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Improved Sensitivity of a Histamine Sensor Using an Engineered Methylamine Dehydrogenase

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Methylamine dehydrogenase (MADH) may be immobilized in a polypyrrole (PPy) film on an electrode surface and used as an amperometric sensor for the determination of histamine. Using site-directed mutagenesis, phenylalanine 55 on the α subunit of MADH was converted to alanine. This αF55A MADH exhibits a 400-fold lower K_m value for histamine than does native MADH when assayed in solution. An αF55A MADH−PPy sensor was constructed, and its properties were compared to that of the native MADH−PPy sensor. The αF55A MADH immobilized on the electrode exhibited Michaelis−Menten behavior in response to varied concentrations of histamine with an ∼3-fold lower K_m value than that exhibited by the immobilized native MADH. The detection limit for the native MADH−PPy sensor was ∼20 μM while the αF55A MADH−PPy sensor exhibited a detection limit of ∼5 μM, a 4-fold increase compared to the native MADH−PPy sensor. This work highlights the potential value of using site-directed mutagenesis to engineer enzymes to alter and improve biosensor performance.

Methylamine dehydrogenase (MADH) catalyzes the oxidative deamination of primary amines.[1] MADH from *Paracoccus denitrificans* possesses an $\alpha_2\beta_2$ structure and subunit molecular weights of 46 700 and 15 500.[2] Each smaller β subunit possesses a tryptophan tryptophylquinone[3] (TTQ) prosthetic group (Figure 1), which is formed by posttranslational modifications of Trp57 and Trp108 of the β subunit. As is true of quinoproteins in general, MADH does not require pyridine nucleotides (NAD^+ or $NADP^+$) and does not react with oxygen.[1,4] The natural electron acceptor for MADH is another redox protein, amicyanin,[5] a type I copper protein. These properties make quinoproteins desirable candidates for the biological component of enzyme-based electrodes. MADH has its highest affinity for short-chain aliphatic amines and methylamine is its preferred substrate.[6] However, it will also recognize other larger primary amines such as histamine.

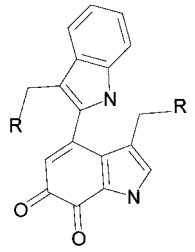

Figure 1. Structure of the tryptophan tryptophylquinone prosthetic group of methylamine dehydrogenase.

We previously reported the immobilization of MADH in a polypyrrole (PPy) film on an electrode surface and the use of this enzyme electrode as a sensor to detect and quantitate histamine levels in solution.[7] That was the first report of a sensor that was based on a TTQ-dependent enzyme. That sensor was able to reliably detect histamine over a concentration range from approximately 25 μM to 4 mM. We have also extensively studied the catalytic and electron-transfer reactions of MADH in solution with a wide range of amine substrates and with artificial and natural electron acceptors.[1,8] Recently we used site-directed mutagenesis to alter the substrate specificity of MADH by conversion of phenylalanine 55 of the α subunit of MADH to alanine.[9] The phenyl group of αPhe55 serves two functions in determining the substrate specificity of MADH. It interacts with the methyl group of the normal substrate, methylamine, to help orient the substrate's amino group for nucleophilic attack of TTQ. It also excludes amines with longer and bulkier carbon chains from the active site.[9] The αF55A mutation transformed MADH into an enzyme that instead exhibits a preference for long-chain amines rather than short-chain amines.

In this paper, we investigate the reactivity of αF55A MADH toward histamine relative to native MADH, both in solution and when immobilized in the enzyme electrode. Compared to native MADH, the K_m value of αF55A MADH for histamine in solution was increased 400-fold while that of the immobilized αF55A

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MADH was increased ~3-fold. The detection limit of the αF55A MADH electrode was 4-fold lower than that of the native MADH electrode. The reasons for these similarities and differences between native and αF55A MADH in solution and immobilized in the electrode are discussed. The results of these studies demonstrate that it is possible to modify and improve the response of an enzyme-based biosensor by site-directed mutagenesis of the enzyme component of an amperometric sensor. This study provides an example of how site-directed mutagenesis of enzymes can be a useful approach for the logical design of enzyme-based biosensors.

EXPERIMENTAL SECTION

Chemicals and Materials. Native MADH was purified from *P. denitrificans* as described previously.[10] The αF55A site-directed mutant of MADH was prepared, expressed in *Rhodobacter sphaeroides*, and purified as described previously.[9]

Nafion perfluorinated ion-exchange resin (5 wt % solution in a mixture of alcohol and water) was obtained from Aldrich. All other chemicals were reagent grade. Deionized, doubly distilled water was used to prepare all aqueous solutions. Glassy carbon (GC) electrodes were obtained from Cypress Systems, Inc. (Lawrence KS).

Preparation of Electrodes. GC electrodes (1.5-mm diameter) were polished successively with 1-, 0.3-, and 0.05-μm alumina suspensions (Buehler). Then they were sonicated for 5 min in deionized, doubly distilled water, followed by extensive rinsing with deionized, doubly distilled water.

Enzyme−PPy films of native MADH and αF55A MADH were formed on the GC electrode by applying a constant potential at 750 mV versus Ag/AgCl (3 M KCl) in an aqueous solution containing 30 mg/mL of either native or αF55A MADH, 0.35 M pyrrole, and 0.05 M potassium ferricyanide. After the formation of the native or αF55A MADH−PPy film, the electrode was further coated with Nafion as described previously.[7] It was previously shown that this concentration of ferricyanide was needed to keep MADH in the oxidized form and to mediate electron transfer from reduced MADH to the electrode surface in the enzyme−PPy film. Ferricyanide is not added during the measurements since the Nafion coating will serve as a barrier that will retain this mediator in the film as well as prevent it from entering during measurements. The thickness of the enzyme−PPy film was controlled by the charge passed during the electropolymerization and was estimated, as described previously,[11] to be 1000 Å by passing 48 mC/cm^2. For the enzyme electrodes used in this study, sufficient charge was passed during electropolymerization to form films that were ~200 Å thick.

Electrochemical Measurements. Electrochemical experiments were carried out with a CH Instruments model CHI 832 electrochemical detector. The electrochemical cell that was used consisted of a mini (1.5-mm diameter) GC electrode as the working electrode, a platinum wire as the auxiliary electrode, and an Ag/AgCl (3 M KCl) as the reference electrode. The amperometric response of histamine at the MADH−PPy film electrode in 0.1 M, pH 7.5 potassium phosphate solution containing 0.2 M KCl was measured by differential pulse amperometry (DPA). The parameters used for DPA measurements are following: $E_i = 0.0$ V, $t_i = 0.8$ s, $E_1 = 0.05$ V, $t_1 = 0.1$ s, $E_2 = 0.35$V, and $t_2 = 0.1$ s. This condition provided the maximum response for the detection of histamine because the $Fe(CN)_6^{4-}$/ $Fe(CN)_6^{3-}$ pair mediated the redox reaction at the electrode surface. All the electrochemical measurements were performed at room temperature (22 ± 2 °C).

Kinetic Studies. Steady-state kinetic assays[10] were performed in potassium phosphate, pH 7.5, at 30 °C. The assay mixture contained 16 nM MADH, 170 μM 2,6-dichlorophenolindophenol (DCIP), 4.8 mM phenazine ethosulfate, and varied concentrations of histamine. The reaction was initiated by the addition of histamine and monitored at 600 nm to determine the rate of reduction of DCIP. Data were fit to the Michaelis−Menten equation (eq 1), where v is the measured initial rate, E is the

$$v/E = k_{cat}[S]/(K_m + [S]) \quad (1)$$

MADH concentration, [S] is the histamine concentration, k_{cat} is the turnover number, and K_m is the Michaelis constant. Concentrations of enzyme are given as that of the holoenzyme, which possesses two identical active sites.

The concentration dependence of the current response of the MADH−PPy electrodes was also analyzed using the same Michaelis−Menten model. The data were fit to eq 2, where I is

$$I = I_{max}[S]/(K_m + [S]) \quad (2)$$

the current response and I_{max} is the maximum current response at saturating concentrations of histamine.

Transient kinetic experiments were performed using an On-Line Instrument Systems (OLIS, Bogart, GA) RSM1000 rapid-scanning stopped-flow spectrophotometer. The experimental procedures for the rapid mixing experiments were as previously described for the reaction of the MADH with methylamine.[12] Reactions were performed in 0.25 M potassium phosphate, pH 7.5. The reaction of oxidized MADH with histamine was monitored by the decrease in absorbance at 450 nm, which corresponds to the conversion of MADH from the oxidized to reduced form. The absorbance changes with time could be fit to the equation for a single-exponential decay. The data for the concentration dependence of k_{red} were analyzed according to the following model (eqs 3 and 4).

$$\mathrm{MADH_{oxidized}} + \mathrm{substrate} \overset{K_d}{\rightleftharpoons} \mathrm{MADH_{oxidized}/substrate} \overset{\substack{k_{red} \\ \rightleftharpoons \\ -k_{red}}}{} \mathrm{MADH_{reduced}/product} \quad (3)$$

$$k_{obs} = \frac{k_{red}[\mathrm{histamine}]}{[\mathrm{histamine}] + K_d} + -k_{red} \quad (4)$$

RESULTS

Effects of the αF55A Mutation on the Response to Histamine in Solution. Steady-state kinetic analysis of αF55A MADH in solution revealed that its kinetic parameters for the oxidation of histamine were dramatically altered relative to native MADH, as a result of the site-directed mutation (Table 1). Its

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Table 1. Differential Responses to Histamine of Native and αF55A MADH Free in Solution and Immobilized in a Polypyrrole Electrode

	native MADH	αF55A MADH
	Electrode Response	
K_m (μM)	1580 ± 320	615 ± 55
detection limit (μM)	19.8 ± 6.7	5.2 ± 1.7
	Solution Response[a]	
K_m (μM)	3800 ± 300	9 ± 1
k_{cat} (s^{-1})	26 ± 1	1.8 ± 0.1
k_{red} (s^{-1})	100 ± 6	3.0 ± 0.6

[a] Steady-state kinetic experiments were performed in 0.1 M phosphate buffer, pH 7.5, with 0.2 M KCl, the same conditions used with the enzyme electrodes. The K_m value determined in solution for native MADH is dependent on ionic strength and is 620 mM in 10 mM phosphate with no added salt. Because of the dependence of K_m on buffer conditions, values reported in different studies may not exactly match if different buffer conditions are used.

affinity for histamine was significantly increased while its turnover number was significantly decreased. The K_m for histamine was ~400-fold smaller, and the k_{cat} was ~15-fold smaller. For comparison, these assays were performed in the same buffer used in the electrochemical studies, 0.1 M potassium phosphate, pH 7.5, plus 200 mM KCl.

To better understand the basis for these changes in steady-state kinetic parameters, transient kinetic studies were performed that directly monitored the reduction of MADH by histamine. The overall oxidation−reduction reaction of MADH with histamine is a multistep process,[13] as outlined in eq 5, where E is oxidized

$$E + S \rightleftharpoons ES \rightleftharpoons E'P_1P_2 \rightleftharpoons E'P_2 + P_1 \rightleftharpoons E' + P_2 \rightleftharpoons E + 2e^- \quad (5)$$

MADH, E′ is reduced MADH, S is the amine substrate, P_1 is the aldehyde product, and P_2 is the ammonia product. It was determined that the rate at which MADH is reduced within the substrate−enzyme complex (k_{red}) is ~30-fold slower with αF55A MADH (Table 1). In fact, this rate constant is approximately the same as the steady-state k_{cat} for the reaction of αF55A MADH. For native MADH, k_{red} is significantly faster than k_{cat}. Previous studies demonstrated that the rate-limiting step in the steady-state reaction of MADH with its preferred substrate, methylamine, is the release of the aldehyde product from the reduced enzyme.[14] These data indicate that the rate-limiting steps in the overall steady-state reaction in solution are different for αF55A MADH and native MADH and that the observed decrease in k_{cat} may be ascribed to the decrease in k_{red}, which is now slower than the aldehyde product release step.

Given the significant changes in kinetic parameters for the reaction of MADH with histamine that were caused by the αF55A mutation, it was of interest to see to what extent the properties of the MADH−PPy-based sensor would be affected by using αF55A MADH instead of native MADH. In particular, it was of interest to see whether the increased affinity for histamine in solution would yield any increase in sensitivity of the sensor and whether the decrease in k_{cat} for the reaction with histamine in solution would result in any decrease in signal intensity for the sensor. It should be noted that previous studies with native MADH were performed with MADH−PPy gold electrodes. The performance of the MADH−PPy GC electrode is essentially the same as that of the gold electrode. With the mutant MADH, it was not possible to form an acceptable αF55A MADH−PPy film on the gold electrode, although there was no such problem with the GC electrode. The reason for this is not known. For accurate comparison of the native and αF55A MADH sensors, GC electrodes were used for both in these studies. The αF55A MADH sensor was evaluated in two different types of studies. (i) The current response of the electrode to a wide range of substrate concentrations was determined and analyzed by a Michaelis−Menten kinetic model (eq 2). (ii) The detection limit of the electrode was determined by examining the signal-to-noise ratio at low concentrations of histamine.

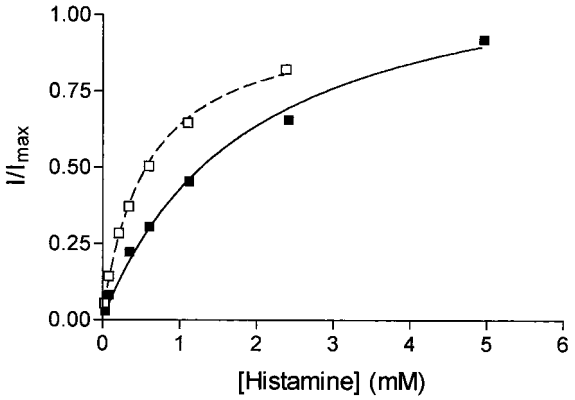

Figure 2. Response curves to histamine of native MADH−PPy (■) and αF55A MADH−PPy (□) electrodes. The electrode was in 0.1 M phosphate buffer, pH 7.5, containing 0.2 M KCl and the indicated concentrations of histamine. The conditions used to perform DPA were as follows: $E_i = 0.0$ V, $t_i = 0.8$ s, $E_1 = 0.05$ V, $t_1 = 0.1$ s, $E_2 = 0.35$ V, and $t_2 = 0.1$ s. The lines represent fits to eq 2. The relative standard deviation of repeated measurements was 3.9%.

Effects of the αF55A Mutation on the Response to MADH Immobilized in a PPy Electrode. The catalytic responses to histamine of both the native and αF55A MADH−PPy electrodes exhibited classical Michaelis−Menten kinetic behavior (Figure 2). The magnitude of the maximum current response obtained by the DPA method will vary depending upon the amount of enzyme that is immobilized on the enzyme surface. For the electrodes used in Figure 2, care was taken to prepare the electrodes by exactly the same protocol to minimize possible differences due to variation in the amount of enzyme present on the electrode. The maximum current responses at saturating concentration of histamine (I_{max} in eq 2) were 47 μA for native MADH and 26 μA for αF55A MADH. These are extrapolated values which are obtained from the fits of the data in Figure 2 to eq 2. This is a representative example of what was routinely observed. The I_{max} values for the native MADH−PPy electrodes were routinely greater than those of the αF55A MADH−PPy electrodes, but only by a factor of ~2. This is similar to what was observed in solution in that the k_{cat} value for native MADH was greater than that of αF55A MADH. However, it is different in that the I_{max} was only decreased 2-fold, whereas k_{cat} in solution was decreased 15-fold.

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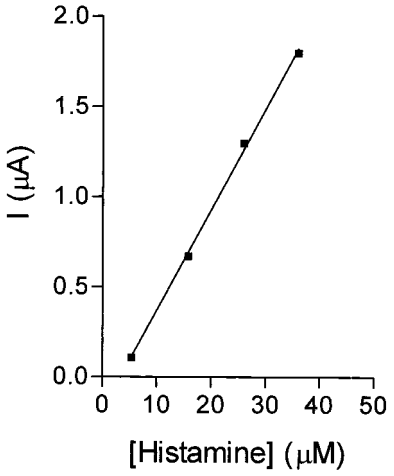

Figure 3. Response of the αF55A MADH−PPy electrode to histamine at the lower limits of detection. The electrode was in 0.1 M phosphate buffer, pH 7.5, containing 0.2 M KCl and the indicated concentrations of histamine. The conditions used to perform DPA were the same as in Figure 2. The data are fit to a straight line by linear regression analysis.

The relative affinity of the immobilized MADH for histamine was clearly altered by the αF55A mutation. The K_m, which in this case the concentration of histamine that yields half the maximum current response, is smaller for the αF55A MADH−PPy electrode than for the native MADH−PPy electrode. This result is similar to what was observed in solution in that the K_m value for native MADH was greater than that of αF55A MADH. However, in solution, the K_m values differed by 400-fold whereas with the electrodes only about a 3-fold difference was observed.

Determination of the Lower Limit of Detection. An important parameter for any sensor is its lower limit of detection for the desired analyte. As a criterion for setting this limit, we have chosen the concentration of histamine at which the signal-to-noise ratio is 3:1. To calculate this parameter, the oscillation (noise) of the baseline current is compared with the magnitude of the change in signal after histamine is added to the test solution. This was measured for several different electrodes. The variability in the lowest detection limits most likely results from variability in the amount of enzyme that is incorporated into the enzyme electrode. For the native MADH−PPy electrodes, the lower limit of detection ranged from 9 to 26 μM, with a mean value of 19.8 ± 6.7 μM. For the αF55A MADH−PPy electrodes, the lower limit of detection ranged from 2.4 to 7.2 μM, with a mean value of 5.2 ± 1.7 μM. Thus, the lower limit of detection was improved ~4-fold as a result of the site-directed mutagenesis of the enzyme. As is characteristic for Michaelis−Menten behavior, the reaction will be first order at the lowest concentrations. This linear portion of the curve for a MADH−PPy electrode is shown in Figure 3. This electrode exhibits a sensitivity of 3.2 ± 0.1 AM^{-1} cm^{-2}.

We previously reported a lower limit of detection for histamine of 25 μM for the native MADH−PPy film on gold electrode by using the amperometric method.[7] That value is actually among the lowest of the electrodes tested. More extensive studies indicate that the mean value for such electrodes is ~50 μM. In the current study, we have used DPA rather than the amperometric method to obtain a mean value for the lower detection limit of the native MADH−PPy electrode of ~20 μM (a 2.5-fold improvement). Thus, this improvement in the method of data collection together with the 4-fold improvement derived from use of the site-directed mutant yields an overall 10-fold increase in sensitivity over the originally reported electrode using native MADH.

Table 2. Response of Histamine and Potential Interferences to αF55A MADH Immobilized in a Polypyrrole Electrode

solute	physiologic concn in blood (μM)[a]	test concn (μM)[b]	response relative to histamine (%)[c]
histamine	0.1−1 (1−240)[d]	100	100
glutamine	450−750	750	0
creatine	8−53	100	0
creatinine	53−124	200	0
urea	2500−7000	5000	0
ascorbic acid	20−100	100	0
putrescine	na[e]	100	15
cadaverine	na	100	13
methylamine	na	500	0

[a] Reference 18. [b] Measurements were made in 0.1 M potassium phosphate, pH 7.5, containing 0.2 M KCl. [c] Zero indicates that the signal-to-noise ratio was less than 3:1. [d] The range in parentheses is observed for certain pathological conditions. [e] na, ot applicable.

Effects of Potential Interferences. It is important to determine whether the use of the mutant MADH has affected the reactivity of the electrode toward potential interferences. For medical applications in which blood or serum will be analyzed, potential interferences, including ascorbic acid and amino-containing compounds, are listed in Table 2. No significant response to these compounds was observed at their physiologic concentrations. Since histamine sensors also have potential applications for evaluation of fish freshness, two other amines that are produced by the microbial degradation of seafood, putrescine (1,4-diaminobutane) and cadaverine (1,5-diaminopentane), were assayed. As seen in Table 2, the αF55A MADH electrode does exhibit some response to these compounds. Methylamine, the substrate for native MADH, also elicited no response from the αF55A MADH electrode.

Electrode Stability. The αF55A MADH electrode exhibited excellent stability with time. Electrodes were stored dry for two weeks at 4 °C. The current response of each electrode to a test solution of 0.2 M histamine was recorded immediately after construction and again after storage. Six electrodes were tested for stability. Since there is some variability among electrodes in the magnitude of the current response, for each electrode, the ratio of the response after storage to the response immediately after construction was determined. For comparison, freshly prepared electrodes were tested and then immediately retested without storage. The mean value of the percent of current response of the electrodes after storage was within 1% of that of retested fresh electrodes.

DISCUSSION

An important aspect of this study is the ability to correlate the results of solution studies of an enzyme with those of the enzyme immobilized on an enzyme electrode.

A key question addressed here is whether the K_m value for an enzyme substrate determined in solution studies correlates with the affinity for that analyte of the enzyme immobilized on the

electrode. As reported previously, the K_m for histamine for the native MADH−PPy electrode is in reasonable agreement with the K_m value that is determined in steady-state kinetic studies in solution. The K_m value for histamine for the αF55A MADH−PPy electrode is smaller, but by only ∼3-fold compared to 400-fold in solution studies. This suggests that some aspect of the immobilization process is affecting the mutant differently from the native MADH. It should be noted that, for a complex kinetic mechanism such as this (see eq 5), K_m is not equivalent to a dissociation constant but a complex term composed of several microscopic rate constants. As discussed earlier, data suggest that the rate-limiting step in the overall reaction mechanism is different for αF55A MADH and native MADH. One or more of these microscopic rate constants may be differentially affected in mutant versus native MADH by the immobilization process. Furthermore, different mediators are used in solution (phenazine ethosulfate) and in the electrode (ferricyanide). These factors may contribute to the differential affects on K_m that are seen in solution and in the electrode. The lower limit of detection of the electrode is also improved for the αF55A MADH−PPy electrode but by a factor of 4, not 400. An explanation for this difference in the lower limit of detection is that with the native MADH−PPy electrode the lower threshold and range of detection is limited by the sensitivity of the enzyme. However, with the αF55A MADH−PPy electrode, the lower threshold and range of detection is limited by the sensitivity of electrode. The mutant enzyme is capable of sensing lower concentrations of histamine, but the signal that is produced is too small to be reliably detected above the background noise. The important point is that site-directed mutagenesis did lower the detection limit for the enzyme electrode. Together with the improvement afforded by using the DPA method, the overall detection limit of the αF55A MADH sensor was 10-fold more sensitive than the original native MADH sensor. With further refinement of the sensor design, it should be possible to detect even lower histamine levels since the mutant enzyme was shown in the solution studies to be capable of sensing significantly lower concentrations of histamine.

There have been relatively few reports of the application of genetic engineering technology, particularly site-directed mutagenesis, to biosensor development. One interesting example involves another quinoprotein, the pyrroloquinoline quinone (PQQ)-dependent glucose dehydrogenase. In this case, site-directed mutagenesis was used to decrease the affinity of the enzyme for glucose.[15] Because of the higher K_m for glucose, the dynamic range of the sensor could be expanded to include higher concentrations. For MADH, we have used site-directed mutagenesis to alter the enzyme in a way to extend the lower limits of detection of an enzyme sensor.

As discussed previously,[7] histamine sensors have potential applications in medicine and the food industry. For medical applications, a sensor would need to be able to detect as low as micromolar levels of histamine (Table 2). The lower detection limit of the αF55A MADH-based biosensor, relative to the native MADH-based biosensor, now more closely approaches that range. It is also important to determine whether the use of the mutant MADH has affected the sensitivity of the electrode to potential interferences. For medical applications in which blood or serum will be analyzed, potential interferences, including ascorbic acid and amino-containing compounds, elicited no significant response at their physiologic concentrations (Table 2). A histamine sensor also has potential applications for evaluation of food freshness, especially seafood. Histamine, which is produced by the microbial degradation of the amino acid histidine, is a marker for seafood freshness and its ingestion in high levels can cause a potentially fatal allergic reaction.[16,17] Two other amines that are produced by the microbial degradation of seafood are putrescine (1,4-diaminobutane) and cadaverine (1,5-diaminopentane). As seen in Table 2, the αF55A MADH electrode does exhibit some response to these compounds. This is not really a problem, however, since these compounds are also biomarkers for freshness.

In addition to refinement of sensor design, the approach of genetic engineering to alter the enzyme component of the biosensor to improve performance has important implications in biosensor technology. The results presented in this report demonstrate that site-directed mutagenesis can provide an effective way to improve the detection limit and substrate specificity of enzymes that are used in biosensors.

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