Electrochemically Mediated Reduction of Horseradish Peroxidase by 1,1′-Ferrocenedimethanol in Organic Solvents

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Cyclic voltammetry is an efficient means of analyzing the catalytic reduction of H₂O₂ at immobilized horseradish peroxidase (HRP)-Eastman AQ 55 electrodes in the presence of 1,1′-ferrocenedimethanol as a one-electron reversible cosubstrate. This system was employed to study the kinetics of the reduction of compound II of HRP in a number of organic solvents. An electrocatalytic response was detected in methanol, ethanol, 1-propanol, 2-propanol, 1-butanol, acetone, 2-butanol, 1,2-propanediol, acetonitrile, ethyl acetate, and ethylene glycol. Unusual bell-shaped variations of the peak or plateau catalytic current with the substrate concentration were observed in all solvents tested. The results obtained in methanol, acetonitrile, and 1-propanol were analyzed using the model developed by Saveant (Limoges, B.; Saveant, J.-M.; Yazidi, D. J. Am. Chem. Soc. 2003, 125, 9192–9203). The values of k₃I₀ and K₃,M, where k₃ = k₃,1k₃,2/(k₃,₁ + k₃,2), I₀ is the surface concentration of active enzyme, and K₃,M = (k₃,₁ + k₃,2)/k₃,₁, were determined. The values of k₃I₀ for the mediated reduction of compound II of HRP in methanol, 1-propanol, and acetonitrile (in the presence of 5% aqueous buffer) were not affected by the solvent dielectric constant but decreased with solvent hydrophobicity. The value of K₃,M obtained in methanol was similar to that obtained for [Os(bpy)₂pyCl]²⁺ in aqueous buffer.

Enzymes have found numerous applications as practical catalysts in chemical synthesis and as recognition elements in biosensors. The realization that enzymes can be catalytically active in organic solvents containing little or no water has expanded their repertoire of use. Some of the advantages in utilizing enzymes in nonaqueous solvents include the high solubility of many hydrophobic substrates in such solvents, the suppression of various side reactions promoted by water, and the comparative simplicity of immobilization procedures due to the insolubility of enzymes in organic solvents. Numerous studies have been conducted in order to gain an understanding of the properties of organic solvents that are key to enzymatic activity. Factors such as water activity, solvent hydrophobicity, solvent polarity, and substrate hydrophobicity can affect the rate of reaction. For example, no enzymatic activity was found for vanillyl alcohol oxidase and HRP in anhydrous water-miscible solvents. More hydrophobic solvents can increase the stability of the ground state of hydrophobic substrates yielding higher Michael–Menten constants for these substrates. The solvent’s partition coefficient (log P) between octanol and water is used as a measure of the solvent’s hydrophobicity. In chloroform (log P 2.0), the apparent K₄₀ values of immobilized tyrosinase using catechol, phenol, and p-cresol as substrates were almost half of those in 1,2-chlorobenzene (log P 3.4). In turn, the polarity of the solvent can play an important role in stabilization of the polar transition states, thus affecting the turnover number. For example, an increase in k₉₀ and catalytic efficiency with the decrease in dielectric constant of the media was observed for trypsin catalysis in water—2-propanol and water—formamide mixtures. The phenomenon was attributed to the stabilization of the negatively charged transition state in a low polar environment.

Many of the studies to date have examined the properties of enzymes such as esterases and lipases, and a number of industrial processes now utilize such enzymes in nonaqueous solvents. In contrast, there have been comparatively few studies on oxidoreductases. Mabrouk extensively studied the structure of horseradish peroxidase (HRP) in organic solvents by means of Raman spectroscopy. It was found that upon suspension in dry acetone, chloroform or acetonitrile, the HRP heme active site lost its native conformation with the coordination number of the iron increasing from 5 to 6. Dordick examined the substrate selectivity of HRP in organic solvents and observed that in all

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solvents the catalytic efficiency decreased as the substrate hydrophobicity increased. Electrochemical studies of the activity of HRP in organic solvents have been reported, with a number of organic-phase biosensors being described. HRP is one of the most extensively studied oxidoreductases. HRP is a plant heme peroxidase, which is stable and readily available. It catalyses the oxidation of a wide variety of organic compounds in the presence of peroxide. Because of the insolubility of enzymes in organic media, electrochemical studies of HRP require immobilization of the enzyme onto the surface of the electrode. As direct electron transfer between HRP and the electrode is comparatively slow and difficult to detect, a mediator, either coimmobilized or freely diffusive, is usually employed to shuttle electrons between the enzyme and the electrode. For example, a silica sol–gel method was employed to immobilize HRP on a carbon paste electrode. A linear response was obtained over the concentration range of 0.001–0.34 mM in methanol. The response of the electrode was unstable in acetone, chloroform, and chlorobenzene. Cruz Vieira and Fatibello-Filho described a biosensor based on a paraffin–graphite paste modified with sweet potato tissue as a source of peroxidase. This enzyme electrode was used for the determination of hydroquinone in cosmetic creams and was tested in ethanol, methanol, acetonitrile, aceton, 1-propanol, THF, 1-butanol, 2-propanol, and chloroform. A linear response was obtained over the hydroquinone concentration range 0.075–1.6 mM. The electrode preparation was found to be unstable in chloroform and tetrahydrofuran. HRP entrapment into Eastman AQ 55 polymer film was employed to determine the concentration of 2-butanone peroxide (BTP) in acetonitrile, acetone, and methanol. The apparent Michaelis–Menten constants for BTP in these solvents showed a correlation with the diffusion coefficient of the mediator in each solvent. Yang and Murray reported an extensive quantitative study of the reduction of PEG-modified HRP with ferrocene derivatives in ethyl acetate and 1,2-dichlorobenzene. A decrease of 1 order of magnitude was reported for the rate of reduction upon transferring the enzyme–mediator system from water to ethyl acetate. Despite the fact that there has been a number of reports describing mediated HRP electrochemistry in organic solvents, the data reported are somewhat fragmentary and mainly qualitative in character. Nevertheless, electrochemistry has proved to be a useful approach in examining the activity of redox enzymes. As each facet of catalysis and inhibition exhibited by an enzyme jointly act to determine the electrochemical response, the rate of oxidation or reduction of an enzyme can be used to determine some of the rate constants of the steps involved. A model that can be used to obtain detailed kinetic data using cyclic voltammetry has been proposed recently. A monolayer of HRP was immobilized onto the surface of a screen-printed electrode by anchoring an avidin–enzyme conjugate to a biotinylated immunoglobulin layer. The response of this system in aqueous solution was then investigated by cyclic voltammetry in order to characterize the catalytic activity and the reaction kinetics of the enzyme in the presence of the natural substrate, H2O2, and of the reversible cosubstrate couple, [Os(bpy)2pyCl]2+/[Os(bpy)2pyCl]+. The reaction mechanism is shown in Scheme 1.

Cyclic voltammograms of the enzyme electrode in the presence of H2O2 displayed significant hysteresis with the current on the anodic sweep being less than that on the cathodic sweep. This phenomenon became more apparent on subtraction of the diffusion current from the catalytic current at high and low concentrations of H2O2, and was ascribed to the slow rate of inactivation/reactivation of the enzyme. A decay in the catalytic response, similar to that obtained in homogeneous solution, was observed while scanning at high concentrations of peroxide. This decay was caused by an irreversible deactivation of the enzyme by H2O2, yielding a verdohemoprotein derivative. The electrocatalytic response of the HRP electrodes was analyzed using the following approximations: (1) the steady-state approximation applied to each enzyme form E, E1, E2, E3; (2) the concentration of H2O2 was sufficiently large that the amount consumed could be considered to be negligible; and (3) reaction 3 was the sole rate-determining step. Using these approximations, the plateau peak current, i_{p,cat}, observed by cyclic voltammetry is given by

\[
i_{p,\text{cat}} = \sqrt{2FS} \frac{C_0^{1/2}}{1 + \frac{k_0}{k_2}C_0}\]

where \(F\) is the Faraday constant, \(S\) the surface area of the electrode surface, \(I^o\) the surface concentration of enzyme, \(K_{3,M} = (k_{3,1} + k_{3,2})/k_{3,3}\) the Michaelis–Menten constant for the mediator, \(k_1 = k_{3,1}k_{3,2}/(k_{3,1} + k_{3,2})\) the rate constant for the reaction between \(E_0\) and the mediator, and \(C_0^p\) and \(C_0^S\) are the concentrations of mediator and peroxide, respectively.

Equation 1 can be rewritten as

\[
i_{p,\text{cat}} = \sqrt{2FS} \left( \frac{1}{2k_3I^o} \left( \frac{C_0}{K_{3,M}} + 1 \right) + \frac{1}{2k_3I^o} \frac{C_0}{k_6} + \frac{C_0}{k_4} \frac{C_0}{k_4} \right)\]

where \(i_{p,\text{cat}}\) was obtained from the current plateaus in the presence of varying concentrations of mediator and peroxide. From eq 2, plots of \(1/i_{p,\text{cat}}\) versus the concentration of peroxide for each concentra-

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tion of mediator should yield straight lines with intercepts equal to

\[
\text{INT} = \frac{1}{2k_3} \frac{1}{C_p} + \frac{1}{K_{3,M}}
\]

\(^{(3)}\)

\(k_3\) and \(K_{3,M}\) were then obtained from the slope and intercept of a plot of INT versus the reciprocal of the mediator concentration.

In this report, we have used this approach to examine the kinetics of HRP reduction in low-water organic media with the aim of improving the understanding of the behavior of HRP under such conditions. HRP was immobilized onto glassy carbon electrodes by entrapment into an Eastman AQ 55 polymer matrix. The response of modified electrodes was studied by cyclic voltammetry in a series of organic solvents (each containing 5% of buffer) in the presence of \(\text{H}_2\text{O}_2\) and 1,1'-ferrocenedimethanol as a mediator. The system was catalytically active in 11 solvents. The response obtained with the enzyme electrode was examined in detail in methanol, acetonitrile, and 1-propanol.

**EXPERIMENTAL SECTION**

**Reagents.** Lyophilized horseradish peroxidase (\(R_z = 2.9\)) was purchased from Fluka and used without further purification. The concentration of HRP was determined spectrophotometrically using the Soret extinction coefficient of 102 mM\(^{-1}\)cm\(^{-1}\) at 403 nm. 1,1'-Ferrocenedimethanol, lithium perchlorate, TRIZMA, tetraethylammonium \(p\)-toluenesulfonate, ethanol, methanol, acetonitrile, tetrahydروفuran, 2-butanol, and 1,2-propanediol were purchased from Aldrich. Hydrogen peroxide, ethyl acetate, 2-propanol, 1-propanol, acetone, and DMSO were purchased from Riedel-de-Haen.

**Electrochemical Experiments.** Cyclic voltammetry and chronamperometry were performed with an Autolab PGSTAT 10 (Ecochemie) interfaced to a personal computer. Ag/AgCl and platinum wire were employed as the reference and counter electrodes, respectively. Measurements were performed in an appropriate organic solvent containing 5% \((v/v)\) TRIS buffer \((50 \text{ mM, pH 6.5})\) and supporting electrolyte \((50 \text{ mM})\). Lithium perchlorate was used as supporting electrolyte in ethyl acetate and 2-butanol; tetraethylammonium \(p\)-toluenesulfonate was used in all other solvents. The reference electrode was separated from the organic media by means of a salt bridge. The potential was scanned at 5 mV/s over the potential range 0.7–0.0 V five times and the current recorded on the last scan. Each CV was acquired only after at least three successive scans showed the same response, allowing the establishment of a steady state between the various forms of enzyme (Scheme 1). Three electrodes were used for each concentration of \(\text{H}_2\text{O}_2\) in each solvent. The value of the catalytic current used for the kinetics calculations was evaluated from the diffusion-subtracted scan in the presence of peroxide. All experiments were performed at room temperature \((20 \pm 2 \text{ °C})\).

**Immobilization Procedure.** HRP was immobilized onto the surface of glassy carbon \((d = 3 \text{ mm})\) electrodes by entrapment into Eastman AQ 55. Prior to immobilization, glassy carbon electrodes (CH Instruments) were polished with 0.1-μm α-alumina followed by 0.05-μm γ-alumina powder (CH Instruments). Polishing was finished on a clean nylon pad. The electrodes were then washed with methanol, followed by water, and dried in air. An aliquot of the immobilization mixture \((5 \muL)\), containing 10% \(v/v\) 2-propanol, 1.6% \(w/v\) Eastman AQ55, and 5.43 mg/mL HRP \((50 \text{ mM TRIS buffer pH 6.5})\), was placed on the electrode surface and allowed to dry in air \((\sim 2 \text{ h})\). Addition of a small quantity of 2-propanol yielded an immobilization mixture with more homogeneous appearance, which increased the stability and reproducibility of the catalytic response of the modified electrodes. Sol-gel HRP immobilization\(^{(15)}\) and Laponite entrapment\(^{(21)}\) were performed using published protocols.

**RESULTS AND DISCUSSION**

**Immobilization of HRP.** Since enzymes are not soluble in organic solvents, it is necessary that HRP be immobilized on the electrode surface. The aim of the immobilization in this case was to stabilize enzyme in a low-water organic medium and to preserve as much of its catalytic activity as possible. A number of different approaches were examined. HRP was simply adsorbed from aqueous solution onto pyrolytic graphite and glassy carbon electrodes. The response of the electrodes prepared in such a manner was not stable in aqueous solution. In addition, the catalytic response of such modified electrodes was low and not reproducible in solvents such as methanol and ethylene glycol. Immobilization in sol–gel\(^{(22)}\) and Laponite\(^{(23)}\) was also examined. In both cases, the resulting enzyme layer was unstable in buffer and a range of nonaqueous solvents.

Entrapment of HRP into Nafion and Eastman AQ 55 polymer matrices was attempted. Nafion and Eastman belong to the same class of poly(ester sulfonic acid) anionomers and have been extensively used in electrochemical studies of positive charged proteins.\(^{(24)}\) Nafion membranes were stable in solutions of aqueous buffer; however, HRP leached from the electrode into solution. As shown previously,\(^{(24)}\) Nafion cannot incorporate cations with molecular mass higher than 10 000 Da. Additionally, it is soluble in alcohols. Eastman AQ 55, due to its less compact structure, can entrap species with molecular weights up to 49 000\(^{(21)}\) and was reported to undergo minimal swelling and to be insoluble in acetonitrile.\(^{(24)}\) The proposed structure of the polymer is depicted in Chart 1.\(^{(24)}\)

During this study, Eastman AQ 55 room-temperature cast membranes were found to be soluble in aqueous buffer. Contra

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**Chart 1. Structure of Eastman AQ 55\(^{(22)}\)**

![Chart 1](image)

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dictory reports have been published regarding the stability of Eastman AQ 55 membranes cast at room temperature in aqueous solution. Wang and Golden\textsuperscript{25} reported that polymer membranes were stable in buffer for at least 3 h, while Gennett and Purdy\textsuperscript{24} observed solubilization of room-temperature dried films. Decreases in activity were previously observed\textsuperscript{24} with HRP–Eastman AQ 29-modified electrodes and were ascribed to leaching of the enzyme from the surface of the electrode. In this study, Eastman AQ 29 and Eastman AQ 55 polymer films cast at room temperature were found soluble in aqueous buffers. However, after being cured at 37 °C overnight, Eastman AQ 29-HRP films were stable in buffer. Nevertheless, addition of hydrogen peroxidase to the reaction vessel led to complete solubilization of the enzyme–polymer film. Thus, the quantitative assessment of HRP–mediator kinetics in aqueous buffer was not possible. Nevertheless, HRP–Eastman AQ 55-modified glassy carbon electrodes were stable in organic solvents and exhibited reproducible enzymatic activity (Figure 1).

All the solvents tested contained 5% (water-miscible) or saturating amounts of aqueous buffer. This amount is a compromise solution between two aims of this study: keep the level of enzyme’s catalysis sufficiently high and keep the aqueous-phase presence in the solvent as low as possible to eliminate the impact of water on the resulting data. The presence of water is highly important for the activity of the enzyme. It has been shown that dry water-miscible solvents tend to dehydrate the enzyme molecules resulting in diminished catalytic activity.\textsuperscript{6} As entrapment of HRP into Eastman AQ did not appear to provide sufficient water conditions for enzymatic catalysis, addition of extra aqueous buffer was required to obtain a catalytic response. However, the aim of this study was to evaluate the effect of the organic solvent on the kinetics. Consequently, it was important to have a minimal amount of water present to minimize its impact on the reaction environment. The results obtained in the previous work\textsuperscript{15} indicated that, at concentrations of 90–95%, the organic solvent alters the transition-state structure and active site microenvironment of HRP, making it feasible to search for the possibility of a correlation between the activity of HRP activity and the properties of the solvents.

The catalytic current (0.89 ± 0.02 μA) measured at HRP-modified electrodes in methanol, containing 0.1 mM mediator and 0.8 mM hydrogen peroxide, was essentially the same upon storage at 4 °C for 72 h (0.90 ± 0.07 μA). However, if the electrode was dried after initially testing its response, there was a significant decrease in the catalytic response to 0.41 μA. Such a result is in qualitative agreement with previous reports on the preparation of subtilisin-modified silica, where virtually all activity was lost if the enzyme preparation was allowed to dry.\textsuperscript{26} Exposure to high concentrations of peroxide (4 mM H_2O_2, catalytic current of 0.81 ± 0.01 μA) resulted in irreversible inactivation of the enzyme—the catalytic current decreased from 1.42 ± 0.04 to 0.77 ± 0.02 μA on testing in the same 0.8 mM H_2O_2–methanol solution. This irreversible deactivation is likely a result of the oxidation of the porphyrin yielding the verdohemoprotein. It should be pointed out that the reversible inactivation (formation of compound III) was observed at lower concentrations of peroxide (up to 1 mM). The operational stability of the electrode depended on the solvent used. For example, 1,2-propanediol was found to decrease the mechanical stability of the membrane due to swelling of the polymer in this solvent. As an unstable and irreproducible electrode response makes it impossible to determine the reaction kinetics of immobilized HRP, only solvents with stable responses were employed for kinetic evaluation.

### Stability of Mediated Reduction of HRP

No direct electron transfer was observed between HRP and the working electrode in all solvents tested. 1,1′-Ferrocenedimethanol was used as a mediator to facilitate the electron transfer, as it is one of the most effective ferrocene-based cosubstrates for HRP. The electron-transfer rate constant for the reduction of HRP by ferrocenedimethanol in aqueous solution was reported to be 3.27 × 10^4 versus 0.73 × 10^4 M⁻¹ s⁻¹ for ferrocenemonocarboxylic acid.\textsuperscript{27}

As with other ferrocenes, 1,1′-ferrocenedimethanol displays reversible electrochemical behavior in aqueous media, is stable in the reduced form, and is neither light nor pH sensitive. It was readily soluble and exhibited one-electron quasi-reversible electrochemistry in all organic solvents examined. The straight lines obtained from Randles–Sevcik plots of the peak current versus the square root of the scan rate were indicative of the diffusion-limited behavior of electroactive species. The diffusion coefficients of 1,1′-ferrocenedimethanol decreased with increasing solvent viscosity (Table 1).

### Table 1. List of Solvent Viscosities and Diffusion Coefficients of 1,1′-Ferrocenedimethanol Obtained in Each Solvent

<table>
<thead>
<tr>
<th>solvent</th>
<th>viscosity, mN s m⁻²</th>
<th>D_{rot}, 10⁻⁶ cm² s⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>methanol</td>
<td>0.544</td>
<td>0.270</td>
</tr>
<tr>
<td>1-propanol</td>
<td>2.522</td>
<td>0.017</td>
</tr>
<tr>
<td>acetonitrile</td>
<td>0.350</td>
<td>6.746</td>
</tr>
</tbody>
</table>


A typical set of voltammograms obtained at a scan rate of 5 mV/s for the HRP–Eastman-modified electrode in methanol with increasing concentration of H₂O₂ is shown in Figure 2. Starting from the quasi-reversible voltammogram of ferrocene dimethanol in the absence of hydrogen peroxide, the reduction peak current increased upon addition of H₂O₂ while the anodic current decreased, displaying a response typical of catalytic behavior.

Upon further increasing the concentration of substrate, the catalytic current decreased. The variation of the peak current in methanol with the concentration of H₂O₂ is shown in Figure 3, and plots of the catalytic current versus H₂O₂ concentration in acetonitrile, ethanol, 1-propanol, 2-butanol, ethyl acetate, and 1,2-propanediol are presented in Figure 4. Trends similar to the one in methanol were observed in these solvents—an initial increase followed by a decrease in the current with increasing H₂O₂ concentration. This trend was not observed for the oxidation of HRP by butanone hydroperoxide, where Michaelis–Menten behavior was reported with butanone hydroperoxide as a substrate over the concentration ranges of 0–1 mM in acetonitrile, 0–0.16 mM in tetrahydrofuran, and 0–0.6 mM in acetonitrile. Nevertheless, the bell-shaped dependence of the catalytic response on peroxide concentration has been reported previously in aqueous media for both immobilized and homogeneous HRP systems. The observed decrease in the current was ascribed to the fast reversible and slow irreversible enzyme deactivation by excess of H₂O₂, which yielded compound III and verdohemoprotein derivative, respectively. It is also important to note that the peroxide concentration, which corresponded to the highest catalytic current, differed from solvent to solvent and shifted to higher values with increasing mediator concentration.

The catalytic current obtained in 2-butanol and ethyl acetate was much higher, while the current in 1,2-propanediol was lower than the current obtained in the other solvents (Figure 4). Such high catalytic currents made ethyl acetate and 2-butanol particularly interesting for the kinetic study of the reduction of compound II of HRP by ferrocenedimethanol. However, the reproducibility of the results in these solvents was low. In the case of 1,2-propanediol, the current was too small to obtain reliable kinetic data.

The response of HRP-modified electrodes was also examined in a range of other organic solvents (Table 2). When the electrode was investigated in ethylene glycol, the catalytic current was unstable and steadily decreased with successive scans (data not shown). The catalytic currents in 1-butanol at low concentrations of ferrocenedimethanol were too low to obtain reliable kinetic data. No catalytic response was obtained in dimethyl sulfoxide, dimethylformamide, dimethylacetamide, formamide, and glycerol. With the first four of these solvents, the absence of catalysis may be a result of lower polymer stability in these solvents or their high denaturating capacity. In glycerol, no electrochemical response was observed at the enzyme-modified electrode with or without hydrogen peroxide. However, on a bare glassy carbon electrode, reversible behavior was obtained for ferrocenedimethanol in this solvent, indicating that the polymer–enzyme film blocked access of the mediator to the surface of the working electrode.

Figure 2. Cyclic voltammograms of 1,1′-ferrocenedimethanol (200 μM) at HRP–Eastman AQ 55-modified electrodes in methanol, at different concentrations of hydrogen peroxide: (A) 0.0 (blue); 0.2 (green); 0.4 (red); 0.8 mM (purple); (B) 0.8 (purple); 1.0 (red); 2.0 (green); 5.0 mM (blue). Scan rate of 5 mV/s.

Figure 3. Cathodic peak currents from Figure 2 versus concentration of H₂O₂.

Figure 4. Plateau current obtained at HRP–Eastman AQ 55-modified electrodes as a function of the concentration of hydrogen peroxide in (A) (×) 2-butanone, (○) ethyl acetate, and (□) 1-butanol, (B) (○) acetonitrile, (□) 1-propanol, (×) 1,2-propanediol, and (*) ethanol. All solutions contained 5% or saturating amounts of buffer; concentration of 1,1'-ferrocenedimethanol of 200 μM.

Table 2. Detection of Electrocatalysis on HRP–Eastman AQ 55-Modified Electrodes in Organic Solvents

<table>
<thead>
<tr>
<th>solvent</th>
<th>electrocatalytic response</th>
<th>solvent</th>
<th>electrocatalytic response</th>
</tr>
</thead>
<tbody>
<tr>
<td>methanol</td>
<td>yes</td>
<td>acetonitrile</td>
<td>yes</td>
</tr>
<tr>
<td>ethanol</td>
<td>yes</td>
<td>ethyl acetate</td>
<td>yes</td>
</tr>
<tr>
<td>1-propanol</td>
<td>yes</td>
<td>DMA</td>
<td>none</td>
</tr>
<tr>
<td>2-propanol</td>
<td>yes</td>
<td>glycerol</td>
<td>none</td>
</tr>
<tr>
<td>1-butanol</td>
<td>yes</td>
<td>formamide</td>
<td>none</td>
</tr>
<tr>
<td>acetone</td>
<td>yes</td>
<td>DMSO</td>
<td>none</td>
</tr>
<tr>
<td>2-butanone</td>
<td>yes</td>
<td>DMF</td>
<td>none</td>
</tr>
<tr>
<td>1,2-propanediol</td>
<td>yes</td>
<td>none</td>
<td>none</td>
</tr>
</tbody>
</table>

reversible behavior was observed on HRP-modified Eastman AQ 55 electrodes.

A series of experiments was conducted in each solvent using three or more concentrations of \( \text{H}_2\text{O}_2 \) at five or six concentrations of mediator. Preliminary experiments revealed that, at a constant concentration of peroxide, the catalytic current leveled off at 200 μM ferrocenedimethanol. The maximum concentration of mediator used for the kinetic experiments was 80 μM. A plateau-shaped CV was obtained at each mediator concentration, consistent with reaction 3 being the sole rate-determining step. The concentrations of the peroxide used were high enough to secure a catalytic response (plateau). At the same time, the peroxide concentration should be kept as low as possible as very high concentrations of oxidizing agent can diminish the catalytic response to undetectable levels due to enzyme inactivation. As expected, the range of \( \text{H}_2\text{O}_2 \) concentration, which fulfilled these requirements, varied from solvent to solvent. Figure 6 displays the data obtained in methanol.

The sigmoidal shape of the voltammograms indicates that the electrochemical response was under catalytic control. The current obtained at a high concentration of \( \text{H}_2\text{O}_2 \) (4 mM) was lower than that obtained at a low concentration (0.8 mM). The good reproducibility obtained is evident in Figure 6, which displays the data obtained with three separate electrodes. The reciprocal of the plateau current from voltammograms was plotted in the form of \( \text{FS}/i_{p,\text{cat}} \) versus substrate concentration (Figure 7a). The intercepts obtained were plotted against the reciprocal of the mediator concentration (Figure 7b) yielding values of \( 4.0 \times 10^{-9} \) cm s\(^{-1} \) and 27.8 μM for \( k_i \Gamma^0 \) and \( K_{3,M} \), respectively. The values obtained for \( k_i \Gamma^0 \) in acetonitrile and 1-propanol are listed in Table 3.

The values of \( k_i \Gamma^0 \) represent the combined effect of the solvent on the activity of the enzyme and its concentration. \( \Gamma^0 \) represents the concentration of active enzyme on the electrode surface. Since the enzyme is insoluble in 95% organic media, the total amount of enzyme on the electrode surface can be assumed to be constant in all solvents. However, as different organic solvents will have different effects on enzyme activity, either the surface concentration of active enzyme (\( \Gamma^0_{\text{act}} \)) or the activity of the immobilized electrode in glycerol. The catalytic response observed in acetone was irreproducible between the electrodes.

Determination of Kinetic Constants. The model proposed by Limoges and Saveant\(^{19} \) was found to successfully describe all of the unusual facets of the electrocatalytic behavior of the HRP-modified electrode that were observed—the bell-shaped dependence of the plateau current on the concentration of substrate (Figure 4), the occurrence of hysteresis, and trace crossing (Figure 5). The hysteresis effect can be ascribed to the delay in the establishment of a steady state between \( E_2,E_2\text{Q} \) and \( E_3 \) enzyme forms, while trace crossing is the result of slow diffusion of \( Q_i \).\(^{20} \)

It should be noted that the model proposed was derived for a system in which a monolayer of HRP was attached to the surface of the electrode.\(^{20} \) Such is not the case here, with it being likely that there are a number of layers of enzyme present. While multilayers may introduce mass transport interferences from the mediator and substrate, the catalytic responses obtained (Figure 7) indicated that such interferences were not substantial. In addition, the purpose of this study was to ascertain the overall course of this study, it was observed that ferrocenedimethanol displayed an irreversible response (in, for example, ethyl acetate) on electrodes on which only HRP was adsorbed, while quasi-
enzyme ($k_3$) or both these parameters are likely to change from solvent to solvent. Unfortunately, kinetic approaches, such as that used in this study, are not able to distinguish between these cases. Nevertheless, the values of $k_3 \Gamma^0$ reported here are measures of the effect of the solvent on the overall rate of reduction of HRP by ferrocenedimethanol. From the data in Tables 1 and 3, no clear correlation between $k_3 \Gamma^0$ and the viscosity or dielectric constant of the solvent was found. The values of $k_3 \Gamma^0$ decreased with the hydrophobicity of the solvent (Table 3). It is important to stress that this trend was obtained in water-miscible solvents only and obviously, taking into account the high catalytic currents obtained in 2-butanone and ethyl acetate, cannot be extrapolated to water-immiscible solvents. For the solvents examined, the decrease in $k_3 \Gamma^0$ suggests that destabilization of the transition-state complex formed by the compound and the mediator occurs with increasing solvent hydrophobicity, possibly due to the distortion of the hydrogen network and the heme geometry by solvent molecules. As reported by Ryu and Dordick, methanol can bind as a sixth ligand to the heme. The formation of a ligand complex was evident from EPR signal at $g = 6$, which in 80% methanol became as sharp as that of hemin chloride, indicating that the heme symmetry became completely tetragonal. Together with this ligand effect,
a number of reports have indicated the importance of the substrate—enzyme hydrogen bond network. Thus, penetration of the solvent molecules into the enzyme active site can change the geometry of the active site and distort the hydrogen bond network in its proximity, which will affect the values of $K_{3,M}$ and $k_3^{-1}$. Substrate hydrophobicity has been shown to influence the rate of reaction of HRP in organic solvents, with partitioning of the substrate from the reaction medium into the heme active site influencing the rate of catalysis. The degree of partitioning of a hydrophobic substrate would be expected to decrease as the hydrophobicity of the solvent was increased, requiring higher concentrations of substrate to saturate the enzyme and increasing the value of $K_{3,M}$. The value of $K_{3,M}$ obtained in methanol (27.8 $\mu$M) was found to be close to that determined for [Os(bpy)$_2$pyCl]$^{2+}$ in aqueous solution (37 $\mu$M). The similarity in the values obtained indicates that binding of the mediator to the heme active site occurs in a similar manner for the osmium complex in aqueous solution and for ferrocenedimethanol in methanol. It was not possible to determine the value of $K_{3,M}$ in acetonitrile and propanol due to the large variations observed for the values of the intercept of eq 3 in these solvents.

Table 3. Kinetic Characteristics and Solvent Properties for the Reaction of HRP with 1,1′-Ferrocenedimethanol

<table>
<thead>
<tr>
<th>solvent</th>
<th>$k_3^{-10^9}$ cm s$^{-1}$</th>
<th>$K_{3,M}$ $\mu$M</th>
<th>$\epsilon$</th>
<th>Log $P$</th>
</tr>
</thead>
<tbody>
<tr>
<td>methanol</td>
<td>4.0 ± 0.2</td>
<td>27.8 ± 1.6</td>
<td>33.6</td>
<td>-0.74</td>
</tr>
<tr>
<td>1-propanol</td>
<td>0.25 ± 0.05</td>
<td>undetermined</td>
<td>22.2</td>
<td>0.34</td>
</tr>
<tr>
<td>acetonitrile</td>
<td>0.6 ± 0.1</td>
<td>undetermined</td>
<td>35.9</td>
<td>-0.34</td>
</tr>
</tbody>
</table>

CONCLUSIONS

HRP–Eastman AQ 55-modified glassy carbon electrodes were prepared, and the catalytic activity toward H$_2$O$_2$ was examined in a range of solvents. Using ferrocenedimethanol as a mediator, the modified electrodes showed catalytic activity in a range of solvents. High activity was seen in 2-butanone and ethyl acetate with no activity observable in dimethyl sulfoxide, dimethylformamide, dimethylacetamide, and glycerol. The catalytic response of the HRP–Eastman AQ 55-modified glassy carbon electrodes demonstrated a bell-shaped dependence of the maximum catalytic current on peroxide concentration in all solvents tested. The concentration of the peroxide at which the highest catalytic current was obtained was found to depend on the mediator concentration and varied in different solvents. A detailed kinetic analysis was performed in acetonitrile, methanol, and 1-propanol. In these solvents, increasing hydrophobicity of the solvent resulted in a decrease in the overall rate of the reaction. The value of $K_{3,M}$ obtained for ferrocenedimethanol in methanol was similar to that obtained for [Os(bpy)$_2$pyCl]$^{2+}$ in aqueous buffer. The catalytic activity of the modified electrodes is currently under examination as a biosensor for the detection of H$_2$O$_2$ in a range of solvents.

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