Supplementary Material for:

Kinetic and Product Distribution Analysis of NO- Reductase Activity in *N. europaea* Hydroxylamine Oxidoreductase

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S1. Determining the concentrations of all species in a reaction mixture: an example

The blue trace in Fig. S1 shows the UV/Vis spectrum of the solution used to obtain main text Figure 4, immediately prior to irradiation by the laser pulse. This solution contained $MV_{ox}$, $MV_{red}$, HAO, and the NO-generating species 1 in phosphate buffer. The $MV_{red}$ was generated by adding an appropriate amount of dithionite to the solution initially containing only $MV_{ox}$, HAO, and 1. The total solution volume was 150 µL. The mixture was prepared by combining aliquots of appropriate stock solutions with known concentrations, so the approximate concentration of each species in the final solution could be calculated directly. However, because the reagents were added in fairly small volumes, significant errors were associated with these concentration measurements. Better concentration measurements were obtained by calculating the best fit to the experimental trace using the matrix form of Beer’s law (red trace, Fig. S1). The path length for the cell in Fig. S1 was 3 mm, the extinction coefficient spectra of the 4 species were obtained independently as described in the main text, and the 4 concentrations were manually adjusted using Mathcad slider controls, until the sum of squares between the experimental and calculated spectra was minimized. All concentrations needed for this article were obtained using the method illustrated in Fig. S1, including those reported in Fig. 4.

S2. Dependence of NO· generation on the initial concentration of NO-generating species 1

As shown in Fig. 5 of the main text, when solutions containing the NO-generating species 1, $MV_{red}$, HAO and Mb are irradiated with a laser pulse, the NO· produced reacts quantitatively with Mb to give MbNO. This process is accompanied by a distinct spectral change, which allows the amount of NO· generated under a particular set of conditions to be quantified. In addition, the amount of $MV_{ox}$ produced by reaction of $MV_{red}$ with the denitrosated radical 2 can also be quantified by following the distinct spectral changes associated with MV oxidation.

The results seen in main text Fig 5 are not dependent on the presence of HAO; as an example, Fig. S2 shows the results of irradiating a solution containing approximately the same amounts of 1, $MV_{red}$ and Mb as main text Fig 5, but no HAO. It is clear from comparing Figs. 5 and S2 that the amount of NO· generated is not affected by the presence of HAO, and neither is the amount of MV oxidized on the sub-ms timescale. This allowed us to investigate, in the absence of HAO, how the concentrations of photogenerated NO· and $MV_{ox}$ vary as a function of [1] initially added to the solution. The results of this investigation are shown in Fig. S3. One can see that the concentrations of MbNO and $MV_{ox}$ initially increase with [1], reaching a maximum value at $[1] \sim 100$ µM, and then start to decrease. The ratio $[MbNO]/[MV_{ox}]$ remains
Figure S2. (a) Spectral change (blue) observed 90 s after a solution initially containing 22 µM 1 18 µM Mb and 53 µM MVred was irradiated with a 308 nm, 10 ns laser pulse. The theoretical trace (red dashed) was calculated from the known extinction coefficients of MVred, MVox, Mb and MbNO, as described in the experimental section. (b) [MVox] vs. time trace corresponding to the data shown in (a). Compare these traces with those of main text Fig. 5.

Figure S3. (a) Amounts of NO· and MVox photogenerated as a function of the [I] initially present; (b) The ratio [MbNO]/[MVox] as a function of [I]. For all experiments, [MVox] = 147 µM; [MVred]₀ = 60 µM; [Mb]₀ = 20 µM. The red line in plot (b) has a slope of -170±75 µM⁻¹, and a y-intercept of 0.864±0.008. Because the dependence of [MbNO]/[MVox] was so minor, and all experiments with HAO were generally carried out at [I] ≤ 100µM, the average ratio (0.85±0.02) was used to determine the initial concentrations of NO· from [MVox]₀.
virtually constant throughout the range investigated (Fig. S3b), perhaps decreasing very slightly. We attribute the observed attenuation in product yield to self-screening by I at high concentrations, a process that can be modeled using Eq. S1.

\[ y = a \cdot x \cdot \exp(-b \cdot x) \]  

**Eq. S1**

In this equation, the parameter \(a\) suggests that photoproduct should increase linearly with \([I]\) in the absence of self-screening, and the parameter \(b\) is the self-screening constant. In accordance with Beer’s law, the degree of self-screening depends exponentially on the concentration of the screening species \(x\). The theoretical fits in Fig. S3a (red traces) are to Eq. S1. The two plots were fitted simultaneously, subject to the constraint that the self-screening constant \(b\) was identical for both NO\(^{•}\) and MV\(_{\text{ox}}\) yields.

For this paper, the most important information obtained from the experiments of Fig. S3 was that the ratio \([\text{MbNO}]/[\text{MV}_{\text{ox}}]\) changed little with the experimental conditions. Similar experiments (see below) also showed that the amount of MV\(_{\text{ox}}\) generated was not affected by the presence or absence of Mb. Taken together, these two observations gave us confidence that the ratio \([\text{NO}^{•}]/[\text{MV}_{\text{ox}}]\) immediately after a laser pulse is the same with or without the presence of Mb. A separate investigation of the photochemistry and photophysics of species I, in the presence of several other low-potential reducing agents, is currently under way.

**S3. Preliminary analysis of the [MV\(_{\text{ox}}\)] vs. time traces obtained for NO\(^{•}\) reduction by MV\(_{\text{red}}\) in the absence of HAO**

Figure S4 shows a series of [MV\(_{\text{ox}}\)] vs time traces for solutions initially containing 34±3 µM MV\(_{\text{red}}\), varying concentrations of \(1\) and no added HAO. The data were fitted simultaneously to two exponentials as given in Eq. S2, subject to the constraint that the rate constants were equal under all four conditions. Clearly this model produces excellent fits, though at the moment we don’t know the chemical significance of this behavior. Figure S5a shows how the initial rate of MV\(_{\text{ox}}\) formation (\(V_{\text{MVox0}}\)) varies as a function of the \([\text{NO}^{•}]\) initially generated by the laser pulse. To correct for minor variations in \([\text{MV}_{\text{red}}]_0\) from one experiment to the next, \(V_{\text{MVox0}}/[\text{MV}_{\text{red}}]_0\) is used as the ordinate in Fig. S5a. The value of \(V_{\text{MVox0}}\) was calculated as \(\text{amp}_1 k_1 + \text{amp}_2 k_2\), which is the derivative of Eq. S2 at \(t=0\). Using the results presented in Fig. S3, the initial concentration of NO\(^{•}\) was calculated as 0.85[\(\text{MV}_{\text{ox}}\)]\(_0\), where \([\text{MV}_{\text{ox}}]_0\) is the concentration of MV\(_{\text{ox}}\) immediately after the laser pulse (\(y_0\) in Eq. S2).

\[ y = y_0 + \text{amp}_1 [1 - \exp(-k_1 t)] + \text{amp}_2 [1 - \exp(-k_2 t)] \]  

**Eq. S2**
Figure S5 shows how $V_{\text{MVox0}}$ varies as a function of $[\text{MV}_{\text{red}}]_0$ when $[\text{NO}^-]_0$ is held approximately constant. Analogously to Fig. 5a, $V_{\text{MVox0}}/[\text{NO}^-]_0$ is used as the ordinate to correct for minor variations in $[\text{NO}^-]_0$ from one experiment to the next. The plots of Figure S5 show that the uncatalyzed reaction of $\text{MV}_{\text{red}}$ with $\text{NO}^-$ is first order in both $[\text{NO}^-]$ and $[\text{MV}_{\text{red}}]$. From the slopes of the Fig. S5 lines one calculates an overall second-order rate constant $k_{\text{uncat}}$ of $(7.5\pm0.3)\times10^3$ M$^{-1}$s$^{-1}$. Interestingly, this value of $k_{\text{uncat}}$ is twice the value determined from Fig. 7 in the main text, and closer to the less precise value obtained from Fig. 6. At present we can’t explain this discrepancy, because we don’t fully understand the uncatalyzed reaction of $\text{MV}_{\text{red}}$ with $\text{NO}^-; this is an ongoing area of investigation in our laboratories. One possible contributing factor is that the overall profile of the uncatalyzed reaction varies with conditions. Thus, at low initial $\text{NO}^-$ concentrations two or more equiv. of $\text{MV}_{\text{red}}$ are oxidized per $\text{NO}^-$ consumed, whereas only one equiv. of $\text{MV}_{\text{red}}$ is oxidized per $\text{NO}^-$ consumed at higher initial $\text{NO}^-$ concentrations (Fig. S4). Note that in any case the reaction profile for the uncatalyzed oxidation of $\text{MV}_{\text{red}}$ by $\text{NO}^-$ is strikingly different from that of the catalyzed reaction, where five equiv. of $\text{MV}_{\text{red}}$ are oxidized per $\text{NO}^-$ consumed.

S4. Error analysis for the data of main text Figures 6-8

Figure S6 compares the main text’s Figure 6 plot, in which $V_{\text{MVox0}}$ is divided by $[\text{NO}^-]_0$, with a direct plot of $V_{\text{MVox0}}$ vs. $[\text{HAO}]$. For this set of experiments the systematic errors that would be incurred by not correcting for the small variations in $[\text{NO}^-]_0$ prove to be relatively minor, and the linear trend is in no way obscured in the uncorrected plot (Fig. S6a). It is not possible to correct for variations in $[\text{MV}_{\text{red}}]_0$ in the same way as for variations in $[\text{NO}^-]_0$ because $[\text{MV}_{\text{red}}]_0$ isn’t present in both terms of main text Eq. 2. However, one can estimate the systematic error propagated into $V_{\text{MVox0}}/[\text{NO}^-]$ by variations in $[\text{MV}_{\text{red}}]_0$ as follows. From main text Eq. 2 one obtains Eq. S3, in which the value of $k_{\text{uncat}}$ is known to be $(3.64\pm0.08)\times10^3$ M$^{-1}$s$^{-1}$ from the analysis of main text Fig. 7. Then, the error in $V_{\text{MVox0}}/[\text{NO}^-]$ is given by Eq. S4, where $\Delta[\text{MV}_{\text{red}}]_0$ is the difference.
between the average $[MV_{red}]_0$ for all the experiments of Fig. S6 and the individual $[MV_{red}]_0$ for a given experiment. The systematic errors are presented as half error bars in Fig. S6b, and are seen to be very small relative to the scatter in the data points.

Figure S7a shows the direct plot of $V_{MVox0}$ vs $[MV_{red}]_0$ that is obtained from the data that were used to produce the main text’s Fig. 7. In this case there is sufficient scatter in the data to effectively obscure any underlying pattern. Replacing $V_{MVox0}$ with $V_{MVox0}/[NO]$ as dependent variable decreases the scatter somewhat (Fig. S7b), but not enough to confidently assign a linear dependence to the plot. It is not possible to correct for variations in $[HAO]$ in the same way as for variations in $[NO]$ because $[HAO]$ isn’t present in both terms of main text Eq. 2. However, one can estimate the systematic error propagated into $V_{MVox0}/[NO]$ by variations in $[HAO]$ as follows. From main text Eq. 2 one obtains Eq. S5, where the value of $k_1$ is known to be $(4.7\pm0.3)\times10^5$ M$^{-1}$s$^{-1}$ from the analysis of main text Fig. 8. Analysis of main text Fig. 8

$$\frac{\partial (\frac{V_{MVox0}}{[NO]_0})}{\partial [HAO]} = 3k_1 + k_{app}[NO]_0$$  \hspace{1cm} \text{Eq. S5}$$

also shows that the $k_{app}[NO]_0 \ll 3k_1$, so that the second term in Eq. S5 can be neglected. Then, the error in $V_{MVox0}/[NO]$ is given by:

$$\Delta \frac{V_{MVox0}}{[NO]} = 3k_1 \Delta [HAO]$$  \hspace{1cm} \text{Eq. S6}$$

where $\Delta [HAO]$ is the difference between the average $[HAO]$ for all the experiments of Fig. S7 and the individual $[HAO]$ for a given experiment. The systematic errors are presented as half error bars in Fig. S7b, and are seen to be substantial relative to the variation between the data


Figure S7. (a) Dependence of $V_{\text{MVox0}}$ on $[\text{MVred}]_0$ at roughly fixed $[\text{HAO}]$ and $[\text{NO}^-]$. (b) Same as (a), except that $V_{\text{MVox0}}/[\text{NO}^-]$ is plotted against $[\text{MVred}]_0$ to correct for the small variations in $[\text{NO}^-]_0$ between data points. The half-error bars show the systematic errors due to the known variations in $[\text{HAO}]$ between data points. (c) Same as (b), except that the values of $V_{\text{MVox0}}/[\text{NO}^-]$ have been corrected for the variations in $[\text{HAO}]$, using the relationship of Eq. S6. The red line here is identical to the dotted line in main text Fig. 7, and the data points track the ends of the half error bars in (b).

In Figure S7c the individual values of $V_{\text{MVox0}}/[\text{NO}^-]$ have been corrected for variations in $[\text{HAO}]$ between experiments, and the plot shows substantially less scatter than those of Fig. S7a or S7b. The same thing is shown in more compact form by the dashed line in main text Fig. 7.

Figure S8a shows the direct plot of $V_{\text{MVox0}}$ vs $[\text{NO}^-]_0$ that is obtained from the data that were used to produce the main text’s Fig. 8. The data of Fig. S8a show a definite curvature, that is more closely modeled by the equation $y=ax+bx^2$ (purple dashed curve) than by a straight line (red).

However, this apparent relationship must be viewed with caution because in the data of Fig S8a $[\text{MVred}]_0$ was very definitely not constant between experiments, and furthermore its value tended to decrease with increasing $[\text{NO}^-]_0$. This is due to the fact that the amount of $[\text{NO}^-]$ generated in a given experiment increases in lockstep with the amount of $\text{MVred}$ oxidized by the radical 2, (main text Scheme 1 and Fig. S3). Thus, if the same amount of $\text{MVred}$ were present prior to the laser pulse in each experiment, a progressively greater fraction of this would be oxidized by 2 as more $\text{NO}^-$ was generated by the laser pulse. This could be corrected for by adjusting the pre-flash concentration of $[\text{MVred}]$ from one experiment to the next, but such a process proved cumbersome and difficult to carry out experimentally. Instead, the pre-flash $[\text{MVred}]$ was maintained roughly constant, and the ordinate data were corrected for systematic variations in $[\text{MVred}]_0$ (post-laser pulse concentrations) as shown in main text Fig. 8 and Fig. S8b. Curvature is much less evident in Fig. S8b than it is in Fig. S8a, but the trend may be hidden by the added scatter associated with extra data manipulations. Thus, we do not rule out the existence of a second-order dependence of the initial rate on $[\text{NO}^-]_0$ (as shown in main text Eq. 2). But, as seen in the Results section of the main text, such a dependence, if really present, is small.

The reader will have inferred, correctly, that the procedure described in this section requires the use of iteration. The following routine was
used. The slope of Fig. S6b was first used to estimate the value of $k_1$, neglecting any contribution from the term $k_2$. This value of $k_1$ was then used in Eqs. S5 and S6 to estimate the magnitudes of the error bars in Fig. S7b, which in turn allowed us to generate and fit the linear plot of Fig. S7c. The value of $k_{\text{uncat}}$ was estimated from the slope of the line in Fig. S7c, and used to correct the data of Fig. S8, which tracks the dependence of $V_{\text{MVox0}}$ on NO$^-$ concentration. Finally, a new value of $k_1$ and associated value of $k_2$ were obtained from the parabolic fit of Fig. S8b. At this point the whole loop could be repeated as many times as necessary to obtain self-consistency. However, in practice, only the first loop proved necessary; new estimates from a second loop were not significantly different from those of the first.

**S5. Some comments about the [MV$_{\text{ox}}$] vs. time traces obtained for NO$^-$ and NH$_2$OH reduction by MV$_{\text{red}}$, in the presence of HAO**

Main text Fig. 4b was obtained under conditions of high HAO and initial MV$_{\text{red}}$ concentrations, and low NO$^-$ concentrations. Under such conditions one might expect [MV$_{\text{ox}}$] vs. time traces to be well approximated by 2-exponential fits (the first exponential would be for reduction of NO$^-$ to NH$_2$OH, and the second for reduction of NH$_2$OH to NH$_4^+$). This is because consumption of all the NO$^-$ would lead to little change in [MV$_{\text{red}}$] (“pseudo-first order conditions”), while the high concentration of HAO would ensure that the catalyzed reaction proceeded much faster than the uncatalyzed one.

Many of the [MV$_{\text{ox}}$] vs. time traces that led to main text Figs. 6-8 were obtained under conditions in which the uncatalyzed reaction would contribute to some extent, and/or pseudo first order conditions were not maintained as NO$^-$ was consumed. Nevertheless, it was usually possible to fit complete [MV$_{\text{ox}}$] vs. time traces reasonably well using two or three exponentials. Such empirical fits were best for the later part of their respective experimental curves, and proved very useful for obtaining estimates of the final MV$_{\text{ox}}$ concentrations. Better estimates of the initial rates were obtained by fitting the first 5 seconds of each data set to the exponential function $y = y_0 + \text{amp}[1-exp(-kt)]$, and then calculating the initial rate as the product $\text{amp} \cdot k$.

Multiple efforts were made to fit all of our complete [MV$_{\text{ox}}$] vs. time traces, using systems of differential equations that took into account both the HAO-catalyzed and the uncatalyzed NO$^-$. 

![Figure S8](image-url)
reactions with MV_red. Unfortunately, though individual traces could often be successfully fitted, we were not able to identify a model that would globally fit all of our data sets using numerically identical parameters. Our failure to obtain a global model stems from our incomplete understanding of the uncatalyzed reaction of MV_red with NO.; as mentioned in Section S3, we are continuing to investigate this reaction in our laboratories.

All of the [MV_{ox}] vs. time traces that were used to generate main text Fig. 11a were obtained with [MV_{red}]_0 >> [NH_2OH]_0. These are pseudo-first order conditions, in that consumption of all available NH_2OH leads to minimal change in [MV_{red}]. Consequently, it is not surprising that the single exponential function \( y = y_0 + \text{amp}[1 - \exp(-kt)] \) provided excellent fits for all such traces (e.g.: Fig. 10). For many of the [MV_{ox}] vs. time traces that were used to generate main text Fig. 11b, the initial concentrations of MV_{ox} and NH_2OH were comparable, and exponential functions did not give good fits to the data. Under such conditions, a variety of empirical functions were used to obtain the best possible fits to the early parts of the respective [MV_{ox}] vs. time traces, and then the initial rates were obtained from the values of the functions’ derivatives at t=0. Importantly, for conditions in which [NH_2OH]_0 >> [MV_{red}]_0, the plots of [MV_{ox}] vs. time were linear, demonstrating that the reaction has zero-order dependence on [MV_{red}].

S6. Detection of ammonia after HAO-catalyzed reduction of hydroxylamine

A solution initially containing 50 µM NH_2OH, 0.25 µM HAO and 80 µM MV_red was allowed to react anaerobically in the glovebox until no further change was observed in the UV/Vis spectrum. The reaction mixture was then removed from the glovebox and mixed with a pipette to oxidize the remaining MV_red. Next, a commercially available ammonia assay was used to determine the ammonia concentration in solution (Sigma ammonia assay reagent, A0853-1VL; Sigma L-glutamate dehydrogenase for ammonia assay, G2626-100MG). This assay uses glutamate dehydrogenase to catalyze the conversion of α-ketoglutaric acid, ammonia and NADPH to L-glutamate and NADP^+. The amount of ammonia initially present is determined from the decrease in absorbance at 340 nm, due to oxidation of NADPH. Solutions with known ammonia concentrations were analyzed first, and used to prepare a standard A_{340} vs [NH_4^+] plot (open circles, Fig. S9). As seen in Fig. S9, this plot is linear in the range 0 - 800 µM. The reaction mixture was then analyzed in the same way as the standards, and A_{340} was found to be 0.99 after the reaction was complete, corresponding to an ammonia concentration of 75 µM (solid orange square, Fig. S9). This value is higher than the expected value of 50 µM, but reasonable given the

Figure S9. Assay for ammonia content in a solution (see accompanying text for details). The data points shown as circles are for standards of known ammonia concentrations, while the orange square shows the ammonia concentration generated when hydroxylamine and MV_red are allowed to react in the presence of HAO.
scatter in the standard curve. The experiment independently confirms that the product of HAO-catalyzed NH$_2$OH reduction by MV$_{red}$ produces NH$_4$\(^+\).