Structural and Kinetic Isotope Effect Studies of Nicotinamidase (Pnc1) from *S. cerevisiae*

Brian C. Smith, Mark A. Anderson, Kelly A. Hoadley, James L. Keck, W. Wallace Cleland, and John M. Denu

SUPPORTING INFORMATION

Contents:

Supporting Discussion

Derivation explaining observed kinetic isotope effects for Pnc1 D51N. For Pnc1 D51N, both the ^{15}N and ^{13}C KIE are ~0.5% compared to ~1.25% for those of Pnc1 WT (Table 3). We believe this is due to an increase in one of the back reaction off rates. A truncated derivation demonstrating how this is possible is shown below. The entire reaction is described by equation S1.

$$\mathrm{E+A} \underset{k_2}{\overset{k_1}{\rightleftarrows}} \mathrm{ES} \underset{k_4}{\overset{k_3}{\rightleftarrows}} \mathrm{ES^*} \xrightarrow{k_5} \mathrm{EX} \underset{k_8}{\overset{k_7}{\rightleftarrows}} \mathrm{EQ^*} \underset{k_{10}}{\overset{k_9}{\rightleftarrows}} \mathrm{EQ} \xrightarrow{k_{11}} \mathrm{E+Q} \qquad \text{(S1)}$$

The KIE are on *V/K* which includes all steps up through and including the first irreversible step where k_1 is substrate binding, k_2 is substrate dissociation, k_3 is the formation of the tetrahedral intermediate, k_4 is the breakdown of the tetrahedral intermediate to reform nicotinamide, and k_5 is the isotope sensitive irreversible step (C–N bond cleavage and the loss of NH_3). *V/K* is expressed by:

$$\frac{V}{K\mathrm{E_t}} = \frac{\dfrac{k_1 k_3 k_5}{k_2 k_4}}{1 + \dfrac{k_5}{k_4} + \dfrac{k_5 k_3}{k_4 k_2}} \qquad \text{(S2)}$$

However when the kinetic data are examined we see the k_{cat} and k_{cat}/K_m values have decreased compared to the wild-type enzyme. This is due to a decrease in binding of nicotinamide in the D51N mutant which leads to an increase in k_2 and a decrease in k_3. As such k_3/k_2 would tend to be very small and can be ignored. Then using the ^{15}N KIE notation the equation becomes:

$$^{15}\left(\frac{V}{K}\right) = \frac{^{15}k_5 + \dfrac{k_5}{k_4}}{1 + \dfrac{k_5}{k_4}} \qquad \text{(S3)}$$

where $^{15}\left(\dfrac{V}{K}\right)$ is the measured KIE and $^{15}k_5$ is the intrinsic KIE. $^{15}\left(\dfrac{V}{K}\right)$ is defined as $\dfrac{^{15}k_5 + \mathrm{C_f} + {}^{15}\mathrm{K_{eq}}}{1 + \mathrm{C_f} + \mathrm{C_r}}$ where C_f and C_r are forward and reverse commitments to catalysis and $^{15}K_{eq}$ is the ^{15}N

equilibrium isotope effect. In this reaction there is no reverse commitment since the C-N bond cleavage is irreversible and thus C_r and $^{15}K_{eq}$ can be disregarded and:

$$^{15}\left(\frac{V}{K}\right)=\frac{^{15}k_5+C_f}{1+C_f} \tag{S4}$$

For this example, an intrinsic isotope effect of 3% will be assumed and then for wild type and D51N we have:

$$1.0122\pm0.0002=\left(\frac{1.0300+C_{fwt}}{1+C_{fwt}}\right),\ \text{and}\ C_{fwt}=1.50\pm0.04 \tag{S5}$$

$$1.0045\pm0.0006=\left(\frac{1.0300+C_{fD51N}}{1+C_{fD51N}}\right),\ \text{and}\ C_{fD51N}=5.50\pm0.82 \tag{S6}$$

Equation 12 shows that $C_f = \frac{k_5}{k_4}$ and so the C_f values are $\left(\frac{k_5}{k_4}\right)_{WT}$ ~ 1.5 and $\left(\frac{k_5}{k_4}\right)_{Mut}$ ~ 5.5

These values show that the reaction partitions forward faster in the D51N mutant, resulting in a smaller KIE. The mechanism of this partitioning is unknown but indicates that k_5 (loss of NH_3) has increased or that breakdown of the tetrahedral intermediate (k_4) has decreased.

Table S1. Primers used in Pnc1 mutagenesis.

Pnc1 mutant	Primers
D8A	5′-CGAGATGAAGACTTTAATTGTTGTTGCTATGCAAAATGATTTTATTTCACC-3′ 5′-GGTGAAATAAAATCATTTTGCATAGCAACAACAATTAAAGTCTTCATCTCG-3′
D8N	5′-CGAGATGAAGACTTTAATTGTTGTTAATATGCAAAATGATTTTATTTCACC-3′ 5′-GGTGAAATAAAATCATTTTGCATATTAACAACAATTAAAGTCTTCATCTCG-3′
D8E	5′-CGAGATGAAGACTTTAATTGTTGTTGAGATGCAAAATGATTTTATTTCACC-3′ 5′-GGTGAAATAAAATCATTTTGCATCTCAACAACAATTAAAGTCTTCATCTCG-3′
D51A	5′-GTGGTCACCAGAGCTTGGCACCCTTCC-3′ 5′-GGAAGGGTGCCAAGCTCTGGTGACCAC-3′
D51N	5′-GTGGTCACCAGAAATTGGCACCCTTCC-3′ 5′-GGAAGGGTGCCAATTTCTGGTGACCAC-3′
H53A	5′-GGTCACCAGAGATTGGGCCCCTTCCAGAC-3′ 5′-GTCTGGAAGGGGCCCAATCTCTGGTGACC-3′
H94A	5′-TGTGGCCCGTAGCCTGTGTGAAAAACACC-3′ 5′-GGTGTTTTTCACACAGGCTACGGGCCACA-3′
K122A	5′-GATTGTCGACGCGGGTTTCTTGACTGACC-3′ 5′-GGTCAGTCAAGAAACCCGCGTCGACAATC-3′
K122R	5′-GATTGTCGACAGGGGTTTCTTGACTGACC-3′ 5′-GGTCAGTCAAGAAACCCCTGTCGACAATC-3′
C167A	5′-AGCTTTGGAGTATGCTGTCAAAGCCACCG-3′ 5′-CGGTGGCTTTGACAGCATACTCCAAAGCT-3′

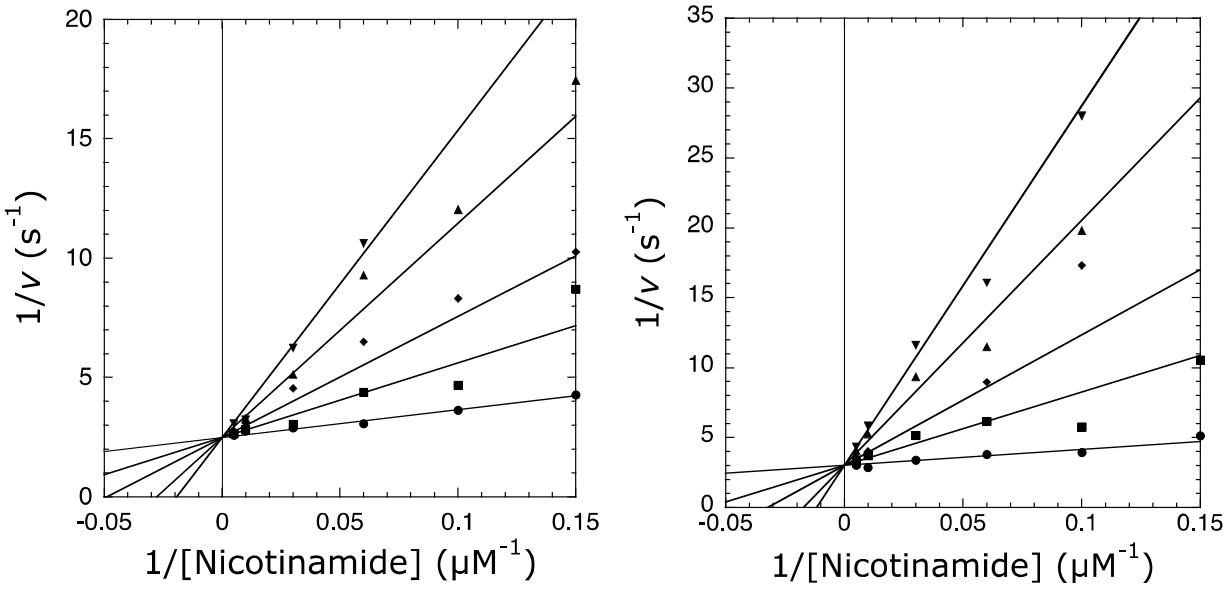

Figure S1. Double-reciprocal inhibition plots. Initial rates were determined as described under Experimental Procedures. (left) Nicotinic acid exhibits competitive inhibition toward nicotinamide during the Pnc1-catalyzed reaction. The following nicotinic acid concentrations were used: 0 (●), 200 (■), 400 (◆), 800 (⬧), and 1200 μM (⧫). (right) Nicotinaldehyde exhibits competitive inhibition toward nicotinamide during the Pnc1-catalyzed reaction. The following nicotinaldehyde concentrations were used: 0 (●), 1 (■), 2 (◆), 4 (⬧), and 6 μM (⧫). Data were fit to competitive inhibition models using KinetAsyst as described in Experimental Procedures. All reactions were performed at pH 7.5.

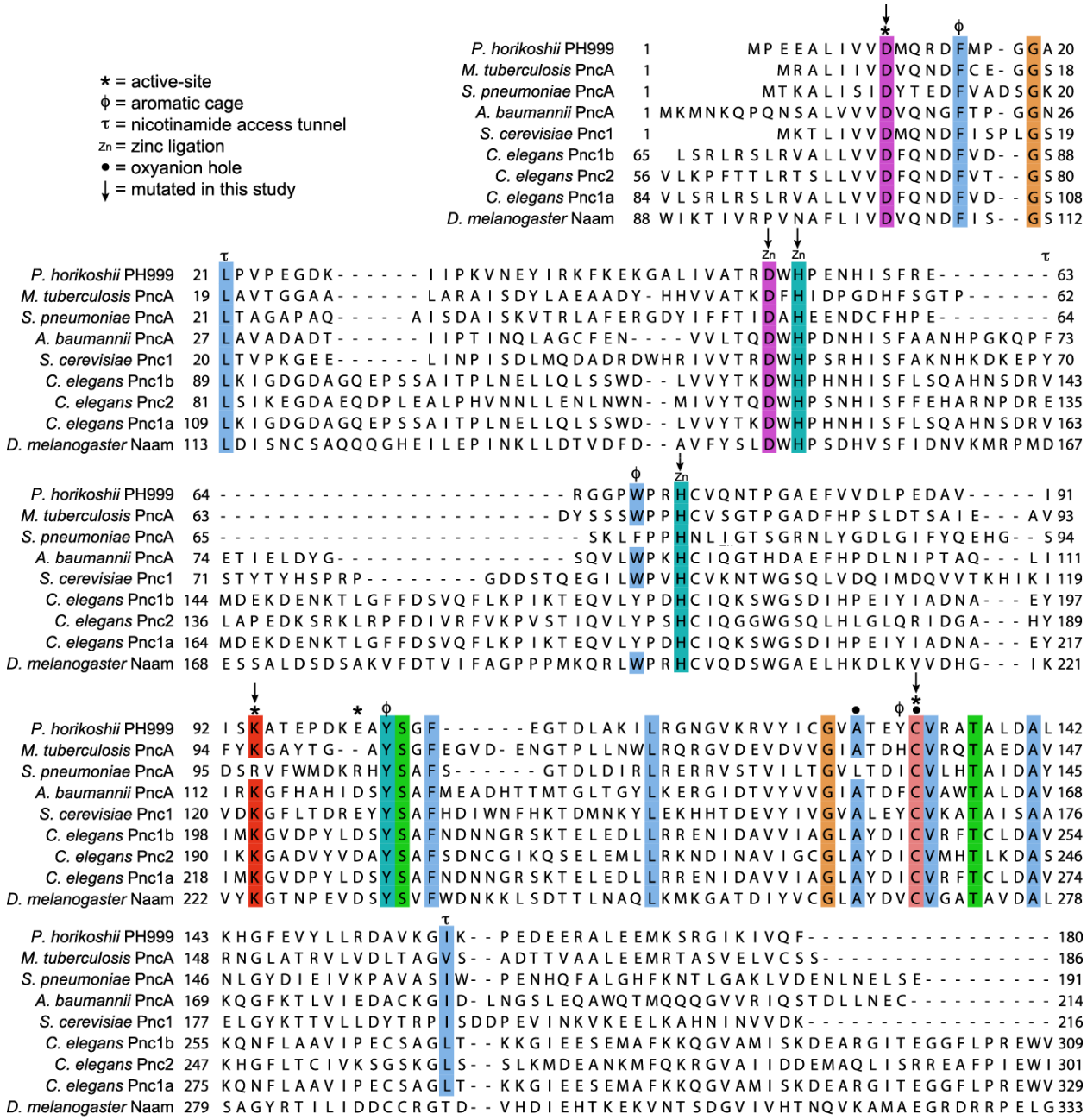

Figure S2. Multiple-sequence alignment of the nicotinamidases for which structures have been solved from *Pyrococcus horikoshii* (PDB entries 1ILW and 1IM5), *Mycobacterium tuberculosis* (PDB entry 3PL1), *Streptococcus pneumoniae* (PDB entries 3O90, 3O91, 3O92, 3O93, and 3O94), *Acinetobacter baumannii* (PDB entries 2WT9 and 2WTA), and *Saccharomyces cerevisiae* (PDB entry 2H0R and this work). Eukaryotic nicotinamidases from *Caenorhabditis elegans* and *Drosophila melanogaster* are also shown for comparison. The alignment was generated using ClustalW (*59*).