

Significance of Ser-188 in human mitochondrial NAD kinase as determined by phosphomimetic and phosphoresistant amino-acid substitutions

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Abbreviations: NADK, NAD kinase; CD, Circular dichroism.

ABSTRACT

Human mitochondrial NAD kinase is a crucial enzyme responsible for the synthesis of mitochondrial NADP⁺. Despite its significance, little is known about the regulation of this enzyme in the mitochondria. Several putative and known phosphorylation sites within the protein have been found using phosphoproteomics, and here, we examined the effect of phosphomimetic mutations at six of these sites. The enzymatic activity was downregulated by a substitution of an Asp residue at Ser-289 and Ser-376, but not a substitution of Ala, suggesting that the phosphorylation of these residues downregulates the enzyme. Moreover, the activity was completely inhibited by substituting Ser-188 with an Asp, Glu, or in particular Ala, which highlights two possibilities: first, that Ser-188 is critical for catalytic activity, and second, that phosphorylation of Ser-188 inhibits the activity. Ser-188, Ser-289, and Ser-376 were found to be highly conserved in the primary structures of mitochondrial NAD kinase homologs in higher animals. Moreover, Ser-188 has been frequently detected in human and mouse phosphorylation site studies, whereas Ser-289 and Ser-376 have not. Taken together, this indicates that Ser-188 (and perhaps the other residues) is an important phosphorylation site that can downregulate the NAD kinase activity of this critical enzyme.

Key words: NAD kinase, mitochondria, phosphomimetic mutation, phosphorylation, phosphoproteomics.

1. Introduction

NADP (NADP⁺ and its reduced form NADPH) is involved in anabolic reactions, as well as in the defense against oxidative stress. It also functions as a substrate in the synthesis of NAADP, a potent intracellular calcium messenger [1,2]. NAD kinase (NADK; EC 2.7.1.23), the sole NADP⁺-biosynthetic enzyme known to catalyze the phosphorylation of NAD⁺ into NADP⁺ using ATP, plays a vital role in the cell. There are two types of NADK in human cells: cytosolic NADK (cytNADK) [3,4,5,6] and mitochondrial NADK (mitNADK) [7,8,9], with mitNADK having a much lower K_m (for NAD⁺, 22 μ M) and activity (V_{max} , 0.091 U/mg) than cytNADK (K_m for NAD⁺, 1.07 mM; V_{max} , 18.5 U/mg) [7]. A homozygous nonsense mutation in the gene encoding the human mitNADK causes dienoyl-CoA reductase deficiency with hyperlysinemia and a failure to thrive [9], emphasizing the critical role of mitNADK. Despite the importance of both NADKs, little is known about how they are regulated *in vivo*.

Phosphorylation is a widespread mechanism for controlling the function of enzymes. Recently, Love *et al.* reported that the N-terminal portion, in particular Ser-64, of human cytNADK is phosphorylated in a Ca²⁺/CaM-dependent manner [4]. However, recombinant purified cytNADK mutants with a phosphomimetic (S64D) or a phosphoresistant (S64A) amino-acid substitution at Ser-64 have similar levels of enzymatic activity [4]. Moreover, *in vitro* phosphorylation of wild-type and S64A cytNADK does not change catalytic activity [4]. Therefore, the physiological role of

human cytNADK phosphorylation remains to be clarified.

Mitochondria are the major sites of reactive oxygen species (ROS) generation, and mitochondrial ROS levels are tightly regulated, because they have critical roles in the adaptation to hypoxia, autophagy, immunity, differentiation, and probably aging [10]. Because NADPH also participates in the regulation of mitochondrial ROS levels [10], the function of mitNADK is most likely tightly regulated in mitochondria in response to intracellular and extracellular signals. Phosphoproteomics has revealed several phosphorylation sites in mitNADK from humans [11,12,13], mice [14,15,16], and rats [17,18], which are summarized in PhosphoSitePlus (Q4G0N4, <http://www.phosphosite.org>) (Fig. 1, Table 1).

To elucidate the role of human mitNADK phosphorylation, we introduced several phosphomimetic amino-acid substitutions into human mitNADK and measured the NADK activity of the purified enzymes. We expected that a few of the phosphomimetic mutations would increase activity, because the activity of mitNADK is very low relative to that of cytNADK [7]. However, we found that two phosphomimetic mutations at Ser-289 and Ser-376 downregulated activity and one at Ser-188 abolished its activity altogether, implying that these phosphorylation events actually downregulate this enzyme in mitochondria.

2. Materials and methods

2.1. Plasmid and expression

Phosphomimetic and phosphoresistant mitNADKs were constructed with PCR using pMK3272 as a template. pMK3272 is the expression plasmid pQE-80L (Qiagen, Venlo, Netherlands) carrying a human mitNADK gene [7]. This gene encodes $\Delta 62$ mitNADK, which lacks the 62 N-terminal amino acids including the mitochondrial targeting sequence [7] (Fig. 1). The expression of wild-type, phosphomimetic (substitution of Ser to Asp or Glu), and phosphoresistant (substitution of Ser to Ala) mitNADK was conducted as described [7]. $\Delta 62$ mitNADK is expressed from pMK3272 as an N-terminal His-Tag (1 MRGSHHHHHHGS 12)–fusion protein. Purification of the expressed protein was performed using a TALON Metal Affinity Resin column (Clontech, Otsu, Japan) [7]. Washing was done with 10 mM Tris-HCl (pH 8.0), 300 mM NaCl, and 30 mM neutralized imidazole, and elution was done with 10 mM Tris-HCl (pH 8.0), 300 mM NaCl, and 150 mM neutralized imidazole. The fractions containing the purified enzymes were pooled and stored at 4°C.

2.2. Assay

NADK activity was assayed at 37°C by the continuous method and the stop method, as described previously [7]. Briefly, NADPH formation was continuously measured at A_{340} in a reaction mixture (1.0 mL) containing 5.0 mM NAD⁺, 5.0 mM ATP, 5.0 mM glucose 6-phosphate, 0.5 U glucose 6-phosphate dehydrogenase (Sigma, St. Louis, MD), 5 mM MgCl₂, 100 mM Tris-HCl (pH 8.0), and an appropriate

concentration of purified protein. In the stop method, glucose 6-phosphate dehydrogenase was diluted with 50% glycerol to reduce background. One unit of enzyme activity was defined as 1.0 $\mu\text{mol NADP}^+$ produced in 1 min at 37°C; specific activity was expressed in U/mg protein, unless otherwise stated. The protein concentration of the purified $\Delta 62\text{mitNADK}$ and its mutants was determined using A_{280} and a molecular coefficient that was calculated using the ExPASy ProtParam tool [19]. The molecular mass of $\Delta 62\text{mitNADK}$ (plus the N-terminal His-Tag) is 44,726.4, and the molecular coefficient is 50,880 $\text{M}^{-1} \text{cm}^{-1}$ ($\Delta 62\text{mitNADK}$ of $A_{280} = 1.138$ corresponds to 1 mg/ml).

2.3. Circular dichroism (CD)

The purified mitNADK (wild type), phosphomimetic mitNADK (S188D), and phosphoresistant mitNADK (S188A) were dialyzed against 10 mM Tris-HCl (pH 8.0) containing 300 mM NaCl. This dialysis external solution was prepared with ultra-pure water. CD of the purified enzyme was analyzed with a CD spectrometer (J-720 Spectropolarimeter, Jasco, Tokyo, Japan). The control was 10 mM Tris-HCl (pH 8.0) containing 300 mM NaCl. Conditions were as follows: protein concentrations, 0.1–0.2 mg/ml; quartz cell length, 0.01 cm; scan range, 190–260 nm; resolution, 0.1 nm; band length, 1 nm; scan speed, 10 nm/min; cumulative number, 3 times; and sample volume, 65 μL .

3. Results and discussion

3.1. Activity of phosphomimetic mitNADK proteins

Phosphoproteomics has revealed several phosphorylation sites within the mitNADK of humans [11,12,13], mice [14,15,16], and rats [17,18], which are summarized in PhosphoSitePlus (Q4G0N4, <http://www.phosphosite.org>) (Fig. 1, Table 1). Among the sites, we focused on Ser and Thr residues, especially on the residues that had been identified as phosphorylation sites in human mitNADK: Thr-183, Ser-188, Ser-345, and Ser-367 [11,12,13]. The detected count for Ser-188 was 207, whereas the counts for Ser-183, Ser-345, and Ser-367 were 6, 1, and 2 in the mass spectrometric analysis conducted by Bian *et al.* [11], implying that Ser-188 is relatively frequently phosphorylated.

We constructed expression plasmids for the human phosphomimetic mitNADK mutants T813D, S188D, S289D, S294D, S345D, T357D, S363D, S367D, and S376D. These mitNADK mutants were expressed in *E. coli* and purified (Fig. S1). The phosphomimetic mitNADK S188D, S289D, S345D, S363D, S367D, and S376D were successfully purified. Phosphomimetic mitNADK S183D failed to express, and two other mutants, T357D and S294D, were difficult to express in large quantity and to purify.

We expected that some phosphomimetic mutations would increase activity, because the activity of mitNADK is low relative to that of cytNADK [7]. However, the

activity of mitNADK S289D and S376D was lower than that of wild type, and the activity of mitNADK S188D was not detected, although mitNADK S363D, S345D, and S367D had the same activity as wild-type mitNADK (Fig. 2A). mitNADK S289E and S188E were also purified (Fig. S1). Again, the activity of mitNADK S289E was lower than that of wild type, and the activity of mitNADK S188E was not detected (Fig. 2A).

To rule out the possibility that Ser-289, Ser-376, and Ser-188 had a significant role in the catalytic activity itself, not in the regulation of the enzyme, we also constructed and purified phosphoresistant mitNADK S188A, S289A, and S376A (Fig. S2). The activity of mitNADK S376A was similar to that of wild type, whereas the activity of mitNADK S289A was 2-fold higher than that of the wild type (Fig. 2B), indicating that the phosphorylation of Ser-376 and Ser-289 reduces activity. The activity of mitNADK S188A was again not detected (Fig. 2B). The purified enzymes (wild-type mitNADK, mitNADK S188D, and mitNADK D188A) had approximately the same CD spectrum (Fig. S3), indicating that the substitutions of Asp or Ala into Ser-188 caused no noticeable structural change. These data highlight two possibilities: first, Ser-188 is critical for catalytic activity, and second, phosphorylation of Ser-188 completely inhibits this activity.

3.2. Conservation of the investigated Ser residues in the primary structure

NADKs can be roughly divided into two groups: human mitNADK homologs and human cytNADK homologs. Moreover, mitNADK homologs have sequence

similarity with plant NADK3 homologs and can be further divided into animal and protist mitNADK homologs [7]. The three key motifs (GGDG, NE/D, and NADK conserved motifs) are conserved in the three groups (mitNADK homologs, plant NADK3 homologs, and cytNADK homologs), and two motifs (motif 1/2 and the C5orf33 additional region) are not found in cytNADK homologs (Fig. 1) [7]. Thr-183 and Ser-188 are found in motif 1/2, and the residues Ser-345, Thr-357, Tyr-360, and Ser-363 are located in the C5orf33 additional region that is specific to mitNADK homologs (Fig. 1).

The conservation of these residues in animal and protist mitNADK homologs and plant NADK3 homologs is listed in Table 2. Multiple alignments of the representative primary structures of mitNADK homologs are shown in Fig. S4. Thr-183, Ser-188, and Ser-289 are highly conserved in animal mitNADK homologs including mouse mitNADK and plant NADK3 homologs (Table 3). Ser-376 is highly conserved (100%) in the 62 mitNADK homologs from higher animals (mammals, birds, reptiles, and fishes). Thr-183 and Ser-188 are also found in protist mitNADK homologs from Apicomplexa including the genera *Plasmodium*, *Theileria*, and *Babesia* (Table 3). Earlier, we demonstrated that phosphomimetic mutation of the highly conserved Ser-188 abolished mitNADK activity, and mutation of the conserved Ser-376 and the highly conserved Ser-289 downregulated this activity (Fig. 2).

However, although Ser-183 and Ser-188 were frequently detected as phosphorylation sites, Ser-289 and Ser-376 were not; phosphorylation of Ser-183 and Ser-188 was detected in human mitNADK in two independent experiments [11,12,13].

Phosphorylation of Ser-188 was detected in mouse mitNADK in three independent experiments [14,15,16], and the phosphorylation of Ser-183 was detected in mouse in one experiment [15]. However, phosphorylation of Ser-289 and Ser-376 was only detected once in mouse and rat mitNADKs, respectively (Table 1) [18]. Taken together, phosphorylation of Ser-188, and perhaps the other sites, could be a significant mechanism for downregulating mitNADK activity.

Acknowledgements

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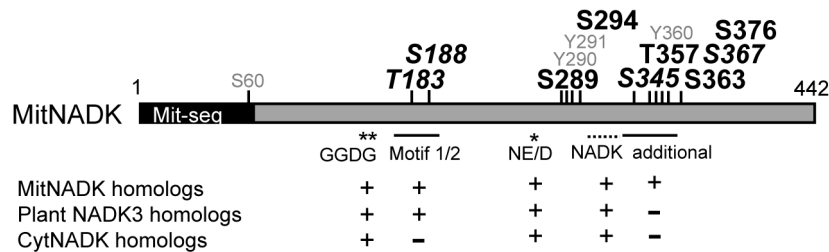


Fig. 1. Putative and detected phosphorylation sites in human mitNADK [11,12,13,14,15,16,17,18], and the structures of NADK homologs. The residues studied here are in bold, and the residues that have been detected in human mitNADK with phosphoproteomics [11,12,13] are in italics (Thr-183, Ser-188, Ser-345, and Ser-367). Recombinant mitNADK ($\Delta 62$ mitNADK) lacks the mitochondrial targeting sequence (Mit-seq, 62 residues) [7]. Primary structures of mitNADK homologs possess the GGDG motif, motif 1/2, the NE/D short motif, the NADK conserved motif (NADK), and the C5orf3 additional region (additional); in plant NADK3 homologs, only the additional region is not present (-), whereas in cytNADK homologs, motif 1/2 and the additional region are not present [7]. Note that plant NADK3 homologs have inserted sequence (NADK3 additional region consisting of 15 amino acids) between motif 1 and motif 2 [7].

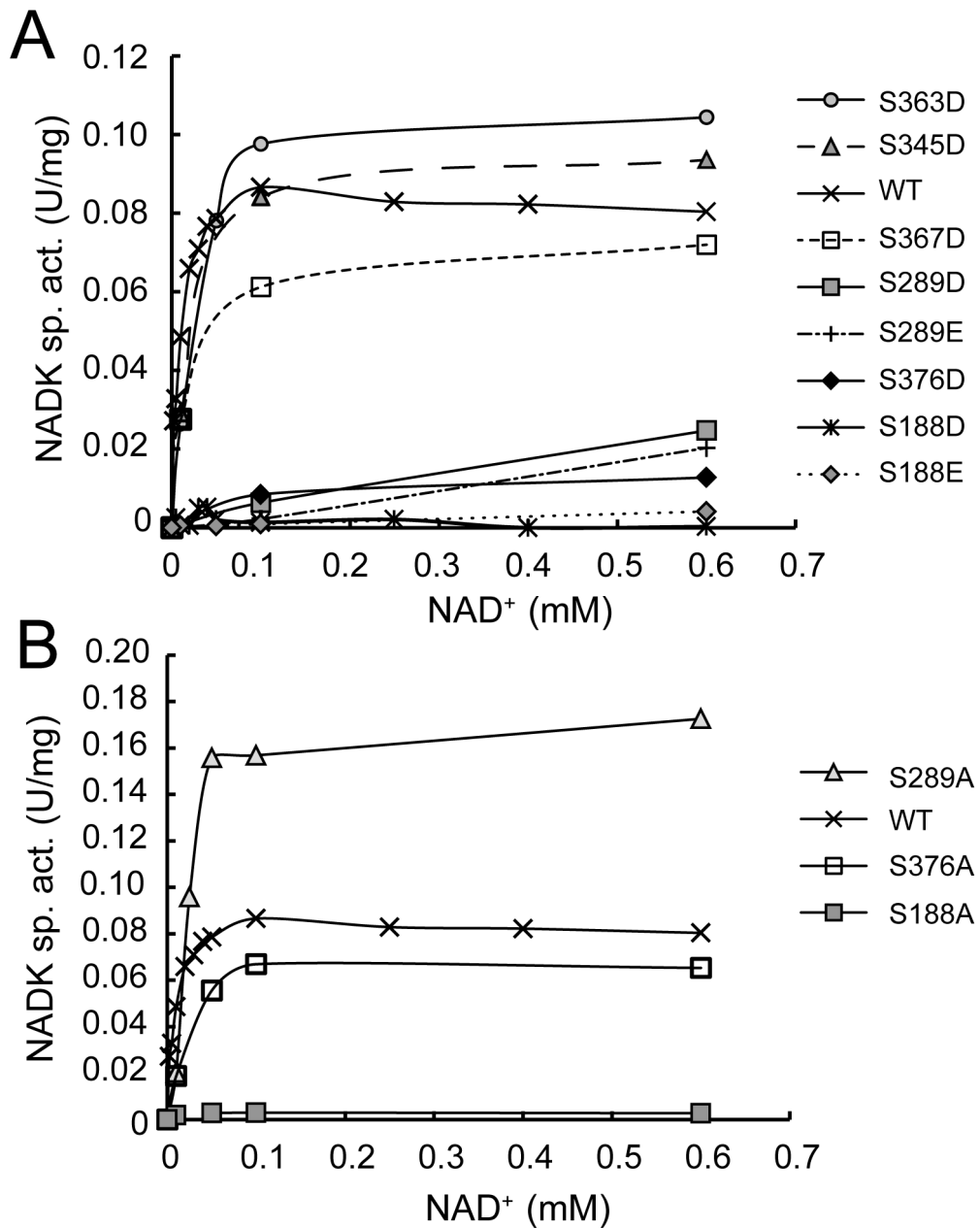


Fig. 2. NADK activity of each (A) phosphomimetic or (B) phosphoresistant mitNADK.

Table 1. The phosphorylation sites of mitNADK in human, mouse, and rat^a

mitNADK site	Corresponding human site	Ref	Disease tissue studied	Relevant cell line / cell type / tissue
Human				
Thr-183	Thr-183	[11]		liver
Ser-188 ^b	Ser-188 ^b			
Ser-345	Ser-345			
Ser-367	Ser-367			
Thr-183	Thr-183	[12]	hepatocellular carcinoma, surrounding tissue	hepatocyte-liver
Ser-188 ^b	Ser-188 ^b			
Ser-367	Ser-367	[13]		HeLa (cervical)
Mouse				
Ser-48	Ser-60	[14]		liver
Ser-176	Ser-188 ^b			
Ser-48	Ser-60	[15]		liver
Thr-171	Thr-183			
Ser-176	Ser-188 ^b			
Ser-377	Ser-367			
Ser-176	Ser-188 ^b	[16]		brain

Ser-299	Ser-289 ^b	(2007)	
Tyr-300	Tyr-290	CST	
Tyr-301	Tyr-291	Curation	
Ser-304	Ser-294	Set: 3621	
<hr/>			
Rat			
Ser-43	Ser-60	[17]	liver
Thr-340	Thr-357	[18]	kidney, inner
Tyr-343	Tyr-360		medulla
Ser-346	Ser-363		
Ser-359	Ser-376 ^b		
<hr/>			

^a All sites were identified with mass spectrometry.

^b Phosphomimetic mutations at these sites downregulated human mitNADK (Fig. 2).

Table 2. Number of homologs in which the phosphorylation site in human mitNADK is conserved. ^{a, b}

	117 animal mitNADK homologs ^c	19 protist mitNADK homologs ^d	50 plant NADK3 homologs ^e
Ser-60	16 (14%)	0 (0%)	0 (0%)
Thr-183 ^{f, i}	108 (92%)	10 (53%) ^j	50 (100%)
Ser-188 ^{g, i}	107 (91%)	10 (53%) ^j	50 (100%)
Ser-289 ^g	104 (89%)	3 (16%)	50 (100%)
Tyr-290	77 (66%)	0 (0%)	0 (0%)
Tyr-291	76 (65%)	2 (11%)	4 (8%)
Ser-294	67 (57%)	0 (0%)	46 (92%)
Ser-345 ^{h, i}	32 (27%)	0 (0%)	0 (0%)
Tyr-357	66 (56%)	7 (37%)	0 (0%)
Tyr-360	65 (56%)	1 (5%)	0 (0%)
Ser-363 ^h	68 (58%)	7 (37%)	0 (0%)
Ser-367 ^{h, i}	46 (39%)	0 (0%)	0 (0%)
Ser-376 ^g	66 (56%) ^k	0 (0%)	0 (0%)

^a The sites shown in Fig. 1.

^b Blast analysis [20] was conducted using the primary structure of human mitNADK as a query, and the obtained data was aligned using ClustalW [21].

^{c, d, e} The homologs with e value $< 3e-16$ ^c, $< 6e-05$ ^d, and < 0.007 ^e.

^f For example, Thr-183 of human mitNADK is conserved as a Ser or Thr residue in 108 animal mitNADK homologs.

^{g, h} Phosphomimetic substitutions of these residues into Asp or Glu downregulated the activity ^g or had no effect on activity ^h.

ⁱ The residues had been identified as phosphorylation sites in human mitNADK with phosphoproteomics [11,12,13].

^j All 10 homologs belong to protist mitNADK homologs from Apicomplexa including the genera *Plasmodium*, *Theileria*, and *Babesia*.

^k Ser-376 is highly (100%) conserved in the 62 mitNADK homologs from higher animals (mammals, birds, reptiles, and fishes).

Supporting information for

Significance of Ser-188 in *human mitochondrial NAD kinase as determined by phosphomimetic and phosphoresistant amino-acid substitutions*

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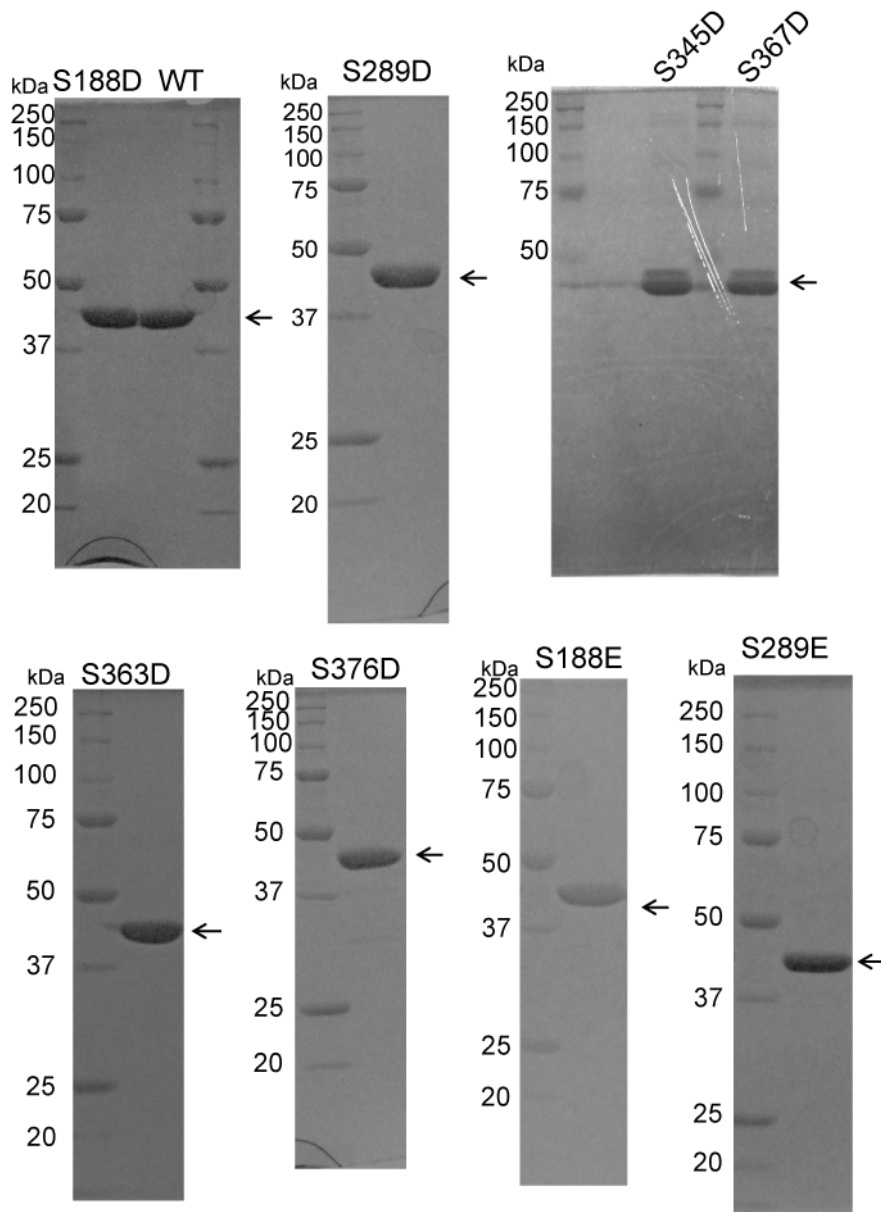


Fig. S1. SDS-PAGE of the purified wild-type (WT) and phosphomimetic mitNADK (3 μ g).

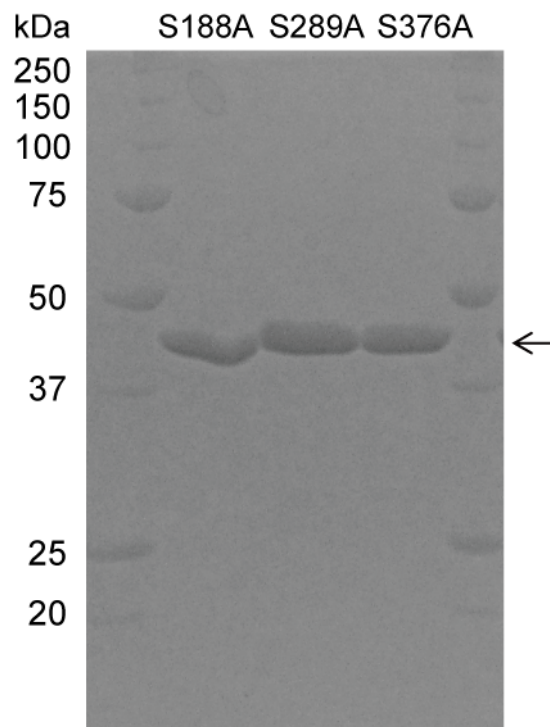


Fig. S2. SDS-PAGE of the purified phosphoresistant mitNADK (3 μ g).

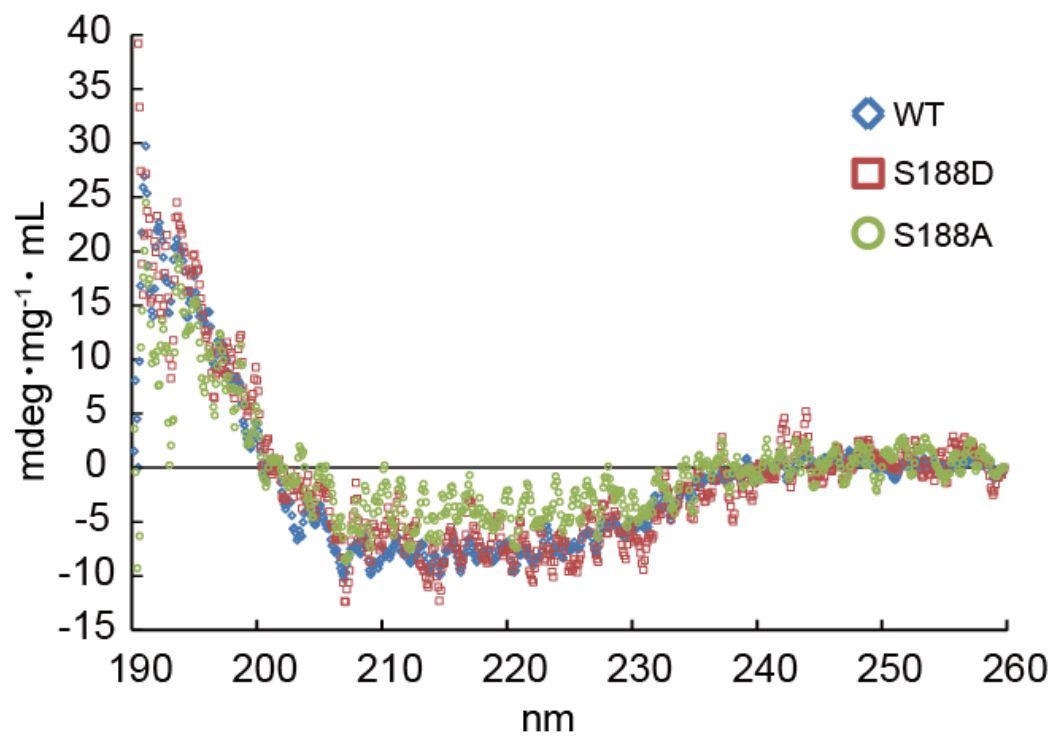


Fig. S3. CD spectrum of the purified enzymes (wild-type [WT] mitNADK, mitNADK S188D, and mitNADK S188A).

	GGDG motif	Motif 1 T-183	Motif 2 S-188	
	159	162		
Human mitNADK	136: VRLVKRREYDEE TVRWADAVIAAGG	DG TMLLAASKVLDRLKPVIGVNTD	PERSEGHCLLPVRYTHS--	FPEALQKFYRGEFRWLWRQRIRLYLEGTGINP :233
Macaca mulatta	136: VRLVKRREYDEE TVRWADAVIAAGG	DG TMLLAASKVLDRLKPVIGVNTD	PERSEGHCLLPVRYTHS--	FPEALQKFYRGEFRWLWRQRIRLYLEGTGINP :233
Sus scrofa	136: VRLVKRREYDEE TVRWADAVIAAGG	DG TMLLAASKVLDRLKPVIGVNTD	PERSEGHCLLPVRYTHS--	FPEALQKFYRGEFRWLWRQRIRLYLEGTGINP :233
Bos taurus	136: VRLVKRREYDEE TVRWADAVIAAGG	DG TMLLAASKVLDRLKPVIGVNTD	PERSEGHCLLPVRYTHS--	FPEALQKFYRGEFRWLWRQRIRLYLEGTGINP :233
Rattus norvegicus	119: VRLVKRREYDEE TVRWADAVIAAGG	DG TMLLAASKVLDRLKPVIGVNTD	PERSEGHCLLPVRYTHS--	FPEALQKFSRGEFRWLWRQRIRLYLEGTGINP :216
Ailuropoda melanoleuca	66: VRLVKRREYDEE AVRWADAVIAAGG	DG TMLLAASKVLDRLKPVIGVNTD	PERSEGHCLLPVRYTHS--	FPEALQKFYRGEFRWLWRQRIRLYLEGTGINP :163
Monodelphis domestica	130: VRLVKRREYDEE TVRWADAVIAAGG	DG TMLLAASKVLDRLKPVIGVNTD	PERSEGHCLLPVRYTHS--	FPEALQKLYRGEFRWLWRQRIRLYLEGTGINP :227
Anolis carolinensis	139: VRLVKRREYDEE TVRWADAVIAAGG	DG TMLLAASKVLDRLKPVIGVNTD	PERSEGHCLLPVRYTHS--	FPEALQKLYRGEFRWQRQRIRLYLEGTGINP :236
Ornithorhynchus anatinus	36: VRLVKRREYDEE TVRWADAVIAAGG	DG TMLLAASKVLDRLKPVIGVNTD	PERSEGHCLLPVRYTHS--	FPEALKRLTHGEFRWLWRQRIRLYLEGTGINP :133
Gallus gallus	49: VRLVKRRDYNEE TVRWADAVISAGG	DG TMLLAASKVDFKFKPVIGVNTD	PERSEGHCLLPVRYTHS--	FPEALQKLYRGEFRWQRQRIRLYLEGTGINP :146
Xenopus tropicalis	106: VRLVKRRDYDEE TVRWADAVISAGG	DG TMLLAASKVDFKFKPVIGVNTD	PERSEGHCLLPVRYTHS--	FPEALQKLYRGEFRWQRQRIRLYLEGTGINL :203
Taeniopygia guttata	187: VRLVKRREYNEE TVRWADAVISAGG	DG TMLLAASKVDFKFKPVIGVNTD	PERSEGHCLLPVRYTHS--	FPEALQKLYRGEFRWQRQRIRLYLEGTGINP :284
Strongylocentrotus purpuratus	55: TRTVDRYHFNDAAVRWADLIVSMGG	DG TFLLAASKVLDQT-PVIGVNTD	PEEGSEGHCLPNRYTFL--	FEDAMKRILSGNFRWRRQRI RVTVGDRMVNK :151
Caenorhabditis elegans	160: SRLVRRFGYTQEAVDADAVFSAAGG	DG TFLMASSKVRTKHKPVIGVNTD	PPQSEGYMCLMRKLPEEN-LAGALKLFSGN	FELNLRQRI RVTVGDDGIS :258
Caenorhabditis briggsae	154: SRLVRRFGYTQEAVDADAVFSAAGG	DG TFLMASSKVRTKHKPVIGVNTD	PPQSEGYMCLMRKLPEEN-LSGALKLFSGN	FELNLRQRI RVTVGDDGIS :252
Brachiostoma floridae	58: KVVIQKHEYTEKVNADVIMSAGG	DG TFLMAASHILTRKPLIGVNTD	PPSRSEGYLCLPKESYSGK--	FSRALDRLLAGKFRWRNRI RVTVGYSH :155
Pediculus humanus corporis	130: IRNVNRFNCTHDD IEWCTSVFPIGG	DG TFLYAARQISNTDKFVIGFNDS	PPSRSEGYLCLPKKYSNN--	ILDALKKLINGDFRWFNRI RVTVGQVYVSC :227
Caenorhabditis elegans	99: TKVVTR-EQLAQYLPKADLVISAGG	DG TFLAAASVVNDNT-PIIGVNTD	PIGSEGHCLCVGGKNPFR---	DLIERLVSGNLKWVQRRI RVTVKESRNSI :192
Caenorhabditis briggsae	99: SRVVTR-QQLAQYLPKADLVISAGG	DG TFLAAASVVNDNT-PIIGVNTD	PIGSEGHCLCVGGKTPFR---	NLIERLVSGNLKWVQRRI RVTVSAKDGA :192
Phytophthora infestans	406: KVVSANQLTHEAVEGTDMSFSAAGG	DG TFLKTASFVNTPI-FVAGLNTD	PKRSEGNLCCYKVDNVTHRFSTALDRLLE	GDYKWLWRQRI RVGMVNDQGYW :504
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	NADK conserved region	C5orf33 additional region			
	S-289 S-294	S-345 S-363 S-367 S-376			
	306 317 319				
C5orf33	289: SY YE I SVDDG-PWEKQKSSGLN	LC TGTGSKAWSFNINR V	ATQAVE DV LNIAKRQGNLS---	LPLNRELVEKVTNEYNESLLY	SPEEPKILFSIREPIANR :384
Macaca mulatta	289: SY YE I SVDDG-PWEKQKSSGLN	LC TGTGSKAWSFNINR V	ATQAVE DV LNIAKRQGNLS---	LPLNRELVEKVTNEYNESLLY	SPEEPKILFSIREPIANR :384
Sus scrofa	289: SY YE I SVDDG-PWEKQKSSGLN	LC TGTGSKAWSFNINR V	ATQAVE DV LNIAIRQGNLS---	LPLNRELVEKVTNEYNESLLY	SPEEPKILFSIREPIANR :384
Bos taurus	289: SY YE I SVDDG-PWEKQKSSGLN	LC TGTGSKAWSFNINR V	APQAVE DV LNIANRQGNLS---	LPLNRELVEKVTNEYNESLLY	SPEEPKILFSIREPIANR :384
Rattus norvegicus	272: SY YE I SVDDG-PWEKQKSSGLN	LC TGTGSKAWSFNINR V	AQAVE DV LNIAIRQGNLT---	LPLNKELVEKVTNEYNESLLY	SPEEPKILFSIREPIANR :367
Ailuropoda melanoleuca	219: SY YE I SVDDG-PWEKQKSSGLN	LC TGTGSKAWSFNINR V	ATQAVE DV LNIAKRQGNLS---	LPLNRELVEKVTNEYNESLLY	SPEEPKILFSIREPIANR :314
Monodelphis domestica	283: SY YE I SIDDG-PWEKQKSSGLN	LC TGTGSKAWSYNINR V	ATQAVE DV LKIAKRQANLD---	LPLKLEKVEKVTNEYNESLLY	SPEEPKMLFSIREPIANR :378
Anolis carolinensis	292: SY YE I SVDDG-PWEKQKSSGLN	LC TGTGSKAWSFNINR V	ANQAVE E ILKIAKGYDSL---	LPLSKELIQVTDGNEYNESLLY	SPEEPKMLFSVREPIANR :387
Ornithorhynchus anatinus	189: SY YE I SIDDG-PWEKQKSSGLN	LC TGTGSKAWSYNINR V	ATQAVE DV LTIARQGNLN---	IPLSRELIEKVTNEYNDLLY	SPEEPKMLFSIREPIANR :284
Gallus gallus	202: SY YE I SVDDG-PWEKQKSSGLN	VC TGTGSKAWSYNINR V	ANQAVE E ILNIAKHGSLN---	MPLNTELVQKVTNAYNESLLY	SPEEPKMFVREPIANR :297
Xenopus tropicalis	274: SY YE I SVDDG-PWEKQKSSGLN	VC TGTGSKAWSYNINR V	MSQSVE ELLNIVRQHSLN---	VSLSDSVIQRVNAYNDSL	VNPEEPKMFVREPIANR :369
Taeniopygia guttata	340: SY YE I SVDDG-PWEKQKSSGLN	VC TGTGSKAWSYNINR V	AHQAVE E ILKIAKHGSLN---	MPLNTELVQKVTNAYNESLLY	SPEEPKMFVREPIANR :435
Strongylocentrotus purpuratus	210: SY YE I SVDDG-PWEKQKSSGLN	VC TGTGSSWSFNINR V	LCLSVK DILKISEYSNEE	TGSLNATEDCTVERIADRFNS	LELDGADPRMAY TVRD PVTNR :308
Caenorhabditis elegans	338: SY YE I GINDA-QMLKQKSSGI	TICTGTGTSW	HNFNINR L TEQCQVQ DLMKIVA	EHCLNLP-QIPHGDKNVASE	ICTKFNQQLIFDPDRKQLAFSVRDPIFNA! :435
Caenorhabditis briggsae	336: SY YE I GINDA-QMLKQKSSGI	TICTGTGTSW	HNFNINR L TEQCQVQ DLMKIVA	EHCLNLP-QIPHENKNVASE	ICTKFNQQLIFDPDRRQLAFSVRDPIFNA! :433
Brachiostoma floridae	224: SY YELSV DGR-RWEKQKSSGLT	VLMRR-----	YNNSLMEDASEPKMTY	TVRDPPIING :274	
Pediculus humanus corporis	283: SHFEMRLNNSNKSVKIKSSGL	CVCTGTGTSW	NLSINR LSQDDVR DILKLY	ISETKGEID--DNFTDLSY	ISHNPNDFKQEPKQILWY TIRDLISLR :380
Caenorhabditis elegans	220: STYNI SIDDS-QTVKQKSSGL	IVSTGTGTSW	YLGMR DENATT SVLEALQSLGVDV---	PI TRNLVKVVSNVEKIPE	PDHFSFAFSIREPIANR :314
Caenorhabditis briggsae	224: STYNI SIDDS-QSVKQKSSGL	IVSTGTGTSW	YLGMR DENATT SVLEALQSLGINV---	PI TRNLVKVVTVNEKIPE	PDHFSFAFSIREPIANR :318
Phytophthora infestans	526: SHYNI GIDQH-QRESHRS	GLIMCTGTGSSAW	YSACC LYREQVA TVLSAMDH	THNET-----	VTELETSINKQVPEPDSRDMGYVVREPIANR :615
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Fig. S4. Multiple alignments of the representative primary structures of mitNADK homologs. Ser-188, Ser-289, and Ser-376, which downregulated NADK activity when they were substituted with phosphomimetic Asp, are in bold. Residues that were detected in human mitNADK are in red.