Anal. Chem. **1998,** *70,* 5042−5046

Biosensor for Direct Determination of Organophosphate Nerve Agents Using Recombinant *Escherichia coli* with Surface-Expressed Organophosphorus Hydrolase. 2. Fiber-Optic Microbial Biosensor

Ashok Mulchandani,* Irina Kaneva, and Wilfred Chen

Department of Chemical and Environmental Engineering, University of California, Riverside, California 92521

A fiber-optic microbial biosensor suitable for direct measurement of organophosphate nerve agents was developed. The unique features of this novel microbial biosensor were the recombinant *Escherichia coli* cells expressing the enzyme organophosphorus hydrolase on the cell surface and the optical detection of the products of enzyme-catalyzed organophosphate hydrolysis. The use of cells with the metabolic enzyme expressed on the cell surface as a biological sensing element provides advantages of no resistance to mass transport of the analyte and product across the cell membrane and low cost due to elimination of enzyme purification, over the conventional microbial biosensors based on cells expressing enzyme intracellularly and enzyme-based sensors, respectively. The use of an optical transducer allows the detection of different organophosphates in a mixture, presently not feasible with acetylcholinesterase-based biosensors. *E. coli* cells expressing organophosphorus hydrolase (OPH) on the cell surface were immobilized in low melting temperature agarose on a nylon membrane and attached to the common end of a bifurcated fiber-optic bundle. OPH-expressing *E. coli* cells catalyzed the hydrolysis of organophosphorus pesticides to form stoichiometric amounts of chromophoric products that absorb light at specific wavelengths. The backscattered radiation of the specific wavelength incident light was measured using a photomultiplier detector and correlated to the organophosphate concentration. The best sensitivity and response time were obtained using a sensor constructed with 1.5 mg of cells operating in pH 9, 50 mM HEPES buffer with 100 mM NaCl and 0.05 mM $CoCl_2$ at 30 °C. At optimized conditions, the biosensor measured paraoxon, parathion, and coumaphos pesticides with high selectivity against triazine and carbamate pesticides in approximately 10 min. The lower detection limits were 3 μM for paraoxon and parathion and 5 μM for coumaphos. When stored in the buffer at 22 °C, the biosensor was stable for over a 1-month period and showed no decline in the response for over 75 repeated usages. The new fiber-optic microbial biosensor is an ideal tool for on-line monitoring of the detoxification process for organophosphate pesticides-contaminated wastewaters but may not be suitable for environmental monitoring.

At a time when public concern about organophosphorus pesticide residues in food, water, and the environment is increasing, the use of these pesticides in agriculture has also increased.[1,2] A large volume of hazardous wastes containing these neurotoxic compounds are generated at both the producer and consumer levels.[3] Additionally, the recently ratified Chemical Weapons Treaty requires the United States to destroy all of its chemical weapons arsenal, including the organophosphorus-based nerve gases, within 10 years.[4,5] The above pressing needs has stimulated the research and development of technologies to effectively detoxify/destroy organophosphorus compounds (OPs).[4−11] The successful use of any laboratory-developed technology for detoxification of the organophosphate neurotoxins will require analytical tools for monitoring concentrations of these neurotoxins.

Acetylcholinesterase (AChE) inhibition-based biosensors have been an active research area over the past decade. Although sensitive and useful as single-use disposable sensors for environmental monitoring, these biosensors are not suitable for on-line process monitoring application. Biosensors based on organophosphorus hydrolase (OPH), an enzyme that catalyzes hydrolysis

* Corresponding author. Mailing address: College of Engineering, Bourns Hall, University of California, Riverside, CA 92521. Phone: (909) 787-6419. Fax: (909) 787-3188. E-mail: adani@engr.ucr.edu.

(1) Food and Agricultural Organization of the United Nations, Rome. *FAO Prod. Yearb.* **1989**, *43*, 320.
(2) United States Department of Agriculture. *Agricultural Statistics*; United States Government Printing Office: Washington, DC, 1992; p 395.
(3) Munnecke, D. M. *J. Agric. Food Chem.* **1980**, *28*, 105−111.
(4) Kolakowski, J. E.; DeFrank, J. J.; Harvey, S. P.; Szafraniec, L. L. *Biocat. Biotransform.* **1997**, *15*, 297−312.
(5) Rastogi, V. K.; DeFrank, J. J.; Cheng, T. C.; Wild, J. R. *Biochem. Biophys. Res. Commun.* **1997**, *241*, 294−296.
(6) Mulbury, W. M.; Del Valle, P. L.; Karns, J. S. *Pestic. Sci.* **1996**, *48*, 149−155.
(7) Grice, K. J.; Payne, G. F.; Karns, J. S. *J. Agric. Food Chem.* **1996**, *44*, 351−357.
(8) LeJeune, K. E.; Mesiano, A. J.; Bower, S. B.; Grimsley, J. K.; Wild, J. R.; Russell, A. J. *Biotechnol. Bioeng.* **1997**, *54*, 105−114.
(9) Chen, W.; Mulchandani, A. *Trends Biotechnol.* **1998**, *16*, 71−76.
(10) Caldwell, S. R.; Raushel, F. M. *Biotechnol. Bioeng.* **1991**, *37*, 103−109.
(11) Munnecke, D. M. *Appl. Environ. Microbiol.* **1976**, *32*, 7−13.

10.1021/ac980643l CCC: $15.00
Published on Web 10/31/1998

of a range of organophosphate esters, pesticides such as parathion, coumaphos, and acephate, and chemical warfare agents such as soman, sarin, VX, and tabun, have been reported.[12−15] The detection scheme based on monitoring the OPH-catalyzed hydrolysis products of OPs offers the advantages of simpler, more direct, and quicker measurements of only the organophosphorus class of nerve agents over that based on the inhibition of acetylcholinesterase activity, which responds to all types of nerve agents and many more toxic chemicals. Additionally, the OPH-based biosensor has the potential of quantifying individual organophosphate pesticides when used as a detector in conjunction with HPLC for chromatographic separation.[13] These biosensors, due to relatively high detection limits, however, may not be suitable for environmental monitoring.

Biosensors based on immobilized cells have been demonstrated to be an alternative biological sensing element to enzymes. Many examples of microbial biosensors for a variety of applications have been reported.[16] Recently, we reported a potentiometric microbial biosensor based on recombinant *E. coli* cells carrying plasmid pJK33 that expressed native OPH intracellularly.[14] The major limitations of this biosensor were its low stability and an initial interference by glucose. These problems were alleviated by using another recombinant *E. coli* cell that had active OPH anchored and displayed on its surface.[13] Although elegant, the inability to discriminate between different organophosphates was a limitation of this potentiometric microbial biosensor.

This paper describes the construction, characterization, and evaluation of a fiber-optic microbial biosensor (FOMB) based on recombinant *E. coli* cells expressing OPH on the cell surface for the direct, selective, and sensitive determination of organophosphate nerve agents.

MATERIALS AND METHODS

Reagents. Low melting temperature agarose was acquired from Gibco. HEPES, glycine and CHES was purchased from Sigma Chemical Co. (St. Louis, MO). Paraoxon, parathion, coumaphos, pentachlorophenol, sevin, sutan, atrazine, and simazine were acquired from Supelco Inc. (Bellefonte, PA). Biodyne A nylon membrane, 5-μm pore size, was a gift from the Pall Corp. (East Hills, NY). All the solutions were made in distilled deionized water.

Bacterial Strains and Growth Conditions. The details of the recombinant *E. coli* strain anchoring and displaying OPH on the cell surface and growth conditions used in this study have been described previously.[13]

Experimental Setup. *Apparatus.* Figure 1 shows a block diagram of the FOMB arrangement. All the components were acquired from Photon Technology International, Inc. (Monmouth, NJ). The light source was a 75-W xenon arc lamp (model 02-5002X), housed in PowerArc lamp housing, that was powered by a constant voltage dc lamp power supply (model LPS-220) with

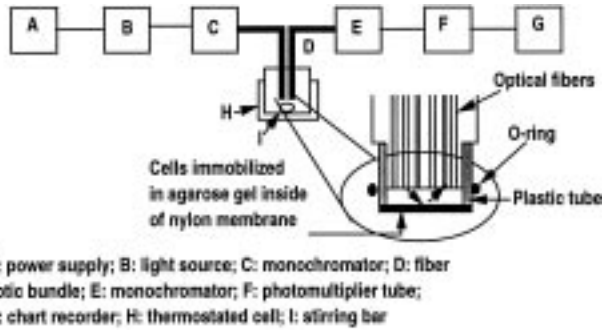

Figure 1. Schematic drawing of the fiber-optic microbial biosensor.

igniter (model LPS-221). Light from this source passed through a monochromator (model 101) set at a desired cut-off wavelength (403 nm for *p*-nitrophenol, hydrolysis product of paraoxon and parathion, and 373 nm for chlorferon, hydrolysis product of coumaphos) before being focused onto the input arm of a 0.5-m bifurcated quartz fiber-optic bundle. Backscattered radiation was then transported through the other arm of the bifurcated fiber bundle to the second monochromator, set at the same wavelength as the first one, before being detected by a photomultiplier detection system (model 814). The output from the detection system was displayed on a strip chart recorder (model BD112, Kipp and Zonen, Holland). To eliminate problems of ambient light interference, the above system was isolated by using thick black curtains.

Sensor Tip. Figure 1 shows the details of the sensor tip. It consisted of the optical fiber bundle common end (7 mm diameter) encased by an 11-mm-o.d. × 12.5-mm-long cylinder on which a tightly fitting plastic tube (11 mm i.d. × 1 mm thick × 13.3 mm long) with two open ends was mounted. To the open end of this plastic tube was attached a nylon membrane with immobilized cells (please see below) by an O-ring. This arrangement provided an approximately 0.8-mm gap between the optical fiber flush end and the immobilized cells, the path length for backscattered irradiation.

Cell Immobilization. A predetermined weight of *E. coli* XL-1 Blue cells expressing OPH suspended in 100 μL of buffer A was mixed with 100 μL of 1% melted low melting temperature agarose at 37 °C. The mixture was immediately applied onto the inner surface of 5-μm-pore size Biodyne A membrane affixed with the O-ring to one of the open ends of the plastic extension tube (described above) and allowed to solidify at room temperature for 1 h.

Experimental Setup and Measurement. The experimental setup is schematically depicted in Figure 1. All measurements were made in 5 mL of an appropriate buffer, maintained at the desired temperature, in a 10-mL working volume jacketed glass cell, equipped with a magnetic stirrer. The temperature of the liquid in the cell was controlled by circulating water in the cell jacket using a circulating water bath (model 1160, VWR Scientific, San Francisco, CA). At the start, the dark (background) current was recorded by closing the entrance slit of the collection monochromator. Subsequently, the entrance slit of this monochromator was opened, and the incident/initial intensity (I_o) was recorded. A 5−10-μL portion of OP nerve agent, dissolved in 5% methanol (paraoxon and parathion) or pure methanol (coumaphos), was then added to the cell, and the trace of the change in

(12) Mulchandani, P.; Mulchandani, A.; Kaneva, I.; Chen, W. *Biosens. Bioelectron.* In press.
(13) Mulchandani, A.; Mulchandani, P.; Kaneva, I.; Chen, W. *Anal. Chem.* **1998**, *70*, 4140−4145.
(14) Mulchandani, A.; Chauhan, S.; Mulchandani, P.; Kaneva, I.; Chen, W. *Electroanalysis* **1998**, *10*, 733−737.
(15) Rainina, E.; Efremenco, E.; Varfolomeyev, S.; Simonian, A. L.; Wild, J. R. *Biosens. Bioelectron.* **1996**, *11*, 991−1000.
(16) Karube, I.; Nakanishi, K. *Curr. Opin. Biotechnol.* **1994**, *5*, 54−59.

Table 1. Effect of Cell Loading on the Fiber-Optic Microbial Biosensor Response

cell dry weight, mg	response, $(-1/I_o)\ dI/dt$, min^{-1} [a]
0.5	0.0034
1.0	0.0046
1.5	0.0073
2.0	0.0062
2.5	0.0052
3.0	0.0046

[a] Response to 0.03 mM parathion in pH 8.5, 50 mM HEPES + 100 mM NaCl + 0.05 mM $CoCl_2$ at 30 °C.

light intensity as a function of time was recorded. The recorded data were used to determine the kinetic response of the FOMB. The kinetic response measurement, the initial rate of change of absorbance, was determined from eq 1, where dI/dt is the initial rate of intensity change (the slope of the tangent to the response curve at $t = 0$).

$$dA/dt = -(1/I_o)\ dI/dt \qquad (1)$$

RESULTS AND DISCUSSION

Effects of Various Parameters on FOMB Response. The sensitivity, change of absorbance per unit change in analyte concentration, and response time of a FOMB are functions of the buffer pH and the cell weight immobilized on the surface of the optical fiber. Experiments were performed to investigate the effects of these variables on the rate of change of the initial response of the electrode to injections of parathion.

The initial rate of change of absorbance was a function of the cell weight immobilized in the low melting temperature agarose gel at the tip of the fiber bundle (Table 1). The rate of absorbance change first increased, reaching a maximum at 1.5 mg cell loading, and then decreased rapidly. This trend is similar to that reported for other microbial biosensors.[17,18] The observed profile can be attributed to the heterogeneous nature of the biosensor configuration, in which either the kinetics of the catalytic reaction or the mass transport is the rate-limiting step in the sensor response. The initial increase is attributed to an increased catalytic activity of the OPH enzyme responsible for OP hydrolysis. However, as the cell loading in the immobilized layer is increased the cells get packed more densely, forming a thicker layer, and slow the mass transport rate. A cell loading of 1.5 mg dry weight was used subsequently for optimal response.

Table 2 shows the pH profile for the FOMB. This profile is similar to that of the free enzyme.[12] This suggests that the observed pH dependence of the sensor response is due to the pH dependence of the OPH activity present on the cell surface. The optimum pH for the FOMB is 0.5 unit higher than that for the free enzyme. Such a shift in optimum pH for immobilized enzymes is not uncharacteristic and can be attributed to the alteration of the physicochemical characteristics of the enzyme upon immobilization.[19] pH 9, which gave the maximum rate of absorbance change, was selected for subsequent studies.

(17) Kingdon, C. F. M. *Appl. Microbiol. Biotechnol.* **1985**, *21*, 176−179.
(18) Luong, J. H. T.; Mulchandani, A.; Groom, C. A. *J. Biotechnol.* **1989**, *10*, 241−252.

Table 2. Effect of pH on the Fiber-Optic Microbial Biosensor Response

pH	buffer type	response $(-1/I_o)\ dI/dt$, min^{-1} [a]
6	HEPES	0.0009
7	HEPES	0.0035
8.5	HEPES	0.0062
9	HEPES	0.0085
8	CHES	0.0054
9	CHES	0.0086
10	CHES	0.0081
8.5	glycine	0.0086
9	glycine	0.0076

[a] Response to 0.03 mM parathion in 50 mM buffer + 100 mM NaCl + 0.05 mM $CoCl_2$ at 30 °C. Cell loading, 1.5 mg dry weight.

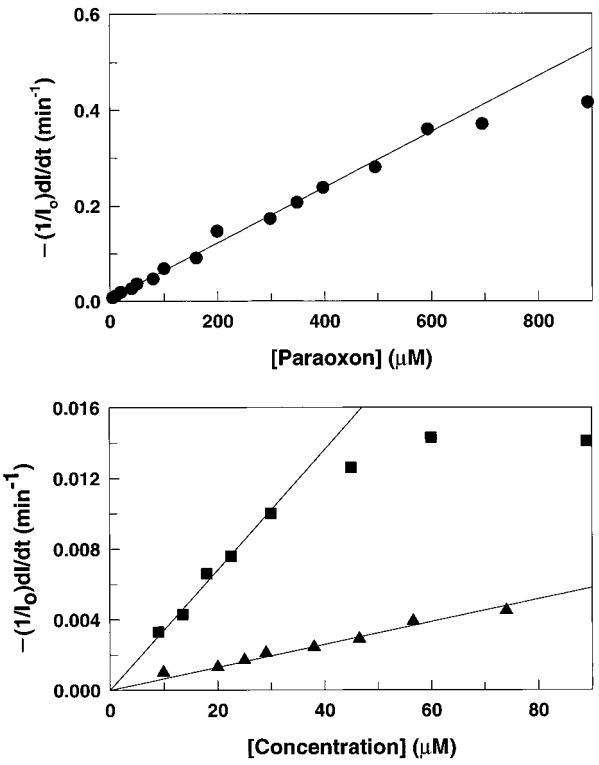

Figure 2. Calibration plots for organophosphates. Operating conditions: 50 mM HEPES + 100 mM NaCl + 0.05 mM $CoCl_2$, pH 9, 30 °C; 1.5 mg of cells. (●) Paraoxon, (■) parathion, (▲) coumaphos.

Table 3. Analytical Characteristics of the Fiber-Optic Microbial Biosensor

compound	sensitivity, $min^{-1}\ \mu M^{-1}$	linear range, mM	detection limit, μM
paraoxon	5.98×10^{-4}	0.0−0.6	3
parathion	3.4×10^{-4}	0.0−0.03	3
coumaphos	6.46×10^{-5}	0.0−0.075	5

Analytical Characteristics of FOMB. *Calibrations for Organophosphates.* Figure 2 and Table 3 show the calibration plots and the important analytical characteristics, respectively, of the FOMB for parathion and paraoxon (like parathion, paraoxon hydrolysis also produces *p*-nitrophenol).

(19) Mulchandani, A.; Male, K. B.; Luong, J. H. T. *Biotechnol. Bioeng.* **1990**, *35*, 739−745.

To demonstrate the ability of the FOMB to determine different OPs in a mixture, the sensor was used to measure coumaphos. The λ_{max} (maximum absorbance wavelength) for chlorferon, the hydrolysis product of coumaphos at pH 9, experimentally determined to be 373 nm, is far from the λ_{max} of 403 nm for *p*-nitrophenol. The calibration plot and important analytical characteristics of FOMB for coumaphos are given in Figure 2 and Table 3, respectively.

The FOMB sensitivity was in the order paraoxon > parathion > coumaphos. This is in agreement with model-predicted[20] trend based on the K_M and V_{max}/K_M of the OPH enzyme for these compounds.[21] Additionally, the extinction coefficient of chlorferon is about 2-fold lower than that for *p*-nitrophenol, which would also affect the FOMB sensitivity for coumaphos.[21] The ability to potentially measure multiple organophosphates directly in a mixture is an advantage of the present FOMB over the OPH-based potentiometric biosensors.[12−15]

The 3−5 μM lower detection limit (3 times the standard deviation of the response obtained for blanks/buffer) of the FOMB is comparable to those for the OPH-based microbial[13−15] and enzyme[12] biosensors. However, this limit is still 10-fold higher than that of the biosensors based on AChE inhibition. This will, therefore, limit the applicability of the present sensor for environmental monitoring to off-line analysis. For any such application of the present FOMB, off-line sample preparation involving solvent extraction and concentration will be necessary. The present FOMB, however, will be an ideal tool for (1) on-line monitoring of detoxification processes for treatment of wastewater generated during production and consumption of the organophosphate-based pesticides and insecticides and disposal of organophosphate-based nerve agents, (2) selectively monitoring only the organophosphate-based pesticides/neurotoxins, and (3) monitoring individual organophosphates simultaneously in a mixture.

Selectivity. The FOMB was highly selective for organophosphates. Other pesticides, such as atrazine, sevin, sutan, pentachlorophenol, and simazine, even at 20 μM concentrations, did not interfere with the monitoring of the three OPs tested. This high selectivity is a great advantage over the AChE-based biosensors, especially for on-line monitoring of detoxification processes. Unlike the present biosensor, AChE-based biosensors are interfered by not only the other pesticides but also other potential inhibitors of AChE activity, such as heavy metals.[13]

Reproducibility. The FOMB response was fairly reproducible (RSD = 7.26% for $n = 14$). Additionally, a very low relative standard deviation of 1.48% between three different calibration plots for paraoxon prepared using the same sensor tip over a 6-day period again demonstrates the high reproducibility of the FOMB. The low RSD of 2.6% ($n = 4$) between the different FOMB sensor tips prepared during the duration of this research work illustrates a good biosensor-to-biosensor reproducibility.

Accuracy. Table 4 shows a comparison between the concentrations of paraoxon determined by the FOMB and the conventional enzymatic assay (based on the concentration of *p*-nitrophenol formed by the enzyme-catalyzed hydrolysis of paraoxon) in simulated samples representing a feed to a biological (enzymatic/microbial) detoxification process.[6−11] The excellent agreement between the two methods (slope = 1.075; $r^2 = 0.999$) indicates excellent accuracy and reliability of the sensor.

Table 4. Comparison of Paraoxon Measurements by Fiber-Optic Microbial Biosensor and Enzyme Assay

measured by enzymatic assay, μM	measured by FOMB, μM
44	46 ± 0.8 ($n = 3$)
35	35 ± 0.2 ($n = 3$)
70	73 ± 4 ($n = 3$)

Table 5. Stability of the Fiber-Optic Microbial Biosensor Response

time, days	response, $(-1/I_o)\ dI/dt$, min^{-1} [a]
0	0.02 ± 0.001 ($n = 4$)
2	0.021 ± 0.001 ($n = 4$)
4	0.022 ± 0.001 ($n = 4$)
15	0.024 ± 0.001 ($n = 4$)
18	0.024 ± 0.002 ($n = 4$)
22	0.023 ± 0.002 ($n = 4$)
26	0.024 ± 0.002 ($n = 4$)
32	0.021 ± 0.007 ($n = 4$)

[a] Response to 0.035 mM paraoxon in 50 mM HEPES + 100 mM NaCl + 0.05 mM $CoCl_2$, pH 9, at 30 °C. Cell loading, 1.5 mg.

Stability. The long-term storage and multiple-use stability of the FOMB sensor tip were investigated by evaluating the response of the sensor to paraoxon and storing the tip back at 22 °C in pH 9, 50 mM HEPES +100 mM NaCl + 0.05 mM $CoCl_2$ buffer. As shown in Table 5, the biosensor had excellent stability for over a month of investigation. During this period, the same tip was used for a total of 75 times. The multiple-use stability of the present FOMB is significantly better than that for AChE-based biosensors, which lose as much as 40% of their original response after as few as three repeated uses. The low stability of AChE-based biosensors can be attributed to the inactivation of AChE every time it is exposed to some of the strong inhibitors.[22−24]

Response Time. The detection of OPs by the FOMB is a simple, single-step, and direct measurement. The analysis time for each sample was less than 10 min (data not shown). This time is comparable to those of the OPH-based enzyme and microbial biosensors.[13−15] In contrast, AChE-based biosensors involve multiple steps (measurement of the initial AChE activity, incubation with the neurotoxin, determination of the inhibited enzyme activity, and regeneration of enzyme activity), and analysis times range anywhere from 15 min for the disposable type (where the enzyme reactivation step is excluded and hence unsuitable for multiple-use on-line process monitoring) to 5 h for reusable types.[13]

CONCLUSIONS

The expression of enzymes on the surface of cell membrane eliminates the need for isolation and purification of enzymes, as

(20) Schultz, J. S. In *Handbook of chemical and biological sensors*. Taylor, R. F., Schultz, J. S., Eds.; Institute of Physics Publishing: Bristol, U.K., 1996; pp 171−201.
(21) Dumas, D. P.; Caldwell, S. R.; Wild, J. R.; Raushel, F. M. *J. Biol. Chem.* **1989**, *33*, 19659−19665.
(22) Marty, J.-L.; Soda, K.; Karube, I. *Electroanalysis* **1992**, *4*, 249−252.
(23) La Rosa, C.; Pariente, F.; Hernandez, L.; Lorenzo, E. *Anal. Chim. Acta* **1994**, *295*, 273−282.
(24) Dzyadevich, S. V.; Soldatkin, A. P.; Shul'ga, A. A.; Strikha, V. I.; El'Skaya, A. V. *J. Anal. Chem.* **1994**, *49*, 874−878.

in the case with enzyme-based sensors, thereby reducing the overall cost. It also alleviates the problems of the loss of the desired enzyme due to proteolysis and mass-transfer barrier due to the cell membrane encountered in microbial biosensors constructed with cells expressing enzyme intracellularly. In a previous report on the application of the *E. coli* cells expressing OPH on the cell surface in a potentiometric microbial biosensor for OPs, we demonstrated that, for all purposes, these cells act more like the OPH enzyme in terms of long-term stability. Hence, this microbial biosensor is similar to any enzyme biosensor, with the cell wall basically acting as an immobilization support for the enzyme. Consequently, theoretical models for enzyme-based biosensors can be applied to predict the direction of future research to improve the sensitivity and detection limit of this microbial biosensor. These models predict that the sensitivity and detection limit of enzyme electrodes based on nonreactive transducer can be improved by either lowering the enzyme K_M or increasing the bimolecular rate constant.[20,25,26] The advancements in enzyme engineering have now made these goals potentially achievable. One such example is the use of site-directed mutagenesis of OPH to improve the rate of hydrolysis of the chemical warfare agent, soman.[27] Thus, it is foreseeable that a microbial biosensor of higher sensitivity and lower detection limit will be realizable by expressing an OPH variant that has a lower K_M and/or higher bimolecular rate constant for the nerve agents to be analyzed.

(25) Caras, S. D.; Janata, J.; Saupe, D.; Schmitt, K. *Anal. Chem.* **1985**, *57*, 1917−1920.

(26) Carr, P. W.; Bowers, L. D. *Immobilized enzymes in analytical and clinical chemistry: Fundamentals and applications;* John Wiley & Sons: New York, 1980; pp 197−310.

(27) Lai, K.; Grimsley, J. K.; Kuhlmann, B. D.; Scapozza, L.; Harvey, S. P.; DeFrank, J. J.; Kolakowski, J. E.; Wild, J. R. *Chimiai* **1996**, *50*, 430−431.

ACKNOWLEDGMENT

This work was supported by a grant from the U.S. EPA (R8236663-01-0).

Received for review June 11, 1998. Accepted September 17, 1998.

AC980643L