

Orientation Specific Positioning of Organophosphorus Hydrolase on Solid Interfaces for Biosensor Applications

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Protein immobilization on solid interfaces is a crucial aspect of their successful application in technologies such as biosensing, purification, separation, decontamination, etc. Although immobilization can improve the long-term and operational stability of proteins, this is often at the cost of significant losses in the catalytic activity of the tethered enzyme. Covalent attachment methods take advantage of reactive groups on the amino acid side chains. The distribution of the solvent exposed side chains on an enzyme's molecular surface often results in an ensemble of orientations when the protein is immobilized on a surface or in a matrix through these side chain linkages. Depending on the attachment mechanism and resulting orientation, access to and from the active site could be restricted. This study describes a methodology for the design and implementation of an orientation specific attachment of an enzyme to a surface plasmon resonance sensor surface. The enzyme, organophosphorus hydrolase, was structurally analyzed to identify surface residues as candidates for modification to optimize active site accessibility and, thus, sensitivity of detection. A single surface lysine on the active site face of the enzyme dimer was selected for elimination, thus allowing for the immobilization of the catalyst in the preferred orientation. Kinetic evaluation of the enzymes determined that the surface lysine-to-alanine variant retained 80% of the wild-type activity with the neurotoxin substrates, paraoxon and demeton-S. After immobilization, surfaces bearing the variant were determined to be more active even though the enzyme coverage on the sensor surface was reduced by 17%.

1. Introduction

With the judicious choice of an enzyme with well-defined kinetics, decontamination and biosensor applications can be afforded both high specificity and high sensitivity. Organophosphorus hydrolase (OPH) is an enzyme capable of degrading a wide array of organophosphate pesticides and nerve agents,^{1–7} and this ability makes it ideal for many detection and decontamination purposes.

Several approaches are available when immobilizing enzymes in decontamination and detection applications, the simplest being a mixture. OPH has been successfully used to decontaminate surfaces when incorporated in fire fighting foams and latex paints.^{8,9} This strategy circumvents the problems of using caustic agents on large areas. Slightly more complicated, but still requiring no

modification of the enzyme, is entrapment. Encapsulation of the enzyme in murine erythrocytes by hypotonic dialysis has been shown to be successful at degrading pesticides.¹⁰ An optical technique utilizing poly(ethylene glycol) hydrogel encapsulated OPH was able to detect paraoxon down to 16 nM (0.004 ppm) using pH sensitive seminaphthofluorescein (SNAFL).¹¹ Cryoimmobilization of *Escherichia coli* (*E. coli*) cells expressing OPH has been used to detect paraoxon down to 1×10^3 nM (0.25 ppm) using a potentiometer biosensor.¹²

Other slightly more sophisticated methods of immobilizing OPH, yet still noncovalent, include using self-assembled chitosan/poly(thiophene-3-acetic acid) layers allowed for the detection of paraoxon down to 1.0 nM concentration.¹³ Another approach used an OPH–cellulose binding domain fusion protein, which was reported to retain kinetic characteristics similar to the free enzyme when immobilized.¹⁴ The studies mentioned above were predominantly noncovalent, relying on hydrophobic interactions, electrostatic attractions, or physical containment to immobilize an enzymatic fraction, whether whole cells or purified enzymes. Although these approaches offer ease of construction, the use of trapping or adsorption mechanisms can make the surface subject to loss of activity through such processes as diffusion or aggregation of the protein. The necessity for tight, essentially irreversible, attachment makes covalent linkage an attractive alternative, and

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one in which retention of biological activity, uniform structure, and long-term stability can be achieved.

Covalent attachment relies on chemical modification of the side chains exposed at the enzyme's surface. Cystamine–glutaraldehyde immobilization has been used in an amperometric detection system,^{15,16} relying on the detection of pH change resulting from the release of protons when OPH hydrolyses substrates. OPH has been immobilized in photosensitive polyethylene glycol (PEG) gels, which employs both covalent attachment and physical entrapment.¹⁷ The reversible inhibition of OPH has been utilized for the development of an optical sensor that detects substrate binding and not hydrolysis of target compounds.¹⁸ In this system, purified enzyme is immobilized on a glass surface through glutaraldehyde activation of lysines. Introduction of a substrate results in the competitive displacement of the porphyrin bound in the active site, and the change in the porphyrin absorption spectrum was observed.

Immobilization strategies are as varied as the intended applications, and all will have advantages and disadvantages that would preclude the use of a single technique. A recent extensive review details the use of OPH, as well as other enzymes, in biomaterials for detection and decontamination of chemical warfare agents.¹⁹ To address the issue of efficiency of the immobilized enzyme, this study presents a method to further refine surface construction by the orientation specific attachment of OPH. Such attachment should facilitate active site access as well as reduce the potential for restriction of movement of important secondary structural elements near the active site.

2. Experimental Section

2.1. Variant Design. To aid in the selection of residues for substitution, two methods were employed to calculate solvent accessibility of the lysine residues from the 1DPM PDB file. First, parameter optimized surfaces analysis (POPS) was used to calculate an approximation of area of the first solvation shell for each lysine residue.²⁰ Second, *pfis* calculations were performed to determine the degree of burial, specifically of the ξ -N, of each lysine residue.²¹

2.2. Site-Directed Mutagenesis. The pUC19 plasmid containing the wild-type (WT) (pOP419) gene⁷ was used as the template, and the mutation was made with the QuickChange site-directed mutagenesis kit (Stratagene, LaJolla, CA). Nonhomologous, nonoverlapping primers were synthesized by Integrated DNA Technologies, Inc. (Coralville, IA) and used for the mutagenesis reactions. The primers used to construct the variant were 5'-AAAGGGGGTTCGCGCAGCCTGTGGTC-3', 5'-GACCACAGGCTGCGCGACCCCTTTCA-3'. The mutated plasmids were sequenced to ensure the fidelity of the PCR reactions. The electrophoretic separation and analysis were performed by the Gene Technology Laboratory of Texas A&M University, the resulting sequence data was analyzed with Vector NTI software (Stratagene, LaJolla, CA).

2.3. Enzyme Purification and Biotinylation. The enzymes were purified, as previously described.²² Purified enzymes were

concentrated to greater than 1 mg/mL for storage at 4 °C in the final column buffer (10 mM KPO₄, 20 mM KCl, 50 μ M CoCl₂, pH 8.3). Protein concentration was determined by using an extinction coefficient of 58 000 M⁻¹cm⁻¹ when measuring absorbance at 280 nm. Purity was verified by SDS-PAGE.

The enzymes WT and K175A (1 mg/mL) were incubated with equal concentrations of biotin (Pierce, Rockford, IL) in 5% DMSO, 10 mM KPO₄, and pH 8.3 overnight on a shaker at 4 °C. The unbound biotin was removed by dialysis, performed overnight against 500 mL of 10 mM KPO₄ (pH 8.3) at 4 °C. Under these reaction conditions, the guanidino group of arginine residues (pK_a > 12) are not considered as candidates for biotinylation.

2.4. Enzyme Activity Assay. The substrates used in this study were paraoxon and demeton-S (ChemService), and the free thiol reporter for the demeton-S assays was 2,2'-dithiodipyridine (2,2'-TP). Michaelis constants (K_M) and the catalytic rates (k_{cat}) for paraoxon and demeton-S were determined by performing enzymatic assays with varying concentrations of substrate and constant enzyme concentrations. The activity of the biotinylated enzymes was measured in a similar fashion with paraoxon as the substrate.

Paraoxon hydrolysis was followed by measuring the appearance of the *p*-nitrophenol anion at 400 nm (ϵ = 17 000 M⁻¹cm⁻¹) in 20 mM CHES (pH 9.0) at 25 °C, and initial velocities were calculated and fit to the Michaelis–Menten equation, allowing for substrate inhibition eq 1:

$$v_0 = \frac{V_{\max}S}{K_M + S\left(1 + \frac{S}{K_i}\right)} \quad (1)$$

Demeton-S hydrolysis was followed by the appearance of the 2,2'-TP anion at 343 nm (ϵ = 7 060 M⁻¹cm⁻¹) in tripart buffer at pH 8.0,²³ and initial velocities were calculated and fit to the Michaelis–Menten eq 2:

$$v_0 = \frac{V_{\max}S}{K_M + S} \quad (2)$$

2.5. SPREETA Preparation and Sensing Layer Construction. The sensor surface was cleaned with piranha solution (3:1 mixture of sulfuric acid and 30% hydrogen peroxide; extreme caution should be exercised with piranha, it is a very strong oxidant and reacts violently with organic matter) followed by rinsing and sonication with Milli-Q water. The sensor was then initialized in air and water, followed by in situ cleaning with NaOH–TritonX. After establishing a baseline with phosphate-buffered saline (PBS), neutra-avidin (1 mg/mL) was nonspecifically adsorbed on the gold surface. Bovine serum albumin (BSA) (1 mg/mL) was used to block the remaining sites, followed by specific immobilization of biotinylated enzymes (WT, K175A –1 mg/mL).

2.6. Calculation of Surface Coverage. The amount of enzyme covering the surface of the sensor was calculated using the equations described by eq 3 and 4.^{24,25} The thickness of the adsorbed layer (ad-layer) is calculated using

$$d_a = \left(\frac{l_d}{2}\right) \left(\frac{n_{\text{eff}} - n_b}{n_a - n_b}\right) \quad (3)$$

where d_a is the thickness of the ad-layer, l_d is the characteristic decay length of an evanescent wave at 307 nm, n_{eff} is the effective RI of the ad-layer (from SPR signal), n_b is the RI of the buffer (1.333), and n_a is the RI of the proteins (1.57). Surface coverage is

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calculated using the thickness and density of the protein:

surface coverage (g/mm²) = thickness (*d_a*)
× density (~1.3g/cm³) surface coverage (molecules/mm²)
= $\left(\frac{\text{surface coverage (g/mm}^2\text{)} \times \text{avagadro number}}{\text{mol. wt}} \right)$ (4)

2.7. Immobilized Enzyme Activity. Paraoxon, 0.048–0.462 mM, was circulated across the surface using a flow rate of 100 μL/min for 2 min. Activity was determined by collecting 200 μL of the flow through and measuring the absorbance at 405 nm of the p-nitrophenol product.

3. Results

3.1. Structural Analysis. In OPH, there are eight lysines per monomer. Of these, K169 is carboxylated and buried in the active site where it serves as a bridging ligand for the binuclear metal center. K82 is a surface residue; however, the ζ-N is only partially exposed. The remaining lysines are accessible and are the more probable attachment sites. Parameter optimized surfaces analysis (POPS) of the 1DPM PDB structure (Table 1) shows that K175 has 209.6 square angstroms of solvent accessible surface area (SASA).²⁰ The percent burial of the ζ-N of K175 and K294 was calculated by *pfis* to be –8.2 and –6.4%, respectively, indicating a completely exposed side chain at those two positions.²¹ The ζ-N

Table 1. Solvent Accessible Surface Area of OPH Lysine Residues in Square Angstroms

Lys residue	hydrophobic	hydrophilic	total
77	94.4	54.9	149.3
82	48.2	13.5	61.7
169	11.8	10.4	22.2
175	142.4	67.2	209.6
185	33.2	36.3	69.5
285	37.2	29.6	66.8
294	102.4	63.0	165.4
339	42.7	58.4	101.2

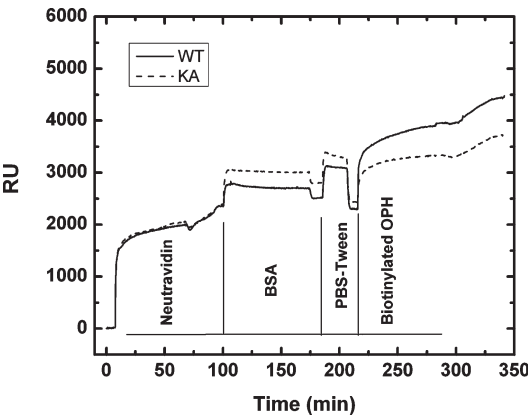


Figure 1. SPR sensorgram of surface assembly.

of K339 and K77 were calculated to be less than 50% buried. Taken together, these data suggest that K175 and K294 are the most accessible, although not necessarily the only, biotinylation sites.

3.2. Enzyme Activity in Solution. The K175A variant is kinetically similar to WT in solution assays, having a *k_{cat}* of 3 100 s^{–1} and *K_M* of 0.05 mM, giving a catalytic efficiency of 6.2 × 10⁷ compared to 1 × 10⁸ for the WT. It is more susceptible to substrate inhibition, with a *K_I* of 6.1 mM versus 17 mM for the WT. With demeton-S, K175A has a *k_{cat}* of 2 s^{–1} and *K_M* of 3.1 mM, giving a catalytic efficiency of 558 compared to 870 for the WT, which has a *k_{cat}* of 35 s^{–1} and a *K_M* of 0.04 mM.

3.3. Surface Construction and Immobilized Activity. From the real time sensorgram of the surface construction (Figure 1), the surface coverage of the enzymes was calculated. K175A covers the sensor surface at 1.8 × 10¹⁰ ± 9.2 × 10⁸ mm^{–2}, and the WT covers the surface at a slightly higher concentration of 2.16 × 10¹⁰ ± 7.3 × 10⁸ mm^{–2}. This suggests that surface

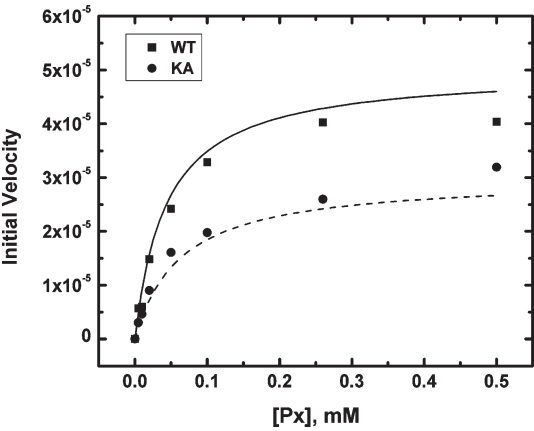


Figure 2. Michaelis–Menten plot for biotinylated WT and K175A in solution.

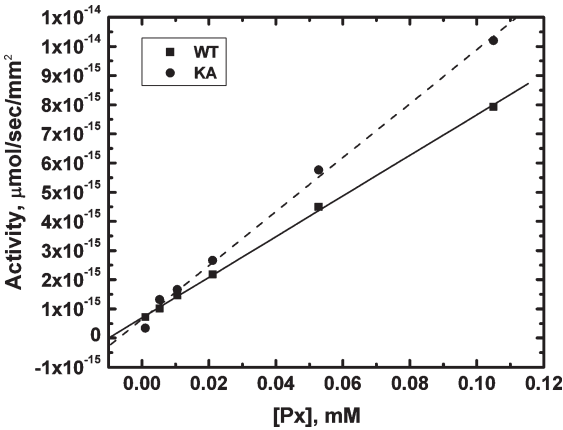


Figure 3. Activity of biotinylated WT and K175A on surface.

Table 2. Kinetic Constants for the Immobilized and Bulk Biotinylated Enzymes

kinetic parameters	solution		surface	
	WT	KA	WT	KA
<i>V_{max}</i>	0.00005 ± 1.5 × 10 ^{–6}	0.00003 ± 1.4 × 10 ^{–6}	0.00044 ± 1.6 × 10 ^{–4}	0.00055 ± 1.6 × 10 ^{–4}
<i>K_m</i> (mM)	0.0434 ± 0.005	0.0624 ± 0.008	0.1854 ± 0.094	0.2278 ± 0.089
<i>k_{cat}</i> (s ^{–1})	206.9	108		
<i>k_{cat}/K_m</i>	4.8 × 10 ⁶	1.7 × 10 ⁶		

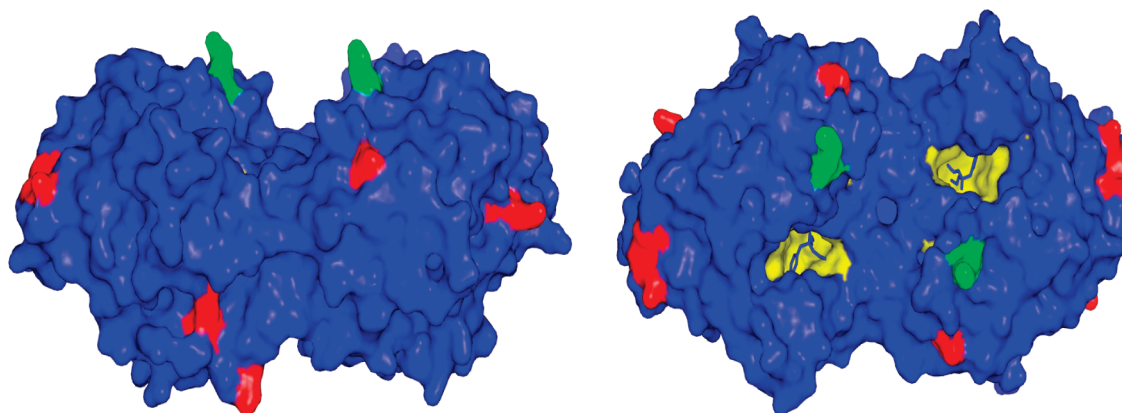


Figure 4. (A) Solvent exposed lysine side chains of OPH. K175 is shown in green, the others are in red. (B) The active site is shown in yellow to show the proximity to K175.

attachment of the K175A variant resulted in a 17% reduction in the number of molecules immobilized on the surface, as compared with that observed for the WT enzyme. In spite of this, the specific activity per mm^2 of surface is virtually identical between the enzymes, with immobilized K175A exhibiting a rate of $5.22 \times 10^{-15} \pm 2.8 \times 10^{-16} \text{ } \mu\text{mol/s/mm}^2$ with the WT measured rate at $4.30 \times 10^{-15} \pm 1.3 \times 10^{-16} \text{ } \mu\text{mol/s/mm}^2$ (paraoxon = 0.05 mM). This is expected only if the mutation of K175 to alanine resulted in an overall reduction of biotinylation of the enzyme population, leading to fewer molecules per surface area, while at the same time providing an improved catalytic environment for those enzymes which were attached.

3.4. Comparison of Enzyme Activity on Surface and in Solution. The activity of the biotinylated WT and K175A was measured both in solution and on the surface. The kinetic constants were determined using a Michaelis–Menten plot of initial reaction rates of free or immobilized enzyme (Table 2). The biotinylated WT has $\sim 48\%$ more activity in solution than that of K175A (Figure 2), whereas comparison of the surface attached enzymes determined the activity of K175A to be 18% higher than that of the WT enzyme. A calibration graph (Figure 3) for the activity of the immobilized enzymes against the paraoxon concentration was obtained. A linear model was fit to the K175A ($y = 6.4 \times 10^{-16} \pm 9.2 \times 10^{-14}x$, $R = 0.9979$) and WT ($y = 6.9 \times 10^{-16} \pm 6.9 \times 10^{-14}x$, $R = 0.9996$) data. This supports the hypothesis that surfaces with enhanced catalytic capacity can be created through the orientation-specified attachment of enzyme, which was made possible in this case through the creation and use of the K175A variant.

4. Discussion

Biotin forms an amide bond with the ζ -N of solvent exposed lysine side chains. Ordering the lysine residues, based on solvent accessible surface areas and the degree of exposure of the ζ -N, allows an evaluation of the likelihood any given side chain will be biotinylated. The more solvent accessible surface area (SASA) and the more exposed the ζ -N, the higher the probability of biotinylation. Using this approach, there are four lysines with an SASA of greater than 50% for the ζ -N. Although two, K165 and K294, were assessed as fully solvent exposed, it is likely that all four are candidates for biotinylation and can serve as a site for surface attachment.

The most exposed surface lysine in OPH is K175, which is also the most proximate to the active site (Figure 4). Immobilization of OPH through this side chain would result in its attachment in an essentially “face down” manner with the active site oriented

toward the sensor surface, potentially resulting in reduced catalytic rates by occluding the active site. Taken together, the data presented in this study: (1) the k_{cat} of the biotinylated K175A enzyme, in solution, is reduced 48% relative to that of the biotinylated WT; (2) the attachment of K175A to the surface is reduced 17% relative to that of WT; and (3) the activity of K175A surfaces are slightly higher than WT surfaces, support a critical role for orientation of the enzyme on the sensor surface. Modeling of the substrate interaction with active site structures indicates that even small changes in the active site structure and/or accessibility of the substrate to the active site may dramatically change the catalytic process.

The activity of K175A is 18% greater than that of the WT enzyme after immobilization on a surface, when the number of protein molecules per mm^2 is taken into account. Taken together, the data presented in this study suggest the increased activity is the result of enzyme orientation, potentially through facing the active site toward the bulk solvent. Alternatively, elimination of surface attachment through residue 175 could allow for a more efficient enzyme by permitting flexibility in secondary structural elements near the active site.

5. Conclusions

In conclusion, the orientation-specific attachment of an enzyme, OPH, was studied for biosensor applications. Substitution of a lysine residue near the active site of the enzyme resulted in an increase in the catalytic efficiency of the enzyme when surface attached, as determined by SPR. By selectively removing attachment sites, in this case a lysine from the protein surface, the enzyme molecules can be site(s) selectively attached, and thus, the orientation of the protein molecules on the surface can be controlled.

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