

Measurement of the Kinetic Isotope Effect for the Oxidation of NADH at a Poly(aniline)-Modified Electrode

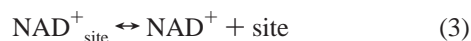
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The kinetic isotope effect has been widely used to investigate the mechanisms of reactions in homogeneous solution. This Communication extends the use of the technique to study the mechanism of a reaction at a chemically modified electrode. At a chemically modified electrode, redox molecules immobilized at the electrode surface are used to mediate between successive one-electron-transfer reactions at the electrode and oxidation or reduction of the species in solution. The electrochemical oxidation of NADH to NAD^+ provides an attractive model system on which to test ideas about the design of modified electrodes.¹ The oxidation of NADH to NAD^+ involves the transfer of two electrons and one proton and cleavage of a C–H bond. Direct electrochemical oxidation of NADH only occurs at high overpotentials and is accompanied by rapid poisoning of the reaction because the electrode surface is fouled by the polymeric products of side reactions which occur because the one-electron oxidation intermediates formed in the reaction are unstable and highly reactive. The design of chemically modified electrodes for the oxidation of NADH is also of great practical interest because the oxidation of NADH at low potentials is an essential requirement in the development of amperometric biosensors based on a wide range of NADH-dependent dehydrogenases.

In earlier work we have shown that electropolymerized poly(aniline) films grown in the presence of large polymeric counteranions are excellent modified electrodes for NADH oxidation in neutral solution at low potential.^{2–4} Using a kinetic model in which a reactive complex first forms between the NADH and polymer and then oxidation of NADH occurs within this reactive complex,^{2–4} we were able to fit the electrochemical data from an extensive set of experiments with films of different thickness, and for poly(aniline) films with different counteranions. According to this model the oxidation of NADH can be represented by



where site represents a reaction site on the polymer, K_{M} is the dissociation constant for the reaction complex, and k_{cat} is the first-order rate constant for oxidation of NADH within the reaction complex. We also suggested that the reason the poly(aniline) composite films are such good mediators for NADH oxidation is that the oxidation can occur by the transfer of a hydride from NADH to one of the nitrogens in the poly(aniline) chain followed by two rapid, successive one-electron oxidations of the poly(aniline) to regenerate the catalytic site, see Figure 1.

To investigate this model in greater detail, we prepared mono- and dideuterated samples of NADH by following standard literature procedures⁵ and studied the kinetics of their reaction at the poly(aniline)–poly(vinyl sulfonate)-modified electrode. The thicknesses

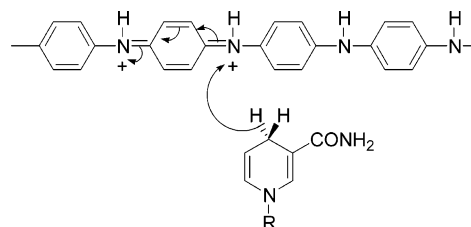


Figure 1. Suggested mechanism for the mediated oxidation of NADH at a poly(aniline)-coated electrode. Reaction of the oxidized, emeraldine form of the polymer with NADH produces NAD^+ and the reduced, leucoemeraldine form of the polymer which is then reoxidized at the electrode in two, sequential one-electron steps to complete the catalytic cycle.

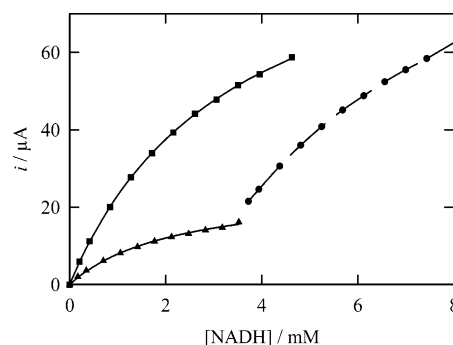


Figure 2. Plot of the oxidation current as a function of the NADH concentration recorded at 0.10 V vs SCE at a poly(aniline)–poly(vinyl sulfonate)-modified electrode (electrode area = 0.38 cm², film thickness = 5 μm) rotated at 9 Hz in 0.1 M citrate/phosphate buffer pH 7.0 at 25 °C. The NADH concentrations have been corrected to allow for concentration polarization in the diffusion layer of the rotating disk. Results for two experiments are shown. In the first experiment aliquots of [4,4-²H₂]NADH were added (▲) first up to a total concentration of 3.5 mM followed by aliquots of [4,4-¹H₂]NADH (●). In the second experiment aliquots of [4,4-¹H₂]NADH were added (■). The solid lines show the best fit of the data to the theory.

of the poly(aniline)–poly(vinyl sulfonate) films used in these experiments were carefully chosen so that we were in a regime where the reaction of NADH occurs throughout the whole of the film since this ensures the maximum sensitivity of the current on k_{cat} .^{2,3} Figure 2 shows plots of the oxidation current for [4,4-¹H₂]NADH and the doubly deuterated compound, [4,4-²H₂]NADH as a function of concentration. In the experiment aliquots of [4,4-²H₂]NADH were added to the cell first and the current recorded. As shown in the figure, the current initially increases and then reaches at plateau at around 3.5 mM [4,4-²H₂]NADH. To be sure that any differences in the currents for the two isotopomers arise from a kinetic isotope effect and are not caused by changes in the activity of the poly(aniline) film between measurements, we then added aliquots of [4,4-¹H₂]NADH to the cell containing 3.5 mM [4,4-²H₂]NADH. The current now increases further with increasing [4,4-¹H₂]NADH concentration clearly indicating that the [4,4-¹H₂]NADH reacts more rapidly at the modified electrode. The electrode was

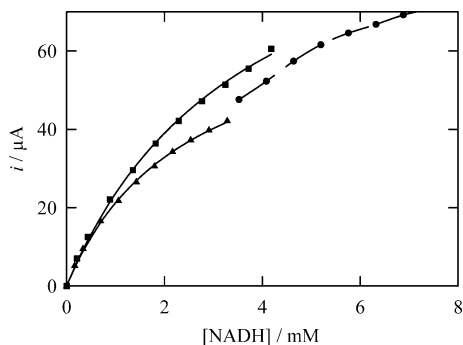


Figure 3. Plot of the oxidation current as a function of the NADH concentration under the same conditions as Figure 2. Results for two experiments are shown. In the first experiment aliquots of [4-¹H, 4-²H]NADH were added (▲) first up to a total concentration of 3.5 mM followed by aliquots of [4-¹H₂]NADH (●). In the second experiment aliquots of [4,4-¹H₂]NADH were added (■). The solid lines show the best fit of the data to the theory.

Table 1. Rate Constants for the Reaction of [4,4-¹H₂]NADH and [4,4-²H₂]NADH at a Poly(aniline)–Poly(vinyl sulfonate)-Modified Electrode Derived from the Analysis of the Data in Figure 2

	$k_{\text{cat}}/\text{s}^{-1}$	$K_{\text{M}}/K_{\text{S}}$, mM	$k_{\text{cat,HH}}/k_{\text{cat,DD}}$
[4,4- ¹ H ₂]NADH	0.100 ± 0.001	3.4 ± 0.1	4.0
[4,4- ² H ₂]NADH	0.026 ± 0.001	2.2 ± 0.2	

Table 2. Rate Constants for the Reaction of [4,4-¹H₂]NADH and [4-¹H, 4-²H]NADH at a Poly(aniline)–Poly(vinyl sulfonate)-Modified Electrode Derived from the Analysis of the Data in Figure 3

	$k_{\text{cat}}/\text{s}^{-1}$	$K_{\text{M}}/K_{\text{S}}$, mM	$k_{\text{cat,HH}}/k_{\text{cat,HD}}$
[4,4- ¹ H ₂]NADH	0.100 ± 0.004	3.5 ± 0.3	1.5
[4- ¹ H, 4- ² H]NADH	0.067 ± 0.003	2.4 ± 0.1	

then washed and the experiment repeated to record the response to [4,4-¹H₂]NADH on its own. As shown in the figure, the response for [4,4-¹H₂]NADH is significantly larger than that for [4,4-²H₂]NADH. Analysis of the data in Figure 2 using the model given in our earlier paper³ gives the values for k_{cat} ⁶ and $K_{\text{M}}/K_{\text{S}}$ in Table 1, where $K_{\text{M}}/K_{\text{S}}$ is the ratio of the dissociation constant for the reaction complex to the partition coefficient for NADH within the polymer film. From the table we see that deuteration of the NADH has little effect on the ratio $K_{\text{M}}/K_{\text{S}}$ but significantly alters the values of k_{cat} . This is consistent with our model and gives a kinetic isotope effect, $k_{\text{cat,HH}}/k_{\text{cat,DD}}$, of 4.0. We also carried out the same experiment using the monodeuterated compound, A-side [4-¹H, 4-²H]NADH,⁷ Figure 3. Again there is a kinetic isotope effect, but in this case it is considerably smaller. Table 2 gives the rate constants derived from these data, and now we find a kinetic isotope effect, $k_{\text{cat,HH}}/k_{\text{cat,HD}}$, of 1.5.⁸ The kinetic isotope effects measured in these experiments

are a combination of the primary kinetic isotope effect and the secondary kinetic isotope effect.⁸ Combining our results from the experiments for [4,4-²H₂]NADH and [4-¹H, 4-²H]NADH, we obtain estimates for the primary and secondary kinetic isotope effects of 4.2 and 0.91.⁹ This value for the primary kinetic isotope effect is within the range of those reported in the literature for the reactions of NADH with a variety of two-electron oxidants in homogeneous solution^{8,11–13} and confirms that transfer of H from the NADH to the polymer occurs in the rate-limiting step within the reaction complex. Our results are inconsistent with rate-limiting electron transfer followed by proton and electron transfer and indicate that the reaction proceeds either by hydride transfer or by hydrogen atom transfer followed by rapid electron transfer.¹⁴ Our results demonstrate that kinetic isotope measurements can be usefully applied to study the mechanisms of reactions at chemically modified electrodes.

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Supporting Information Available: Details of preparation of deuterated NADH samples, polymerization of poly(aniline) films, and measurement conditions. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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- (6) Values of k_{cat} were calculated by assuming that each imine unit in the emeraldine form of the polymer acts as a site for reaction and calculating the number of imine units from the voltammetry of the film. This probably underestimates k_{cat} because the large size of the NADH molecule will mean that adjacent sites will be blocked when the NADH is adsorbed.
- (7) Our deuteration scheme selectively adds the first deuterium to the A-face of the NADH molecule. See Supporting Information for details.
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- (9) The values for the primary and secondary kinetic isotope effects were calculated from the values of $k_{\text{cat,HH}}/k_{\text{cat,DD}}$ and $k_{\text{cat,HH}}/k_{\text{cat,HD}}$ using the equations given by Powell and Bruce⁸ which assume no difference in the reaction rate for the two faces of the molecule. For a change from sp³ to sp² a secondary kinetic isotope effect greater than 1 is expected. The calculated value for the apparent secondary kinetic isotope effect of 0.91 could be explained by a difference in either K_{M} or k_{cat} by a factor of 1.2 to 1.3 for the two faces of NADH. Such an effect has been reported for the reaction of NADH with 4-cyano-2,6-dinitrobenzenesulfonate¹⁰ where a factor of 1.2 difference in reaction rate was found.
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