 2007). Votchitseva et al. (2006) report a *K*~M~ of OPH (60 μM), which is similar to the values exhibited by the mutants in this work. Omburo et al. (1992) reported the *K*~M~ of wild-type OPH to be dependent on the divalent metal ions present in the culture media used for protein expression, ranging from 40 μM with high Ni2+ content, 130 μM with high Co2+ content and 400 μM with high Cd2+ content.

All of the protein constructs were able to self-assemble to form hydrogels as evidenced by visual inspection and through evaluation using a vial inversion test (Fig. 5A). However, there was a difference in the minimum protein concentration required for hydrogel formation. A single N-terminal H and S fusion to OPH was sufficient for hydrogel assembly, demonstrating that hydrogel formation of the HS-OPH construct is mediated both by cross-linking of the appended H domains as well as protein–protein interactions that occur upon OPH dimerization. This agrees with previous observations that have demonstrated that dimeric or multimeric proteins require only a single H fusion to form hydrogels, while monomeric proteins require two terminal H fusions to form hydrogels (Wheeldon et al., 2007; Wheeldon et al., 2008). Protein with an additional C-terminal H domain forms hydrogels at a lower weight percent, and this reflects the increased cross-linking abilities that occur from the added H-domains.

The addition of an N-terminal 6His tag to either HS-OPH or HS-OPH-H further decreased the protein weight required for hydrogel formation. The construct with the lowest concentration required for hydrogel formation was the 6His-HS-OPH-H mutant. It is possible that the 6His domain
interacts with the divalent metal in OPH, adding an additional physical cross-link. This assertion is consistent with the suggestions that polyhistidine tag protein fusions can mediate structural assembly and oligomerization (Efremenko et al., 2007; Salgado et al., 2008). It could also be possible that two histidine tags bind a single metal ion to form a physical cross-link between protein constructs.

Comparison of the protein concentrations used in the inversion tests (Fig. 5A) show that the inversion tests are not simply reporting protein concentration effects. The protein with the lowest concentration for hydrogel formation (6His-HS-OPH-H) forms a hydrogel material at 1.5 mM, while the other three constructs at higher protein concentrations (ranging from 1.7 to 3.2 mM) do not form hydrogels, and appear instead to be viscous liquids upon inversion.

A sample of the 6His-HS-OPH-H hydrogel was subjected to 5 months of cold storage in a buffered solution, and the sample remarkably retained its three-dimensional shape and did not erode into the buffer. This result is in stark contrast to the relatively fast erosion rate of the HSH peptide (Shen et al., 2006) and the slower, but measurable, erosion rate of the hydrogels formed using fluorescent proteins (Wheeldon et al., 2007). The superior stability of the 6His-HS-OPH-H hydrogel may possibly be attributed to the additional cross-linking provided by the addition of the 6His tag.

Importantly, all of the OPH protein constructs with terminal H domains (both N-terminal only, and N- and C-terminal) were catalytically active in hydrogel form (Fig. 5A and B). A change in color corresponding to the production of p-nitrophenolate, the hydrolysis product of para-thion, is visible after reaction in bulk hydrogel samples.

Additionally, we used 6His-OPH to demonstrate the utility of the constructs as a surface modification. A film of hydrogel coated on a glass slide produces a catalytically active surface coating (Fig. 5C) that could potentially be used as an OP decontaminating or protective coating.

In order to further explore the potential utility of the new constructs, we investigated alternative methods for hydrogel formation. To create hydrogels for rigorous investigation, multiple purifications steps are used, and hydrogels were formed by lyophilization and rehydration, a procedure that is both time consuming and expensive. For larger scale applications, hydrogel formation at various stages of purification was explored. Protein purification was proven to be unnecessary for hydrogel formation, as hydrogels were formed with protein obtained from clarified crude cell lysates, concentrated via simple membrane filtration (Fig. 5B). The ability to form hydrogels without the need for purification or modification will significantly lower the cost of utilizing this technology.

The stability of the engineered proteins will be critical for certain applications. A hydrogel sample that was subjected to cold storage in a buffer solution remained in a hydrogel state and retained its catalytic activity. A lyophylized protein sample was stored for at least 6 months at −20°C and upon rehydration it was able to form a hydrogel capable of catalyzing the OP degradation. The stability of the OP-based hydrogels is likely due in part to the inherent stability of the OPH enzyme, which has been reported to exhibit a Gibbs free energy change of over 40 kcal/mol upon unfolding (Grimsley et al., 1997). The stability of enzyme with the helical appendages is greater than what has been reported for the wild-type OPH stored in similar conditions (Yair et al., 2008), but it is consistent with a report that demonstrated that cross-linked OPH retains catalytic activity after 3 months of storage (Laohanachareon et al., 2008). Remarkably, the hydrogel left in a buffered solution did not erode away, which suggests that the OPH-based hydrogels are more robust than any of the bifunctional proteins that we previously reported (Wheeldon et al., 2007; Wheeldon et al., 2008; Wheeldon et al., 2009).

OPH has great potential in many applications and technologies, but the high costs of preparation and processing is a major barrier to its widespread adaptation (Yair et al., 2008). OPH is prone to form inclusion bodies in high-level E. coli expression systems (Cha et al., 2000), and efforts to immobilize OPH onto surfaces and into different materials further increase costs. Here we present a simple protein engineering strategy that results in the creation of self-assembling OPH-active hydrogels, which are highly expressed in the soluble fraction and are readily purified. Hydrogel formation via self-assembly can be controlled by adjusting protein concentration, and does not require the addition of chemical cross-linking agents. This may be especially advantageous in applications involving gelation on surfaces where contact with chemical cross-linking reagents should be avoided, such as application to the skin. For some large-scale applications, the catalytic biomaterials can be created by simple concentration of crude cell extract, without the need for additional purification of the proteins. The protein required for spontaneous hydrogel formation is high (ranging from 8 to 18% gel weight), but this may be reduced by increasing the number of helical appendages to the system, such as by including the HSH peptide to create mixed hydrogels (Wheeldon et al., 2007). The self-assembling protein-based OPH hydrogels have the potential to create new protective gel coatings, act as catalysts in decontamination reactors, and to be used as simple surface modifications for OP biosensors.

Supplementary data
Supplementary data are available at PEDS online.

Acknowledgements
We thank Prof. David Tirrel (California Institute of Technology) for the expression plasmid pQE9AC10Acsy and Prof. David Wood (Princeton University) for the pE1OPD plasmid.

Funding
This work was supported by the Air Force Office of Scientific Research Multidisciplinary University Research Initiative [FA9550-06-1-0264 to S.B.] and the U.S. Department of Education Ronald E. McNair Postbaccalaureate Achievement Program [P217A070257 to H.D.L.].

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