Electrochemistry of redox proteins and enzymes

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1 Background: redox thermodynamics

1.1 The Nernst equation

Consider a reaction mixture containing the oxidised and reduced forms of two different species (1 and 2):

\[ \text{Ox}_1 + \text{Red}_2 \rightleftharpoons \text{Red}_1 + \text{Ox}_2 \] (1)

The free energy of the reaction ($\Delta_r G$) is given by

\[ \Delta_r G = \Delta_r G^0 + RT \ln \frac{[\text{Red}_1][\text{Ox}_2]}{[\text{Ox}_1][\text{Red}_2]} \]

$R$ is the gas constant, $T$ the absolute temperature and $\Delta_r G^0$ is the (tabulated) standard free energy of the reaction.

If equilibrium between the different species is reached, $\Delta_r G = 0$, and the ratio of concentrations (the reaction quotient) is linked to $\Delta_r G^0$ by the relation:

\[ K_{eq} = \frac{[\text{Red}_1]_{eq}[\text{Ox}_2]_{eq}}{[\text{Ox}_1]_{eq}[\text{Red}_2]_{eq}} = \exp \left( -\frac{\Delta_r G^0}{RT} \right) \] (2)

Initially upon mixing $\text{Ox}_1$ and $\text{Red}_2$, the concentrations of various species in the solution do not satisfy eq. (2), and $\Delta_r G$ is non zero: the system is not at equilibrium. Thermodynamics predicts that reaction (1) will proceed in the forward direction until the reaction quotient equals $K_{eq}$.

During the reaction, $\text{Red}_2$ is oxidised and gives electrons to $\text{Ox}_1$.

\[ \text{Ox}_1 + n\text{e}^- \rightarrow \text{Red}_1 \]
\[ \text{Red}_2 \rightarrow \text{Ox}_2 + n\text{e}^- \]

It is possible to measure the flux of electrons from $\text{Red}_2$ to $\text{Ox}_1$, and therefore the rate of the overall reaction, by placing the species in a two-compartment cell ($\text{Ox}_1$ in one compartment and $\text{Red}_2$ in the other). Place in each side an electrode at which the species can interact. When the two electrodes are connected together, a current flows, while the system evolves towards equilibrium.

The current flow between the two electrodes results from a potential difference between them, the value of which can be predicted using the Nernst equation.

\[ E = E^0 + \frac{RT}{nF} \ln \frac{[\text{Ox}]}{[\text{Red}]} \] (3)

Applying this equation to each of the two electrodes, the potentials $E_1$ and $E_2$ are given by:

\[ E_1 = E_1^0 + \frac{RT}{nF} \ln \frac{[\text{Ox}_1]}{[\text{Red}_1]} \] (4a)
\[ E_2 = E_2^0 + \frac{RT}{nF} \ln \frac{[\text{Ox}_2]}{[\text{Red}_2]} \] (4b)

---

1 "Standard conditions" means that the activity of all constituents is unity, and the pressure equals one atmosphere.

2 Thermodynamics predicts only the direction of the reaction. In reality, a “favorable” reaction might not happen because its rate is very small, in which case the equilibrium cannot be reached.

3 Walther Hermann Nernst (1864-1941) was born in West Prussia. He obtained his Ph.D. at Würzburg (Friedrich Kohlrausch), beginning his career as a physicist.

He graduated in 1887 with a thesis on electromotive forces produced by magnetism in heated metal plates. Nernst’s first outstanding work was his theory of the electromotive force of the voltaic cell (1888). Nernst applied the principles of thermodynamics to the chemical reactions proceeding in a battery. In 1889, he showed how the characteristics of the current produced could be used to calculate the free energy change in the chemical reaction producing the current. The Nernst Equation, which related the voltage of a cell to its properties, also has very important biophysical implications. In 1905, he was appointed Professor of physical chemistry in the University of Berlin. His theorem, known as the Third Law of Thermodynamics, was developed in 1906. In 1914 Walther Hermann Nernst and Max Planck succeeded in bringing Albert Einstein to Berlin.
$E_0^\circ$ is the standard reduction potential of the redox couple Ox\textsubscript{1}/Red\textsubscript{1}. $F = 96500$ C is the Faraday\textsuperscript{4} constant. The potential difference between the electrodes in compartments 1 and 2 is therefore:

$$V = E_2 - E_1 = E_2^\circ - E_1^\circ + \frac{RT}{nF} \ln \frac{[\text{Ox}_2][\text{Red}_1]}{[\text{Red}_2][\text{Ox}_1]}$$

(5)

The electrons are going to flow from the cell whose electrode potential is the lowest to the other until the potential difference $V$ is zero and the concentrations satisfy

$$K_{eq} = \frac{[\text{Red}_1]_{eq}[\text{Ox}_2]_{eq}}{[\text{Ox}_1]_{eq}[\text{Red}_2]_{eq}} = \exp \left( -\frac{nF}{RT} (E_2^\circ - E_1^\circ) \right)$$

This is equivalent to eq. (2), since reduction potentials and free energies are linked by

$$\Delta_r G^\circ = -nFE_0^\circ$$

Reaction (1) will proceed forward significantly ($K_{eq}$ will be large) only if $E_1^\circ > E_2^\circ$. (If $E_2^\circ$ is “high”, Ox\textsubscript{1} is called a strong oxidant and Red\textsubscript{1} is a weak reductant.) The Nernst equation can therefore be used to determine the direction in which a redox reaction will proceed spontaneously.\textsuperscript{5}

1.2 Reference potential and reference electrodes

If you are interested in studying only one half-reaction, it is convenient to make sure that the potential difference between the two electrodes reflects the potential of the electrode you’re interested in. This can be done by using in the other compartment an electrode designed to have a constant potential: this is called a “reference electrode”.

The Standard Hydrogen Electrode is one of these. It consists of a platinum electrode immersed in a pH=0 electrolyte below one atmosphere of H\textsubscript{2}.

By convention, the potential of the Standard Hydrogen Electrode is zero. All reduction potentials tabulated in the literature are quoted versus the SHE.

A real SHE is rarely practical. Instead one uses convenient reference electrodes such as:

- Hg/Hg\textsubscript{2}Cl\textsubscript{2}/KCl (Standard Calomel Electrode, SCE, E(SCE)=241mV vs. SHE.) This is the most commonly used reference electrode
- Hg/Hg\textsubscript{2}SO\textsubscript{4}/K\textsubscript{2}SO\textsubscript{4}, used when chloride ions must be avoided. E(SCE)=615mV vs. SHE.
- Ag/AgCl (E=800mV vs. SHE)

Because standard conditions\textsuperscript{1} (pH 0!) are not suitable for biological reactions, reduction potentials are usually stated for pH 7, and termed $E_0^\prime$ or $E_{m,7}$.

Figure 1 gives an idea of the range of reduction potentials spanned by biologically important redox couples.

1.3 Influence of coupled reactions (e.g. protonation or ligand binding) on reduction potentials

Redox reactions can be coupled to other chemical equilibria such as ligand binding (e.g. protons, substrate, inhibitor... ) or conformational changes.

\textsuperscript{4}Michael Faraday (1791-1867). His scientific work laid the foundations of all subsequent electro-technology. From his experiments came devices which led directly to the modern electric motor, generator and transformer. Faraday was also the greatest scientific lecturer of his day, who did much to publicize the great advances of nineteenth-century science and technology. During the 1830s, Faraday worked on developing his ideas on electricity. He enunciated a new theory of electro-chemical action between 1832 and 1834 one of the results of which was that he coined many of the words now so familiar - electrode, electrolyte, anode, cathode and ion to name but five. His work led him to reject the traditional theory that electricity was an imponderable fluid or fluids. Instead, he proposed that electricity was a form of force that passed from particle to particle of matter.

\textsuperscript{5}It may not be enough to compare standard reduction potentials however, since the sign of ln $\frac{[\text{Ox}_2][\text{Red}_1]}{[\text{Ox}_1][\text{Red}_2]}$ in eq. (5) can change the sign of $V$ (and $\Delta_r G$) and thus the direction of the current flow.
A very common (and physiologically important) coupled reaction is proton transfer, as represented in the square scheme fig. 2. $K_{Ox}$ and $K_{Red}$ are the acidity constants for Ox and Red. Utilising the principle of thermodynamic cycles (the sum of $\Delta r G^0$ values round the square is zero), these acidity constants can be linked to the reduction potentials of the protonated and un-protonated redox couples. Note that potentials alone cannot be summed; they must be scaled by $n$. Usually Red is a much better base than Ox, so it has a higher $pK$, i.e. $pK_{Ox} < pK_{Red}$.

- For $pH < pK_{Ox}$, both Ox and Red are protonated.

$Ox : H + ne^- \rightarrow Red : H$

The reduction potential is $pH$ independent, and equals $E_{acid}^0$ (fig. 2).

- For $pH$ between $pK_{Ox}$ and $pK_{Red}$, Ox is not protonated but Red is.

$Ox + ne^- + H^+ \rightarrow Red : H$

The reduction potential is $pH$ dependent: it decreases by $2.3RT/nF$ Volts per pH unit ($\frac{23}{n}mV/pH$ at $25^\circ C$).

In general, for a redox process involving $n$ electrons and $m$ protons, the maximal $pH$-dependence is

$$\frac{2.3RT}{F} \frac{m}{n} V/pH$$

- For $pH > pK_{Red}$, neither Ox nor Red are protonated, the redox process is

$Ox + ne^- \rightarrow Red$
Figure 3: (A) Simplified Pourbaix diagram for a 1e\textsuperscript{−} : 1H\textsuperscript{+} reaction. (B) Reduction potential vs. pH for amicyanin from Paracoccus versatus at 25°C. The values of pK\textsubscript{Red} and pK\textsubscript{Ox} can easily be measured from the pH dependence. Line is best fit to eq. (7), with \( n = 1 \). Amicyanin is a type I blue copper protein which exhibits a Cu\textsuperscript{II}/Cu\textsuperscript{I} redox process. From ref. [17].

Figure 4: Pourbaix-like diagram for the change in the reduction potential of the flavin cofactor of E. coli fumarate reductase as a function of the succinate concentration (increasing from right to left) at 20°C, pH 7. Line is best fit to eq. (7), with \( n = 2 \), but with [succinate] instead of [H\textsuperscript{+}]. From ref. [18].

The reduction potential is pH independent, and equals \( E\textsubscript{0}^\text{alk} \) (fig. 2).

For an \( n \)-electron, one-proton process, the whole pH-dependence of the reduction potential is given by:

\[
E\textsuperscript{0}' ([H\textsuperscript{+}]) = E\textsuperscript{0}^\text{alk} + \frac{2.3RT}{nF} \log_{10} \left( \frac{1 + \frac{[H\textsuperscript{+}]}{K\textsubscript{Red}}}{1 + \frac{[H\textsuperscript{+}]}{K\textsubscript{Ox}}} \right)
\]

This equation is demonstrated in the appendix, on page 28.

Redox-linked protonations are conveniently represented by a Pourbaix diagram,\textsuperscript{6} a plot of \( E\textsuperscript{0}' \) as a function of pH, as schematised in fig. 3A.

It is essential to understand that these thermodynamic considerations apply for any ligand which binds a redox center: provided that the dissociation constants from the reduced and oxidised forms are different, the reduction potential depends on the concentration of ligand, and the dissociation constants can be measured by determining how the reduction potential depends on the concentration of ligand.

\textsuperscript{6}Marcel Pourbaix (1904-1998). Marcel Pourbaix was born in Myshega (Russia). He studied in Brussels and graduated from the Faculty of Applied Sciences of the Université Libre de Bruxelles in 1927, with which he was associated from 1937 for the rest of his career.

By 1938, he had devised the potential-pH diagrams for which he became famous. In 1939, just before the outbreak of World War II, he presented to the Faculty his doctoral dissertation, accompanied by a thesis entitled “Thermodynamics of Dilute Aqueous Solutions. Graphical Representation of the Role of pH and Potential”. The war and some confusion among the jury on the sign of electrode potential impeded the completion of the graduation process and, on the suggestion of Prof F.E.C. Scheffer (Delft), the thesis was presented to the Technical University Delft. This Delft thesis had a major influence on corrosion science. During the fifties and early sixties, Marcel Pourbaix and his collaborators produced potential-pH diagrams for all the elements and published the “Atlas of Electrochemical Equilibria” [19] in French in 1963 and in English in 1966.
### Table 1: Standard reduction potentials at pH 7, 25°C, of common mediators.

<table>
<thead>
<tr>
<th>Mediator</th>
<th>( E^{\circ} ) (mV vs SHE)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Benzyl viologen</td>
<td>-360</td>
</tr>
<tr>
<td>Lapachol</td>
<td>-172</td>
</tr>
<tr>
<td>Methylene blue</td>
<td>11</td>
</tr>
<tr>
<td>Ferricyanide ((\text{K}_3\text{Fe(CN)}_6))</td>
<td>360</td>
</tr>
<tr>
<td>Hexachloroiridate ((\text{Na}_2\text{IrCl}_6))</td>
<td>870</td>
</tr>
</tbody>
</table>

Application: the effect of pH on the reduction potentials of the copper site in a blue copper protein [17]

As an example, fig. 3B, from ref [17] shows the dependence on pH of the reduction potential of the type-I copper site of amicyanin, determined using protein film voltammetry (see below). The data can be analysed in terms of electron transfer being coupled to a single protonatable group with \( pK_{\text{Red}} = 6.3 \) and \( pK_{\text{Ox}} \leq 3.2 \). Protonation occurs on one of the two Histidines which anchor the copper ion to the protein.

Application: the effect of substrate binding on the reduction potentials of the active site flavin in fumarate reductase [18]

Figure 4 illustrates the succinate-concentration-dependence of the reduction potential of the active site flavin in *E. coli* fumarate reductase. In this case, the reduction potential decreases as the concentration of succinate is raised (from right to left in this figure) because binding of succinate to the oxidised form of the enzyme is tighter than to the reduced form (\( pK_{\text{Ox}} > pK_{\text{Red}} \)).

Other applications

See e.g. refs. [20–23] for the effect of protonation or binding of metal or exogenous thiolate on the reduction potential of Fe-S clusters.

### 2 Electron transfer kinetics

In the redox enzymes involved in respiration and photosynthesis, electrons are transferred along chains of redox centers (see e.g. ref [24–26] for a review). It was crucial to understand which parameters determine the *rates* of electron transfer (ET).

Let’s consider a certain ET step, from a donor \( D \) to an acceptor \( A \).

\[
D_{\text{red}} + A_{\text{ox}} \overset{k_{D \rightarrow A}}{\rightleftharpoons} D_{\text{ox}} + A_{\text{red}}
\]

(8)

The rate of electron transfer (ET) from \( D \) to \( A \) depends on the reduction potentials of \( D \) and \( A \), but also on other parameters. This was discovered in the 1960’s, by Marcus\(^7\) who developed a model based on a molecular description of ET between small molecules in solution. He showed

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\(^7\)Rudolph Arthur Marcus (http://www.geocities.com/bioelectrochemistry/marcus.htm) was born on July 21, 1923 in Montreal, Canada, he became a naturalized citizen of the US in 1958 and won the 1992 Nobel Prize for Chemistry for his work on the theory of electron-transfer reactions in chemical systems. His theory shed light on diverse and fundamental phenomena such as photosynthesis, cell metabolism, and simple corrosion. In a series of papers published between 1956 and 1965, he investigated the role of surrounding solvent molecules in determining the rate of redox reactions—oxidation and reduction reactions in which the reactants exchange electrons—in solution. He determined that subtle changes occur in the molecular structure of the reactants and the solvent molecules around them; these changes influence the ability of electrons to move between the molecules. He established that the relationship between the driving force of an electron-transfer reaction and the reaction’s rate is described by a parabola. Thus, as more driving force is applied to a reaction, its rate at first increases but then begins to decrease. This insight aroused considerable skepticism until it was confirmed experimentally in the 1980s.
that this process requires the formation of a transient complex, in which the kinetics of the ET step can be described by an equation of the form

\[ k_{D\rightarrow A} = C \exp \left( -\frac{(\Delta_r G^0 + \lambda)^2}{4\lambda RT} \right) \]  

(9)

- \( \Delta_r G^0 \) is the standard free energy of the reaction, which is related to the standard reduction potentials of the donor D and the acceptor A according to \( \Delta_r G^0 = F(E^0_D - E^0_A) \).
- The parameter \( \lambda \), called “reorganization energy,” is all the greater that large molecular rearrangements accompany the transfer (both the geometries of the molecules that are oxidized or reduced and the polarization of the surrounding solvent should be considered).
- The expression of the preexponential factor \( C \) depends on the strength of the electronic coupling between the acceptor and the donor. If it is strong enough (“adiabatic” transfer), \( C \) simply equates \( kT/h \), as given by the classical transition state theory. When it is weak (this is so for long-distance, “nonadiabatic” ET), \( C \) depends on the overlap of the molecular wave functions of D and A, and therefore on the nature of the redox centers, on their distance and on the intervening medium. An exponential decrease of \( C \) with distance is expected. In the literature, nonadiabatic transfers are often referred to as electron tunneling processes.

The relation between thermodynamics and kinetics is understood by calculating an equilibrium constant from the ratio \( k_{D\rightarrow A}/k_{A\rightarrow D} \) (then compare with eq. 2):

\[ \frac{k_{D\rightarrow A}}{k_{A\rightarrow D}} = \frac{C \exp \left( -\frac{(\Delta_r G^0 + \lambda)^2}{4\lambda RT} \right)}{C \exp \left( -\frac{(-\Delta_r G^0 + \lambda)^2}{4\lambda RT} \right)} = \exp \frac{-\Delta_r G^0}{RT} \]  

(10)

Now consider the electron transfer between a molecule and a metallic electrode

\[ \text{Red} = \text{Ox} + e^- \text{(electrode)} \]  

(11)

The equation that gives the rate of ET is a complex function of the reduction potential of the molecule \( E^{0'} \), the electrode potential \( E \), and the reorganization energy \( \lambda \) [2, 6] but a simplified rate equation, known as the “Butler Volmer equation,” predicts that the rates of oxidation and reduction are independent on \( \lambda \) (provided \( \lambda \) is large) and increase and decrease (respectively) exponentially as the electrode potential increases:

\[ k_{ox} = k_0 \exp \frac{F}{RT}(E - E^{0'}) \]  

(12)

The preexponential factor \( k_0 \) depends on the coupling between the electrode and the redox molecule. The greater \( k_0 \) the faster the electron exchange between the electrode and the redox molecule; of course, this parameter has no physiological relevance.

The relation between thermodynamics and kinetics is understood by calculating the ratio \( k_{ox}/k_{red} \) (then compare with eq. 3):

\[ \frac{k_{ox}}{k_{red}} = \frac{k_0 \exp \frac{F}{RT}(E - E^{0'})}{k_0 \exp \frac{F}{RT}(E^{0'} - E)} = \exp \frac{F}{RT}(E - E^{0'}) \]  

(13)

3 Mediated potentiometry (under equilibrium conditions)

The reduction potential of a redox couple is determined by recording the ratio \([\text{Ox}]/[\text{Red}]\) observed after allowing the system to equilibrate with the electrode at different potentials.
The potential is usually varied by adding titrants of an oxidant or a reductant. Unlike small molecules, protein redox centers do not generally react rapidly with the measuring electrode and equilibrium is not established quickly. To overcome this problem, small redox agents called mediators are added to the solution to transport electrons between the active site and the electrode. For best results, these should have reduction potentials close to that of the active site being studied; mixtures of mediators are often employed to cover a wide range. (A short list of mediators and their reduction potentials is given in table 1. A useful list of “Reduction Potentials of One-electron Couples Involving Free Radicals in Aqueous Solution” is available on the web at http://www.rcdc.nd.edu/compilations/Red/Intro_Red.htm)

The ratios [Ox]/[Red] are typically determined by examination of the optical or EPR spectra. According to the Nernst equation (3), a plot of $E$ versus log[Ox]/[Red] should be a linear, with a slope $2.3RT/nF$. The electrode potential equals $E^0'$ when [Ox]/[Red]= 1.

Very often, the concentration of Ox or Red is measured as a function of the electrode potential, and the plot is fitted with:

$$\text{[Red]} \propto \frac{1}{1 + \exp\left(\frac{nF}{RT}(E - E^0')\right)}$$  \hspace{1cm} (14a)

$$\text{[Ox]} \propto \frac{\exp\left(\frac{nF}{RT}(E - E^0')\right)}{1 + \exp\left(\frac{nF}{RT}(E - E^0')\right)}$$  \hspace{1cm} (14b)

Obviously, this approach requires that the redox center has a distinct spectroscopic signature.

**Application: measuring the reduction potential of a blue copper site in azurin [27]**

Fig. 5 is the result of a potentiometric titration of the “blue” (type-I) copper site in an azurin mutant, followed by UV-vis spectroscopy. The oxidized copper site absorbs at $\approx$600nm due to a Cys-S to Cu ligand-to-metal-charge-transfer.

### 4 Introduction to direct methods (“dynamic” electrochemistry)

#### 4.1 Electrodes for electron transfer to/from proteins

In an increasing number of cases, direct electrochemistry of redox proteins (without the need for mediators) is possible, and this opens the way for detailed studies not only of the thermodynamics but also the kinetics of reactions. To be successful, electrodes must exchange electrons quickly with the proteins, and preserve their native properties. These electrodes may resemble natural environments or reaction partners for the protein.

Interactions between protein and electrode may be tailored to be weak or strong. Weak interactions might ideally give rise to diffusion-controlled voltammetry (section 5), whereas with strong
interactions, the experiment may address just a small sample (“film”) of protein molecules on the electrode (section 6).

Various types of electrode surfaces for which protein voltammetry is commonly observed:
- Metal (Au, Pt, Ag) surfaces on which a monolayer of adsorbate is self assembled (“Self Assembled Monolayers” or SAMs). The adsorbate is a bifunctional molecule of the type X-Y, where X is a substituent that anchors the molecule on the metal electrode surface (e.g. a thiol) and Y is a functional group that interacts with the protein (typically carboxyl for cytochromes c, or amino for acidic proteins such as plastocyanin or ferredoxins.)
- Various electrode materials onto which a surfactant film or layers of polyelectrolytes incorporate large membrane-bound proteins, but catalytic activity is generally greatly impaired!
- Pyrolytic graphite edge or basal plane electrodes provide hydrophilic or hydrophobic interactions, respectively. The former is often used with co-adsorbates (aminocyclitols, polymixyn, polyllysine) which probably form cross-linkages between the protein and the electrode surface.

### 4.2 Electrochemical equipment

The experiment is carried out using an electrochemical analyser in conjunction with the cell. The cell consists of three electrodes. The reference electrode is often contained in a side arm linked to the main compartment by a capillary tip called a Luggin (after the glassblower who invented it). The tip is positioned close to the working electrode. To avoid passing current through the reference electrode, a third electrode, called auxiliary or counter electrode, is used. The working electrode can also be rotated (section 5.2) to control mass transport of solution species. See fig. 6. The analyser measures the current registered in response to the potential that is applied. In general, the potential of the working electrode (versus the reference electrode) is modulated (e.g. in a linear sweep) and the current flowing between the working electrode and the counter electrode is recorded. Since the electrode potential is swept forward and back, the technique is called “cyclic voltammetry”. A voltammogram is a plot of the current as a function of the electrode potential.

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*This would change its potential and also damage it.*
4.3 Vocab and conventions

A cathodic process is a reduction, the cathode is the electrode onto which the reduction occurs. A anodic process is an oxidation, occurring at the anode.

Usually, a cathodic current is counted as negative and an oxidation results in a positive current. American people and software often use the opposite convention for the sign of the current (See e.g. fig. 8).

For the following descriptions (sections 5 and 6.1), we will assume that electron transfer is fast, in which case the redox reaction is said to be reversible, and the Nerst equation can be used despite the fact that the system is not at equilibrium.

5 Diffusion controlled Voltammetry

5.1 Diffusion controlled voltammetry at stationary electrodes

Imagine the case of a solution containing only the reduced form of a soluble electroactive species. The potential is swept linearly in time, as shown in fig. 7A, starting from a low potential. The current response as a function of time is plotted in fig. 7B, and the cyclic voltammogram (current against potential) in fig. 7C.

While the potential is lower than $E^0$, no oxidation occurs and no current is measured [see (a)
in figs. 7]. When the electrode potential approaches $E^{0'}$, Red starts being oxidised into Ox, giving electrons to the electrode. This is measured as a (positive) current which increases as more and more species are oxidised (b). However, the electrode oxidises only species close to it, and the interface is soon depleted. The current then goes through a maximum before decreasing (c). It doesn’t go to zero since diffusion still brings (slowly) Red from the bulk of the solution.

While a positive current is being measured, Ox produced by the reaction accumulates near the electrode and diffuses slowly towards the bulk.

After the scan is reversed, (d), the current is still positive and decreasing: Red species are still being oxidised since the electrode potential is above the reduction potential. Near the reduction potential, the Ox species which have accumulated are now reduced and a negative current is observed (e) until the concentration of Red near the interface drops down (f), and so does the current.

This results in a peak-like response in both directions.9

For an $n$-electron reaction, the separation between cathodic (reduction) and anodic (oxidation) peaks is given by

$$\Delta E_p = E_{p,a} - E_{p,c} = 2.218 \frac{RT}{nF} = \frac{57}{n} \text{ mV at 298K}$$

○ The peak current is proportional to the bulk concentration of species $C$, and to the square root of the potential scan rate $\nu$:

$$i_p = 2.69 \times 10^5 n^{3/2} A D^{1/2} \times C \sqrt{\nu}$$

This is the Randles-Sevcik equation. $A$ is the electrode surface, $D$ is a diffusion coefficient. A linear plot of $i_p$ against $\sqrt{\nu}$ is the criterion used to identify when the redox species are diffusing from the bulk to the electrode.

○ The reduction potential is given by the average of the cathodic and anodic peak potentials.

$$E^{0'} \approx \frac{E_{p,a} + E_{p,c}}{2}$$

A system that conforms to these criteria is said to be reversible and diffusion controlled.

**Application: measuring the reduction potential of a cytochrome**

Figure 8 shows a voltammogram for the reversible oxidation and reduction of a cytochrome. Note that the concentration of the protein sample must be high.

5.2 Diffusion controlled voltammetry at rotating electrodes

The peak shape of the diffusion-limited voltammogram at a macro electrode is due to the depletion of electroactive species near the electrode surface as they are consumed by the redox reaction.

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9The modeling of voltammograms might be complex because the current response depends on the diffusion of the species in the solution, and the equations coupling reaction of the species and their diffusion into the solution are in general difficult to solve.
Figure 9: Cyclic voltammetry at a rotating disc electrode of an electroactive species in solution. The electrode potential is swept linearly in time (A), and the current recorded as a function of time (B). A convenient way of displaying the results is to plot the current as a function of the potential (C).

There are many electrochemical techniques in which the electrode moves with respect to the solution. In the most popular, the electrode (called a “rotating disc electrode”) is rotating along its axis in the solution. This introduces a convective movement of the solution which increases the efficiency of the transport of species from the bulk towards the electrode. Because the depletion layer cannot spread anymore in the solution, the current reaches a limiting value $i_{\text{lim}}$ at high driving force, fig. 9.

- The Levich equation predicts that the limiting current is proportional to the concentration of electroactive species $C$ and to the square root of the electrode rotation rate $\omega$:

$$i_{\text{lim}} = 0.620nFA\nu_s^{-1/6}D^{2/3} \times C\sqrt{\omega}$$

(18)

In this equation, $\nu_s$ is the kinematic viscosity of the solution.\(^{11}\)

A plot of $i_{\text{lim}}^{-1}$ against $\omega^{-1/2}$ is called a Koutecky-Levich plot.

- The reduction potential $E^{0'}$ is simply given by the half-wave potential $E_{1/2}$, the potential at which the current reaches half its limiting value.\(^{12}\)

$$E^{0'} = E_{1/2}$$

(19)

\(^{10}\)The zone of the solution adjacent to the electrode where the concentration of species differ from that in the bulk.

\(^{11}\)The kinematic viscosity is the ratio of the viscosity over the density. E.g., the viscosity of pure water at 20°C is $10^3\mu\text{Pa-s}$, and its density $\approx 1\text{g/cm}^3$; this gives a kinematic viscosity $\nu_s \approx 10^{-2}\text{cm}^2/\text{s}$.

\(^{12}\)A Heyrovsky-Ilkovich plot, $\log_{10}[i_{\text{lim}} - i]/i$ against $E$, is usually used to analyze sigmoidal polarographic waves recorded under steady-state diffusion-limited conditions [1]. For an $n$-electron electrochemical reaction that is reversible (i.e. the Nernst equation is obeyed at all times), the plot is linear with a slope $\pm nF/2.3RT$, and the semi-logarithmic plot intercepts the line for $(i_{\text{lim}} - i)/i = 1$ at $E = E^{0'}$. 

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Figure 10: Cyclic voltammograms of amicyanin from *P. denitrificans* at a 3µm gold microelectrode. $\nu = 10\text{mV/s}$. The amicyanin concentration in 50mM phosphate buffer (pH7) were (a) 130µM, (b) 90µM, (c) 50µM and (d) 0µM. Note the sigmoidal shape of the voltammogram despite the fact that the electrode is stationary, and the increase in limiting current when the concentration of protein is raised. From ref. [29].

The scan rate does not enter the measurement if it is small because the current depends on the electrode potential but is independent of time: the voltammogram is said to be at steady state.

5.3 Diffusion controlled voltammetry at stationary micro-electrodes

The considerations above apply only for “macro”-electrodes (*i.e.* when the diameter of the electrode is larger than the typical size of the diffusion layer). With a “micro”-electrode, whose typical size is of the order of a few micrometers, the voltammogram has a sigmoidal shape despite the fact that the transport is diffusion-limited, fig. 10.

Microelectrodes have been used in the context of cell biology; their size make them suitable to detect electroactive species released by a single cell. See e.g. refs [30] for a recent review.

6 Voltammetry of adsorbed proteins: Protein Film Voltammetry (PFV)

When the protein sample is immobilised on the electrode surface, diffusion can be eliminated and much greater thermodynamic and kinetic resolution can be obtained with extremely small sample quantities.

This approach was developed by F. Armstrong (now in oxford) in the 1970’s with small redox proteins, and since the beginning of the 1990’s with large redox enzymes. Over the last years, this technique was used to study all sorts of aspects of the mechanism of redox proteins and enzymes: proton transfer [31] (*cf* page 17), inter and intramolecular electron transfer [32,33], oxygen sensitivity in hydrogenases [7], diffusion along substrate channels [34,35] (*cf* page 24) etc. (see ref. [6] for review).

The rest of this manual will focus on the principles and applications of protein film voltammetry.

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13 [http://www.chem.ox.ac.uk/icl/ffaingroup/index.html](http://www.chem.ox.ac.uk/icl/ffaingroup/index.html)
6.1 “Non-catalytic” (or “stoichiometric”) voltammetry at slow scan rates

Figure 11 shows the shape of a cyclic voltammogram when the redox species is adsorbed onto an electrode. Starting again at an electrode potential lower than $E^0$, the redox centers are fully reduced [see (a) in fig. 11]. Sweeping the potential towards high values, the protein starts being oxidised when the electrode potential approaches the reduction potential (b); a positive current is then measured, which drops down to zero at high potential when all the adsorbed proteins are oxidised (c). On the reverse scan, a reductive (negative) current is observed when the electrode potential matches the reduction potential of the protein (d) until all the sample is reduced and the current vanishes.

For an ideal, reversible system, the signal consists of symmetrical oxidation and reduction peaks centered at the reduction potential $E^0$.

$$E^0 = E_{p,c} = E_{p,a}$$  \(20\)

The area under the peak gives the charged passed for that redox couple ($nF$ electrons per mole of adsorbed centers). It should be the same for the oxidative and for the reductive peaks.

The peak current is proportional to the scan rate $\nu$, to the surface concentration of electroactive species $\Gamma$ and to the square of $n$.

$$i_p = \frac{n^2 F^2 A \Gamma \nu}{4RT}$$  \(21\)

$\text{Therefore, the electroactive coverage must be high enough for a current to be observed. Typically, a coverage higher than 5pmol/cm}^2$ will suffice.
Figure 12: Cyclic voltammogram for *Pseudomonas aeruginosa* azurin adsorbed at a pyrolytic graphite electrode. The dashed line is the baseline which has to be subtracted to remove the contribution of the non-faradaic current. Inset is the baseline subtracted current (the faradaic current). 0°C, pH 8.5, \( \nu = 20 \text{mV/s} \). \( \Gamma_A \approx 5.5 \text{pmol} \).

Figure 13: Cyclic voltammogram for *Sulfolobus acidocaldarius* 7Fe ferredoxin adsorbed at a pyrolytic graphite electrode. The dashed line is the baseline which has to be subtracted to remove the contribution of the non-faradaic current. 0°C, pH 8.5, \( \nu = 20 \text{mV/s} \). \( \Gamma_A \approx 3.5 \text{pmol} \). [J. McEvoy and F. Armstrong, unpublished results].

Figure 14: Cyclic voltammograms (raw data out of scale, base-line subtracted and deconvoluted signals) for *E. coli* fumarate reductase (FrdAB) adsorbed at a pyrolytic graphite edge electrode. This enzyme contains 3 Fe-S clusters and a flavin cofactor. 20°C, pH 7 & 9, \( \nu = 10 \text{mV/s} \). @ pH 7, FADox/FADred \(-50 \text{mV vs SHE}, [2\text{Fe}_2\text{S}]^{2+/+} \approx -40 \text{mV}, [4\text{Fe}_4\text{S}]^{2+/+} \approx -305 \text{mV}, [3\text{Fe}_4\text{S}]^{+/0} \approx -65 \text{mV} \). \( \Gamma_A \approx 0.4 \text{pmol} \). Note the strong pH dependence of the FAD signal.

A linear plot of \( i_p \) against \( \nu \) proves that the redox species are adsorbed onto the electrode.

- The peak width at half height, \( \delta \), is:
  \[
  \delta \approx 3.53 \frac{RT}{nF} \tag{22}
  \]

\( (91 \text{mV at } 25^\circ \text{C}, \text{ or } 83 \text{mV at } 0^\circ \text{C}. ) \) Therefore cooperative two-electron transfers give signals with up to four times the height and half the width of one electron transfers; they are therefore more easily distinguished.

**Applications: various redox proteins and a multicenter enzyme**

In experiments, there is an additional contribution to the current due to the “charging of the electrode”. Figure 12 shows a non-catalytic voltammogram allowing the determination of the redox potential of the copper site of azurin. The dashed line is the baseline which has to be subtracted to remove the contribution of the non-faradaic current.
The 7Fe ferredoxin of *Sulfolobus acidocaldarius* contains one [3Fe-4S] cluster, with redox transitions [3Fe4S$^+$/0] and [3Fe4S$^{0/2-}$] and one [4Fe-4S] cluster with a 2+/+ redox transition, fig. 13. When different centers are present in a protein, the different redox transitions appear as multiple peaks, the areas of which reveal the stoichiometry of the redox processes. E.g. the area under the low potential [3Fe4S$^{0/2-}$] peak is twice as much as that of the high potential [3Fe4S$^+$/0] peak. This figure illustrates the fact that 2-electron redox processes give prominent signals.

The fumarate reductase from *E. coli* contains 3 Fe-S clusters and one flavin cofactor. The signal associated with the flavin is easily distinguished from the three one-electron peaks due to the Fe-S clusters (fig. 14). The data clearly show that the reduction potential of the FAD is strongly pH dependent, as expected for a reduction process coupled to protonation.

**Advantages of PFV versus equilibrium titration & bulk voltammetry.**

Voltammetry is now becoming a routine technique to measure reduction potentials, and offers several advantages over bulk titrations.

- **No need for a spectroscopic “handle”**. There is no requirement for a distinctive and unambiguous change in some spectroscopic parameter. The counterpart of this advantage is obvious: voltammetry provides no structural information.
- **Sample economy**. A few pmol of protein/enzyme are adsorbed onto the electrode (although making a film requires a larger amount than that, typically ten to one hundred times as much, this is still much less than required for bulk titration)
- **Quick measurements**. Recording a cyclic voltammogram takes usually a few seconds to a few minutes.
- **Instantaneous dialysis**. The electrode can be transferred in a solution of different composition/pH and the measurement repeated with the same sample. The protein might even survive long enough in a hostile environment for measurements to be performed before the electrode is taken back into a more gentle solution.
- **Easier analysis & modeling of data**. The baseline (the charging current) is easily removed, and interpretation of non-catalytic data might not require solving any transport (diffusion/convection) equation.
- **In situ measurements**. Reduction potentials can be measured as a function of temperature [36–39], or even pressure [40].

### 6.2 Fast scan voltammetry: equilibrium and beyond.

The measurement of a reduction potential (an equilibrium property) theoretically requires that the system is at equilibrium. This appears to be in contradiction to measuring a current: indeed, the flow of electrons when the protein is oxidised or reduced results from the fact that the system is driven out of equilibrium when the electrode potential is changed around the (equilibrium) reduction potential.

In practice, this does not matter too much if the scan rate (and therefore the current) is slow enough (typically lower than $\approx 10 mV/s$) that the system is *nearly* at equilibrium.

When the scan rate is raised, however, departure from equilibrium can be observed, and a great deal of information about the kinetics of redox processes can be gained by looking at the scan rate dependence of the voltammograms.

**Effect of electron transfer rate.**

Theoretical voltammograms for an uncoupled one-electron redox process are plotted in fig. 15A. At slow scan rate, the oxidation peak occurs at $E^0$. If the scan rate is high, because it takes time for the electron transfer between the electrode and the redox center to occur, the maximal current is observed *after* the redox potential has been reached, i.e. at higher electrode potential. The same reasoning applied to the reductive process predicts that $E_{p,c}$ is lower than $E^0$. 

16
Figure 15: Effect of scan rate on the voltammetry of a redox species undergoing a one-electron, no-proton redox process. A: (theoretical) cyclic voltammograms at different scan rates (from 1000V/s to 10mV/s). The difference between $E_{p,a}$ and $E_{p,c}$ increases as the scan rate is raised. B: “Trumpet plot”: $E_{p,a}$ (filled squares) and $E_{p,c}$ (empty squares) as a function of the log of the scan rate. The lower an electron transfer rate is, the trumpet plot will shift to low scan rates.

$$k_{on} = k_0 /[H^+]_{eq}$$

Figure 16: L-shape scheme used to interpret the fast scan voltammetry of a [3Fe-4S] cluster.

When measurements are performed over a large range of scan rates, the results can be displayed by plotting $E_{p,a}$ and $E_{p,c}$ as a function of scan rate, on a log scale (fig. 15B). This is called a trumpet plot. The more efficient the electron transfer between the electrode and the active site (the greater $k_0$ in eq. 12), the higher the scan rate at which the oxidative and reductive peaks start to separate.\(^{15}\)

### Effect of coupled reactions.

The rate of electron transfer between the electrode and the active site might not have physiological relevance; however, fast scan voltammetry can be used to resolve and quantify the chemical processes coupled to electron transfer. From a biological point of view, proton transfer is certainly the most important reaction coupled to electron transfer, and determining the mechanism by which light- or redox-driven pumps translocate protons is an exciting challenge.

### Application: measuring the rates of proton transfer to and from a buried FeS cluster [31]

As an example, fig. 17 illustrates the voltammetric study of the [3Fe-4S]\(^{+/0}\) one-electron one-proton reaction (fig. 16), for a mutant of *A. vinelandii* ferredoxin I. High-resolution crystal structures reveal that the [3Fe-4S] is buried with no access to water molecules, and that a carboxylate group from an aspartate (D15) is located close to the cluster on the protein surface. The mechanism of proton transfer between the cluster and solvent has been determined using PFV in conjunction with site-directed mutagenesis [31,43].

The experiments depicted in fig. 17 were performed at pH 5.4, much higher than $pK_{ox}$ and 1.3 pH unit lower than the $pK_{red} = 6.7$ of the [3Fe-4S] cluster, the reduced form of which is therefore protonated at equilibrium.

\(^{15}\)For the effect of distance between the electrode and the redox site on the rate of electron transfer, see e.g. refs. [25,26,41,42].

---
Figure 17: Effect of scan rate on the voltammetry of a redox species undergoing a one-electron, one-proton redox process. The data are for the $[3\text{Fe-4S}]^{+/0}$ redox couple of a slow proton-transfer mutant of *Azodobacter vinelandii* ferredoxin I (D15E) [31, 43]. pH 5.4, 0°C.
Figure 18: A schematic catalytic cycle illustrating the flux of electrons from succinate to the electrode via the FAD cofactor of fumarate reductase.

- At slow scan rates, oxidation and reduction peaks for the [3Fe-4S]^{+/-0} appear at the same electrode potential. Under these “close-to-equilibrium” conditions, the reduction is followed by protonation, and oxidation proceeds along the reverse route.
- When the scan rate is increased, oxidation and reduction peaks start to separate.
- (2) At scan rates between 1 and 10V/s (the scans were started from the high potential limit, and only the first scan is considered), the reductive peak is still clearly visible, but the oxidation peak vanishes, because the cluster is trapped in the protonated form: it is quickly protonated upon reduction, but the rate of de-protonation, $k_{off}$ in fig. 16, is too small for the cluster to be de-protonated during the oxidative scan. The deprotonation of the cluster gates its reoxidation.
- (3) At very high scan rates, 20V/s and above, both peaks are observed, but the average reduction potential is lower than at low scan rate, and matches the alkaline limit: this is because the scan is reversed before the reduced cluster is protonated.

These experiments allow the determination of the rates of (de)protonation, because the time scale of potential modulation can be changed over orders of magnitude (1 min at 10mV/s to 1 ms at 1000V/s) to match that of the chemical events.

Several examples of voltammetric studies of coupled reactions, involving cytochromes and Fe-S clusters, have been reported (see [15] and references therein).

6.3 Catalytic voltammetry with adsorbed redox enzymes [6]

In the absence of substrate and at sufficiently high coverage, a redox enzyme immobilized onto an electrode may give peak-like signals corresponding to the reversible transformation of its redox centers (fig. 14). Upon adding substrate, the non-turnover peaks are transformed to sizeable waves: reaction with substrate transforms the active site, which is regenerated by electron exchange with the electrode in a succession of catalytic cycles. Current equates directly to rate of turnover, and so the relationship between driving force (potential) and catalytic activity is traced in a single voltammetric experiment. Note that catalysis may be studied even if coverage is too low to observe non-catalytic signals (as is often the case).

As an example, the non-catalytic voltammetry of E. coli fumarate reductase has been shown in fig. 14. In vivo, this enzyme reduces fumarate to succinate,

$$\text{COO}^- + 2H^+ + 2e^- \rightleftharpoons \text{OOC}^- \text{COO}^-, $$

but it can also oxidize succinate in the presence of an artificial electron partner.

If this enzyme is adsorbed onto an electrode, in a solution containing succinate, and if the electrode potential is high enough, the flavin cofactor is oxidised, giving 2 electrons to the electrode, fig. 18.

$$\text{FAD}_{\text{Red}} \rightarrow \text{FAD}_{\text{Ox}} + 2e^-$$
The oxidised enzyme can bind succinate, and the oxidation of succinate results in the reduction of the flavin:

$$\text{FAD}^{\text{Ox}} + \text{succinate} \rightarrow \text{FAD}^{\text{Red}} + \text{fumarate}$$

The reduced FAD can be re-oxidised giving electrons to the electrode and so on. This results in a steady-state flux of electrons from succinate in solution to the electrode, via the adsorbed enzyme, which is measured as a steady-state current. This catalytic current is directly proportional to the rate of turnover, and is a direct measure of the activity of the enzyme.

At low electrode potential, in a solution containing fumarate, a reductive (negative) current is observed that is proportional to the rate of fumarate reduction by the enzyme. Figure 19B illustrates this bi-directional catalysis by *E. coli* fumarate reductase when the solution contains both fumarate and succinate.

**Application: the Michaelis constant of fumarate reductase [18]**

In an assay of the enzyme activity in solution, the enzyme is oxidised by a high potential dye (*e.g.* ferricyanide). The rate of turnover can be measured as a function of the concentration of
substrate, and the results allow the determination of Michaelis-Menten\textsuperscript{16} parameters ($k_{\text{cat}}$, the maximal turnover rate, and $K_m$, the Michaelis constant):

\[
\text{turnover rate} = \frac{k_{\text{cat}}}{1 + \frac{K_m}{[\text{substrate}]}},
\]

(23)

The same parameters can be determined from voltammetric experiments looking at the substrate-concentration dependence of the limiting current \cite{18}, see fig. 20, e.g. for succinate oxidation,

\[
i_{\text{lim}} = nFA\Gamma \frac{k_{\text{cat}}}{1 + \frac{K_m}{[\text{succinate}]}}
\]

(24)

In practice, this kind of measurement might be far from easy.\textsuperscript{17}

\textbf{Mass-transport controlled catalytic voltammetry [45]}

Figure 21A illustrates the voltammetry for hydrogen oxidation by \textit{Allochromatium vinosum} [Ni-Fe] hydrogenase. In this case, the limiting current increases dramatically as the rotation rate is raised because during turnover the concentration of hydrogen near the electrode decreases, the enzyme is able to consume $H_2$ faster than it is brought to the electrode by the convective motion of the solution. The greater the rotation rate, the more efficient the transport of hydrogen from the bulk solution towards the enzyme, and the greater the current. At infinite rotation rate, the catalytic

\textsuperscript{16}Leonor Michaelis (January 16, 1875 / October 8, 1949) was a German biochemist and physician. He was born in Berlin (Germany) and studied medicine in Freiburg. \textbf{Maud Menten} (March 20, 1879 / July 26, 1960) was a Canadian medical scientist. In 1912 she moved to Berlin where she worked with Leonor Michaelis, obtaining a Ph.D. in 1916. She conducted the first electrophoretic separation of proteins in 1944. Their most famous work was on enzyme kinetics, based on earlier findings of Victor Henri.

\textsuperscript{17}(i) The limiting current is proportional to $\Gamma$, the total amount of enzyme adsorbed. This can be determined (with a very relative accuracy) only when the electrode coverage is high enough for non-catalytic signals to be measured in the absence of substrate. (ii) The measurement of $K_m$ could be performed without knowing the exact electroactive coverage. This requires however that the adsorbed film is stable enough as a function of time for the coverage to be constant when limiting currents are measured with the same film in solutions of different substrate concentrations. We have proposed methods for correcting for film desorption [44] (iii) Last, eq. (24) does not take into account mass transport of substrate in solution; this is fine if there is no depletion of substrate near the electrode. This should be checked for by looking at the rotation rate dependence of the limiting current, or using the rotation rate dependence to determine $i_{\text{lim}}(\omega = \infty)$. 

\[\text{Figure 21: (A) Influence of electrode rotation rate on the catalytic current measured for hydrogen oxidation by \textit{Allochromatium vinosum} [Ni-Fe] hydrogenase at different electrode rotation rates ($\omega$, given in units Revolution Per Minute). (B) Koutecky-Levich plot (eq. 25) showing small but non-zero intercept at infinite rotation rate, allowing $k_{\text{cat}}$ to be estimated. 0.1 bar $H_2$, $\nu = 100 \text{mV/s}$, $T = 45^\circ \text{C}$, pH=6.5. From ref. [45].}\]
current is finite: mass transport is no longer rate limiting, and the extrapolated current reveals the intrinsic efficiency of the enzyme. The Koutecky-Levich plot in fig 21B therefore follows:

$$\frac{1}{i_{\text{lim}}(\omega)} = \frac{1}{nFA\Gamma k_{\text{cat}}} + \frac{\text{constant}}{\sqrt{\omega}}$$

This equation handles departure from mass-transport control at high $\omega$ [compare to eq. (18), page 12].

Interestingly, this study of hydrogenase showed that the turnover number of the enzyme is significantly higher than that observed in solution assays, using oxidizing dyes [45]; in the latter case, it becomes evident that turnover in solution is limited by electron transfer from the soluble electron partner, and that the electrode is a much faster electron acceptor than the soluble dye.

Potential dependent catalytic activity.

A major advantage of catalytic protein film voltammetry, with respect to traditional assays of enzyme activity (homogeneous kinetics with soluble redox dyes) is that it provides the electrode potential as an invaluable control parameter. By varying the electrode potential, we can change the thermodynamic driving force for the reaction and the redox state of the enzyme under turnover conditions while we are simultaneously measuring the activity as a current. This has various applications, as exemplified below.

Application: the reduction potentials of the active site flavin bound to substrate in fumarate reductase [18]

We now examine the shape (as opposed to merely the magnitude) of the catalytic voltammograms for succinate oxidation by E. coli fumarate reductase shown in Fig. 20. At low electrode potential, the enzyme and its active site are reduced and unable to oxidize succinate, there is no activity. At very high potential the current tends to a limit (a plateau) which represents the maximal turnover rate when the enzyme is fully oxidized. In between the two, the activity increases when the electrode potential becomes high enough that the active site flavin becomes oxidized. Hence the position and precise shape of the voltammogram reports on the reduction potential of the active site flavin, under turnover conditions and in the presence of substrate. In ref [18], these data were actually used for determining the dependence of the reduction potential of the active site flavin on succinate concentration plotted in fig. 4.

Application: (in)activation of hydrogenase [7]

The [Ni-Fe] hydrogenase from A. vinosum is known to deactivate in oxidizing conditions, (e.g. after exposure to oxygen) due to the over-oxidation of the active site. The enzyme has to be reduced (e.g. incubated with hydrogen) for the activity to be recovered. This makes tricky the assay of the enzyme in solution, since it involves keeping the enzyme in very oxidising conditions; the enzyme might therefore inactivate over the time course of the solution assay!
This inactivation was not observed in the electrochemical experiments depicted in fig. 21 because the scan rate was fast enough: the enzyme is taken to high potential (where the limiting current is measured) and back to low potential before the inactivation reaction can proceed.

Figure 22 is a catalytic voltammogram recorded essentially in the same conditions as in fig. 21 but for a slow scan rate (recording a whole catalytic voltammogram at this scan rate takes more than an hour and a half.) The activity of the enzyme, measured as a positive current, decreases above -200mV. This kind of study therefore allows the energetics of the oxidative reaction causing the “switch off” to be probed (the potential of the switch corresponds to that of the redox transition towards an oxidised, inactive active site). Moreover, one can design experiments in which a high electrode potential is held, and the slow decrease in current resulting from the inactivation measured: this allows the kinetics of the inactivation to be studied, too.

**Potential dependent catalytic activity [10, 48]**

Surprisingly, among the different redox enzymes which have been studied by PFV, quite a few of them exhibit complex activity profiles as a function of the driving force (the electrode potential) (See ref. [10] and references therein).

Succinate dehydrogenase, the membrane-extrinsic domain of Complex II, efficiently catalyzes fumarate reduction, but only over a narrow potential range because activity decreases abruptly once a critical driving force is reached, i.e., at sufficiently low potential. This reversible switch (of possible physiological relevance) may arise from a conformational change linked to the oxidation state of FAD. By contrast, the fumarate reduction activities of several fumarate reductases are marked by complex sigmoidal increases upon successive reductions of the prosthetic groups.

As a last example, fig. 23 shows catalytic voltammograms corresponding to the reduction of dimethyl sulfoxide by *E. coli* DMSO reductase [47]. At high pH, the activity of the enzyme drops down below -300mV, but this effect is independent of scan rate: the switch on and off is fast on the time scale of the experiment. Although the concept of pH optima for enzyme activity is well established, the possibility that activity of redox enzymes might be optimised within a finite, and perhaps narrow, range of potential has not been explored. While pH optima give mechanistic information on protonation equilibria during catalysis, potential optima provide information on the roles played by different oxidation states of redox-active sites. Certain redox transitions may regulate electron flow to or from the active site. Or substrate binding or atom transfer may occur only when the active site is in a certain oxidation state. These relationships are difficult to observe by conventional techniques but can be revealed by protein film voltammetry, due to its ability to measure subtle changes in catalytic activity as the electrode potential is varied.
Catalytic chronoamperometry, to resolve rapid changes in activity

Another great advantage of catalytic protein film voltammetry is the high temporal resolution of the measurement. The activity being measured as a current, it can easily be sampled every 0.1 sec or even faster. This is particularly convenient e.g. for detecting rapid changes in turnover rates that follow changes in inhibitor concentration, as illustrated hereafter.

Application: measuring the rate of diffusion along the substrate channel of hydrogenase [34,35]

We have developed a method to characterize quantitatively the kinetics of inhibition of hydrogenase, which consists in monitoring the H₂-oxidation current after the concentration of CO or O₂ first suddenly increases when an aliquot of solution saturated with inhibitor is injected in the electrochemical cell, and then slowly returns to zero as the buffer is flushed by a stream of H₂ (ref [49] and section 2.4.2 in ref [6]). The concentration of H₂ is nearly constant, and the inhibitor concentration follows an exponential decay. The concentration of inhibitor need not be independently measured because its change against time is defined by the amount of inhibitor that is injected and by the time constant of the decay, which is determined by fitting the change in current. If binding and/or release of the inhibitor is slow, the change in activity is delayed from the time of injection and the rates of inhibition can be measured [34].

Fig. 24 shows that, in the case of the WT (at 40°C), the decrease in activity after CO injection is too fast to be resolved. Also, the recovery of activity follows exactly the decrease in CO concentration as the latter is flushed away from the cell. The binding and release of CO is much slower in certain mutants of the enzyme, where amino acids whose side chains point inside the substrate channel have been substituted. We showed that this is a direct consequence of the rates of diffusion along the channel being slowed by the mutations. Hence protein film voltammetry provides a unique tool for studying how the structure of the channel affect the diffusion rates [34].

O₂ also inhibits the enzyme by reacting at the active site after it diffuses along the substrate access channel, and we could show that the structure of the channel determines the sensitivity of the enzyme to oxidative dammage [35].

7 PFV Quiz

In fig. 25, I have plotted two voltammograms recorded with a film of E. coli fumarate reductase in contact with a solution containing only succinate at a concentration [S] =50mM (approximately 200 times the Michaelis constant). The plain line corresponds to a stationary (non-rotating) electrode, whereas the dashed voltammogram was recorded with the electrode rotating at 3000rpm.

(i) Why is the current at high potential rotation-rate independent in this case? (ii) Why are the shapes of the voltammograms different?
An extensive description of most of the electrochemical techniques will be found in refs [1, 2]. Ref. [3] is a textbook on enzyme kinetics. Refs [4–14] are reviews of the use of voltammetry to probe the energetics and dynamics of redox proteins and enzymes. In ref [7], emphasis is made on the study of hydrogenases. Ref [8] summarizes recent advances in the electrochemistry of membrane-bound redox enzymes. The mediated protein electron transfer voltammetry of enzymes, and its application to biosensors are discussed in [15]. Ref. [16] goes beyond the scope of faradaic bioelectrochemistry.

References


A Appendix

The demonstration of eq. 7 is the following.

We write the Nernst equation first for the alkaline couple Ox/Red, and then for both forms (protonated and deprotonated) of the redox couple:

\[
E = E^0_{\text{alk}} + \frac{RT}{nF} \ln \left( \frac{[\text{Ox}]}{[\text{Red}]} \right) \quad (26a)
\]

\[
E = E^0'([H^+]) + \frac{RT}{nF} \ln \left( \frac{[\text{Ox}] + [\text{OxH}]}{[\text{Red}] + [\text{RedH}]} \right) \quad (26b)
\]

We rewrite eq. 26b as follows:

\[
E = E^0'([H^+]) + \frac{RT}{nF} \ln \left( \frac{[\text{Ox}]}{[\text{Red}]} \right) \left( 1 + \frac{[H^+]}{K_{\text{Ox}}} \right) \left( 1 + \frac{[H^+]}{K_{\text{Red}}} \right) \quad (27a)
\]

\[
E = E^0'([H^+]) + \frac{RT}{nF} \ln \left( \frac{[\text{Ox}]}{[\text{Red}]} \right) + \frac{RT}{nF} \ln \left( \frac{1 + \frac{[H^+]}{K_{\text{Ox}}}}{1 + \frac{[H^+]}{K_{\text{Red}}}} \right) \quad (27b)
\]

Equating eq. 27b and eq. 26a gives eq. 7:

\[
E^0'([H^+]) = E^0_{\text{alk}} + 2.3 \frac{RT}{nF} \log_{10} \left( \frac{1 + \frac{[H^+]}{K_{\text{Red}}}}{1 + \frac{[H^+]}{K_{\text{Ox}}}} \right) \quad (28)
\]

Check that \(E^0'([H^+])\) tends to \(E^0_{\text{alk}}\) when \([H^+]\) is small.

Using

\[
E = E^0_{\text{acid}} + \frac{RT}{nF} \ln \left( \frac{[\text{OxH}]}{[\text{RedH}]} \right) \quad (29)
\]

instead of eq. 26a gives

\[
E^0'([H^+]) = E^0_{\text{acid}} + 2.3 \frac{RT}{nF} \log_{10} \left( \frac{1 + \frac{[H^+]}{K_{\text{Red}}}}{1 + \frac{[H^+]}{K_{\text{Ox}}}} \right) \quad (30)
\]

Check that \(E^0'([H^+])\) tends to \(E^0_{\text{acid}}\) when \([H^+]\) is large.

The relation between \(E^0_{\text{alk}}\) and \(E^0_{\text{acid}}\) is simply obtained by equating 26a and 29:

\[
E^0_{\text{acid}} = E^0_{\text{alk}} + \frac{RT}{nF} \ln \frac{K_{\text{Ox}}}{K_{\text{Red}}} \quad (31)
\]

Check that with \(pK_{\text{Ox}} < pK_{\text{Red}}, E^0_{\text{acid}} > E^0_{\text{alk}}\)