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Investigation of Enzyme-Mediated Electron Transfer Using DigiSim®

This article discusses interpretation of the cyclic voltammograms of an enzyme-catalyzed mechanism using BASi DigiSim® simulation software.

Many biosensors for physiologically important molecules (e.g., glucose) are based on the coupling of enzyme-catalyzed reactions with heterogeneous electron transfer reactions. Although an empirical approach can be used for optimizing the performance of such biosensors, a more systematic approach has also been adopted based on characterization of the fundamental reactions involved. Cyclic voltammetry is a very useful technique for qualitative characterization of mechanisms in which electron transfer reactions are coupled to chemical reactions, although digital simulation is often required to reveal some of the more subtle aspects of the voltammetric behavior and to extract quantitative data such as rate constants. In this note, we will discuss a recent publication (1) in which the diaphorase-catalyzed oxidation of NADH was investigated using BASi DigiSim® simulation software for cyclic voltammetry.

Enzyme Kinetics

The most commonly used model for enzyme kinetics is the Michaelis-Menten model, which is based on the following equation:

$$E + S \underset{k_2}{\overset{k_1}{\rightleftharpoons}} ES \overset{k_3}{\rightarrow} E + P$$

The enzyme E forms an intermediate complex ES with the substrate S. ES can then either dissociate to regenerate E and S or can proceed to form the product P. The rate of the catalytic reaction V is given by the equation:

$$V = k_3[ES]$$

An equation for the concentration of ES can be derived by assuming that the system is in steady state; that is, the rate of formation of ES is equal to the rate of breakdown.

$$[ES] = \frac{[E][S]}{\frac{k_2 + k_3}{k_1}} = \frac{[E][S]}{K_M}$$

where K_M is the Michaelis constant.

If we assume that concentration of the enzyme is much lower than that of the substrate, we can make further approximations to generate an equation for the rate of the catalytic reaction based on total enzyme concentration $[E_T]$:

$$V = \frac{k_3[E_T]}{K_M /[S] + 1}$$

Although further derivations can be made to this equation to generate the Michaelis-Menten equation, it is the above equation that has been commonly used in attempts to extract enzyme kinetics from cyclic voltammetry data, and hence this form will be used in this note.

Many enzyme-based biosensors involve oxidoreductase enzymes; that is, the net reaction is an homogeneous electron transfer reaction. Therefore, regeneration of the catalytic activity of the enzyme requires electron transfer to or from the active site of the enzyme. Direct electron transfer between an electrode and an active site is often not possible, since the active site is frequently buried within the enzyme. In such cases, small molecules (mediators) are used to shuttle electrons between the electrode surface and the active site. This is often referred to as mediated electron transfer, and it introduces additional steps to the simple enzyme kinetics model shown above, since we must incorporate the enzyme-mediator interaction. Equation 1 now becomes:

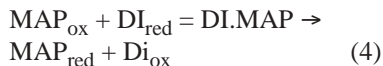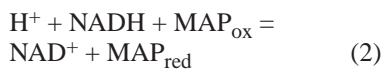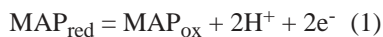$$V = \frac{k_3[E_T]}{K_{MS} /[S] + K_{MM} /[M] + 1}$$

where K_{MS} and K_{MM} are the Michaelis constants for the substrate and the mediator, respectively.

Previous reports (2,3) of the characterization of enzyme-catalyzed electron transfer by cyclic voltammetry have been based on the Michaelis-Menten model, including a recent study based on digital simulation using the explicit finite difference method (3). These studies have extracted values for the Michaelis constants and the catalytic rate constant from the cyclic voltammetry data using either linear regression analysis or working curves. However, these methods required some approximations for this approach to be valid. A more recent approach has used DigiSim to extract quantitative data from cyclic voltammograms (1). This approach is fundamentally different since the overall enzyme reaction is broken down into the fundamental reactions.

The particular reaction investigated in this study was the diaphorase(DI)-catalyzed oxidation of NADH using *p*-methylamino-phenolsulfate (MAP) as the mediator. The basic electro-chemistry of this system is shown in *F1*. *F1a* shows the cyclic voltammogram of NADH at a glassy carbon electrode. As expected, there is no direct oxidation of NADH, due to the large overpotential required. The cyclic voltammogram of the MAP mediator (*F1b*) shows a reversible process. The addition of NADH to the MAP solution causes a change in the voltammogram from an asymmetric peak to a sigmoidal curve (*F1c*) that is characteristic of a catalytic reaction following electron transfer; that is, MAP itself can catalyze the oxidation of NADH. The addition of diaphorase to the solution again generates a sigmoidal voltammogram (*F1d*), but with a much larger limiting current, which is to be expected due to the greater catalytic activity of the enzyme.

The following mechanism was proposed on the basis of the above

observations:

$$MAP_{red} = MAP_{ox} + 2H^+ + 2e^- \quad (1)$$

$$H^+ + NADH + MAP_{ox} = NAD^+ + MAP_{red} \quad (2)$$

$$H^+ + DI_{ox} + NADH = DI.NADH \rightarrow DI_{red} + NAD^+ \quad (3)$$

$$MAP_{ox} + DI_{red} = DI.MAP \rightarrow MAP_{red} + Di_{ox} \quad (4)$$

Reaction 1 represents the electron transfer reaction between the mediator and the electrode surface (note that this two-electron, two-proton reaction must be simulated as two sequential one-electron transfers when using DigiSim [4]). Reactions 2 and 3 are the competing homogeneous catalytic electron transfer reactions (although these would have to be simulated as second-order reactions by DigiSim), and reaction 4 represents regeneration of the active site of the catalyst by homogeneous electron transfer to the mediator. Although the breakdown of the two enzyme-substrate complexes are represented above as irreversible reactions, the principle of microscopic reversibility (which is obeyed by DigiSim) does not allow completely irreversible reactions. Therefore, when simulating such a reaction using DigiSim, a very large equilibrium constant is used in order to make the reaction *effectively* irreversible.

The first stage in simulating the above mechanism and obtaining kinetic and thermodynamic parameter values by fitting simulated data to experimental data was to simulate the heterogeneous electron transfer reaction between MAP and the electrode surface (using the approach mentioned above). The next step was to consider the competing catalytic oxidation of NADH by MAP; that is, electron transfer reactions followed by a second-order catalytic reaction. As with any simulation study, experimental and simulated data were compared for a range of scan rates (2 - 100 mVs^{-1}) and NADH concentrations (0.5 - 20 mM) with a fixed concentration of MAP (0.1 mM). A rate constant for this reaction (k_{comp}) of 10 M^{-1}s^{-1} was estimated. (The equilibrium constant for this reaction can be calculated from the known redox potentials of NADH and MAP, and hence it could be entered as a fixed parameter in the fitting operation.)

The parameters calculated for these two steps could now be used in the simulation for the catalytic oxidation by DI. As with the equilibrium constant for reaction 2, the equilibrium constants were calculated from the appropriate known redox potentials. Experimental and simulated cyclic voltammograms for a range of experimental conditions (scan rate = 2 - 100 mVs^{-1}, [MAP] = 1 μM - 1 mM, [NADH] = 10 mM, and [DI] = 5 μM). An excellent fit between the experimental and simulated data (*F2*) was obtained over this range of experimental conditions for the following kinetic parameter values:

k_1	$1 \times 10^5\,M^{-1}s^{-1}$
k_{-1}	$2.15 \times 10^2\,s^{-1}$
k_2	$40\,s^{-1}$
k_3	$1.1 \times 10^5\,M^{-1}s^{-1}$
k_{-3}	$0.23\,s^{-1}$
k_4	$1 \times 10^7\,s^{-1}$

It should be noted that DigiSim requires the equilibrium constant and the forward rate constant for each chemical reaction. Since the equilibrium constants listed for reactions 3 and 4 were known (*vide supra*), optimized values of k_{-1} and k_{-3} were calculated automatically from optimized values of k_1 and k_3, respectively. Once these kinetic parameters had been extracted, the Michaelis constants for NADH (K_{MS}) and MAP (K_{MM}) could be calculated using the equations below:

$$K_{MS} = \frac{k_{-1} + k_2}{k_1}$$

$$K_{MM} = \frac{k_2}{k_4}\left(\frac{k_{-3} + k_4}{k_3}\right)$$

In this example, $k_4 \gg k_{-3}$, so K_{MM} reduces to k_2/k_3. Values of 0.36 and 2.15 were calculated for K_{MM} and K_{MS}, respectively.

This study illustrates that DigiSim can readily be used for simulation of cyclic voltammograms of mediated enzyme-catalyzed electron transfer reactions. Since the mechanism has to be entered in terms of the fundamental heterogeneous electron transfer and homogeneous chemical reactions, additional reactions such as the competing catalytic reactions can readily be incorporated. Approaches based on the extraction of an overall catalytic rate constant would not detect these subtle variations in the mechanism.

References

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2. P.N. Bartlett and K.F.E. Pratt, *Biosens. Bioelectron.* 8 (1993) 451 and references therein.
3. K. Yokoyama and Y. Kayanuma, *Anal. Chem.* 70 (1998) 3368 and references therein.
4. A.W. Bott, *Curr. Seps.* 16 (1997) 61.

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F1.

Experimental cyclic voltammograms for a solution containing a) 0.01 M NADH, b) 0.1 mM MAP, c) 0.01 M NADH + 0.1 mM MAP, and d) 0.01 M NADH + 0.1 mM MAP + 5 μM DI. Scan rate = 2 mVs^{-1}, Tris buffer (pH = 8.5), all potentials measured with reference to Ag/AgCl. Figure adapted with permission from (1).
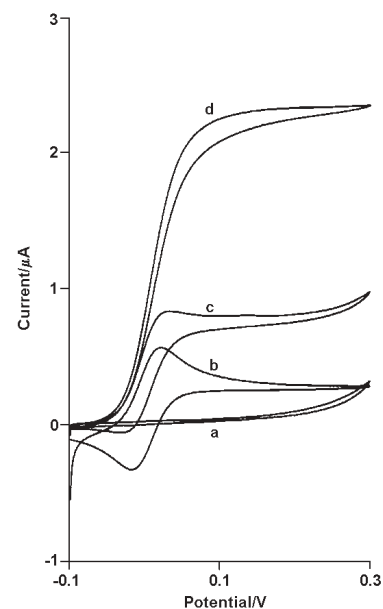
F2.

Comparison of experimental data from *F1d* (after background subtraction) with simulated data (kinetic parameters given in text). Figure adapted with permission from (1).