Comparison of Spectrophotometric and Amperometric Rate Parameters of Enzymatic Reactions

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INTRODUCTION

Amperometric sensors have seen extensive biomedical application in recent years due to the ease with which they can be coupled to biologically important enzymatic redox reactions. Such oxidoreductase-catalyzed reactions invariably involve an electron-transfer step which can be transduced, by a suitably designed amperometric sensor, into a current which may serve to monitor substrate and/or product concentrations. X-ray crystallographic studies1 and indirect evidence from mediator-modified enzymes2,3 suggest that catalytic sites of redox enzymes are often deeply embedded, minimizing the possibility of direct charge transfer from the site of the reaction to an electrode surface. In the large group of redox enzymes which depends on the soluble pyridine nucleotide cofactor, NAD(P)+/NAD(P)H, this limitation could, in principle, be overcome because the cofactor serves as a soluble charge carrier and is capable of electron transfer at an electrode. However the process

NAD(P)H = NAD(P)+ + 2 e− + H+  

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entails a large anodic overpotential at all known electrode materials and may result in decreased selectivity when other electrooxidizable species are present in solution. Moreover, NADH adsorbs strongly at platinum electrodes and proceeds through a complicated radical mechanism.4 These difficulties can be circumvented through the use of an amperometric couple capable of homogeneous redox reaction, in aqueous solution, with the pyridine nucleotide moiety and having facile heterogeneous electron-transfer kinetics at the electrode surface. A generalized three-step sequence may consist, for instance, of the reactions

enzymatic  s-H + NAD(P)+ = s + NAD(P)H \hspace{1cm} (2)

chemical  NAD(P)H + ox. = NAD(P)+ + red. \hspace{1cm} (3)

electrochemical red. = ox. + e− \hspace{1cm} (4)

where s-H and s represent the reduced and oxidized forms of the substrate, respectively, and ox. and red. represent the oxidized and reduced forms of the amperometric couple.

The ADH-catalyzed oxidation of alcohols is a bireactant, biproduct reaction involving a ternary alcohol-NAD-ADH complex.5 This is similar to the enzyme mechanism proposed for alcohol oxidase-catalyzed oxidation of ethanol we have discussed in an earlier publication.6 Conventional single-substrate enzyme kinetics rely on the assumption that the overall process

\[ E + S \rightleftharpoons P \]  

5

proceeds from the substrate (S) to the product (P) in the presence of the enzyme (E) via the sequence

\[ E + S \rightleftharpoons ES \rightleftharpoons k_{-1} \]  

6

\[ ES \rightarrow E + P \]  

7

where ES is a binary substrate–enzyme complex (the "Michaelis–Menten" intermediate). This model leads to the well-known relationship

\[ V_0 = \frac{V_{\text{max}}[S]}{K_M + [S]} \]  

8

where \( V_0 \) is the initial velocity of reaction 7 whose rate-determining step is reaction 9. \( V_{\text{max}} \) denotes the limiting maximal velocity that would be observed if all the enzyme is present as the complexed ES form, [S] is the substrate concentration, and \( K_M \) is the so-called Michaelis–Menten constant. The parameter \( K_M \) expresses the relationship between the steady-state concentrations of reactants and intermediates,7 and it can be shown that

\[ K_M = \frac{k_2 + k_{-1}}{k_1} = \frac{[E][S]}{[ES]} \]  

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When the substrate concentration equals the value of \( K_M \),

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the initial velocity, \( V_0 \), is half of the saturation velocity, or \( \frac{1}{2} V_{\text{max}} \).

Multisubstrate systems such as reaction 2 of the type

\[ A + B = P + Q \]  \hspace{1cm} (10)

may proceed via ternary intermediate complexes through a sequential mechanism such as

\[ E + A = EA \quad \text{or} \quad E + B = EB \]  \hspace{1cm} (11)

\[ EA + B = EAB \quad \text{or} \quad EB + A = EAB \]  \hspace{1cm} (12)

\[ EAB \rightarrow E + \text{product(s)} \]  \hspace{1cm} (13)

Reaction sequence 11–13 is characterized by the feature that both the substrate (A) and the cosubstrate (B) first bind with the enzyme (E) in a ternary complex (EAB) before any products are released. If the transformation of the ternary complex into products is relatively slow and that of complex formation is rapid, the following simplified velocity equation may be obtained for such a system:

\[ V_0 = \frac{V_{\text{max}}}{1 + (K_{M1})_A + (K_{M2})_B + (K_{M1}K_{M2})_A[B]} \]  \hspace{1cm} (14)

where two Michaelis–Menten constants, \((K_{M1})_A\) and \((K_{M2})_B\), and corresponding substrate (A) and cosubstrate (B) concentrations are implicated, and the last term of the denominator relates to the mutual interaction of A and B at the binding site. Under conditions where the concentration of substrate A is much greater than its Michaelis constant, \((K_{M1})_A\), velocity eq 14 can be reduced to

\[ V_0 = \frac{V_{\text{max}}}{1 + (K_{M2})_B[B]} = \frac{V_{\text{max}}[B]}{(K_{M2})_B + [B]} \]  \hspace{1cm} (15)

A similar approach with \((K_{M2})_B\) will result in a velocity equation in terms of substrate A and \((K_{M2})_B\). Such equations correspond to the velocity equation of a single-substrate reaction and allow simplified Michaelis constant evaluation.

As the reaction proceeds, substrate concentrations gradually decrease and, as the product concentration builds up, the unidirectionality of the reaction no longer exists. This invalidates the assumptions used to derive the \( V_o \) equations. Hence, it is essential to make measurements within the time interval during which reaction conditions do not deviate significantly from those required for the velocity equations.

A variety of approaches have been used to monitor the progress of pyridine nucleotide-dependent enzymatic reactions. Spectrophotometric absorption of ultraviolet radiation by the reduced form of the cofactor, NAD(P)H, is the most commonly accepted method. However, few published studies have intercompared amperometrically and spectrophotometrically obtained enzyme kinetic parameters. Talbott found good agreement between spectrophotometrically determined values reported in the literature and his electrochemically determined Michaelis constant, \( K_m \), for the substrate \( \beta\)-D-glucose but not for the cosubstrate of glucose oxidase, a bisubstrate, biproduct enzyme which does not involve a ternary complex. Other researchers have noted discrepancies as well. It is of general interest to compare results obtained spectrophotometrically and amperometrically, both for analytical purposes and as a way of probing the possibility that mediators effect changes in the kinetic mechanism of the "natural" enzymatic reaction.

**EXPERIMENTAL SECTION**

**Apparatus.** Electrochemical experiments were performed in a water-jacketed, thermostated cell at a platinum rotated disk electrode (Pt-RDE, area = 3.2 \( \times \) 10\(^{-4}\) m\(^2\)) with a platinum wire auxiliary electrode and saturated calomel reference electrode (SCE). Constant-potential chronoamperometric experiments were carried out with an IBM EC/225 voltammetric analyzer set at the desired potential. Data were acquired with a Rapid System 4 X 4 digit oscilloscope and/or a Yokogawa XVT recorder.

**Reagents.** All solutions were prepared with distilled water in which the conductivity was determined to be less than 11 \( \mu \)S:\( 1\). Potassium hexacyanoferrate(III) (Baker), ethanol (Pharmco), \( \beta \)-nicotinamide adenine dinucleotides (NAD\(^+\) and NADH, Sigma), and yeast alcohol dehydrogenase, EC 1.1.1.1 (Sigma), were used without further purification. The supporting electrolyte was 0.2 M KCl in pH 8.8, 0.05 M pyrophosphate buffer.

**Procedure.** Unless otherwise stated, experiments were performed at 298 K under anaerobic conditions. Yeast alcohol dehydrogenase (Y-ADH) in concentrations appropriate to the experimental design (100 \( \mu \)M for initial velocity experiments, 400 \( \mu \)M for ethanol concentration determinations) was added to the electrochemical cell which held 10 mL of a buffered solution of ethanol and NAD\(^+\). Ethanol concentrations of 0.01–300 mM (100 \( \mu \)M) and NAD concentrations of 0.02–8 mM (10 \( \mu \)M), were used. Except as noted, the KFe(CN)\(_6\) concentration was kept at 50 mM. Prior to each recording the working electrode was polished 3 min with 0.05-\( \mu \)m alumina (Buehler), rinsed in distilled water, and sonicated 10 s in distilled water. The cell was covered, and its contents were sparged prior to and blanketed during each experiment with N\(_2\). The Pt-RDE was rotated at 900 or 1600 rpm with a Pine Instrument Co. analytical rotator. The potentiostat was set to maintain a potential difference of \(-500 \text{ mV vs SCE} \) at which the anodic current was zero. Ethanol was added to the electrode solution, and the anodic current was recorded as a function of time. Experiments to determine enzyme kinetic parameters were patterned after the initial velocity studies described by Segel.\(^7\) Slope measurements were made on the steepest part of the current–time response, representing the first 10–30 s of the reaction.

**RESULTS AND DISCUSSION**

**Relationship of Current–Time Behavior to Hexacyanoferrate(II) Concentration.** The ternary reaction sequence for the ADH-catalyzed oxidation of ethanol was enzymatic

\[ C_2H_5OH + NAD^+ = CH_3CHO + NADH + H^+ \]  \hspace{1cm} (16)

chemical

\[ NADH + 2Fe(CN)_6^{3-} = NAD^+ + 2Fe(CN)_6^{4-} + H^+ \]  \hspace{1cm} (17)

electrochemical

\[ Fe(CN)_6^{4-} = Fe(CN)_6^{3-} + e^- \text{ (anodic current)} \]  \hspace{1cm} (18)

Reactions 16 and 17 are homogeneous reactions occurring in aqueous solution, while reaction 18 is heterogeneous, with electron transfer across the electrode–solution interface. Oxidation of ethanol, indicated by the forward direction of reaction 16, proceeds to virtual completion at pH 8.8. Reaction 18 is fast compared to reactions 16 and 17 at the applied potential (500 mV vs SCE) where the rate is mass transport limited. Figure 1 shows the relative concentration profile of the reactants and products in this sequence. Note that the Fe(CN)\(_6\)\(^{4-}\) concentration increases at twice the rate of ethanol depletion and that, after an initial decrease, the

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NAD\(^+\) concentration remains constant since virtually all NADH produced is immediately reoxidized by Fe(CN)\(_6^{3-}\) via reaction 17. Hence, the concentration of Fe(CN)\(_6^{3-}\) or NADH were varied while the other was held constant. Certain residual ethanol and NADH, are partly responsible for the observed current excess. On the other hand, some oxidation of ADH by hexacyanoferrate(III) may account for the excess current, which is proportional to the amount of enzyme present. We have encountered high oxidation currents and loss of enzyme activity with another enzyme, alcohol dehydrogenase, in the presence of hexacyanoferrate(III) (unpublished data), and Durliat et al.\(^{13}\) have reported loss of enzyme activity with hexacyanoferrate(III) concentrations greater than 10 mM with lactate dehydrogenase. However, this effect with ADH is small and does not invalidate determination of ethanol concentration or kinetic parameters discussed below. Initial reaction rates were determined from the slope of the current–time graph during the first 30 s of the reaction (initial velocity region of Figure 1), using the Levich equation:

\[
i_i = 0.620 nFAD^{2/3}a^{1/2}v^{-1/6}C^*\]

in which \(D\), the diffusion coefficient of Fe(CN)\(_6^{3-}\), is 6.5 \(\times\) 10\(^{-10}\) m\(^2\)/s at 298 K (Adams\(^{14}\)), \(a\), kinematic viscosity, is 1.0 \(\times\) 10\(^{-6}\) m\(^2\)/s, \(A\) is the area of the Pt-RDE, \(C^*\) is the bulk concentration of Fe(CN)\(_6^{3-}\), \(n\) = 1 is the number of electrons transferred in the electrooxidation of Fe(CN)\(_6^{3-}\), and \(F\) is the Faraday constant. Since the amount of Fe(CN)\(_6^{3-}\) generated in reaction 17 is stoichiometrically related to the amount of ethanol reacted

\[
\Delta C^{*}=C^{*}_{Fe(CN)_{6}^{3-}}=2\Delta C^{*}_{ethanol}
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\(i_i\) represents a measure of the amount of ethanol that has been oxidized. The rate of change of the limiting current then gives the rate of change of concentration, i.e., the reaction rate:

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\frac{\Delta i}{\Delta t} = 0.620 nFAD^{2/3}a^{1/2}v^{-1/6}\frac{\Delta C^*}{\Delta t}
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Initial rate analysis of experiments in which concentrations of Fe(CN)\(_6^{3-}\) or NADH were varied while the other was held constant meets many of the criteria for desirable redox couples, as listed by Coury et al.\(^{10}\) well-defined, reversible voltammetry, adequate aqueous solubility, availability in high purity, sufficient chemical stability, lack of cross-reactivity with the enzymatic substrate, and ability to accept electrons from the reduced enzyme or the reduced cofactor. In addition, at moderate concentrations Fe(CN)\(_6^{3-}\) does not significantly affect the activity of the enzyme itself. The two-reaction sequence (reactions 17 and 18) has been studied at glassy carbon and carbon paste electrodes and applied to lactate dehydrogenase assays in serum.\(^{11}\) Hexacyanoferrate(III) has been employed as a mediator or amperometric couple by other researchers with flavin enzymes.\(^{12,13}\) At a platinum electrode, hexacyanoferrate(II) (\(E_{1/2} = 220\) mV vs SCE) is oxidized at a potential several hundred millivolts less anodic than NADH, significantly reducing the probability that side reactions (electrooxidation of other biological components which may be present in the sample) will occur. Nevertheless, some side reactions do occur. Analysis of the steady-state current obtained for these experiments which were allowed to proceed to virtual completion (equilibrium region of Figure 1) revealed an anodic current 2–3% in excess of the current predicted for 100% efficiency according to the Levich equation. The percent current excess is greatest for experiments using high enzyme concentrations and amounts to 0.6 \(\mu\)A/mg of ADH. It is possible that impurities in the crystalline Y-ADH preparation, such as residual ethanol and NADH, are partly responsible for the observed current excess. For the other hand, some oxidation of ADH by hexacyanoferrate(III) may account for the excess current, which is proportional to the amount of enzyme present. We have encountered high oxidation currents and loss of enzyme activity with another enzyme, alcohol dehydrogenase, in the presence of hexacyanoferrate(III) (unpublished data), and Durliat et al.\(^{13}\) have reported loss of enzyme activity with hexacyanoferrate(III) concentrations greater than 10 mM with lactate dehydrogenase. However, this effect with ADH is small and does not invalidate determination of ethanol concentration or kinetic parameters discussed below. Initial reaction rates were determined from the slope of the current–time graph during the first 30 s of the reaction (initial velocity region of Figure 1), using the Levich equation:

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M-15-1 at 298 and 279 K, respectively. The Arrhenius plot of
the initial reaction velocities (Figure 3). The maximum
initial reaction velocity, $V_{\text{max}}$, seen in Figure 3 was lower than
the value determined spectrophotometrically with comparable
NAD, ADH, and ethanol concentrations. The primary
experimental difference was the presence of various concen-
trations of Fe(CN)$_3^-$ in the reaction mixture of the electro-
chemical experiments. Possibly, hexacyanoferrate(II) in-
teracts with the enzyme in a concentration-independent
manner and limits the maximum reaction velocity. The
enzyme activity ($V_{\text{max}}$) specified on the assay data sheet
accompanied the Y-ADH purchased from Sigma was con-
dumed spectrophotometrically with those determined elec-
rochemically thus must take this factor into consideration.

Kinetic parameters determined amperometrically in our
laboratory are summarized in Table I, together with spec-
trophotometric literature values. Michaelis constants, $K_M$
for ethanol and NAD were determined from initial reaction
rates at the Pt-RDE. Analysis by Eadie-Hofstee plots and
Lineweaver-Burk plots yielded similar values. A survey of
the literature shows that these have been extensively studied
spectrophotometrically. Dickinson and Monger,16 working at
pH 7.05, obtained $K_M$ for ethanol and NAD to be 0.109 mM and
21.7 mM. Hayes and Velick17 determined the Michaelis
constants, $K_M$, at pH 7.9 to be 0.17 mM (NAD$^+$) and 22 mM
(ethanol). Burstein et al.18 reported $K_M$ values of 1 and 3
mM for NAD$^+$ and ethanol, respectively, for ADH in pH 9.2
buffer solution. The considerable variability of the Michaelis
constants reported above appears to be at least partially
related to pH. A comparison of kinetic parameters deter-
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Table I. Comparison of Spectrophotometric and Amperometric Kinetic Parameters

<table>
<thead>
<tr>
<th>kinetic param</th>
<th>spectrophotometric value</th>
<th>electrochemical value</th>
<th>method of data analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>$V_{\text{max}}$</td>
<td>100% of specified ADH assay value</td>
<td>85% of specified ADH assay value</td>
<td>Eadie-Hofstee</td>
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<tr>
<td>$K_M$(ethanol), mM</td>
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<td>3.2</td>
<td>Eadie-Hofstee</td>
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<td>$K_M$(NAD$^+$), mM</td>
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<td>0.80</td>
<td>Eadie-Hofstee</td>
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<tr>
<td>$k_{\text{cat}}, \text{s}^{-1}$</td>
<td>450</td>
<td>448</td>
<td>Eadie-Hofstee</td>
</tr>
</tbody>
</table>

$\Delta [\text{NADH}, \mu M]/\Delta t$ per $\mu M$ enzyme

* Information supplied by manufacturer. $^a$ Sample standard deviation, $s = \sqrt{(n\sum x^2 - (\sum x)^2)/n(n - 1)}$; $b = 0.3$, $n = 16$. $^c$ $s = 0.08$, $n = 10$. $^d$ $s = 42$, $n = 14$. [3]

primarily by the rate at which NADH was produced in reaction
16. Thus the rate-determining reaction in the sequence is
reaction 16 and $\Delta V/\Delta t$ is a valid measure of the rate of enzyme-
catalyzed ethanol oxidation under the prevailing experimental
conditions. Concentration-dependent enzyme activity inhibi-
tion could not be discerned up to 150 mM Fe(CN)$_3^-$.

Enzyme Kinetic Parameters. Ethanol concentrations
between 0.01 and 1 mM show an excellent linear current response ($R = 0.9992$), as can be seen from Figure 2. The
current was measured 30 min after enzyme was added to the
reaction mixture, at which time the reaction had nearly gone
to completion (the so-called “equilibrium region” of Figure
1). This linearity is further documented in the Michaelis
plot of initial reaction velocities (Figure 3). The maximum
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Albery19 worked out the steady-state kinetic equations for a
conducting organic salt-membrane electrode and applied it
to ADH-catalyzed alcohol oxidation.20 He lists an “electro-
chemical Michaelis constant”, $K_M$, for ethanol of 2.7 mM at

17. Burstein, B.; Cunliff, H.; Lager, M. D.; Gell, G.; Thomas, D.
19. Albery, W. J.; Bartlett, P. N.; Cass, A. E. G.; Sim, K. W. J.
pH 9.0. This is in good agreement with our results ($K_M$ (ethanol) = 3.2 mM at pH 8.8) as determined from Eadie-Hofstee plots (Figure 4), in spite of experimental differences. The research reported here differs from the Albery experiment in that neither the enzyme nor the mediator were physically immobilized near the electrode surface. Thus, our system is dependent only on the well-documented diffusion coefficient of Fe(CN)$_6^{3-}$ in water and is not affected by diffusion through membrane material. It is apparent that at pH closer to 7, the Michaelis constant for ethanol is much greater than in basic solution. This suggests that the enzyme has greater affinity for ethanol in basic solution, as anticipated from the fact that ethanol oxidation is optimal at pH 8.8 while equilibrium is strongly shifted in favor of aldehyde reduction$^{21}$ at pH 7. The reverse trend for NAD$^+$ is difficult to explain but may in fact reflect variation due to experimental design. For many dehydrogenases the $K_M$ for NAD$^+$ falls in the range 0.1–1 mM. As pH increased from 7.05 to 9.2, the reported $K_M$ (NAD) increased from 0.109 to 1 mM. The $K_M$(NAD) = 0.80 mM value obtained amperometrically at pH 8.8 in our laboratory (Figure 5) is in general agreement with this pattern of pH dependency. Our amperometrically determined catalytic constant, $k_{cat}$, also known as the turnover number, was in excellent agreement with spectrophotometrically determined values.$^{16,17}$

The averaged kinetic results of more than a dozen experiments are presented in Table I alongside spectrophotometrically determined parameters. Literature values used in this table are referenced in the discussion and were selected from research conducted at pH 9 ± 0.2 whenever available.

**CONCLUSIONS**

A comparison of amperometrically (in the presence of an artificial mediator) and spectrophotometrically determined enzyme kinetic parameters shows overall good agreement between the two methods. We feel that the concordance between available spectrophotometric measurements in the literature and electrochemical measurements is generally so satisfactory that it indicates that the relevant enzymatic mechanisms of the natural enzymatic reaction and the one occurring in the presence of the mediator are the same. Only the maximum reaction velocity, $V_{max}$, determinations showed differences in excess of 10% which warrant further investigation.

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