SUPPORTING INFORMATION

Beyond the Plateau: pL-Dependence of Proton Inventories as a Tool for Studying Ribozyme and Ribonuclease Catalysis

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S1. Derivation of General Population Weighted Gross-Butler equation in acid/base catalysis

In this section, we review and discuss of how equilibrium isotope effects on the general acid and base alter the species distributions of enzymes in the ionization state necessary for catalysis and simulate the resulting effects on predicted experimental PI results. Next, individual ϕ^{TS} contributions due to general acid and base catalysis are incorporated following the same species distribution.

In **Scheme 1** in main manuscript, acid (LA^+) and base (B^-) residues in the active site contribute to catalysis. The active protonation state $_{HA^+}E_{B^-}$ reacts with rate constant k to form products. The values of $pK_{a,A}$ and $pK_{a,B}$ are the values for the active site acid and base functional groups, respectively. We assume that the equilibrium isotope effect on the acid and base functional groups (including chemical species such as OH^- or H_3O^+) are independent.

As described previously[1], it is useful for deriving species plots for equilibrium processes to denote the quantity Q as a partition function defined as the sum of all enzyme forms divided by a reference state here designated as the active form of the enzyme. In 100% H_2O solution (**n** = 0), Q is sum of all four protonation status of enzymes ($[_{HA^+}E_{B^-}$ (I)] +$[_{HA^+}E_{BH}$ (II)] + $[_AE_{BH}$ (III)] +$[_AE_{B^-}$ (IV)]) divided by the concentration of an active enzyme, $[_{HA^+}E_{B^-}$ (I)].

$$Q_H = 1 + ([HA^+E_{BH}]/[HA^+E_{B-}]) + ([AE_{BH}]/[HA^+E_{B-}]) + ([AE_{B-}]/[HA^+E_{B-}]) \quad \textbf{(S1)}$$

$$= 1 + \frac{[H^+]}{K_{a,B}} + \frac{[H^+]}{K_{a,B}} \cdot \frac{K_{a,A}}{[H^+]} + \frac{K_{a,A}}{[H^+]} = 10^{\log 1} + 10^{\log \frac{[H^+]}{K_{a,B}}} + 10^{\log \frac{K_{a,A}}{K_{a,B}}} + 10^{\log \frac{K_{a,A}}{[H^+]}}$$

$$\therefore Q_H = 1 + 10^{pK_{a,B} - pH} + 10^{pK_{a,B} - pK_{a,A}} + 10^{pH - pK_{a,A}} \quad \textbf{(S2)}$$

As shown in **Scheme 1**, the concentrations of HA^+, B^-, and HA^+B^- are determined from species (I) and (II), (I) and (IV), and (I), respectively. The fraction of total enzyme with the acid and base in their correctly protonated forms, $f(HA^+)$ and $f(B^-)$ can be written in terms of the following equilibrium expressions:

$$f(HA^+) = f(I) + f(II) = \frac{1 + 10^{pK_{a,B} - pH}}{Q_H} \quad \textbf{(S3)}$$

$$f(B^-) = f(I) + f(IV) = \frac{1 + 10^{pH - pK_{a,A}}}{Q_H} \quad \textbf{(S4)}$$

The fraction of the enzyme in the correctly protonated form is $f(_{HA^+}E_{B^-}) = \frac{1}{Q_H}$ that in turn can be expressed as a function of the equilibrium expressions for $f(HA^+)$ and $f(B^-)$ as described previously[1].

$$f(_{HA^+}E_{B^-}) = f(HA^+)f(B^-) = \left(\frac{1 + 10^{pK_{a,B} - pH}}{Q_H}\right)\left(\frac{1 + 10^{pH - pK_{a,A}}}{Q_H}\right) = \frac{Q_H}{Q_H^2} = \frac{1}{Q_H} \qquad \textbf{(S5)}$$

The pK_a values for the acid and base depend on the nature of the functional groups involved and can be near neutrality or lie outside the range of pH values that can be measured experimentally. Nonetheless the observed rate constant is a function of the product $f(HA^+)f(B^-)$.

In 100 % D_2O solution (n = 1) the pK_a values of the acid and base can be defined as,

$$pK_{a,A_D} = pK_{a,A} + \Delta pK_{a,A} \qquad \textbf{(S6)}$$

$$pK_{a,B_D} = pK_{a,B} + \Delta pK_{a,B} \qquad \textbf{(S7)}$$

where $\Delta pK_{a,A}$ and $\Delta pK_{a,B}$ are pK_a shifts caused by equilibrium isotope effects on acid and base functional groups, respectively. Q_D is sum of all four possible protonated status of enzymes ($[_{DA^+}E_{B^-}$ (I)] +$[_{DA^+}E_{BD}$ (II)] + $[_{A}E_{BD}$ (III)] +$[_{A}E_{B^-}$ (IV)]) divided by the concentration of active enzyme, $[_{DA^+}E_{B^-}$ (I)], resulting in equation S8. The concentration of DA^+ is the sum of $_{DA^+}E_{B^-}$ (I) and $_{DA^+}E_{BD}$ (II) (Eq. S9), and concentration of B^- is sum of $_{DA^+}E_{B^-}$ (I) and $_{A}E_{B^-}$ (IV) (Eq. S10). Concentration of $_{DA^+}E_{B^-}$ (I) is a product of multiplication of equations S9 and S10, as shown in equation S11.

$$Q_D = 1 + 10^{pK_{a,B_D} - pD} + 10^{pK_{a,B_D} - pK_{a,A_D}} + 10^{pD - pK_{a,A_D}} \qquad \textbf{(S8)}$$

$$f(DA^+) = (1) + (2) = \frac{1 + 10^{pK_{a,B_D} - pD}}{Q_D} \qquad \textbf{(S9)}$$

$$f(B_D^-) = (1) + (4) = \frac{1 + 10^{pD - pK_{a,A_D}}}{Q_D} \qquad \textbf{(S10)}$$

$$f(_{DA^+}E_{B^-}) = f(DA^+)f(B_D^-) = \left(\frac{1 + 10^{pK_{a,B_D} - pD}}{Q_D}\right)\left(\frac{1 + 10^{pD - pK_{a,A_D}}}{Q_D}\right) = \frac{1}{Q_D} \qquad \textbf{(S11)}$$

In mixed H_2O/D_2O solutions the active forms of the enzyme will include a combination of both $_{HA}E_{B^-}$ and $_{DA}E_{B^-}$. As outlined, above, the fraction of an active enzyme can be expressed as a

product of the fractions in the active protonation states ($f(LA^+)$ and $f(B)_L$ (L = H or D). There are four configurations (due to H^+/D^+ exchange: HH, DH, HD, and DD. The concentration of active enzyme is the sum of all four species: $f(HA^+)f(B_H^-)$, $f(DA^+)f(B_H^-)$, $f(HA^+)f(B_D^-)$, and $f(DA^+)f(B_D^-)$. Fractions of enzyme forms with protonated species HA^+ and B_H^- are proportional to 1-**n**, and fractions of species affected by deuteration (DA^+ and B_D^-) are proportional to **n**. Because k_{obs} is proportional to $f(LA^+)f(B_L^-)$ an equation describing k_{obs} at **n** can be written.

$$k_n = k\left[(1-n)^2 f(HA^+)f(B_H^-) + n(1-n)f(DA^+)f(B_H^-) + n(1-n)f(HA^+)f(B_D^-) + n^2 f(DA^+)f(B_D^-)\right] \quad \textbf{(S12)}$$

This expression describes the observed pL versus log k profile at any value of **n** for an enzyme using acid/base catalysis in the absence of any ϕ_{TS} on the catalytic step.

For enzymes in which ϕ^{TS} is significant the rate constant will be a product from both fractions of active enzyme and transition state fractionation factor ϕ_i^{TS}(Transition fractionation factor at i^{th} site). In a mechanism in which two protons are transferred simultaneously, two transition state fractionation factors can be defined: a first TS fractionation factor due to the protonation of 5' leaving group (ϕ^{TA}) and a second TS fractionation factor due to nucleophile deprotonation (ϕ^{TB}). Then, the rate constant at pL (L = H or D) can be written as follows,

$$k_n = k\left[(1-n)^2 f(HA^+)f(B_H^-) + n(1-n)\phi^{TA}f(DA^+)f(B_H^-) + n(1-n)\phi^{TB}f(HA^+)f(B_D^-) + n^2\phi^{TA}\phi^{TB}f(DA^+)f(B_D^-\right. \quad \textbf{(S13)}$$

Using the expressions for the distributions of species $f(HA^+)f(B_H^-)$, $f(DA^+)f(B_H^-)$, $f(HA^+)f(B_D^-)$ and $f(DA^+)f(B_D^-)$ results in the following,

$$k_n = k\left[(1-n)^2\frac{1}{Q_H} + n(1-n)\phi^{TA}\left(\frac{1+10^{pK_{a,B_D}-pL}}{Q_D}\right)\left(\frac{1+10^{pL-pK_{a,A}}}{Q_H}\right) + n(1-n)\phi^{TB}\left(\frac{1+10^{pK_{a,B}-pL}}{Q_H}\right)\left(\frac{1+10^{pL-pK_{a,A_D}}}{Q_D}\right) + n^2\phi^{TA}\phi\right. \quad \textbf{(S14)}$$

$$\therefore \frac{k_n}{k_0} = (1-n)^2 + n(1-n)\phi^{TA}\left(1+10^{pL-pK_{a,A}}\right)\left(\frac{1+10^{pK_{a,B_D}-pL}}{Q_D}\right)$$

$$+ n(1-n)\phi^{TB}(1 + 10^{pK_{a,B} - pL})\left(\frac{1 + 10^{pL - pK_{a,A_D}}}{Q_D}\right) + n^2\phi^{TA}\phi^{TB}\frac{Q_H}{Q_D} \quad \textbf{(S15)}$$

S2. Evaluation of the GPW-GB equation at the boundary conditions of high and low pLs

As described in supplementary information S.1., equation (3) in main text expresses the proton inventory (PI) as a function of equilibrium and transition state fractionation factors. Under conditions of high and low pH the acid and base, respectively, will be entirely in their active protonated forms. Under these conditions the GPW-GB should simplify to more familiar forms of the familiar form of the linear and quadratic GB equation.

At the extreme of low pH (i.e. pH 4), as shown on **Figures 2B** and **3B** in the text, the f(LA^+) approaches 1. In addition, the term $1 + 10^{pK_{a,B_D} - pL}$ approximates Q_D.

$$Q_D = 1 + 10^{pK_{a,B_D} - pL} + 10^{pK_{a,B_D} - pK_{a,A_D}} + 10^{pL - pK_{a,AD}} \cong 1 + 10^{pK_{a,B_D} - pL} \textbf{(S16)}$$

Then, $\frac{k_n}{k_0}$ is approximated by,

$$\frac{k_n}{k_0} = (1-n)^2 + n(1-n)\phi^{TA} + n(1-n)\phi^{TB}\left(\frac{Q_H}{Q_D}\right) + n^2\phi^{TA}\phi^{TB}\left(\frac{Q_H}{Q_D}\right) \textbf{(S17)}$$

$$\therefore \frac{k_n}{k_0} = (1 - n + n\phi^{TB}Q)(1 - n + n\phi^{TA}), \text{ where } Q = \frac{Q_H}{Q_D} \qquad \textbf{(S18)}$$

From this it is evident that the PI at very low pH will be quadratic when both ground state (counting the population of active enzymes) and transition state fractionation factors are present. As shown by simulated PIs (Figure 3 and 4), if there is no transition state isotope effect ($\phi^{TA} = \phi^{TB} = 1$) the PI becomes linear at low pH and the general equation simplifies to,

$$\frac{k_n}{k_0} = (1 - n + nQ). \quad \textbf{(S19)}$$

In contrast, at the boundary condition of very high pH the fraction of base ($f(B^-)_L$) is close to 1 and the term $\left(1 + 10^{pL - pK_{a,A}}\right)$ approaches Q_H.

$$\frac{k_n}{k_0} = (1-n)^2 + n(1-n)\phi^{TA}\left(1 + 10^{pL-pK_{a,A}}\right)\left(\frac{1}{Q_D}\right) + n(1-n)\phi^{TB}\left(\frac{1 + 10^{pL-pK_{a,A_D}}}{Q_D}\right) + n^2\phi^{TA}\phi^{TB}\frac{Q_H}{Q_D} \quad \textbf{(S20)}$$

$$\frac{k_n}{k_0} = (1-n)^2 + n(1-n)\phi^{TA}\left(\frac{Q_H}{Q_D}\right) + n(1-n)\phi^{TB} + n^2\phi^{TA}\phi^{TB}\frac{Q_H}{Q_D} \quad \textbf{(S21)}$$

Substituting $\frac{Q_H}{Q_D}$ to Q,

$$\therefore \frac{k_n}{k_0} = (1 - n + n\phi^{TA}Q)(1 - n + n\phi^{TB}) \quad \textbf{(S22)}$$

Note that simulated PI results at high pH (**Figures 2** and **3** in the main text) are linear whether or not ϕ^{TS} effects are included.

S3. Effect of cooperative interactions between active site acid/base ionizations

An important limitation noted is the text is that protonation/deprotonation of the general acid and general base shown in **Scheme 1** assumes that the underlying protonation states are uncorrelated. Thus, the fraction of enzyme in the active protonation state, $f_{(HA^+E_{B^-})}$, is the product of the fractions $f(HA^+)$ and $f(B^-)$. However, in presence of cooperative interactions between the ionized forms of acid and base the distribution of active enzyme concentrations in mixed D_2O/H_2O solutions cannot be written the sum of products of acid and base species as described previously by Frankel and Bevilacqua[2]. In this section we illustrate the impact and limitations of this complexity on the ability to resolve PI results using a GPW-GB approach and comment on the conditions under which PI results may still provide useful mechanistic information when cooperative interactions between active site titratable groups are present.

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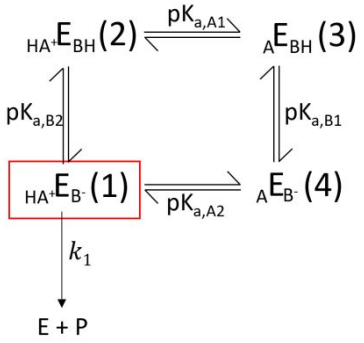


Scheme S1. Thermodynamic box of cooperative model with acid and base catalysis reaction in 100% H_2O. Adapted from Frankel and Bevilacqua (2018)[2].

1) 100 % H_2O

In the cooperative model shown in **Scheme S1**, when either acid (or base) functional group is neutrally charged, pK_a for base (or acid) is perturbed, which causes pK_a shifting (ΔpK_{coop}). In Scheme S1., $pK_{a,A2}$ and $pK_{a,B2}$ are showing pK_a upward shifting and downward shifting due to that cooperativity.

$$pK_{a,A2} = pK_{a,A1} + \Delta pK_{coop} \quad \textbf{(S23)}$$

$$pK_{a,B2} = pK_{a,B1} - \Delta pK_{coop} \quad \textbf{(S24)}$$

As in noncooperative model described previously, the sum of population of four species (Q) can be expressed as equation S26.

$$Q = 1 + ([HA^+EBH]/[HA^+EB^-]) + ([AEBH]/[HA^+EB^-]) + ([AEB^-]/[HA^+EB^-])$$

$$= 1 + \frac{[H^+]}{K_{a,B2}} + \frac{[H^+]}{K_{a,B2}} \cdot \frac{K_{a,A1}}{[H^+]} + \frac{K_{a,A2}}{[H^+]} = 10^{\log 1} + 10^{\log \frac{[H^+]}{K_{a,B2}}} + 10^{\log \frac{K_{a,A1}}{K_{a,B2}}} + 10^{\log \frac{K_{a,A2}}{[H^+]}} \quad \textbf{(S25)}$$

$$\therefore Q_H = 1 + 10^{pK_{a,B2} - pH} + 10^{pK_{a,B2} - pK_{a,A1}} + 10^{pH - pK_{a,A2}} \quad \textbf{(S26)}$$

In the thermodynamic box above, population of acid and base are from (1) and (II), and (I) and (IV), respectively. Then, fractions of acid, base, and active enzyme population are shown in equations **S27, S28, and S29**.

$$f(HA^+) = f(I) + f(II) = \frac{1 + 10^{pK_{a,B2} - pH}}{Q_H} \quad \textbf{(S27)}$$

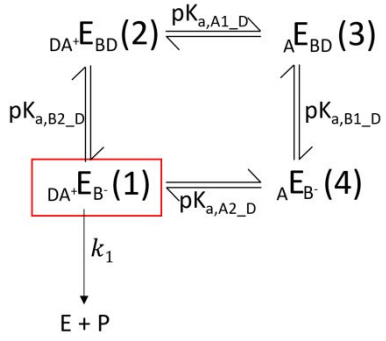
$$f(B^-) = f(I) + f(IV) = \frac{1 + 10^{pH - pK_{a,A2}}}{Q_H} \quad \textbf{(S28)}$$

$$f(_{HA^+}E_{B^-}) = \frac{1}{Q_H} = \frac{1}{1 + 10^{pK_{a,B2} - pH} + 10^{pK_{a,B2} - pK_{a,A1}} + 10^{pH - pK_{a,A2}}} \quad \textbf{(S29)}$$

As previously noted, in contrast to noncooperative model, the population of active enzyme, $_{HA^+}E_{B^-}$, cannot be $f(HA^+)f(B^-)$.

$$f(HA^+)f(B^-) = \left(\frac{1 + 10^{pK_{a,B2} - pH}}{Q_H}\right)\left(\frac{1 + 10^{pH - pK_{a,A2}}}{Q_H}\right) \neq \frac{1}{Q_H} \quad \textbf{(S30)}$$

$$\therefore f(HA^+)f(B^-) \neq f(_{HA^+}E_{B^-}) \quad \textbf{(S31)}$$



Scheme S2. Thermodynamic box of cooperative model with acid and base catalysis reaction in D_2O. Adapted from Frankel and Bevilacqua (2018)[2].

2) 100 % D_2O

In 100 % D_2O solution (n = 1) the pK_a values of the acid and base can be defined as,

$$pK_{a,A_{1\_D}} = pK_{a,A} + \Delta pK_{a,A} \quad \textbf{(S32)}$$

$$pK_{a,B_{1\_D}} = pK_{a,B} + \Delta pK_{a,B} \quad \textbf{(S33)}$$

where $\Delta pK_{a,A}$ and $\Delta pK_{a,B}$ are pK_a shifts caused by equilibrium isotope effects on acid and base functional groups, respectively.

Considering pK_a shifts caused by neutral charge of opponent functional group, $pK_{a,A_{2\_D}}$ and $pK_{a,B_{2\_D}}$ are expressed below:

$$pK_{a,A_{2\_D}} = pK_{a,A_{1\_D}} + \Delta pK_{coop} = (pK_{a,A1} + \Delta pK_{a,A}) + \Delta pK_{coop\_D} \quad \textbf{(S34)}$$

$$pK_{a,B_{2\_D}} = pK_{a,B_{1\_D}} - \Delta pK_{coop} = (pK_{a,B1} + \Delta pK_{a,B}) - \Delta pK_{coop\_D} \quad \textbf{(S35)}$$

Same logic as 100% H_2O, we can write the sum of concentrations of all possible protonation status in terms of pD and pK_a values,

$$Q_D = 1 + 10^{pK_{a,B2\_D} - pD} + 10^{pK_{a,B2\_D} - pK_{a,A1\_D}} + 10^{pD - pK_{a,A2\_D}} \quad \textbf{(S36)}$$

Acid and base fractions are:

$$f(DA^+) = f(I) + f(II) = \frac{1 + 10^{pK_{a,B2\_D} - pD}}{Q_D} \quad \textbf{(S37)}$$

$$f(B^-) = f(I) + f(IV) = \frac{1 + 10^{pD - pK_{a,A2\_D}}}{Q_D} \quad \textbf{(S38)}$$

$$f({}_{DA^+}E_{B^-}) = \frac{1}{Q_D} = \frac{1}{1 + 10^{pK_{a,B2\_D} - pD} + 10^{pK_{a,B2\_D} - pK_{a,A1\_D}} + 10^{pD - pK_{a,A2\_D}}} \quad \textbf{(S39)}$$

$$f(DA^+)f(B^-) = \left(\frac{1 + 10^{pK_{a,B2\_D} - pD}}{Q_D}\right)\left(\frac{1 + 10^{pD - pK_{a,A2\_D}}}{Q_D}\right) \neq \frac{1}{Q_D} \quad \textbf{(S40)}$$

In mixed H_2O/D_2O solutions, there are four possible configurations: ${}_{HA^+}E_{B\text{-}(H)}$, ${}_{DA^+}E_{B\text{-}(H)}$, ${}_{HA^+}E_{B\text{-}(D)}$, and ${}_{DA^+}E_{B\text{-}(D)}$. Although it seems reasonable to accounting for these configurations and transition state fractionation factors, we found limitations of this cooperative model, described below.

1) Accounting for the D_2O ratio, n

As shown in equations **S31** and **S40**, in the cooperative model, the fraction of active enzyme is not same as a product of $f(LA^+)f(B^-)$. In other words, active enzyme concentrations in mixed D_2O/H_2O solutions cannot be written the sum of multiplications of acid and base species (i.e., $f(HA^+)f(B_H^-)$, $f(DA^+)f(B_H^-)$, $f(HA^+)f(B_D^-)$, and $f(DA^+)f(B_D^-)$). Instead, the accurate mathematical expression of total concentration of active enzymes is the sum of fraction $f({}_{LA^+}E_{B\text{-}(L)}) = f({}_{HA^+}E_{B\text{-}(H)}) + f({}_{DA^+}E_{B\text{-}(H)}) + f({}_{HA^+}E_{B\text{-}(D)}) + f({}_{DA^+}E_{B\text{-}(D)})$. Taking an account for D_2O ratios, the first and the last terms, $f({}_{HA^+}E_{B\text{-}(H)})$ and $f({}_{DA}E_{B\text{-}(D)})$, are proportional to 1-n, and n, respectively. However, for the second and third terms, $f({}_{DA^+}E_{B\text{-}(H)})$ and $f({}_{HA^+}E_{B\text{-}(D)})$, it cannot be determined whether or not these terms

are proportional to solely D_2O (n) or H_2O (1-n) ratios, or, quadratic mixed ratio, n(1-n). Given to the complication of D_2O ratio counting, we considered the effect of including only $f_{(HA^+E_{B-(H)})}$ and $f_{(DA^+E_{B-(D)})}$ as active enzyme forms. Then, the active enzyme population becomes as the sum of $f_{(HA^+E_{B-})}$ (Eq. **S29**) and $f_{(DAE_{B-})}$ (Eq. **S39).** $f_{(LA^+E_{B-(L)})}$ can be expressed as equation **S41**.

$$f_{(LA^+E_{B-(L)})} = \frac{1-n}{Q_H} + \frac{n}{Q_D} \textbf{ (S41)}$$

$$k_n = k\left(\frac{1-n}{Q_H} + \frac{n}{Q_D}\right) \textbf{ (S42)}$$

pL rate profiles were simulated via equation **S42** (k = 1 for simplicity), shown in **Figure S1**. The simulation of log k vs pL look similar to Figures 2E and 3E. However, in cooperative model 'the plateau' region is wider, and the magnitude of equilibrium isotope effects (EIE) is smaller than non-cooperative model calculation.

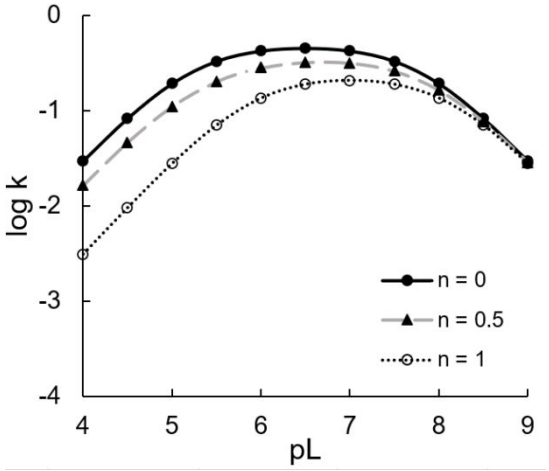


Figure S1. pL rate profiles with $pk_{a,A1} = 5.5$, $pk_{a,B1} = 7.5$, $\Delta pk_{a,A} = \Delta pk_{a,B} = 0.5$, $\Delta pK_{coop\_H2O} = 2$, and $\Delta pK_{coop\_D2O} = 1.5$. Filled circles, filled triangles, and empty circles are data points at n = 0, 0.5, and 1, respectively.

From equation S42, we can divide k_n by k_0 to simulate proton inventories.

$$k_n/k_0 = \frac{\frac{1-n}{Q_H} + \frac{n}{Q_D}}{\frac{1}{Q_H}} = 1 - n + n\left(\frac{Q_H}{Q_D}\right) \textbf{ (S43)}$$

It is important to note that even though Eq. S43 looks like Eq. S19 (boundary condition at low pL in non-cooperative model), the definition of terms Q_H and Q_D are not the same because

cooperativity of acid and base were included in Eq S43. PI simulations from pL 4 (filled circles) to pL 9 (filled squares), calculated by using equation S43. Because of complication of D_2O ratio counting for $f(_{DA^+}E_{B-(H)})$ and $f(_{HA^+}E_{B-(D)})$ terms, the proton inventories become linear at all pL values. Lastly, active enzyme fractions ($f(_{LA^+}E_{B-(L)})$ are no longer given by $f(LA^+)*f(B^-_{(L)})$, and thus ϕ^{TA} and ϕ^{TB} cannot be incorporated. **Figure S2** illustrates how the results are affected due to this inaccuracy. As cited in the text methods such as molecular dynamics simulations to determine the distributions of ionized forms of the enzyme and the distribution of ionized forms may be possible.

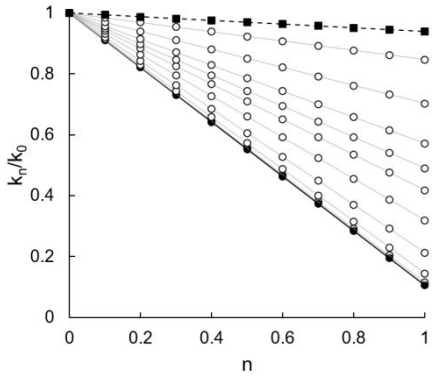


Figure S2. Proton Inventories simulations generated via equation S43. pL range is from 4 (filled circles) to 9 (filled squares). For this plot, $pK_{a,A1} = 5.5$, $pK_{a,B1} = 7.5$, $\Delta pK_{a,A} = \Delta pK_{a,B} = 0.5$, $\Delta pK_{coop\_H2O} = 2$, and $\Delta pK_{coop\_D2O} = 1.5$.

SI. References

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