Supporting Information

Kinetic Understanding of N_2 Reduction versus H_2 Evolution at the E_4(4H) Janus State in the Three Nitrogenases

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Steady-State Kinetic Analysis of Scheme D

The equations that describe the time-dependence of the populations of the three states comprising the high-flux **Schemes C** are given in **eq S1**, where, setting the population derivatives to zero expresses the steady-state condition, namely populations are invariant in time.

$$\frac{dE_2(2H)}{dt} = -\left(k_{ac} + k_{HP}\right)E_2(2H) + k_{HP}E_4(4H) + k_{cat}E_4(2N2H) \xrightarrow{SS} 0$$

$$\frac{dE_4(4H)}{dt} = -\left(k_{HP} + k_{re}P(N_2)\right)E_4(4H) + k_{ac}E_2(2H) \xrightarrow{SS} 0 \qquad \textbf{S1}$$

$$\frac{dE_4(2N2H)}{dt} = -k_{cat}E_4(2N2H) + k_{re}P(N_2))E_4(4H) \xrightarrow{SS} 0$$

$$E_4(2H) + E_4(4H) + E_4(2N2H) = E_0^0$$

The three kinetic equations, in combination with the 'conservation' condition that the populations of the three states sum to the total enzyme population, E_0^0, can be solved to obtain formulas for the steady-state populations of the three states involved in the scheme.

$$\frac{E_2(2H)}{E_0^0} \xrightarrow{SS} \frac{\left(\frac{k_{HP}}{k_{ac}}\right)+\left(\frac{k_{re}}{k_{ac}}\right)P_{N2}}{1+\left(\frac{k_{HP}}{k_{ac}}\right)+\left(\frac{k_{re}}{\bar{k}}\right)P_{N2}}$$

$$\frac{E_4(4H)}{E_0^0} \xrightarrow{SS} \frac{1}{1+\left(\frac{k_{HP}}{k_{ac}}\right)+\left(\frac{k_{re}}{\bar{k}}\right)P_{N2}}$$

$$\frac{E_4(2N2H)}{E_0^0} \xrightarrow{SS} \frac{\left(\frac{k_{re}}{k_{cat}}\right)P_{N2}}{1+\left(\frac{k_{HP}}{k_{ac}}\right)+\left(\frac{k_{re}}{\bar{k}}\right)P_{N2}}$$

$$\frac{1}{\bar{k}} = \frac{1}{k_{ac}} + \frac{1}{k_{cat}}$$

S2

The rates of formation of products are obtained by multiplying the populations involved with the appropriate rate constants,

$$\frac{dNH_3}{dt} = -2\frac{dN_2}{dt} = k_{cat}E_4(2N2H) \xrightarrow{SS} k_{NH3}\bullet E_0^0 \qquad \frac{dH_2}{dt} = k_{HP}E_2(2H) + \left(k_{HP} + k_{re}P_{N2}\right)E_4(4H) \xrightarrow{SS} k_{H2}\bullet E_0^0$$

S3

The final steady-state rate constants, k_{NH_3}, k_{H_2}, are given in the text. As the populations, and thus product formation, are zeroth order in time, the accumulation of product is simply proportional to the rate constant, the total enzyme concentration, and time.

As discussed in the text, **Scheme C**, and the rate constants presented in the main text, incorporate HP at both E_2(2H) and E_4(4H). However, as the flux increases, whether or not N_2 is present and/or P_{N_2} becomes large, the population of E_2(2H) becomes negligible,

$$\frac{E_2(2H)}{E_4(4H)+E_4(2N2H)} = \frac{\left(\frac{k_{HP}}{k_{ac}}\right)+\left(\frac{k_{re}}{k_{ac}}\right)P_{N2}}{1+\left(\frac{k_{re}}{k_{cat}}\right)P_{N2}} \quad \begin{cases} \left(\frac{k_{HP}}{k_{ac}}\right) \xrightarrow{hi\ flux} 0;\ P_{N2}\to 0 \\ \left(\frac{k_{cat}}{k_{ac}}\right) \xrightarrow{hi\ flux} 0;\ P_{N2}\to \infty \end{cases}$$

S4

and as a result, HP at E_2(2H) is quenched and **Scheme C** evolves into **Scheme D**. In the absence of N_2, of course E_4(2N2H) cannot form and its population vanishes. At high N_2 pressures, E_4(4H) itself gets depopulated as the system is 'pushed' to E_4(2N2H) by the second-order reaction with N_2/*re* of H_2,

$$\frac{E_4(4H)}{E_4(2N2H)} = \frac{1}{k_{re}P_{N2}} \xrightarrow{P_{N2}\to\infty} 0$$ **S5**

As a final comment, the formulation presented here, with populations and rate constants for **Schemes C, D** is designed for the description of high-flux experiments and is only valid in that regime because it assumes E_0 doesn't ever accumulate; such accumulation would occur at low flux, which thus would require populations to be calculated with **Scheme B**. *However*, the flux dependence of the ratio of rate constants, $r = k_{H2}/k_{N2}$, presented in **eq 6** is valid as the system falls out of the high-flux regime becauses it is the *ratio* of product-formation rate constants, and thus depends only on the relative populations of the reacting states, namely the two E_4 and E_2 in **Schemes B-D**.

H_2 oa and the Steady-State Kinetics

As described in **Fig 1**, the *re/oa* process is a dynamic equilibrium, with the forward *re* process driven by N_2 binding and the reverse reaction induced by H_2 binding.[1–6] The kinetic analysis in the main text ignores the *oa* reaction, because the turnover is not carried out under an atmosphere containing H_2. But such situations can arise, as for example in our turnover experiments under an atmosphere of N_2, C_2H_2, and added D_2, which showed that E_4(2H,2D) forms by *oa* (it reacts with C_2H_2 to form *cis*-$C_2H_2D_2$). Moreover, *some* H_2 is generated during the turnover experiments. Therefore, we here analyze the influence of H_2 *oa* on the kinetics, and in so doing show that it is proper to ignore this in treating the reported experiments.

The presence of H_2/D_2 and its *oa* reaction with E_4(2N2H) clearly would inhibit the net formation and reaction of E_4(2N2H). This is straightforwardly incorporated as an extension of the reactions of E_4(2N2H) (**Fig 2**), as shown in **Fig S1**. The E_4(2N2H) intermediate is formed by

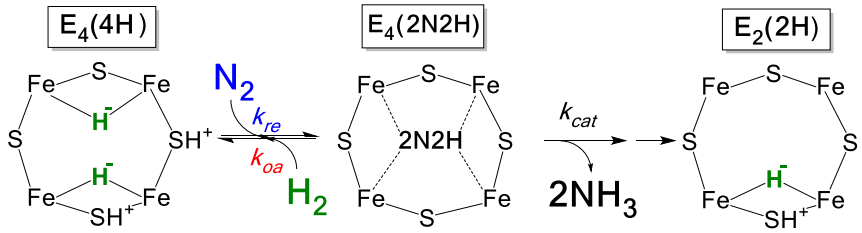
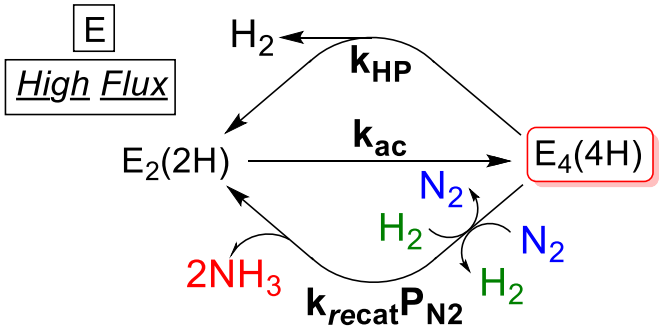


Figure S1: Extennsion of kinetic schemes of **Fig 2** to include the *re/oa* equilibrium where E_4(2N2H) is formed by H_2 *re* with N_2 binding, but this can be reversed by the H_2 *oa* process with N_2 release.

N_2 binding and lost through a competition between the second-order process of H_2 *oa* and first-order progress to NH_3 formation. This intermediate itself be may then be taken to be in steady-state, leading to the further simplified **Scheme E** in which E_4(4H) reacts with N_2 with the composite second-order steady-state rate constant, k_{recat}, and, upon directly returning to E_0, in turn is promptly reduced to E_2(2H). The kinetic cycle thus involves only E_4(4H) and E_2(2H), with k_{recat} given in **eq S1**, k_{HP} and k_{ac} defined as in the other Schemes. The *oa* reaction with H_2 is incorporporated into the



steady-state rate constant, k_{recat}, which is a function of the H_2 partial pressure.

$$k_{recat} = \frac{k_{cat}k_{re}}{k_{cat} + k_{oa}P_{H2}} = \frac{k_{re}}{1 + \frac{(k_{oa}P_{H2})}{k_{cat}}} \qquad \textbf{S6}$$

We recently measured[1] the equilibrium constant for the *re/oa* equilibrium for Mo-nitrogenase to be, $K_{re/oa} \sim 2$, implying that $k_{oa} \sim k_{re}/2 = 42$ $atm^{-1}sec^{-1}$ using the value for Mo-nitrogenase in **Table 1**. The experimental vessel used in turnover experiments has a headspace volume of ~ 8 mL, and the maximum amount of H_2 produced in a turnover experiment (at $P_{N_2} = 0$), ~ 1700 nmoles, corresponds to a partial pressure of $P_{H_2} \sim 5x10^{-3}$ atm. As a first-approximation, we may take the average pressure during H_2 accumulation in such an experiment to be half that, ~ $2.5x10^{-3}$ atm (a precise integration of the differential equations that arise from Scheme **E** is possible, but does not change the discussion here significantly). With these values, then during a turnover experiment, the *oa* of H_2 yields a maximum contribution to the denominator of **eq S1** of, $k_{oa} \cdot P_{H2} < 0.1$ sec^{-1}. As the analysis in the main text shows that, $k_{cat}\bar{k} \geq = 11$ sec^{-1}, the analysis here shows that the 'inhibition' of the forward *re* reaction caused by *oa* of H_2 formed during catalysis can cause *no more* than ~1% variation in the value of k_{re} for Mo-nitrogenase determined by ignoring *oa*. *Moreover*, as extensively discussed in the main text, H_2 formation is suppressed when reaction with N_2 is significant (**Fig 5**), so even this value is an over-estimate of the impact of *oa* for Mo-nitrogenase.

The maximum amount of H_2 produced during a Fe-nitrogenase titration is ~1/2 that for Mo-nitrogenase, but k_{re}, and thus k_{oa} are ~ 10-fold greater, resulting in an (overestimate) upper bound of < 5% decrease in the reported k_{re} for Fe-nitrogenase, well below the uncertainty in the measurement.

Thus, although the inclusion of H_2 *oa* would be essential for analysis of turnover with significant *added* pressures of H_2/D_2, this analysis validates the simplification introduced in Schemes **B-D** of ignoring inhibition of N_2 fixation by *oa* of H_2 formed during steady-state turnover.

Data for Figures 4-6 and Table 1

Table S1: Specific activities of P_{N_2} titration, equation used in SigmaPlot, and derived constants from **Figure 4**.

Mo-nitrogenase			
P_{N_2} (atm)	**Specific Activity - nmol NH_3/nmol protein/s**	**SigmaPlot Equation**	**Derived constants**
0.005	0.09	$y=(1/4)*((a*x)*(1+z*b*x))/(1+b*x)$ y = nmol NH_3/nmol protein/s a = k_{re} b = K_a z = k_{HP}/k_{ac} x = P_{N_2}	k_{re} ($s^{-1}Atm^{-1}$) = 83(5) $K_a = k_{re}/\bar{k}$ (Atm^{-1}) = 7.3(5)
0.01	0.13		
0.02	0.31		
0.04	0.54		
0.06	0.84		
0.08	1.00		
0.1	1.27		
0.2	1.77		
0.6	2.33		
1	2.42		
Fe-nitrogenase			
P_{N_2} (atm)	**Specific Activity - nmol NH_3/nmol protein/s**	**SigmaPlot Equation**	**Derived constants**
0.05	0.08	$y=(1/4)*((a*x)*(1+z*b*x))/(1+b*x)$ y = nmol NH_3/nmol protein/s a = k_{re} b = K_a z = k_{HP}/k_{ac} x = P_{N_2}	k_{re} ($s^{-1}Atm^{-1}$) = 8.5(5) $K_a = k_{re}/\bar{k}$ (Atm^{-1}) = 1.8(2)
0.1	0.19		
0.2	0.30		
0.3	0.40		
0.4	0.51		
0.6	0.65		
0.8	0.69		
1	0.75		

Table S2: Values for ratio of H_2 produced to N_2 reduced per P_{N_2}, equation used in SigmaPlot, and derived rate constants from **Figure 5**.

Mo-nitrogenase

P_{N_2} (atm)	Ratio H_2/N_2	SigmaPlot Equation	Derived constants
0.005	181.87	y=(1+(1/4)*(1+4*a)*b*x)/((1/4)*b*x)) y = ratio H_2/N_2 a = k_{HP}/k_{ac} b = ρ x = P_{N_2}	ρ = k_{re}/k_{HP} (Atm^{-1}) = 5.1(1)
0.01	127.99		
0.02	50.99		
0.04	26.92		
0.06	15.69		
0.08	12.74		
0.1	8.96		
0.2	4.92		
0.6	2.18		
1	1.81		

Fe-nitrogenase

P_{N_2} (atm)	Ratio H_2/N_2	SigmaPlot Equation	Derived constants
0.05	101.65	y=(1+(1/4)*(1+4*a)*b*x)/((1/4)*b*x) y = ratio H_2/N_2 a = k_{HP}/k_{ac} b = ρ x = P_{N_2}	ρ = k_{re}/k_{HP} (Atm^{-1}) = 0.77(5)
0.1	43.64		
0.2	25.39		
0.3	17.75		
0.4	12.30		
0.6	8.88		
0.8	8.05		
1	6.62		

Table S3: Values for ratio of H_2 produced to N_2 reduced per *M*Fe:Fe molar ratio, equation used in SigmaPlot, and derived values from **Figure 6**.

Mo-nitrogenase			
MoFe:Fe	**Ratio H_2/N_2**	**SigmaPlot Equation**	**Derived value**
0.05	1.81	y=(1+(1/4)*(1+4*(a*x))*b*c)/((1/4)*b*c) y = ratio H_2/N_2 a = scale factor b = ρ c = P_{N_2} x = *M*Fe:Fe ratio	*a* = 0.22
0.5	2.29		
1	2.44		
2	3.13		
4	4.96		
8	10.53		
16	17.22		
Fe-nitrogenase			
FeFe:Fe	**Ratio H_2/N_2**	**SigmaPlot Equation**	**Derived value**
0.0333	6.71	y=(1+(1/4)*(1+4*(a*x))*b*c)/((1/4)*b*c) y = ratio H_2/N_2 a = scale factor b = ρ c = P_{N_2} x = *M*Fe:Fe ratio	*a* = 0.19
0.1	6.80		
0.25	7.27		
0.5	7.31		
1	8.07		
4	13.19		

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