Supplementary Information for:

A Beginner's Guide to Thermodynamic Modelling of [FeFe] Hydrogenase

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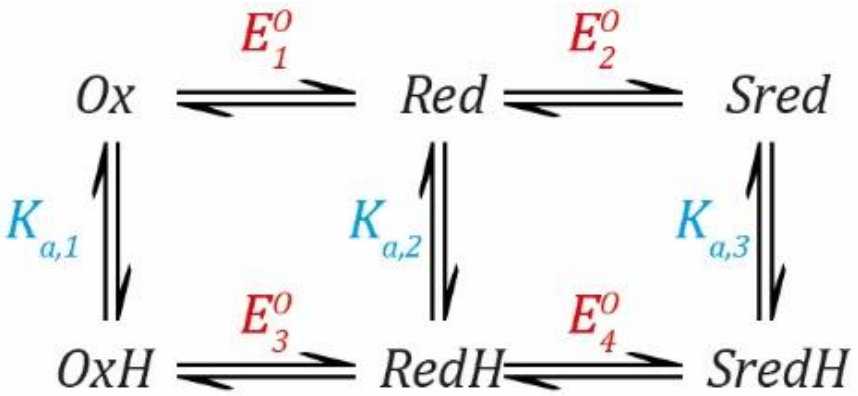
Section S1 – Derivation of equations for a simple model for the [FeFe] hydrogenase

In the main text, *Section 2.3* we described a simple model for the [FeFe] hydrogenase including two redox steps and one protonation step in which *Ox* can be reduced to *Red* and further to *Sred*, and each of these states can be protonated (**Scheme 3** of the main text). The scheme is reproduced here at **Scheme S1**:



Scheme S1. Scheme showing the reactions in a simple model for the [FeFe] hydrogenase. The oxidised and deprotonated species *Ox* can be reduced to *Red* with a redox potential of E_1^O. *Red* can then be further reduced to *Sred* with a redox potential of E_2^O. The oxidised protonated species *OxH* can be reduced to give *RedH* with a redox potential of E_3^O and *RedH* can be further reduced to give *SredH* with a redox potential of E_4^O.*OxH*, *RedH* and *SredH* can be deprotonated to *Ox*, *Red* and *Sred*, respectively, with acid dissociation constants $K_{a,1}$, $K_{a,2}$ and $K_{a,3}$, respectively. The scheme is identical to **Scheme 3** of the main text.

Using the Nernst equation (S1) and the equation for a simple acid dissociation constant (S2):

$$E = E^O - \frac{RT}{nF} ln(\frac{[Red]}{[Ox]}) \tag{S1}$$

$$K_a = \frac{[Ox][H^+]}{[OxH]} \tag{S2}$$

We can define equations for each of the steps in **Scheme S1**:

$$E = E_1^O - \frac{RT}{nF} \ln(\frac{[Red]}{[Ox]}) \tag{S3}$$

$$E = E_2^O - \frac{RT}{nF} \ln(\frac{[Sred]}{[Red]}) \tag{S4}$$

$$E = E_3^O - \frac{RT}{nF} \ln(\frac{[RedH]}{[OxH]}) \tag{S5}$$

$$E = E_4^O - \frac{RT}{nF} \ln(\frac{[SredH]}{[RedH]} \tag{S6}$$

$$K_{a,1} = \frac{[Ox][H^+]}{[OxH]} \tag{S7}$$

$$K_{a,2} = \frac{[Red][H^+]}{[RedH]} \tag{S8}$$

$$K_{a,3} = \frac{[Sred][H^+]}{[SredH]} \tag{S9}$$

Equation S3 can be rearranged to get [*Red*] in terms of [*Ox*] as follows:

$$[Red] = [Ox] \times exp\frac{nF}{RT}(E_1^O - E)$$

$$[Red] = [Ox] \times \alpha \tag{S10}$$

Where:

$$\alpha = exp\frac{nF}{RT}(E_1^O - E)$$

Equation S4 can be rearranged to get [*Sred*] in terms of [*Red*] as follows:

$$[Sred] = [Red] \times exp\frac{nF}{RT}(E_2^O - E)$$

$$[Sred] = [Red] \times \beta \tag{S11}$$

Where:

$$\beta = exp\frac{nF}{RT}(E_2^O - E)$$

Equation S5 can be rearranged to get [*RedH*] in terms of [*OxH*] as follows:

$$[RedH] = [OxH] \times exp\frac{nF}{RT}(E_3^O - E)$$

$$[RedH] = [OxH] \times \gamma \tag{S12}$$

Where:

$$\gamma = exp\frac{nF}{RT}(E_3^O - E)$$

Equation S6 can be rearranged to get [*SredH*] in terms of [*RedH*] as follows:

$$[SredH] = [RedH] \times exp\frac{nF}{RT}(E_4^O - E)$$

$$[SredH] = [RedH] \times \delta \tag{S13}$$

Where:

$$\delta = exp\frac{nF}{RT}(E_4^O - E)$$

And Equations S7-9 can be rearranged to get [*OxH*], [*RedH*] and [*SredH*] in terms of [*Ox*], [*Red*] and [*Sred*], respectively, as follows:

$$[OxH] = [Ox]\frac{[H^+]}{K_{a,1}} \tag{S14}$$

$$[RedH] = [Red]\frac{[H^+]}{K_{a,2}} \tag{S15}$$

$$[SredH] = [Sred]\frac{[H^+]}{K_{a,3}} \tag{S16}$$

Next, we define the sum of the concentrations of all states as 1:

$$[Ox] + [Red] + [Sred] + [OxH] + [RedH] + [SredH] = 1$$

Next, we substitute [*Sred*] and [*SredH*] using Equations S11 and S13, respectively:

$$[Ox] + [Red] + [Red] \times \beta + [OxH] + [RedH] + [RedH] \times \delta = 1$$

$$[Ox] + [Red] \times (1 + \beta) + [OxH] + [RedH] \times (1 + \delta) = 1$$

Next we substitute [*RedH*] using Equation S12, respectively:

$$[Ox] + [Red] \times (1 + \beta) + [OxH] + [OxH] \times \gamma \times (1 + \delta) = 1$$

$$[Ox] + [Red] \times (1 + \beta) + [OxH] \times \{1 + \gamma(1 + \delta)\} = 1$$

Next we substitute [*Red*] and [*OxH*] using Equations S10 and S14, respectively:

$$[Ox] + [Ox] \times \alpha \times (1 + \beta) + [Ox]\frac{[H^+]}{K_{a,1}} \times \{1 + \gamma(1 + \delta)\} = 1$$

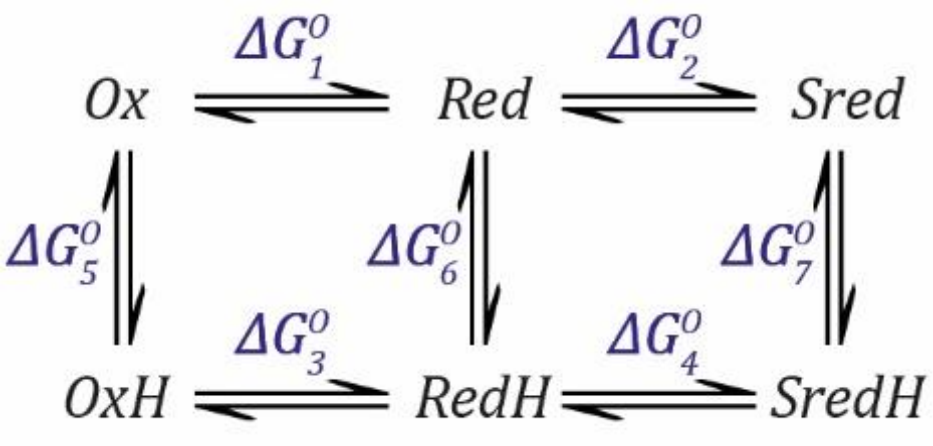
$$[Ox] \times (1 + \alpha(1 + \beta) + \frac{[H^+]}{K_{a,1}}\{1 + \gamma(1 + \delta)\}) = 1$$

$$[Ox] = \frac{1}{1 + \alpha(1 + \beta) + \frac{[H^+]}{K_{a,1}}\{1 + \gamma(1 + \delta)\}}$$

Using $[H^+] = 10^{-pH}$ and $K_{a,1} = 10^{-pK_{a,1}}$ we can substitute $\frac{[H^+]}{K_{a,1}}$ for $10^{(pK_{a,1}-pH)}$ and arrive at Equation 22 of the main text:

$$\boxed{[Ox] = \frac{1}{1 + \alpha(1 + \beta) + 10^{(pK_{a,1}-pH)}\{1 + \gamma(1 + \delta)\}}} \tag{22}$$

As noted in the main text, we did not need to use all the parameters to arrive at the results, ($K_{a,2}$ and $K_{a,3}$ were not used), but we can derive these parameters from the defined parameters using the conjoined thermodynamic cycles in this model. The rules still apply so that the total free energy when going from one state to another is independent of the pathway. Therefore, we can define the situation as follows in **Scheme S2** (same as **Scheme 4** of the main text):



Scheme S2. Scheme showing the free energy changes for the reactions in a simple model for the [FeFe] hydrogenase. The oxidised and deprotonated species *Ox* can be reduced to *Red* with a free energy change ΔG_1. *Red* can be further reduced to *Sred* with a free energy change ΔG_2. The oxidised protonated species *OxH* can be reduced to give *RedH* with a free energy change ΔG_3. *RedH* can be further reduced to *SredH* with a free energy change ΔG_4. *OxH, RedH and SredH* can be deprotonated to *Ox*, *Red* and *Sred*, respectively, with free energy changes ΔG_5^O, ΔG_6^O and ΔG_7^O, respectively (-ΔG_5^O, -ΔG_6^O and –ΔG_7^O for the respective protonation processes). The scheme is identical to **Scheme 4** of the main text.

Since the free energy change is the same regardless which pathway is taken, going from *Ox* to *RedH* via *Red* or via *OxH* have the same overall free energy change, as does going from *Red* to *SredH* via *Sred* or via *RedH*. Importantly, it should be noted that the K_a is defined for a deprotonation, and so when we protonate we need to subtract the ΔG^0associated with this step. Therefore, we can define the following equations:

$$\Delta G_1^O - \Delta G_6^O = \Delta G_3^O - \Delta G_5^O \tag{S17}$$

$$\Delta G_2^O - \Delta G_7^O = \Delta G_4^O - \Delta G_6^O \tag{S18}$$

Where:

$$\Delta G_1^O = -nFE_1^O \tag{S19}$$

$$\Delta G_2^O = -nFE_2^O \tag{S20}$$

$$\Delta G_3^O = -nFE_3^O \tag{S21}$$

$$\Delta G_4^O = -nFE_4^O \tag{S22}$$

$$\Delta G_5^O = -RTlnK_{a,1} \tag{S23}$$

$$\Delta G_6^O = -RTlnK_{a,2} \tag{S24}$$

$$\Delta G_7^O = -RTlnK_{a,3} \tag{S25}$$

For $K_{a,2}$ we substitute Equations S19, S21, S23 and S24 into Equation S17:

$$-nFE_1^O - (-RTlnK_{a,2}) = -nFE_3^O - (-RTlnK_{a,1})$$

$$-nFE_1^O + RTlnK_{a,2} = -nFE_3^O + RTlnK_{a,1}$$

We can then rearrange this equation to find out how $K_{a,2}$ depends on the other variables (Equation 27 in the main text):

$$RTlnK_{a,2} - RTlnK_{a,1} = nFE_1^O - nFE_3^O$$

$$RTln\frac{K_{a,2}}{K_{a,1}} = nF(E_1^O - E_3^O)$$

$$\frac{K_{a,2}}{K_{a,1}} = exp\{\frac{nF}{RT}(E_1^O - E_3^O)\}$$

$$\boxed{K_{a,2} = K_{a,1}exp\{\frac{nF}{RT}(E_1^O - E_3^O)\}} \quad (27)$$

For $K_{a,3}$ we substitute Equations S20, S22, S24 and S25 into Equation S18:

$$-nFE_2^O - (-RTlnK_{a,3}) = -nFE_4^O - (-RTlnK_{a,2})$$

$$-nFE_2^O + RTlnK_{a,3} = -nFE_4^O + RTlnK_{a,2}$$

We can then rearrange this equation to find out how $K_{a,3}$ depends on the other variables (Equation 28 in the main text):

$$RTlnK_{a,3} - RTlnK_{a,2} = nFE_2^O - nFE_4^O$$

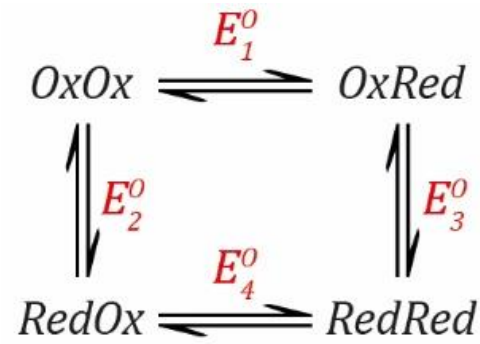
$$RTln\frac{K_{a,3}}{K_{a,2}} = nF(E_2^O - E_4^O)$$

$$\frac{K_{a,3}}{K_{a,2}} = exp\{\frac{nF}{RT}(E_2^O - E_4^O)\}$$

$$\boxed{K_{a,3} = K_{a,2}exp\{\frac{nF}{RT}(E_2^O - E_4^O)\}} \quad (28)$$

Section S2 – Derivation of equations for a redox anticooperativity model

In *Section 2.4* of the main text we introduced the concept of redox anticooperativity where reduction of two adjacent clusters influence each other's redox potentials. In Scheme S3 the simple model describing this situation is shown (**Scheme 5** of the main text):



Scheme S3. Scheme showing the reactions in a simple two step redox model. The doubly oxidised species *OxOx* can be reduced to *OxRed* with a redox potential of E_1^O or to *RedOx* with a redox potential of E_2^O. *OxRed* and *RedOx* can then be further reduced to *RedRed* with redox potentials of E_3^O and E_4^O, respectively.

Using this scheme and the Nernst equation (S1) we can define four equations describing the system:

$$E = E_1^O - \frac{RT}{nF}\ln(\frac{[OxRed]}{[OxOx]}) \tag{S26}$$

$$E = E_2^O - \frac{RT}{nF}\ln(\frac{[RedOx]}{[OxOx]}) \tag{S27}$$

$$E = E_3^O - \frac{RT}{nF}\ln(\frac{[RedRed]}{[OxRed]}) \tag{S28}$$

$$E = E_4^O - \frac{RT}{nF}\ln(\frac{[RedRed]}{[RedOx]}) \tag{S29}$$

Equation S26 can be rearranged to get [*OxRed*] in terms of [*OxOx*] as follows:

$$[OxRed] = [OxOx] \times exp\frac{nF}{RT}(E_1^O - E)$$

$$[OxRed] = [OxOx] \times \alpha \tag{S30}$$

Where:

$$\alpha = exp\frac{nF}{RT}(E_1^O - E)$$

Equation S27 can be rearranged to get [*RedOx*] in terms of [*OxOx*] as follows:

$$[RedOx] = [OxOx] \times exp\frac{nF}{RT}(E_2^O - E)$$

$$[RedOx] = [OxOx] \times \beta \tag{S31}$$

Where:

$$\beta = exp\frac{nF}{RT}(E_2^O - E)$$

Equation S28 can be rearranged to get [*RedRed*] in terms of [*OxRed*] as follows:

$$[RedRed] = [OxRed] \times exp\frac{nF}{RT}(E_3^O - E)$$

$$[RedRed] = [OxRed] \times \gamma \tag{S32}$$

Where:

$$\gamma = exp\frac{nF}{RT}(E_3^O - E)$$

Equation S29 can be rearranged to get [*RedRed*] in terms of [*RedOx*] as follows:

$$[RedRed] = [RedOx] \times exp\frac{nF}{RT}(E_4^O - E)$$

$$[RedRed] = [RedOx] \times \delta \tag{S33}$$

Where:

$$\delta = exp\frac{nF}{RT}(E_4^O - E)$$

Next, we define the sum of the concentrations of all states as 1:

$$[OxOx] + [OxRed] + [RedOx] + [RedRed] = 1$$

Next, we substitute [*RedRed*] using Equation S32:

$$[OxOx] + [OxRed] + [RedOx] + [OxRed] \times \gamma = 1$$

$$[OxOx] + [OxRed] \times (1 + \gamma) + [RedOx] = 1$$

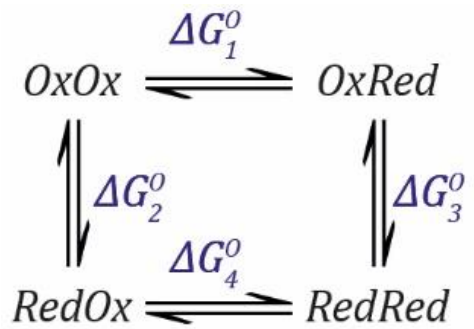
Next, we substitute [*OxRed*] and [*RedOx*] using Equations S30 and S31, and rearrange to get Equation 29 from the main text:

$$[OxOx] + [OxOx] \times \alpha(1 + \gamma) + [OxOx] \times \beta = 1$$

$$[OxOx] \times \{1 + \alpha(1 + \gamma) + \beta\} = 1$$

$$[OxOx] = \frac{1}{1 + \alpha(1 + \gamma) + \beta} \tag{29}$$

As before, in order to arrive at this result, we only needed to consider the three reduction events defined by E_1^O, E_2^O and E_3^O. E_4^O can be calculated considering the thermodynamic square and that the free energy change when going from one state to another is not dependent on the path chosen. Therefore, we can define the situation as in **Scheme S4** (**Scheme 6** in the main text):



Scheme S4. Scheme showing the free energies associated with reactions in a simple two-step redox model. The doubly oxidised species *OxOx* can be reduced to *OxRed* or *RedOx* with free energy changes ΔG_1^O and ΔG_2^O, respectively. *OxRed* and *RedOx* can then be further reduced to *RedRed* with free energy changes ΔG_3^O and ΔG_4^O, respectively.

The free energy change when going from *OxOx* to *RedRed* via *OxRed* is the same as that when going via *RedOx*. Therefore, we can define the equation:

$$\Delta G_1^O + \Delta G_3^O = \Delta G_2^O + \Delta G_4^O \tag{S34}$$

Where:

$$\Delta G_1^O = -nFE_1^O \tag{S35}$$

$$\Delta G_2^O = -nFE_2^O \tag{S36}$$

$$\Delta G_3^O = -nFE_3^O \tag{S37}$$

$$\Delta G_4^O = -nFE_4^O \tag{S38}$$

We then substitute Equations S35-S38 into Equation S34:

$$-nFE_1^O + (-nFE_3^O) = (-nFE_2^O) + (-nFE_4^O)$$

$$nFE_1^O + nFE_3^O = nFE_2^O + nFE_4^O$$

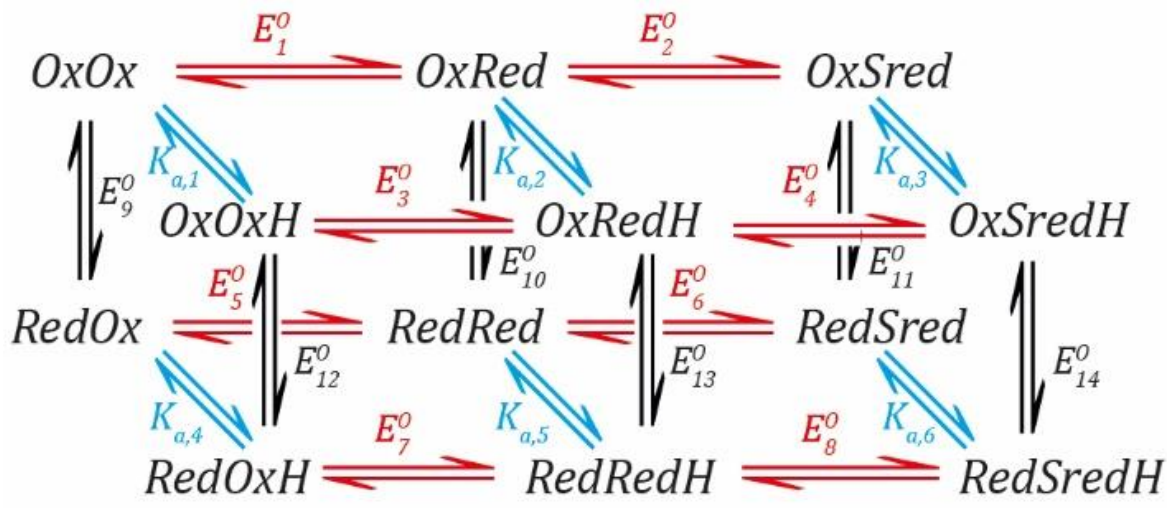
We can then rearrange this equation to find out how E_4^O depends on the other variables and arrive at Equation 34 from the main text:

$$nFE_4^O = nFE_1^O - nFE_2^O + nFE_3^O$$

$$E_4^O = E_1^O - E_2^O + E_3^O \tag{34}$$

Section S3 – Derivation of equations for a redox anticooperativity model for the active [FeFe] hydrogenase

In *Section 2.5* we described a highly complex model containing both PCET and redox anticooperativity. Each species can now exist in three redox states of the H-cluster (*Ox*, *Red* and *Sred*), two protonation states, and also two different redox states of the proximal F-cluster, making the model "three-dimensional" as in **Scheme S5** (**Scheme 7** of the main text):



Scheme S5. Model for active [FeFe] hydrogenase including redox anticooperativity. The steps and redox potentials indicated in red are reduction of the H-cluster first from *Ox* to *Red*, then *Red* to *Sred*. The steps and K_a values indicated in blue are protonation of the H-cluster in the *Ox*, *Red* and *Sred* states. The steps and redox potentials indicated in black are the reduction of the proximal F-cluster from *Ox* to *Red*.

Using the Nernst equation (S1) and the equation for a simple acid dissociation constant (S2) we can define the following equations describing the system:

$$E = E_1^O - \frac{RT}{nF}\ln(\frac{[OxRed]}{[OxOx]}) \tag{S39}$$

$$E = E_2^O - \frac{RT}{nF}\ln(\frac{[OxSred]}{[OxRed]}) \tag{S40}$$

$$E = E_3^O - \frac{RT}{nF}\ln(\frac{[OxRedH]}{[OxOxH]}) \tag{S41}$$

$$E = E_4^O - \frac{RT}{nF}\ln(\frac{[OxSredH]}{[OxRedH]} \tag{S42}$$

$$E = E_5^O - \frac{RT}{nF}\ln(\frac{[RedRed]}{[RedOx]}) \tag{S43}$$

$$E = E_6^O - \frac{RT}{nF}\ln(\frac{[RedSred]}{[RedRed]}) \tag{S44}$$

$$E = E_7^O - \frac{RT}{nF}\ln(\frac{[RedRedH]}{[RedOxH]}) \tag{S45}$$

$$E = E_8^O - \frac{RT}{nF}\ln(\frac{[RedSredH]}{[RedRedH]} \tag{S46}$$

$$E = E_9^O - \frac{RT}{nF}\ln(\frac{[RedOx]}{[OxOx]}) \tag{S47}$$

$$E = E_{10}^O - \frac{RT}{nF}\ln(\frac{[RedRed]}{[OxRed]}) \tag{S48}$$

$$E = E_{11}^O - \frac{RT}{nF}\ln(\frac{[RedSred]}{[OxSred]}) \tag{S49}$$

$$E = E_{12}^O - \frac{RT}{nF}\ln(\frac{[RedOxH]}{[OxOxH]} \tag{S50}$$

$$E = E_{13}^O - \frac{RT}{nF}\ln(\frac{[RedRedH]}{[OxRedH]}) \tag{S51}$$

$$E = E_{14}^O - \frac{RT}{nF}\ln(\frac{[RedSredH]}{[OxSredH]} \tag{S52}$$

$$K_{a,1} = \frac{[OxOx][H^+]}{[OxOxH]} \tag{S53}$$

$$K_{a,2} = \frac{[OxRed][H^+]}{[OxRedH]} \tag{S54}$$

$$K_{a,3} = \frac{[OxSred][H^+]}{[OxSredH]} \tag{S55}$$

$$K_{a,4} = \frac{[RedOx][H^+]}{[RedOxH]} \tag{S56}$$

$$K_{a,5} = \frac{[RedRed][H^+]}{[RedRedH]} \tag{S57}$$

$$K_{a,6} = \frac{[RedSred][H^+]}{[RedSredH]} \tag{S58}$$

Equation S39 can be rearranged to get [*OxRed*] in terms of [*OxOx*] as follows:

$$[OxRed] = [OxOx] \times exp\frac{nF}{RT}(E_1^O - E)$$

$$[OxRed] = [OxOx] \times \alpha_1 \tag{S59}$$

Where:

$$\alpha_1 = exp\frac{nF}{RT}(E_1^O - E)$$

Equation S40 can be rearranged to get [*OxSred*] in terms of [*OxRed*] as follows:

$$[OxSred] = [OxRed] \times exp\frac{nF}{RT}(E_2^O - E)$$

$$[OxSred] = [OxRed] \times \alpha_2 \tag{S60}$$

Where:

$$\alpha_2 = exp\frac{nF}{RT}(E_2^O - E)$$

Equation S41 can be rearranged to get [*OxRedH*] in terms of [*OxOxH*] as follows:

$$[OxRedH] = [OxOxH] \times exp\frac{nF}{RT}(E_3^O - E)$$

$$[OxRedH] = [OxOxH] \times \alpha_3 \tag{S61}$$

Where:

$$\alpha_3 = exp\frac{nF}{RT}(E_3^O - E)$$

Equation S42 can be rearranged to get [*OxSredH*] in terms of [*OxRedH*] as follows:

$$[OxSredH] = [OxRedH] \times exp\frac{nF}{RT}(E_4^O - E)$$

$$[OxSredH] = [OxRedH] \times \alpha_4 \tag{S62}$$

Where:

$$\alpha_4 = exp\frac{nF}{RT}(E_4^O - E)$$

Equation S43 can be rearranged to get [*RedRed*] in terms of [*RedOx*] as follows:

$$[RedRed] = [RedOx] \times exp\frac{nF}{RT}(E_5^O - E)$$

$$[RedRed] = [RedOx] \times \alpha_5 \tag{S63}$$

Where:

$$\alpha_5 = exp\frac{nF}{RT}(E_5^O - E)$$

Equation S44 can be rearranged to get [*RedSred*] in terms of [*RedRed*] as follows:

$$[RedSred] = [RedRed] \times exp\frac{nF}{RT}(E_6^O - E)$$

$$[RedSred] = [RedRed] \times \alpha_6 \tag{S64}$$

Where:

$$\alpha_6 = exp\frac{nF}{RT}(E_6^O - E)$$

Equation S45 can be rearranged to get [*RedRedH*] in terms of [*RedOxH*] as follows:

$$[RedRedH] = [RedOxH] \times exp\frac{nF}{RT}(E_7^O - E)$$

$$[RedRedH] = [RedOxH] \times \alpha_7 \tag{S65}$$

Where:

$$\alpha_7 = exp\frac{nF}{RT}(E_7^O - E)$$

Equation S46 can be rearranged to get [*RedSredH*] in terms of [*RedRedH*] as follows:

$$[RedSredH] = [RedRedH] \times exp\frac{nF}{RT}(E_8^O - E)$$

$$[RedSredH] = [RedRedH] \times \alpha_8 \tag{S66}$$

Where:

$$\alpha_8 = exp\frac{nF}{RT}(E_8^O - E)$$

Equation S47 can be rearranged to get [*RedOx*] in terms of [*OxOx*] as follows:

$$[RedOx] = [OxOx] \times exp\frac{nF}{RT}(E_9^O - E)$$

$$[RedOx] = [OxOx] \times \alpha_9 \tag{S67}$$

Where:

$$\alpha_9 = exp\frac{nF}{RT}(E_9^O - E)$$

Equation S48 can be rearranged to get [*RedRed*] in terms of [*OxRed*] as follows:

$$[RedRed] = [OxRed] \times exp\frac{nF}{RT}(E_{10}^O - E)$$

$$[RedRed] = [OxRed] \times \alpha_{10} \tag{S68}$$

Where:

$$\alpha_{10} = exp\frac{nF}{RT}(E_{10}^O - E)$$

Equation S49 can be rearranged to get [*RedSred*] in terms of [*OxSred*] as follows:

$$[RedSred] = [OxSred] \times exp\frac{nF}{RT}(E_{11}^O - E)$$

$$[RedSred] = [OxSred] \times \alpha_{11} \tag{S69}$$

Where:

$$\alpha_{11} = exp\frac{nF}{RT}(E_{11}^O - E)$$

Equation S50 can be rearranged to get [*RedOxH*] in terms of [*OxOxH*] as follows:

$$[RedOxH] = [OxOxH] \times exp\frac{nF}{RT}(E_{12}^O - E)$$

$$[RedOxH] = [OxOxH] \times \alpha_{12} \tag{S70}$$

Where:

$$\alpha_{12} = exp\frac{nF}{RT}(E_{12}^{O} - E)$$

Equation S51 can be rearranged to get [*RedRedH*] in terms of [*OxRedH*] as follows:

$$[RedRedH] = [OxRedH] \times exp\frac{nF}{RT}(E_{13}^{O} - E)$$

$$[RedRedH] = [OxRedH] \times \alpha_{13} \tag{S71}$$

Where:

$$\alpha_{13} = exp\frac{nF}{RT}(E_{13}^{O} - E)$$

Equation S52 can be rearranged to get [*RedSredH*] in terms of [*OxSredH*] as follows:

$$[RedSredH] = [OxSredH] \times exp\frac{nF}{RT}(E_{14}^{O} - E)$$

$$[RedSredH] = [OxSredH] \times \alpha_{14} \tag{S72}$$

Where:

$$\alpha_{14} = exp\frac{nF}{RT}(E_{14}^{O} - E)$$

And Equations S53-58 can be rearranged to get [*OxOxH*], [*OxRedH*], [*OxSredH*], [*RedOxH*], [*RedRedH*] and [*RedSredH*] in terms of [*OxOx*], [*OxRed*], [*OxSred*], [*RedOx*], [*RedRed*] and [*RedSred*], respectively, as follows:

$$[OxOxH] = [OxOx]\frac{[H^+]}{K_{a,1}} \tag{S73}$$

$$[OxRedH] = [OxRed]\frac{[H^+]}{K_{a,2}} \tag{S74}$$

$$[OxSredH] = [OxSred]\frac{[H^+]}{K_{a,3}} \tag{S75}$$

$$[RedOxH] = [RedOx]\frac{[H^+]}{K_{a,4}} \tag{S76}$$

$$[RedRedH] = [RedRed]\frac{[H^+]}{K_{a,5}} \tag{S77}$$

$$[RedSredH] = [RedSred]\frac{[H^+]}{K_{a,6}} \tag{S78}$$

Next, we define the sum of the concentrations of all states as 1:

$$[OxOx] + [OxRed] + [OxSred] + [OxOxH] + [OxRedH] + [OxSredH] + [RedOx] + [RedRed] + [RedSred] + [RedOxH] + [RedRedH] + [RedSredH] = 1$$

Next, we substitute [*OxSred*], [*OxSredH*], [*RedSred*] and [*RedSredH*] using Equations S60, S62, S64 and S66, respectively:

$$[OxOx] + [OxRed] + [OxRed] \times \alpha_2 + [OxOxH] + [OxRedH] + [OxRedH] \times \alpha_4 + [RedOx] + [RedRed] + [RedRed] \times \alpha_6 + [RedOxH] + [RedRedH] + [RedRedH] \times \alpha_8 = 1$$

$$[OxOx] + [OxRed] \times (1 + \alpha_2) + [OxOxH] + [OxRedH] \times (1 + \alpha_4) + [RedOx] + [RedRed] \times (1 + \alpha_6) + [RedOxH] + [RedRedH] \times (1 + \alpha_8) = 1$$

Next we substitute [*OxRedH*] and [*RedRedH*] using Equation S61 and S65, respectively:

$$[OxOx] + [OxRed] \times (1 + \alpha_2) + [OxOxH] + [OxOxH] \times \alpha_3 \times (1 + \alpha_4) + [RedOx] + [RedRed] \times (1 + \alpha_6) + [RedOxH] + [RedOxH] \times \alpha_7 \times (1 + \alpha_8) = 1$$

$$[OxOx] + [OxRed] \times (1 + \alpha_2) + [OxOxH] \times \{1 + \alpha_3 \times (1 + \alpha_4)\} + [RedOx] + [RedRed] \times (1 + \alpha_6) + [RedOxH] \times \{1 + \alpha_7 \times (1 + \alpha_8)\} = 1$$

Next we substitute [*RedRed*] and [*RedOxH*] using Equations S63 and S76, respectively:

$$[OxOx] + [OxRed] \times (1 + \alpha_2) + [OxOxH] \times \{1 + \alpha_3 \times (1 + \alpha_4)\} + [RedOx] + [RedOx] \times \alpha_5 \times (1 + \alpha_6) + [RedOx]\frac{[H^+]}{K_{a,4}} \times \{1 + \alpha_7 \times (1 + \alpha_8)\} = 1$$

$$[OxOx] + [OxRed] \times (1 + \alpha_2) + [OxOxH] \times \{1 + \alpha_3 \times (1 + \alpha_4)\} + [RedOx] \times (1 + \alpha_5 \times (1 + \alpha_6) + \frac{[H^+]}{K_{a,4}} \times \{1 + \alpha_7 \times (1 + \alpha_8)\}) = 1$$

Next we substitute [*OxRed*], [*OxOxH*] and [*RedOx*] using Equations S59, S73 and S67, respectively:

$$[OxOx] + [OxOx] \times \alpha_1 \times (1 + \alpha_2) + [OxOx]\frac{[H^+]}{K_{a,1}} \times \{1 + \alpha_3 \times (1 + \alpha_4)\} + [OxOx] \times \alpha_9 \times (1 + \alpha_5 \times (1 + \alpha_6) + \frac{[H^+]}{K_{a,4}} \times \{1 + \alpha_7 \times (1 + \alpha_8)\}) = 1$$

$$[OxOx] \times \{1 + \alpha_1 \times (1 + \alpha_2) + \frac{[H^+]}{K_{a,1}} \times \{1 + \alpha_3 \times (1 + \alpha_4)\} + \alpha_9 \times \left(1 + \alpha_5 \times (1 + \alpha_6) + \frac{[H^+]}{K_{a,4}} \times \{1 + \alpha_7 \times (1 + \alpha_8)\}\right)\} = 1$$

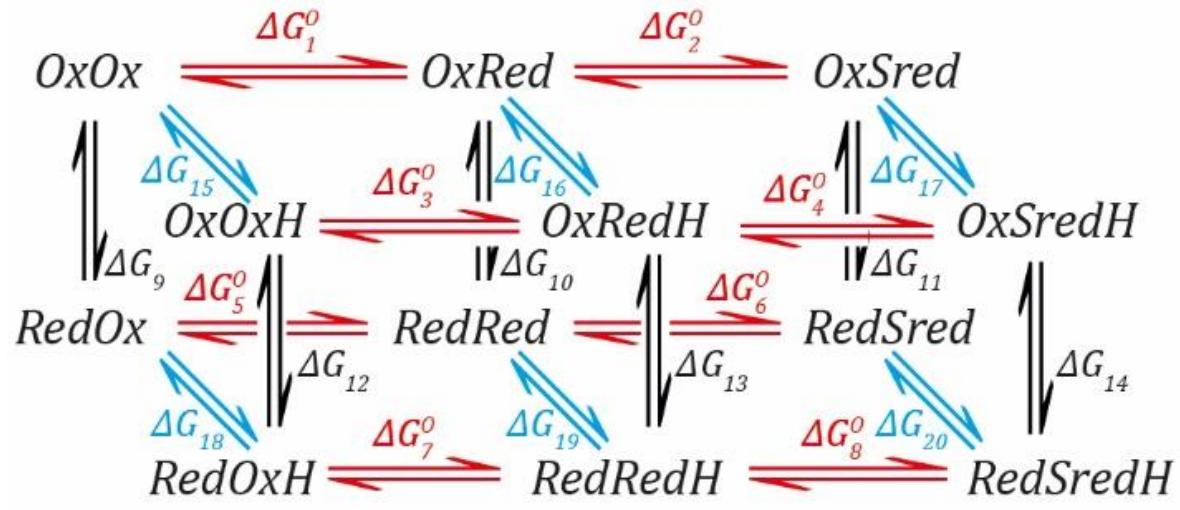
$$[OxOx] = \frac{1}{1 + \alpha_1 \times (1 + \alpha_2) + \frac{[H^+]}{K_{a,1}} \times \{1 + \alpha_3 \times (1 + \alpha_4)\} + \alpha_9 \times \left(1 + \alpha_5 \times (1 + \alpha_6) + \frac{[H^+]}{K_{a,4}} \times \{1 + \alpha_7 \times (1 + \alpha_8)\}\right)}$$

Using $[H^+] = 10^{-pH}$, $K_{a,1} = 10^{-pK_{a,1}}$ and $K_{a,4} = 10^{-pK_{a,4}}$ we can substitute $\frac{[H^+]}{K_{a,1}}$ for $10^{(pK_{a,1}-pH)}$, $\frac{[H^+]}{K_{a,4}}$ for $10^{(pK_{a,4}-pH)}$ and arrive at Equation 35 of the main text:

$$\boxed{[OxOx] = \frac{1}{1 + \alpha_1 \times (1 + \alpha_2) + 10^{(pK_{a,1}-pH)}\{1 + \alpha_3 \times (1 + \alpha_4)\} + \alpha_9 \times \left(1 + \alpha_5 \times (1 + \alpha_6) + 10^{(pK_{a,4}-pH)}\{1 + \alpha_7 \times (1 + \alpha_8)\}\right)}} \quad (35)$$

Where $\alpha_n = exp\frac{nF}{RT}(E_n^O - E)$

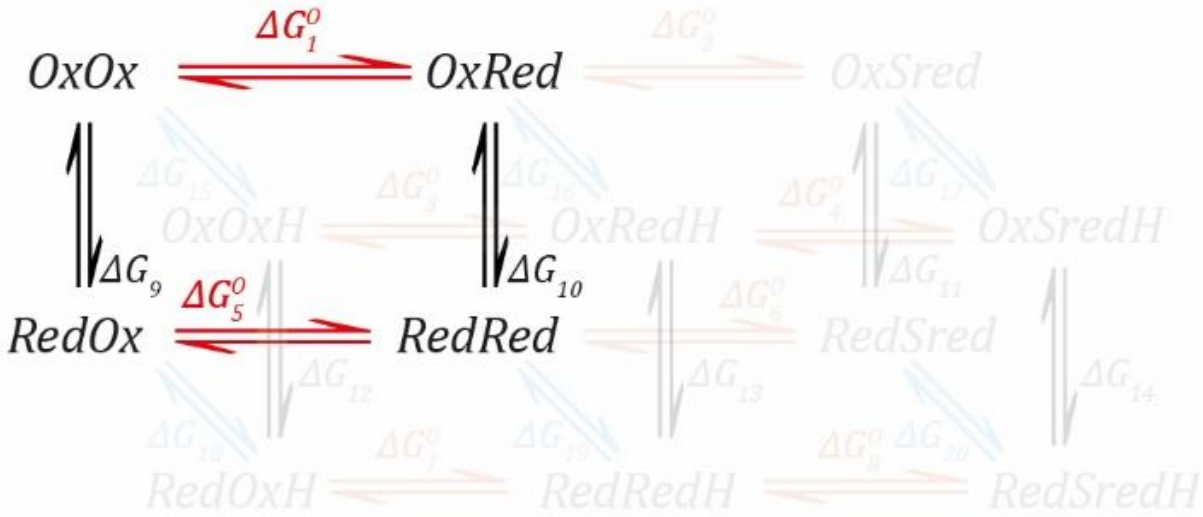
As with the previous models, it was only necessary to use a limited number of the parameters. The rest can be calculated by considering the thermodynamic cycles. We used E_1^O - E_9^O as well as $K_{a,1}$ and $K_{a,4}$. Therefore, we need to calculate E_{10}^O - E_{14}^O, $K_{a,2}$, $K_{a,3}$, $K_{a,5}$, and $K_{a,6}$.



Scheme S6. Model for active [FeFe] hydrogenase including redox anticooperativity with the free energy changes involved. The ΔG^0values corresponding to reduction events at the H-cluster (ΔG_1^O to ΔG_8^O) are coloured in red, the ΔG^0values corresponding to reduction events at the proximal F-cluster (ΔG_9^O to ΔG_{14}^O) are coloured in black, and the ΔG^0values corresponding to deprotonation events at the H-cluster (ΔG_{15}^O to ΔG_{20}^O) are coloured in blue.

From **Scheme S6**, we can focus on specific subcycles containing the steps for which the thermodynamic parameters are not defined:

1) For ΔG_{10}^O:

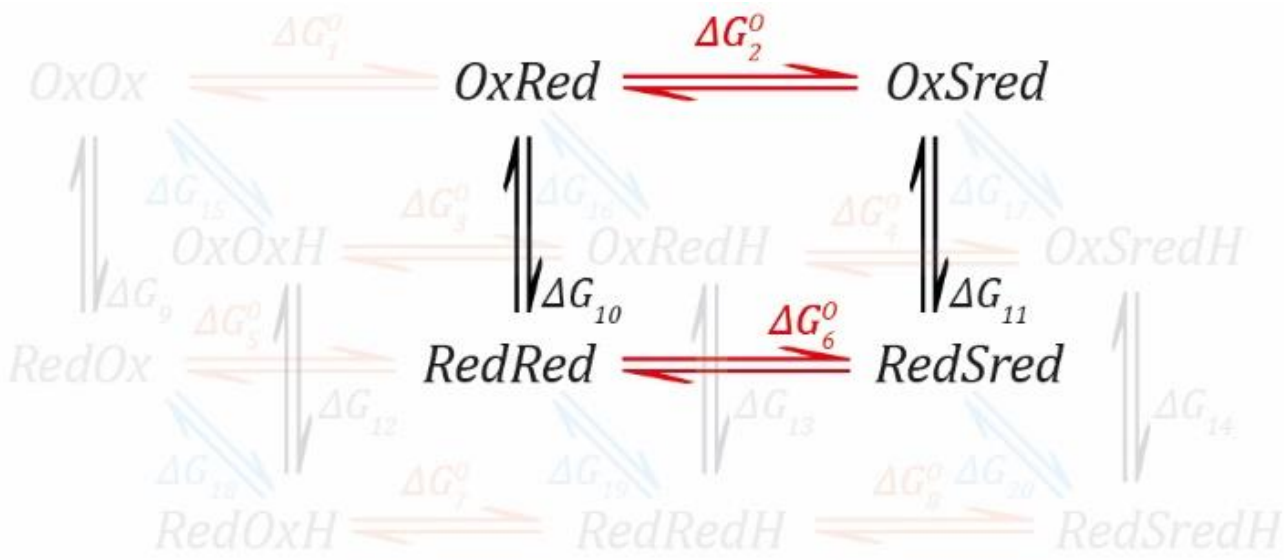


Scheme S7. Focus on the subcycle containing ΔG_{10}^O.

Considering that when going between states the free energy change is the same regardless of the pathway, the following equation can be derived:

$$\Delta G_1^O + \Delta G_{10}^O = \Delta G_5^O + \Delta G_9^O \quad \text{(S79)}$$

2) For ΔG_{11}^{O}:



Scheme S8. Focus on the subcycle containing ΔG_{11}^{O}.

Considering that when going between states the free energy change is the same regardless of the pathway, the following equation can be derived:

$$\Delta G_2^O + \Delta G_{11}^O = \Delta G_6^O + \Delta G_{10}^O \quad \text{(S80)}$$

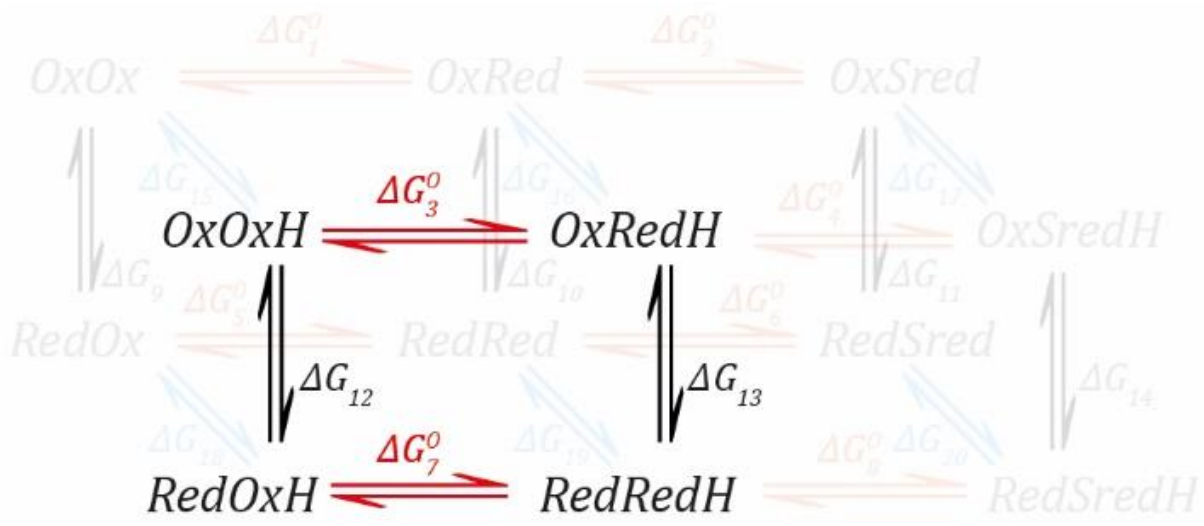
3) For ΔG_{12}^{O}:

Scheme S9. Focus on the subcycle containing ΔG_{12}^{O}.

Considering that when going between states the free energy change is the same regardless of the pathway, the following equation can be derived:

$$\Delta G_{12}^O - \Delta G_{15}^O = \Delta G_9^O - \Delta G_{18}^O \quad \text{(S81)}$$

4) For ΔG_{13}^{O}:

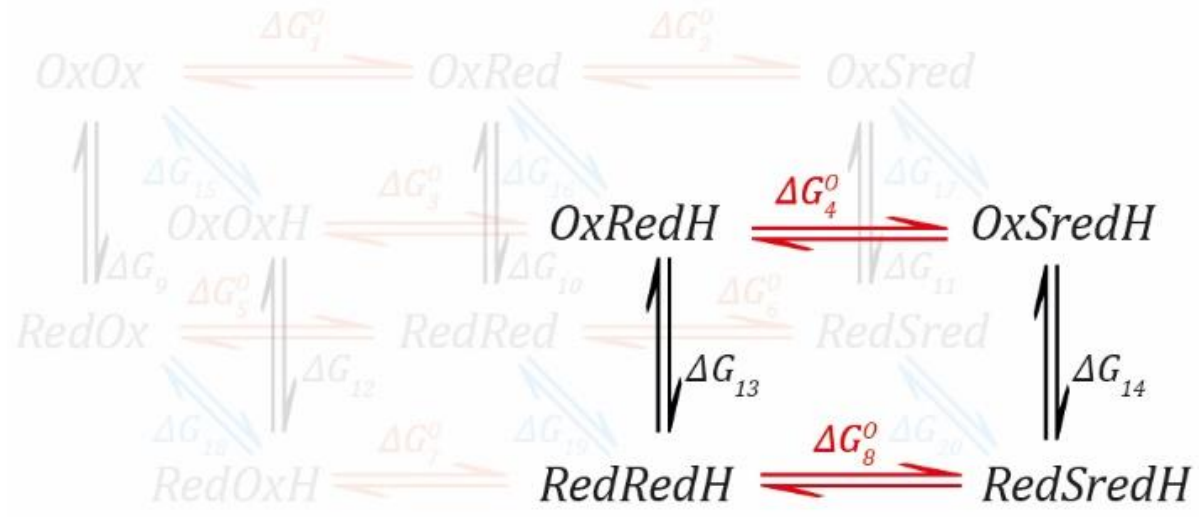


Scheme S10. Focus on the subcycle containing ΔG_{13}^{O}.

Considering that when going between states the free energy change is the same regardless of the pathway, the following equation can be derived:

$$\Delta G_{3}^{O} + \Delta G_{13}^{O} = \Delta G_{7}^{O} + \Delta G_{12}^{O} \quad \text{(S82)}$$

5) For ΔG_{14}^{O}:

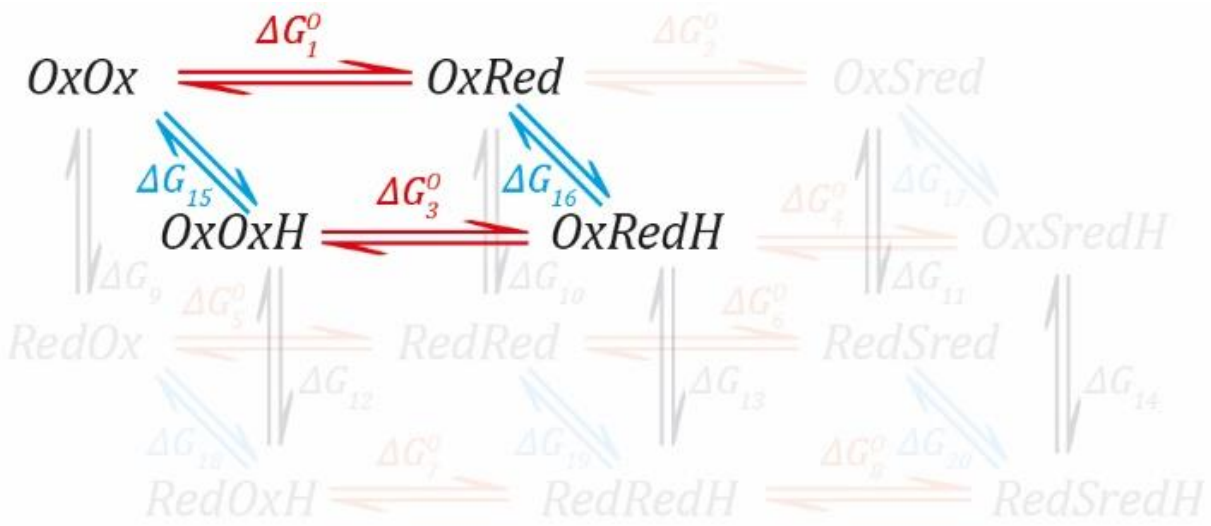


Scheme S11. Focus on the subcycle containing ΔG_{14}^{O}.

Considering that when going between states the free energy change is the same regardless of the pathway, the following equation can be derived:

$$\Delta G_{4}^{O} + \Delta G_{14}^{O} = \Delta G_{8}^{O} + \Delta G_{13}^{O} \quad \text{(S83)}$$

6) For ΔG_{16}^{O}:

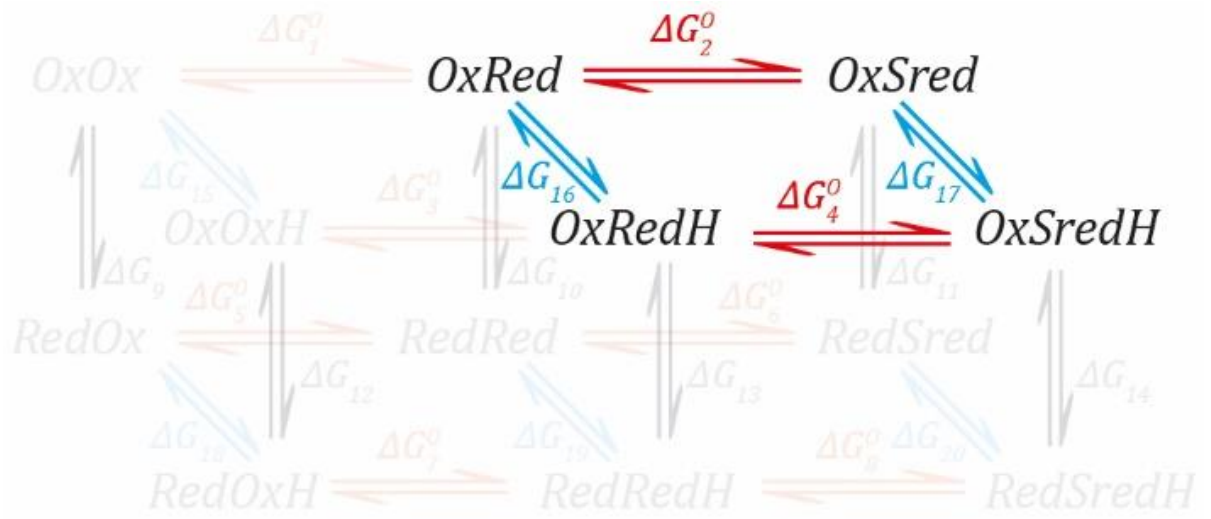


Scheme S12. Focus on the subcycle containing ΔG_{16}^{O}.

Considering that when going between states the free energy change is the same regardless of the pathway, the following equation can be derived:

$$\Delta G_{1}^{O} - \Delta G_{16}^{O} = \Delta G_{3}^{O} - \Delta G_{15}^{O} \quad \text{(S84)}$$

7) For ΔG_{17}^{O}:

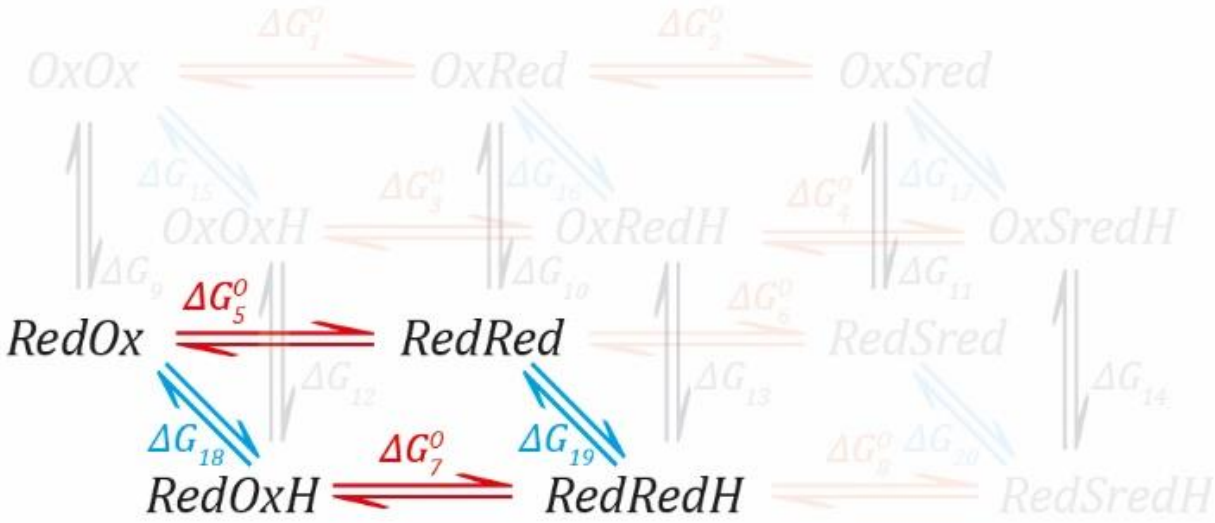


Scheme S13. Focus on the subcycle containing ΔG_{17}^{O}.

Considering that when going between states the free energy change is the same regardless of the pathway, the following equation can be derived:

$$\Delta G_{2}^{O} - \Delta G_{17}^{O} = \Delta G_{4}^{O} - \Delta G_{16}^{O} \quad \text{(S85)}$$

8) For ΔG_{19}^{O}:

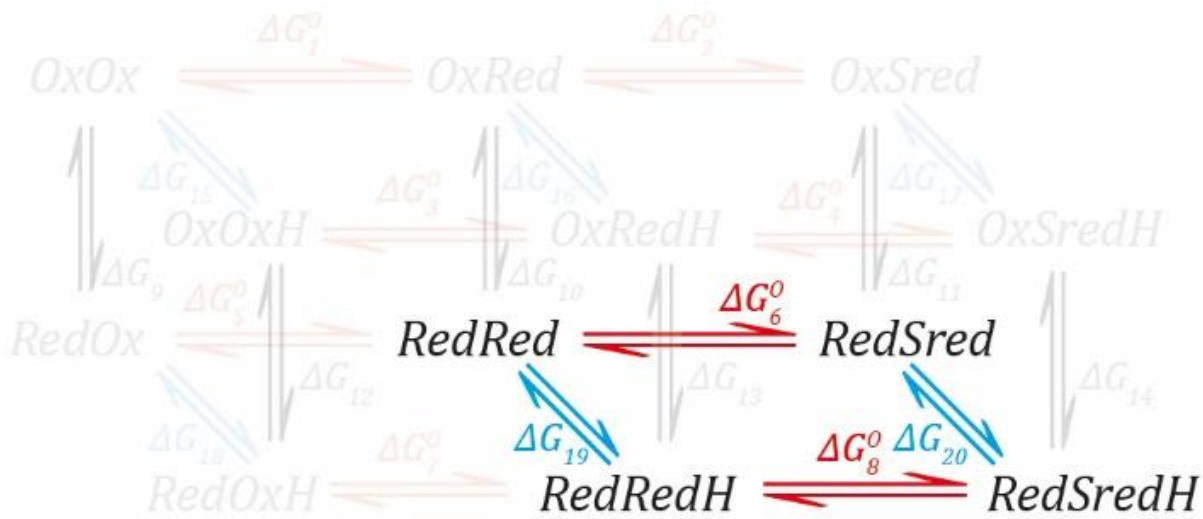


Scheme S14. Focus on the subcycle containing ΔG_{19}^{O}.

Considering that when going between states the free energy change is the same regardless of the pathway, the following equation can be derived:

$$\Delta G_5^O - \Delta G_{19}^O = \Delta G_7^O - \Delta G_{18}^O \quad \text{(S86)}$$

9) For ΔG_{20}^{O}:



Scheme S15. Focus on the subcycle containing ΔG_{20}^{O}.

Considering that when going between states the free energy change is the same regardless of the pathway, the following equation can be derived:

$$\Delta G_6^O - \Delta G_{20}^O = \Delta G_8^O - \Delta G_{19}^O \quad \text{(S87)}$$

Where:

$$\Delta G_1^O = -nFE_1^O \tag{S88}$$

$$\Delta G_2^O = -nFE_2^O \tag{S89}$$

$$\Delta G_3^O = -nFE_3^O \tag{S90}$$

$$\Delta G_4^O = -nFE_4^O \tag{S91}$$

$$\Delta G_5^O = -nFE_5^O \tag{S92}$$

$$\Delta G_6^O = -nFE_6^O \tag{S93}$$

$$\Delta G_7^O = -nFE_7^O \tag{S94}$$

$$\Delta G_8^O = -nFE_8^O \tag{S95}$$

$$\Delta G_9^O = -nFE_9^O \tag{S96}$$

$$\Delta G_{10}^O = -nFE_{10}^O \tag{S97}$$

$$\Delta G_{11}^O = -nFE_{11}^O \tag{S98}$$

$$\Delta G_{12}^O = -nFE_{12}^O \tag{S99}$$

$$\Delta G_{13}^O = -nFE_{13}^O \tag{S100}$$

$$\Delta G_{14}^O = -nFE_{14}^O \tag{S101}$$

$$\Delta G_{15}^O = -RTlnK_{a,1} \tag{S102}$$

$$\Delta G_{16}^O = -RTlnK_{a,2} \tag{S103}$$

$$\Delta G_{17}^O = -RTlnK_{a,3} \tag{S104}$$

$$\Delta G_{18}^O = -RTlnK_{a,4} \tag{S105}$$

$$\Delta G_{19}^O = -RTlnK_{a,5} \tag{S106}$$

$$\Delta G_{20}^O = -RTlnK_{a,6} \tag{S107}$$

1) Substituting Equations S88, S92, S96 and S97 into Equation S79 and rearrange this equation for E_{10}^O giving Equation 36 from the main text:

$$\Delta G_1^O + \Delta G_{10}^O = \Delta G_5^O + \Delta G_9^O$$

$$-nFE_1^O + (-nFE_{10}^O) = (-nFE_5^O) + (-nFE_9^O)$$

$$nFE_1^O + nFE_{10}^O = nFE_5^O + nFE_9^O$$

$$nFE_{10}^O = nFE_5^O - nFE_1^O + nFE_9^O$$

$$\boxed{E_{10}^O = E_5^O - E_1^O + E_9^O} \tag{36}$$

2) Substituting Equations S89, S93, S97 and S98 into Equation S80 and rearrange this equation for E_{11}^{O} giving Equation 37 from the main text:

$$\Delta G_2^O + \Delta G_{11}^O = \Delta G_6^O + \Delta G_{10}^O$$

$$-nFE_2^O + (-nFE_{11}^O) = -nFE_6^O + (-nFE_{10}^O)$$

$$nFE_2^O + nFE_{11}^O = nFE_6^O + nFE_{10}^O$$

$$nFE_{11}^O = nFE_6^O - nFE_2^O + nFE_{10}^O$$

$$\boxed{E_{11}^O = E_6^O - E_2^O + E_{10}^O} \quad (37)$$

3) Substituting Equations S96, S99, S102 and S105 into Equation S81 and rearrange this equation for E_{12}^{O} giving Equation 38 from the main text:

$$\Delta G_{12}^O - \Delta G_{15}^O = \Delta G_9^O - \Delta G_{18}^O$$

$$-nFE_{12}^O - \left(-RTlnK_{a,1}\right) = -nFE_9^O - (-RTlnK_{a,4})$$

$$nFE_{12}^O - RTlnK_{a,1} = nFE_9^O - RTlnK_{a,4}$$

$$nFE_{12}^O = nFE_9^O + RTlnK_{a,1} - RTlnK_{a,4}$$

$$\boxed{E_{12}^O = E_9^O + \frac{RT}{nF} ln \frac{K_{a,1}}{K_{a,4}}} \quad (38)$$

4) Substituting Equations S90, S94, S99 and S100 into Equation S82 and rearrange this equation for E_{13}^{O} giving Equation 39 from the main text:

$$\Delta G_3^O + \Delta G_{13}^O = \Delta G_7^O + \Delta G_{12}^O$$

$$-nFE_3^O + (-nFE_{13}^O) = -nFE_7^O + (-nFE_{12}^O)$$

$$nFE_3^O + nFE_{13}^O = nFE_7^O + nFE_{12}^O$$

$$nFE_{13}^O = nFE_7^O - nFE_3^O + nFE_{12}^O$$

$$E_{13}^O = E_7^O - E_3^O + E_{12}^O$$

$$\boxed{E_{12}^O = E_9^O + \frac{RT}{nF} ln \frac{K_{a,1}}{K_{a,4}}} \quad (39)$$

5) Substituting Equations S91, S95, S100 and S101 into Equation S83 and rearrange this equation for E_{14}^{O} giving Equation 40 from the main text:

$$\Delta G_4^O + \Delta G_{14}^O = \Delta G_8^O + \Delta G_{13}^O$$

$$-nFE_4^O + (-nFE_{14}^O) = -nFE_8^O + (-nFE_{13}^O)$$

$$nFE_4^O + nFE_{14}^O = nFE_8^O + nFE_{13}^O$$

$$nFE_{14}^{O} = nFE_{8}^{O} - nFE_{4}^{O} + nFE_{13}^{O}$$

$$\boxed{E_{14}^{O} = E_{8}^{O} - E_{4}^{O} + E_{13}^{O}} \quad (40)$$

6) Substituting Equations S88, S90, S102 and 103 into Equation S84 and rearrange this equation for $K_{a,2}$ giving Equation 41 from the main text:

$$\Delta G_{1}^{O} - \Delta G_{16}^{O} = \Delta G_{3}^{O} - \Delta G_{15}^{O}$$

$$-nFE_{1}^{O} - \left(-RTlnK_{a,2}\right) = -nFE_{3}^{O} - (-RTlnK_{a,1})$$

$$nFE_{1}^{O} - RTlnK_{a,2} = nFE_{3}^{O} - RTlnK_{a,1}$$

$$RTlnK_{a,2} - RTlnK_{a,1} = nFE_{1}^{O} - nFE_{3}^{O}$$

$$RTln\frac{K_{a,2}}{K_{a,1}} = nF(E_{1}^{O} - E_{3}^{O})$$

$$\frac{K_{a,2}}{K_{a,1}} = exp\left[\frac{nF}{RT}(E_{1}^{O} - E_{3}^{O})\right]$$

$$\boxed{K_{a,2} = K_{a,1}exp\left[\frac{nF}{RT}(E_{1}^{O} - E_{3}^{O})\right]} \quad (41)$$

7) Substituting Equations S89, S91, S103 and 104 into Equation S85 and rearrange this equation for $K_{a,3}$ giving Equation 42 from the main text:

$$\Delta G_{2}^{O} - \Delta G_{17}^{O} = \Delta G_{4}^{O} - \Delta G_{16}^{O}$$

$$-nFE_{2}^{O} - \left(-RTlnK_{a,3}\right) = -nFE_{4}^{O} - (-RTlnK_{a,2})$$

$$nFE_{2}^{O} - RTlnK_{a,3} = nFE_{4}^{O} - RTlnK_{a,2}$$

$$RTlnK_{a,3} - RTlnK_{a,2} = nFE_{2}^{O} - nFE_{4}^{O}$$

$$RTln\frac{K_{a,3}}{K_{a,2}} = nF(E_{2}^{O} - E_{4}^{O})$$

$$\frac{K_{a,3}}{K_{a,2}} = exp\left[\frac{nF}{RT}(E_{2}^{O} - E_{4}^{O})\right]$$

$$\boxed{K_{a,3} = K_{a,2}exp\left[\frac{nF}{RT}(E_{2}^{O} - E_{4}^{O})\right]} \quad (42)$$

8) Substituting Equations S92, 94, S105 and S106 into Equation S86 and rearrange this equation for $K_{a,5}$ giving Equation 43 from the main text:

$$\Delta G_{5}^{O} - \Delta G_{19}^{O} = \Delta G_{7}^{O} - \Delta G_{18}^{O}$$

$$-nFE_{5}^{O} - \left(-RTlnK_{a,5}\right) = -nFE_{7}^{O} - (-RTlnK_{a,4})$$

$$nFE_5^O - RTlnK_{a,5} = nFE_7^O - RTlnK_{a,4}$$

$$RTlnK_{a,5} - RTlnK_{a,4} = nFE_5^O - nFE_7^O$$

$$RTln\frac{K_{a,5}}{K_{a,4}} = nF(E_5^O - E_7^O)$$

$$\frac{K_{a,5}}{K_{a,4}} = exp\left[\frac{nF}{RT}(E_5^O - E_7^O)\right]$$

$$\boxed{K_{a,5} = K_{a,4}exp\left[\frac{nF}{RT}(E_5^O - E_7^O)\right]} \quad (43)$$

9) Substituting Equations S93, S95, S106 and S107 into Equation S87 and rearrange this equation for $K_{a,6}$ giving Equation 44 from the main text:

$$\Delta G_6^O - \Delta G_{20}^O = \Delta G_8^O - \Delta G_{19}^O$$

$$-nFE_6^O - \left(-RTlnK_{a,6}\right) = -nFE_8^O - (-RTlnK_{a,5})$$

$$nFE_6^O - RTlnK_{a,6} = nFE_8^O - RTlnK_{a,5}$$

$$RTlnK_{a,6} - RTlnK_{a,5} = nFE_6^O - nFE_8^O$$

$$RTln\frac{K_{a,6}}{K_{a,5}} = nF(E_6^O - E_8^O)$$

$$\frac{K_{a,6}}{K_{a,5}} = exp\left[\frac{nF}{RT}(E_6^O - E_8^O)\right]$$

$$\boxed{K_{a,6} = K_{a,5}exp\left[\frac{nF}{RT}(E_6^O - E_8^O)\right]} \quad (44)$$