Inflated Kinetic Isotope Effects in the Branched Mechanism of *Neurospora crassa* 2-Nitropropane Dioxygenase

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Derivation of $(k_{cat}/K_m)_{nox}$. The initial rate equation for the branched mechanism of Scheme 2 that applies when the reaction is followed by monitoring nitronate release during enzymatic turnover with nitroethane is given by:

$$\frac{v_o}{e} = [ES^-]k_{13} \quad (1)$$

Where ES^- is the enzyme-ethylnitronate complex.

The $[ES^-]$ can be converted into kinetic rate constants by applying the method of King and Altman(*1*) to express the initial rate equation as:

$$\frac{v_o}{e} = \frac{AO_2k_1k_3k_7k_9k_{11}k_{13} + Ak_1k_3k_6k_9k_{11}k_{13}}{A(k_1k_3k_5k_9k_{11}\ldots + k_1k_6k_9k_{11}k_{13}) + O_2(k_2k_4k_7k_9k_{11}\ldots + k_3k_7k_9k_{11}k_{13}) + AO_2(k_1k_3k_5k_7k_9\ldots + k_1k_7k_9k_{11}k_{13}) + k_2k_4k_6k_9k_{11}\ldots + k_3k_6k_9k_{11}k_{13}} \quad (2)$$

Where A = [Nitroethane].

Since the bimolecular oxidation of the flavosemiquinone becomes significantly faster than its formation (O_2 $k_7 >> k_5$) at high concentrations of oxygen, as was the case in the experiments reported here (*2*), the net flux of the enzyme-nitronate intermediate

through the flavin reduction step becomes practically irreversible. The expression for $(k_{cat}/K_m)_{nox}$ after canceling common terms in both the numerator and denominator is therefore given by:

$$\left(\frac{k_{cat}}{K_m}\right)_{nox} = \frac{k_1 k_3 k_{13}}{k_3 k_5 + k_2 k_5 + k_2 k_4 + k_2 k_{13} + k_3 k_{13}} \tag{3}$$

Based on the lack of solvent viscosity effects previously measured for $(k_{cat}/K_m)_{ox}$ (*2*) $k_2 >> k_3$. Grouping these terms in the denominator and canceling k_3 gives:

$$\left(\frac{k_{cat}}{K_m}\right)_{nox} = \frac{k_1 k_3 k_{13}}{k_2 k_5 + k_2 k_{13} + k_2 k_4} \tag{4}$$

Or as described in the text:

$$\left(\frac{k_{cat}}{K_{NE}}\right)_{nox} = k_{13}\left(\frac{k_1 k_3}{k_2\left(k_4 + k_5 + k_{13}\right)}\right) \tag{5}$$

Derivation of $(k_{cat}/K_m)_{ox}$. The initial rate equation for the branched mechanism of Scheme 2 that applies when the reaction is followed by monitoring oxygen consumption during enzymatic turnover with nitroethane is given by:

$$\frac{v_o}{\mathrm{e}} = [\mathrm{E}P]k_{11} \tag{6}$$

Where EP is the enzyme-peroxynitroethane complex.

The [EP] can be converted into kinetic rate constants by applying the method of King and Altman (*1*) to express the initial rate equation as:

$$\frac{v_o}{e} = \frac{AO_2 k_1 k_3 k_5 k_7 k_9 k_{11}}{\begin{array}{l} A(k_1 k_3 k_5 k_9 k_{11} \ldots + k_1 k_6 k_9 k_{11} k_{13}) + O_2(k_2 k_4 k_7 k_9 k_{11} \ldots + k_3 k_7 k_9 k_{11} k_{13}) \\ + AO_2(k_1 k_3 k_5 k_7 k_9 \ldots + k_1 k_7 k_9 k_{11} k_{13}) + k_2 k_4 k_6 k_9 k_{11} \ldots + k_3 k_6 k_9 k_{11} k_{13} \end{array}} \tag{7}$$

Where A = [Nitroethane].

The expression for $(k_{cat}/K_m)_{ox}$ after canceling common terms in both the numerator and denominator is therefore given by:

$$\left(\frac{k_{cat}}{K_m}\right)_{ox} = \frac{k_1 k_3 k_5}{k_2 k_4 + k_2 k_5 + k_2 k_{13} + k_3 k_5 + k_3 k_{13}} \tag{8}$$

Based on the lack of solvent viscosity effects previously measured for $(k_{cat}/K_m)_{ox}$ (*2*) $k_2 >> k_3$. Grouping these terms in the denominator and canceling k_3 gives:

$$\left(\frac{k_{cat}}{K_m}\right)_{ox} = \frac{k_1 k_3 k_5}{k_2 k_4 + k_2 k_5 + k_2 k_{13}} \tag{9}$$

Or as described in the text:

$$\left(\frac{k_{cat}}{K_{NE}}\right)_{ox} = k_5\left(\frac{k_1 k_3}{k_2\left(k_4 + k_5 + k_{13}\right)}\right) \tag{10}$$

Derivation of $^{D}(k_{cat}/K_m)_{ox}$. In the mechanism of Scheme 2, the kinetic steps k_3, k_4 and k_{13} are isotope sensitive. Taking the ratio of $(k_{cat}/K_m)_{ox}$ with nitroethane and [1,1-$^2H_2$]nitroethane therefore gives:

$$^{D}\left(\frac{k_{cat}}{K_m}\right)_{ox} = \frac{k_1 k_3 k_5}{k_2 k_4 + k_2 k_5 + k_2 k_{13}} \times \frac{k_2 k_{4D} + k_2 k_5 + k_2 k_{13D}}{k_1 k_{3D} k_5} = \frac{k_3 k_{4D} + k_3 k_5 + k_3 k_{13D}}{k_{3D} k_4 + k_{3D} k_5 + k_{3D} k_{13}} \tag{11}$$

Or:

$$^{D}\left(\frac{k_{cat}}{K_m}\right)_{ox} = {}^{D}k_3\left(\frac{k_{4D} + k_5 + k_{13D}}{k_4 + k_5 + k_{13}}\right) \tag{12}$$

Where the subscript D denotes the rate of the kinetic step with [1,1-$^2H_2$]nitroethane as substrate.

Dividing each term in the parenthesis by k_4 gives:

$$^{D}\left(\frac{k_{cat}}{K_m}\right)_{ox} = {}^{D}k_3\left(\frac{\frac{k_{4D}}{k_4}+\frac{k_5}{k_4}+\frac{k_{13D}}{k_4}}{1+\frac{k_5}{k_4}+\frac{k_{13}}{k_4}}\right) \tag{13}$$

Or:

$$^{D}\left(\frac{k_{cat}}{K_m}\right)_{ox} = \frac{{}^{D}K_{eq3}+{}^{D}k_3\left(\frac{k_5}{k_4}\right)+{}^{D}k_3\left(\frac{k_{13D}}{k_4}\right)}{1+\frac{k_5}{k_4}+\frac{k_{13}}{k_4}} \tag{14}$$

Where $^{D}K_{eq3} = \frac{^{D}k_3}{^{D}k_4}$

Dividing each term by $\frac{k_5}{k_4}$ gives:

$$^{D}\left(\frac{k_{cat}}{K_m}\right)_{ox} = \frac{{}^{D}K_{eq3}\left(\frac{k_4}{k_5}\right)+{}^{D}k_3+k_{13D}\left(\frac{^{D}k_3}{k_5}\right)}{\frac{k_4}{k_5}+1+\frac{k_{13}}{k_5}} \tag{15}$$

Since $^{D}k_{13} = \left(\frac{k_{13}}{k_{13D}}\right)$, $^{D}k_{13}$ can be written as $\frac{k_{13}}{^{D}k_{13}}$. Inserting this expression into (15) gives:

$$^{D}\left(\frac{k_{cat}}{K_m}\right)_{ox} = \frac{{}^{D}K_{eq3}\left(\frac{k_4}{k_5}\right)+{}^{D}k_3+\frac{^{D}k_3}{^{D}k_{13}}\left(\frac{k_{13}}{k_5}\right)}{\frac{k_4}{k_5}+1+\frac{k_{13}}{k_5}} \tag{16}$$

Defining P as $\frac{k_{13}}{k_5}$ and C_r as $\frac{k_4}{k_5}$ gives eq 14 of the main text:

$$^{D}\left(\frac{k_{cat}}{K_m}\right)_{ox} = \frac{^{D}k_3 + {}^{D}k_3\left(\frac{1}{^{D}k_{13}}P\right) + {}^{D}K_{eq3}C_r}{1 + C_r + P} \quad (17)$$

Derivation of $^{D}(k_{cat}/K_m)_{nox}$. In the mechanism of Scheme 2, the kinetic steps k_3, k_4 and k_{13} are isotope sensitive. Taking the ratio of $(k_{cat}/K_m)_{nox}$ with nitroethane and [1,1-$^2H_2$]nitroethane therefore gives eq 18:

$$^{D}\left(\frac{k_{cat}}{K_m}\right)_{nox} = \frac{k_1k_3k_{13}}{k_2k_5 + k_2k_{13} + k_2k_4} \times \frac{k_2k_5 + k_2k_{13D} + k_2k_{4D}}{k_1k_{3D}k_{13D}} = \frac{k_3k_{13}k_5 + k_3k_{13}k_{13D} + k_3k_{13}k_{4D}}{k_5k_{3D}k_{13D} + k_{13}k_{3D}k_{13D} + k_4k_{3D}k_{13D}}$$

Or:

$$^{D}\left(\frac{k_{cat}}{K_m}\right)_{ox} = {}^{D}k_3\,{}^{D}k_{13}\left(\frac{k_{4D} + k_5 + k_{13D}}{k_4 + k_5 + k_{13}}\right) \quad (19)$$

Since this expression is eq 13 multiplied by $^{D}k_{13}$, the expression for $(k_{cat}/K_m)_{nox}$ is the that for $(k_{cat}/K_m)_{ox}$ multiplied by $^{D}k_{13}$ as shown in the main text:

$$^{D}\left(\frac{k_{cat}}{K_m}\right)_{nox} = {}^{D}k_{13}\left[\frac{^{D}k_3 + {}^{D}k_3\left(\frac{1}{^{D}k_{13}}P\right) + {}^{D}K_{eq3}C_r}{1 + C_r + P}\right] \quad (20)$$

References

1. King, E. L., and Altman, C. (1956) A Schematic Method of Deriving the Rate Laws for Enzyme-Catalyzed Reactions. *J. Phys. Chem. 60*, 1375-1378.
2. Francis, K., and Gadda, G. (2006) Probing the chemical steps of nitroalkane oxidation catalyzed by 2-nitropropane dioxygenase with solvent viscosity, pH, and substrate kinetic isotope effects. *Biochemistry 45*, 13889-13898.