SUPPORTING INFORMATION

for

Characterization of Nicotinamidases: Steady-State Kinetic Parameters, Class-wide Inhibition by Nicotinaldehydes and Catalytic Mechanism

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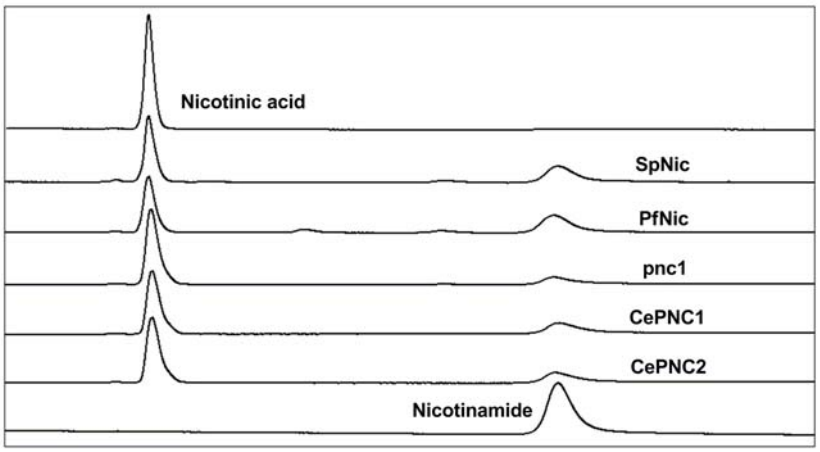
Plasmid Construction and Protein Expression. Standard molecular biology techniques were used to clone the genes of interest. Briefly, the nicotinamidase gene for *Streptococcus pneumoniae* was amplified by PCR from genomic DNA (ATCC 6314D) using primers (5'–CAT ATG ATG ACA AAG GCT TTA ATT TCG ATT–3' and 5'–GGA TCC TTA CTC AAA AAG TTC ATT TAG ATT TTC ATC–3') engineered to contain the NdeI and BamHI restriction endonuclease recognition sites. The PCR product was inserted into the pSTBlue vector (Novagen) using the AccepTor vector kit (Novagen). The gene was digested with NdeI and BamHI and ligated into similarly digested Pet28a; the presence of the gene was verified by sequencing. The protein obtained was called SpNic. The vector of Pet28a containing wild type SpNic was used to act as the template in the site-directed mutagenesis for constructing mutants of R97A, K103A, C136A, C136S following the manual of QuickChange® Site-Directed Mutagenesis Kit (Agilent Technologies, Genomics). The sequences of mutants were verified by sequencing. The protein obtained was called R97A, K103A, C136A, and C136S respectively. The nicotinamidase gene from *Borrelia burgdorferi* was cloned from a construct pBBE22 provided by Steven J. Norris, Ph.D. of University of Texas Health Science Center at Houston. As previously reported (*S1*) in order to produce a functional nicotinamidase an additional 48 base pairs upstream of the annotated start codon were required. PCR was used to amplify the extended gene using primers engineered to include NdeI and BamHI cut sites (5'–CAT ATG ATG CCA GTA TCT AAC AGT AAT GAA ATA ATT TCT TTG ATT–3' and 5'–GGA TCC TTA TAT ATT AAG CTT ACT TTG GCT GTC GAA GAT GTC C–3'). The PCR product was inserted into the pSTBlue vector (Novagen) using the AccepTor vector kit (Novagen). The gene was

digested with NdeI and BamHI and ligated into similarly digested Pet28a; the presence of the gene was verified by sequencing. The protein obtained was called BbNic. The nicotinamidase gene for *Plasmodium falciparum* was amplified from cDNA provided by Kirk Deitsch of Weill Medical College using primers engineered to included the EcoRI and HindIII restriction endonuclease sites (5'−AGA ATT CAT GAA ATG CCT TGT TAT AGT TGA TGC ACA A–3' and 5'– GCA AGC TTA TGA CAA AAG TTT TGA TGA GTT AAT AAA TTT GAT TCC–3'). The PCR product was inserted into the pSTBlue vector (Novagen) using the AccepTor vector kit (Novagen). The gene was digested with NdeI and BamHI and ligated into similarly digested Pet28a; the presence of the gene was verified by sequencing. The protein obtained was called PfNic. In all cases the constructs were transformed into Codon+ cells (Stratagene) and grown with shaking at 37^{o}C to an O.D. of 0.6 before inducing with 0.5 mM IPTG. After 6 h of growth post-induction at 37^{o}C the cells were harvested and lysed using lysozyme followed by three successive freeze-thaw cycles. After treatment with DNAse, the cell debris was pelleted and the supernatant was added to pre-equilibrated Ni-NTA nickel resin (Qiagen). After successive washes with 5 mM and 10 mM imidazole in 10 mM Tris, the protein was eluted from the nickel resin with 250 mM imidazole in 10 mM Tris. The elutions were immediately tested for activity and purity of the samples was assessed by SDS-polyacrylamide gel electrophoresis. Active fractions with purity greater than 90% were pooled and aliquots were flash frozen in 20% glycerol and 2 mM DTT. *C. elegans* PNC1 and PNC2 (CePNC1 and CePNC2) were expressed and purified as described (*S2*) and the expression plasmid of *S. cerevisceae* Pnc1 was received as a gift from Dr. Jeffrey

Smith of the University of Virginia and was expressed and purified as described previously (*S3*).

To construct mutants of nicotinamidase from *S. pneumoniae*, the following primers were used: R97A, 5’ CAAGAGCATGGTTCAGACAGTGCGGTCTTTTGG ATGGATAAACGC 3’ (forward), 5’ GCGTTTATCCATCCAAAAGACCG CACTGTCTGAACCATGCTCTTG 3’ (reverse); K103A, 5’ GTCGTGTCTT TTGGATGGATGCGCGCCATTACTCAGCTTTTTC 3’ (forward), 5’ GAAAAAGCTG AGTAATGGCGCGCATCCATCCAAAAGACACGAC 3’ (reverse); C136A, 5’ CAGGTGTCTTGACGGATATCGCGGTCCTACATACAGCTATAGATG 3’ (forward), 5’ CATCTATAGCTGTATGTAGGACCGCGATATCCGTCAAGACACC TG 3’ (reverse); C136S, 5’ CAGGTGTCTTGACGGATATCAGTGTCCTACAT ACAGCTATAG 3’ (forward), 5’ CTATAGCTGTATGTAGGACACTGATATC CGTCAAGACACCTG 3’ (reverse). The constructs with mutations were made following the instructions of QuickChange® Site-Directed Mutagenesis Kit (Agilent Technologies, Genomics), and the sequence of each mutant was shown as correct after sequencing. The approach for expressing each mutant was the same as the wild type of nicotinamidase of *S. pneumoniae*.

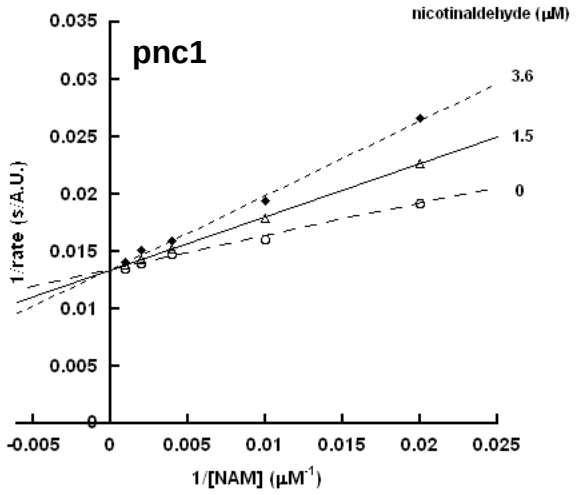
Figure S1.



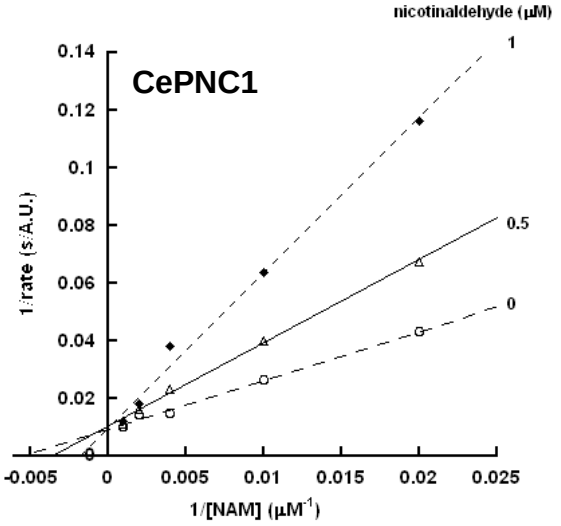
HPLC chromatograms showing the conversion of nicotinamide to nicotinic acid by nicotinamidases. A typical reaction was run on a 50 μL scale containing 200 μM of nicotinamide in 100 mM phosphate buffer at pH 7.3. The reaction was initiated by addition of nicotinamidase enzyme and incubated at 25 ^{o}C to allow approximately 50% nicotinamide conversion. The reaction was quenched by addition of 6 μL of 10% trifluoroacetic acid to a final pH 3 and then allowed to incubate on ice for 30 min. Prior to injection on HPLC, the sample was centrifuged at 13,000 g for 2 min to remove precipitates. Authentic chemical standards of nicotinamide and nicotinic acid were injected to determine retention times for substrate and product.

Figure S2

A



B



C

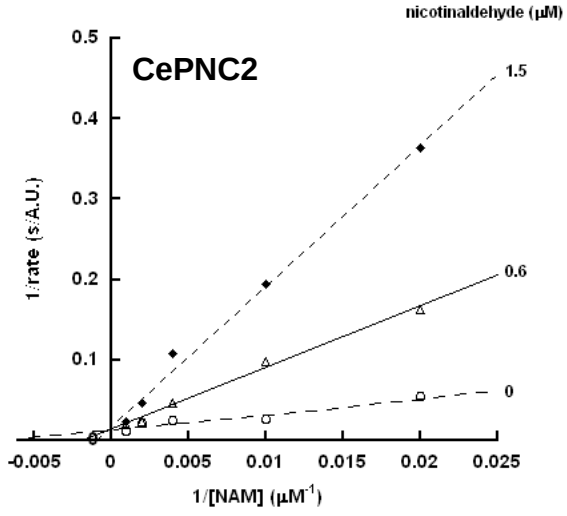


Figure S2. The Lineweaver-Burke plots for competive inhibition by nicotinaldehyde of Pnc1 (A), CePNC1 (B) and CePNC2 (C). The GDH coupled assay described in the main text (Materials and Methods) was used for the competitive analysis. Inhibition reactions were performed in 150 μL volumes containing 1 mM α-ketoglutarate, 250 μM NADPH, 2.25 units of GDH, and different concentrations of nicotinamide (50, 100, 250, 500 and 1000 μM) in 100 mM phosphate buffer, pH 7.3, with inhibitor concentrations from 0 to 2× or 5× estimated K_i as indicated in Figure S2. Reactions were initiated by addition of nicotinamidase enzymes (final concentrations: Pnc1, 200 nM; CePNC1, 100 nM; CePNC2, 133 nM). Double reciprocal plots of 1/*v* versus 1/[Nicotinamide] at different fixed concentrations of the inhibitor were plotted, the intersection of the lines at 1/*v* axis indicated competitive inhibition. All data points were fitted to double reciprocal plots using Kaleidagraph®. A.U.: Arbitrary Units.

Figure S3

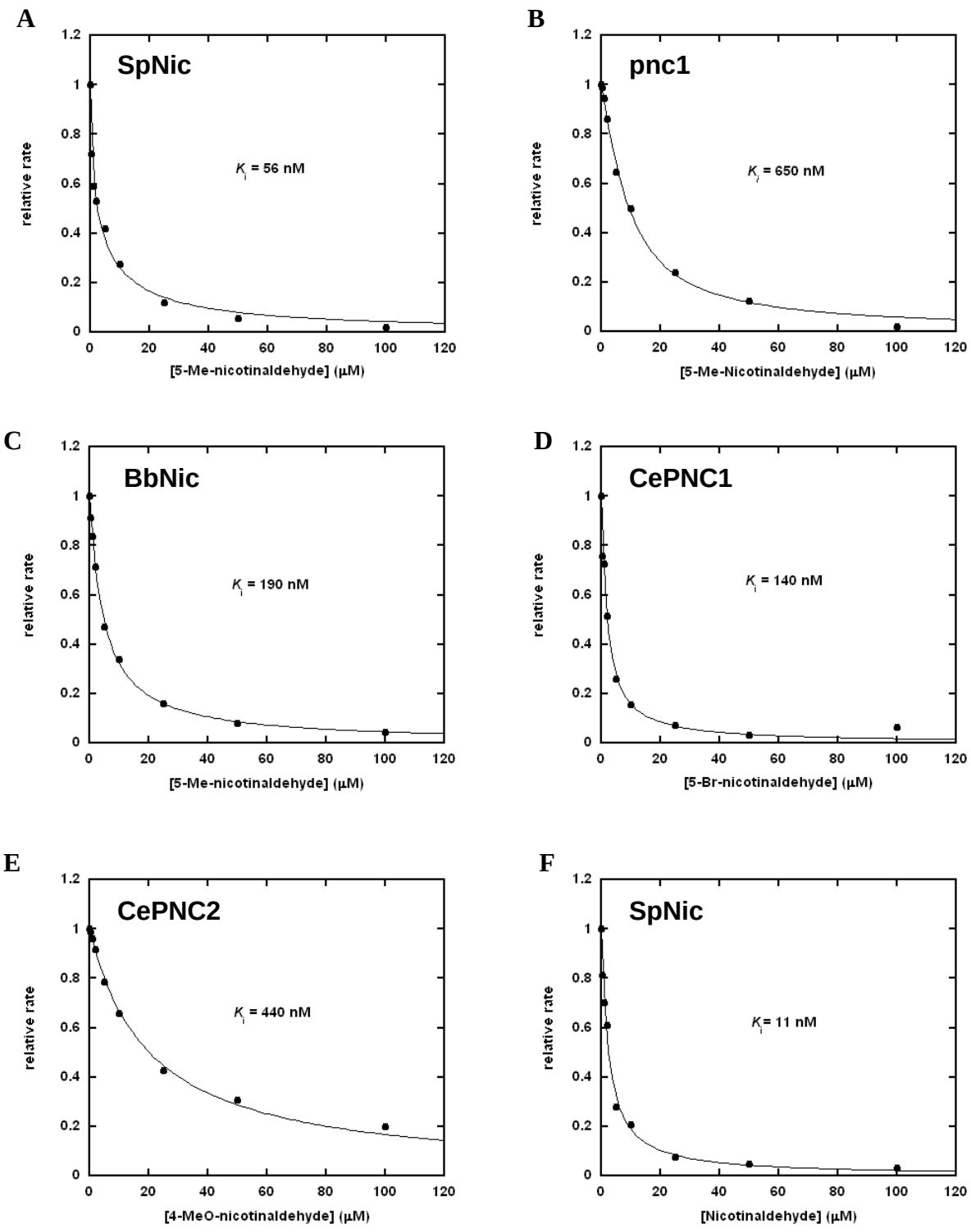
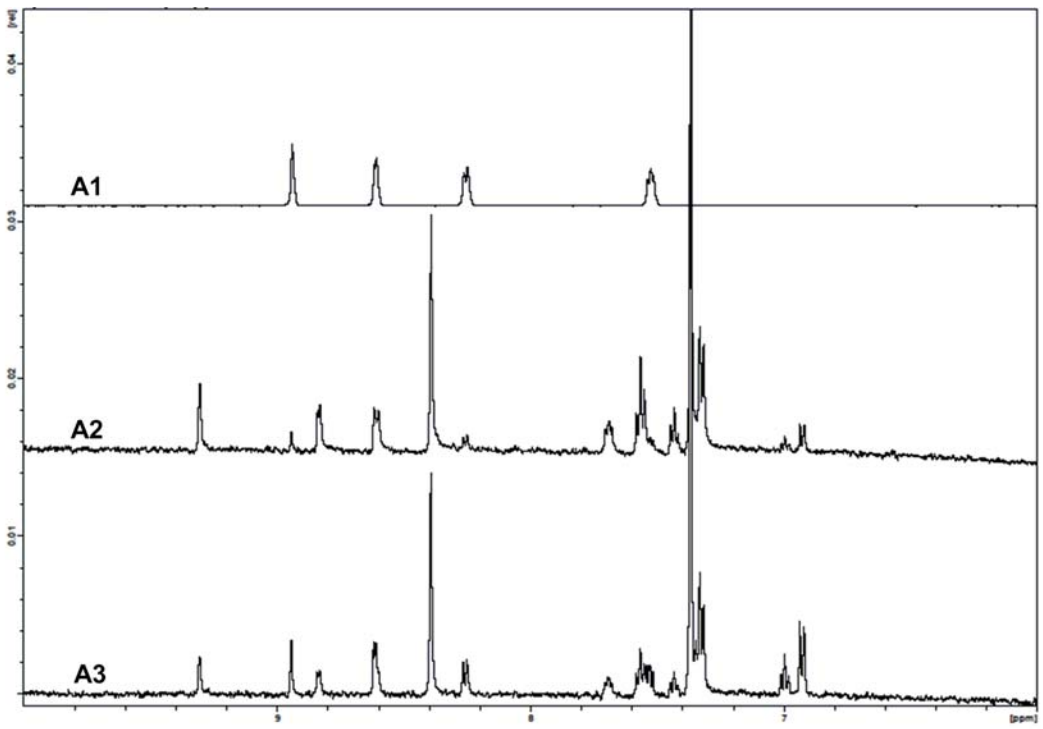

A
SpNic
K_i = 56 nM
relative rate
[5-Me-nicotinaldehyde] (μM)
B
pnc1
K_i = 650 nM
relative rate
[5-Me-Nicotinaldehyde] (μM)
C
BbNic
K_i = 190 nM
relative rate
[5-Me-nicotinaldehyde] (μM)
D
CePNC1
K_i = 140 nM
relative rate
[5-Br-nicotinaldehyde] (μM)
E
CePNC2
K_i = 440 nM
relative rate
[4-MeO-nicotinaldehyde] (μM)
F
SpNic
K_i = 11 nM
relative rate
[Nicotinaldehyde] (μM)

Figure S3. Curves showing the inhibition of the nicotinamidase enzymes by 5-*O*-methylnicotinaldehyde (A, SpNic), 5-methylnicotinaldehyde (B, Pnc1; C, BbNic), 5-bromonicotinaldehyde (D, CePNC1), 4-*O*-methylnicotinaldehyde (E, CePNC2) and nicotinaldehyde (F, SpNic). The reactions were run in the presence of either 200 μM nicotinamide (for SpNic and Pnc1)or 1 mM nicotinamide (for PfNic, BbNic and CePNC1) or 2 mM nicotinamide (for CePNC2) and various concentrations of the inhibitor in 1 mM α-ketoglutarate, 250 μM NADH and 1.5 units of GDH per 100 μL of reaction volume in 100 mM phosphate buffer, pH 7.3. After initiation by addition of the enzyme (final concentrations: SpNic, 12 nM; PfNic, 13.5 nM; Pnc1, 210 nM; BbNic, 140 nM; CePNC1, 10 nM; CePNC2, 133 nM), the reactions were monitored by fluorescence and initial rates of reaction were calculated. The curves show relative rate with respect to inhibitor concentration. K_i values are reported in Table 4 in the main text. Morrisons equation ,Equation 2, and Equation 3 were used for fitting plotted data and for obtaining the K_i value as described in Materials and Methods.

Figure S4

A



B

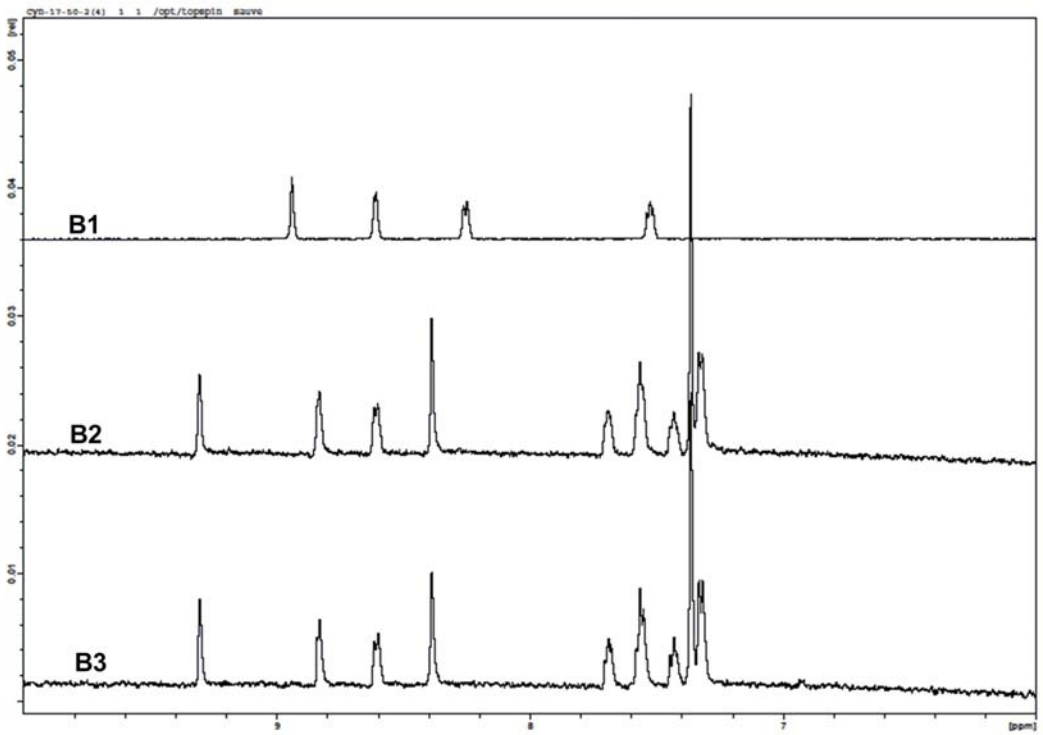


Figure S4. NMR spectra showing phenyl nicotinate is a substrate for PfNic.

A) the reaction was run on a 600 μL scale containing 1.54 μM of PfNic in 100 mM phosphate buffer at pD 7.5. The reaction was incubated at 25ºC for 5 min and initiated by addition of phenyl nicotinate (final concentration 500 μM). A1 is nicotinic acid in 100 mM phosphate buffer pD 7.5; A2 is the enzymatic reaction after 1 h incubation; A3 is the enzymatic reaction after 6 h incubation. After 6 h incubation, the majority of the phenyl nicotinate has been converted to nicotinic acid.

B) the reaction was run on a 600 μL scale containing 10 μM of nicotinaldehyde and 1.54 μM of PfNic in 100 mM phosphate buffer at pD 7.5. The reaction was incubated at 25ºC for 5 min and initiated with the addition of phenyl nicotinate (final concentration 500 μM). B1 is nicotinic acid in 100 mM phosphate buffer pD 7.5; B2 is the enzymatic reaction after 1 h incubation; B3 is the enzymatic reaction after 6 h incubation. 10 μM of nicotinaldehyde was able to inhibit the enzymatic activity of PfNic, so after 6 h incubation, phenyl nicotinate was intact.

Figure S5

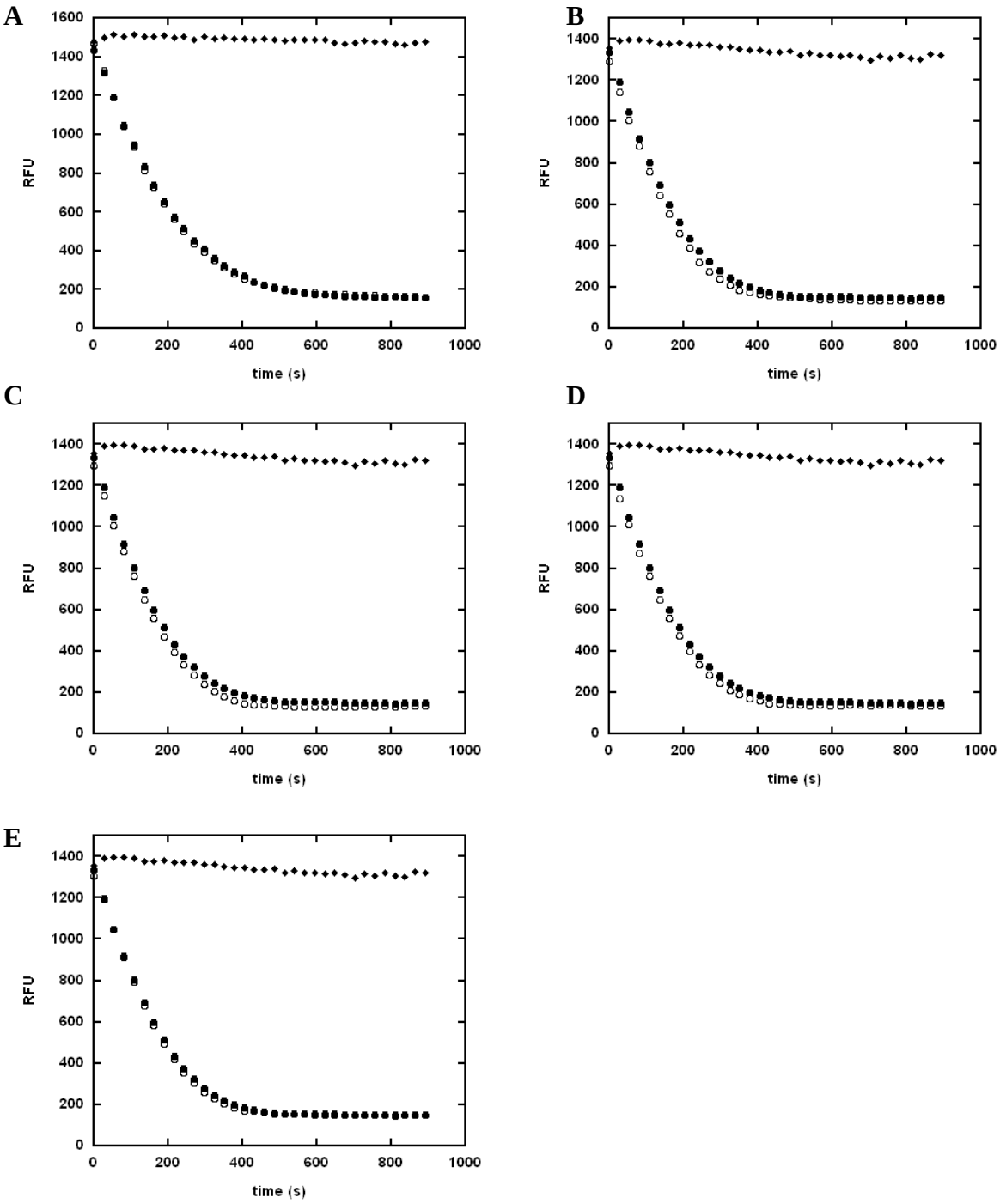


Figure S5. Curves showing that glutamate dehydrogenase (GDH) is not inhibited by nicotinaldehyde and derivatives. The reactions were run in the presence of 1 mM α-

ketoglutarate, 250 μM NADH and 1.5 units of GDH per 100 μL of reaction volume in 100 mM phosphate buffer, pH 7.3. With (open circle) and without (closed circle) 100 μM nicotinaldehyde or derivatives. Reactions were initiated with the addition of 100 μM NH_4OAc. Negative control (diamond) containing 100 μM nicotinaldehyde or derivatives was initiated with the addition of phosphate buffer. The reactions were monitored by fluorescence of NADPH as described in the text. A. Nicotinaldehyde; B. 5-Bromo-nicotinaldehyde; C. 5-*O*-methyl-nicotinaldehyde; D. 5-methyl-nicotinaldehyde; E. 4-*O*-methyl-nicotinaldehyde.

Table S1. Relative Rates of Nicotinamidases in the Presence of Metal Ions

Sample	BbNic	PfNic	Pnc1	SpNic	CePNC1	CePNC2
untreated	1.00	1.00	1.00	1.00	1.00	1.00
Zn^{2+}	0.96	1.14	1.04	1.09	1.21	0.58
Fe^{2+}	0.93	0.95	1.01	1.15	1.02	1.08
Mn^{2+}	0.93	0.95	1.22	2.68	1.21	2.37
Fe^{3+}	0.91	0.97	1.05	0.94	0.96	0.96

To determine the effect of added metal ions to nicotinamidase activity, reactions were run in the presence of 1 mM nicotinamide, 1 mM α-ketoglutarate, 250 μM NADH and 1.5 units of GDH per 100 μL of reaction volume in 100 mM phosphate buffer, pH 7.3. After addition of 500 μM of $FeSO_4$, $MnCl_2$, or $FeCl_3$, the reactions were initiated with the addition of nicotinamidases (final concentrations: BbNic, 140 nM; PfNic, 135 nM; Pnc1, 200 nM; SpNic, 84 nM; CePNC1, 10 nM; CePNC2, 267 nM), and were monitored by fluorescence and initial rates of reactions calculated compared to controls not containing added metal ion. Control experiments were run in parallel to ensure that GDH was not affected by metal ions.

For $Zn(OAc)_2$, which affects GDH activity at 500 μM, reactions were analyzed by HPLC. Reactions were run with 1 mM nicotinamide, with/without 500 μM of $Zn(OAc)_2$ in 100 mM potassium phosphate, pH 7.3. Reactions were initiated with nicotinamidases (final concentrations: BbNic, 1.7 μM; PfNic, 1.35 μM; Pnc1, 200 nM; SpNic, 335 nM; CePNC1, 100 nM; CePNC2, 2.7 μM), and incubated at 37 °C for 5 min. Reactions were quenched with the addition of 8 μL of 10% TFA. Samples were centrifuged at 13, 000 g for 2 min to remove precipitates and reaction mixtures analyzed by HPLC.

Chromatograms were analyzed at wavelength of 260 nm. Reactions were quantified by integrating areas of peaks corresponding to nicotinamide and nicotinic acid and compared to controls with no metal ion present.

Reference

S1. Pruser, J. E., Lawrenz, M. B., Caimano, M. J., Howell, J. K., Radolf, J. D., and Norris, S. J. (2003) *Mol. Microbiol. 48*, 753-764.

S2. Vrablik, T. L., Huang, L., Lange, S. E., and Hanna-Rose, W. (2009) Nicotinamidase modulation of NAD^+ biosynthesis and nicotinamide levels separately affect reproductive development and cell survival in C. elegans. *Development 136*, 3637-46.

S3. Gallo, C. M., Smith, D. L., Jr., and Smith, J. S. (2004) Nicotinamide clearance by Pnc1 directly regulates Sir2-mediated silencing and longevity. *Mol. Cell. Biol. 24*, 1301-12.