1 **Title**

2 NADPH regulates human NAD kinase, a NADP$^+$-biosynthetic

3 enzyme

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1 **Abstract**

2   NAD kinase (NADK, EC 2.7.1.23) is the sole $NADP^+$-biosynthetic enzyme that

3 catalyzes phosphorylation of NAD^+ to yield $NADP^+$ using ATP as a phosphoryl

4 donor, and thus, plays a vital role in the cell and represents a potentially powerful

5 antimicrobial drug target. Although methods for expression and purification of

6 human NADK have been previously established (F. Lerner, M. Niere, A. Ludwig, M.

7 Ziegler, Biochem. Biophys. Res. Commun. 288 (2001) 69-74), the purification

8 procedure could be significantly improved. In this study, we improved the method

9 for expression and purification of human NADK in *Escherichia coli* and obtained a

10 purified homogeneous enzyme only through heat treatment and single column

11 chromatography. Using the purified human NADK, we revealed a sigmoidal kinetic

12 behavior toward ATP and the inhibitory effects of NADPH and NADH, but not of

13 $NADP^+$, on the catalytic activity of the enzyme. These inhibitory effects provide

14 insight into the regulation of intracellular NADPH synthesis. Furthermore, these

15 attributes may provide a clue to design a novel drug against *Mycobacterium*

16 *tuberculosis* in which this bacterial NADK is potently inhibited by $NADP^+$.

17

18 **Keywords**

19 NAD kinase; human; drug design; $NADP^+$; NADPH; NADH

20

21 **Abbreviations**

22 NADK, NAD kinase; LB, Luria-Bertani; SDS-PAGE, sodium dodecyl

23 sulfate-polyacrylamide gel electrophoresis

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1 **Introduction**

2

3   NAD^+ and $NADP^+$ are cofactors involved in oxidative–reductive reactions [1, 2].

4 The function of NAD(H) (NAD^+ and NADH) is distinguishable from that of

5 NADP(H) ($NADP^+$ and NADPH). NAD(H) mainly functions in catabolic reactions,

6 while NADP(H) is involved in anabolic reactions and defense against oxidative

7 stress [3]. NAD^+ also functions as a substrate for mono- and poly-ADP-ribosylation

8 and is involved in cyclic ADP-ribose synthesis [2] and histone deacetylation [4].

9 $NADP^+$ functions as a substrate in the synthesis of nicotinic acid adenine

10 dinucleotide phosphate, which is a potent intracellular Ca^{2+}-mobilizing messenger [1,

11 2, 5].

12   NAD kinase (NADK, EC 2.7.1.23) is the sole $NADP^+$-biosynthetic enzyme that

13 catalyzes phosphorylation of NAD^+ to yield $NADP^+$ using ATP as a phosphoryl

14 donor [1], and thus, plays a vital role in the cell. Accordingly, NADK has been found

15 to be essential for many microbes, including *Mycobacterium tuberculosis* [6],

16 *Bacillus subtilis* [7], *Staphylococcus aureus* [8], *Haemophilus influenzae* [8],

17 *Escherichia coli* [8, 9], *Streptococcus pneumoniae* [8], *Salmonella enterica* [9, 10],

18 and *Saccharomyces cerevisiae* [11, 12], thus representing a potentially powerful

19 antimicrobial drug target [13, 14]. Several bacterial recombinant NADKs have been

20 characterized in detail, including those from *E. coli* [15], *B. subtilis* [16], and *M.*

21 *tuberculosis* [17, 18]. Moreover, tertiary structures of NADKs from the pathogenic

22 bacteria *M. tuberculosis* [19, 20] and *Listeria monocytogenes* [21] have been

23 published. However, the tertiary structure of human NADK has not yet been

24 reported.

1   Lerner *et al.* [22] established methods for expression and purification of human

2   NADK and determined some properties of this enzyme [22]. Although they provided

3   a detailed purification procedure, the procedure still has needed several steps such as

4   heat   treatment   followed   by   DEAE   column   chromatography   and

5   nickel–nitrilotriacetic acid column chromatography. Thus, an improvement in the

6   methods for expression and purification of human NADK would facilitate further

7   study of this vital enzyme. Further determination of the structure and catalytic

8   properties of human NADK would provide a good foundation for designing a novel

9   antimicrobial drug [13].

10   In this article, we describe improved methods to express and purify human

11   NADK, thus enabling to obtain larger amounts of the purified homogeneous enzyme.

12   In addition, we reveal some kinetic properties of human NADK that will provide

13   insight into the regulation of intracellular NADPH synthesis and possibly a clue to

14   design a novel antimicrobial drug.

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16   **Materials and methods**

17

18   Strains and plasmids

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20   As a host for plasmid amplification, *E. coli* DH5α and JM109 were routinely

21   cultured at 37 °C in Luria-Bertani (LB) medium containing 1% tryptone, 0.5% yeast

22   extract, 1% NaCl, and ampicillin (100 μg/ml) (pH 7.2). *E. coli* M15 (pREP4) and

23   SG13009 (pREP4) were purchased from Qiagen (Hilden, Germany), and *E. coli*

24   Rosetta-gami, Rosetta-gami(DE3), Rosetta-gami(DE3)pLysS, Rosetta-gami 2(DE3),

1 and Rosetta-gami B(DE3) were obtained from Novagen (Darmstadt, Germany). The

2 culture conditions for these *E. coli* strains are described below.

3   Human NADK cDNA was amplified by PCR using human leukocyte cDNA

4 (QUICK-Clone cDNA, Clontech, shiga, Japan) as a template and primers

5 [HsNADKBamHI-f, CG<u>GGATCC</u>ATGGAAATGGAACAAGAAAAAATGAC (the

6 *Bam*HI   site   is   underlined)   and   HsNADK-C(NoRe)-r,

7 CTAGCCCTCCTCCTCCTCCTC], and was inserted into the *Hinc*II site of pUC18,

8 resulting in pMK1966. Human NADK cDNA obtained by digestion of pMK1966

9 with *Bam*HI was inserted into the *Bam*HI site of pQE-30 (Qiagen), yielding

10 pMK2071. The primary structure encoded by the cloned cDNA was identical to that

11 of the cDNA sequence (with Phe $(Glu)_9$ Gly as C-terminus) deposited in the NCBI

12 database (accession no. NP_001185922), except that the former contains one

13 additional Glu residue at its C-terminus, i.e., it has Phe $(Glu)_{10}$ Gly.

14   A DNA fragment encoding for an N-terminal sequence consisting of 87 amino

15 acid residues of human NADK was excised from the full-length cDNA clone

16 pMK2071, resulting in pMK2784 that encodes for deleted human NADK with an

17 N-terminal sequence, [88]MHIQDPASQRL[98]. This deletion was generated by inverse

18 PCR   using   primers   (5′-CACATTCAGGACCCCGCGAG-3′   and   5′-

19 CATATGTCCGTGATGGTGATGGTG-3′). The recombinant proteins encoded by

20 pMK2071 and pMK2784 contained a His-tag (MRGSHHHHHHGS) at their

21 N-terminal sites.

22   To express human NADK cDNA, pMK2071 was introduced into *E. coli* M15

23 (pREP4), SG13009 (pREP4), and Rosetta-gami, yielding MK2105, MK2106, and

24 MK2107, respectively. pMK2784 was introduced into *E. coli* Rosetta-gami(DE3),

1 Rosetta-gami(DE3)pLysS, Rosetta-gami 2(DE3), and Rosetta-gami B(DE3),

2 yielding MK2801, MK2802, MK2803, and MK2804, respectively.

3

4 Expressions

5

6   The cells were precultured overnight in LB media supplemented with 100 µg/ml

7 ampicillin and 25 µg/ml kanamycin (MK2105 and MK2106) or with 100 µg/ml

8 ampicillin and 34 µg/ml chloramphenicol (MK2107, MK2801, MK2802, MK2803,

9 and MK2804). The cells were collected, inoculated in LB media supplemented with

10 the same antibiotics, and aerobically cultured at 37 °C until A_{600} reached 0.50–1.0.

11 Isopropyl-β-D-thiogalactopyranoside was then added to obtain a final concentration

12 of 0.025 or 1.0 mM in order to induce expression of human NADK. Cell cultivation

13 was continued further at 16 or 37 °C under aerobic conditions for 4, 8, 12, or 24 h. To

14 purify human NADK, MK2107 and MK2802 were cultivated at 37 °C for 8 h

15 (MK2107)   or   12   h   (MK2802)   after   the   addition   of

16 isopropyl-β-D-thiogalactopyranoside at 1.0 mM. To prepare the crude extract, cells

17 were collected by centrifugation, suspended in 10 mM Tris-HCl (pH8.0), and

18 sonicated to yield cell lysate. Before and after sonication, phenylmethylsulfonyl

19 fluoride was added to obtain a final concentration of 1.0 mM. After centrifugation of

20 the lysate at 20,000 $\times g$ and 4 °C for 10 min, the supernatant was used as crude

21 extract.

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1   Assays

2

3   NADK activity of human NADK was assayed at 37 °C by the modified

4   continuous method [23]. Briefly, NADPH formation was continuously measured at

5   A_{340} in a reaction mixture (1.0 ml) containing 5.0 mM NAD^+, 5.0 mM ATP, 5.0 mM

6   glucose-6-phosphate, 0.5 U glucose-6-phosphate dehydrogenase (Sigma, St. Louis,

7   MD, USA), 5.0 mM $MgCl_2$, and 100 mM Tris-HCl (pH 8.0). In the purified enzyme

8   assay, $MgCl_2$ was used at 10 mM. Enzyme activity was assayed by a stop method

9   [23] to determine inhibitory effects and optimum pH and temperature conditions.

10   Briefly, the amount of $NADP^+$ formed was enzymatically determined using 0.5 U

11   glucose-6-phosphate dehydrogenase after the reaction was terminated by immersing

12   the test tube in boiling water for 5 min. Glucose-6-phosphate and

13   glucose-6-phosphate dehydrogenase were removed from the reaction mixture as

14   described above. When the inhibitory effects of NADP(H) and NADH were

15   investigated, control reactions without human NADK were simultaneously

16   performed in every assay.

17   NADH kinase activity was assayed in a reaction mixture (1.0 ml) containing 2.0

18   mM NADH, 5.0 mM ATP, 10.0 mM $MgCl_2$, and 100 mM Tris-HCl (pH 8.0) [23].

19   One unit of enzyme activity was defined as 1.0 μmol NADP(H) produced in 1 min at

20   37 °C, and specific activity was expressed in U/mg protein. Protein concentrations of

21   the crude extract and purified human NADK from MK2107 were determined by the

22   Bradford method [24]. Protein concentrations of purified human NADK from

23   MK2802 were determined using the extinction coefficient (33,920) at A_{280}. The

24   extinction coefficients were calculated using the ProtParam tool on the ExPASy

1 server (http://ca.expasy.org/tools/protparam.html).

2

3 Purification

4

5 Centrifugation was performed at 20,000 ×g and 4 °C for 10 min. Human NADK

6 was expressed from MK2107 cells cultured in 2 l LB (0.5 l per 2 l Erlenmeyer flask)

7 as described above and treated as described [22]. This was followed by Mono Q

8 chromatography. The partially purified human NADK was applied to Mono Q 5/50

9 GL (0.5 × 5.0 cm) (GE Healthcare, Buckinghamshire, England) equilibrated with

10 buffer A (20 mM Tris-HCl (pH 8.5), 0.10 mM NAD$^+$, 0.50 mM dithiothreitol, 1.0

11 mM ethylenediaminetetraacetic acid, and 0.10 mM pepstatin A). After washing the

12 column with 18 ml buffer A, elution was performed with a linear gradient of NaCl

13 from 0 to 1.0 M in buffer A. Fructions that were obtained by an elution at 370–470

14 mM NaCl were combined, dialyzed against buffer A, and used as purified human

15 NADK.

16 Human NADK was purified from MK2802 as stated below. Crude extract was

17 prepared from MK2802 cells cultured in 13.5 l LB (1.5 l per 2 l Sakaguchi flask) and

18 was treated as described above. Samples were heated at 60 °C for 5 min and

19 centrifuged. The supernatant was applied to a TALON column (1.5 × 22 cm)

20 (Clontech) equilibrated with 10 mM Tris-HCl (pH8.0). The column was washed with

21 2 l of 10 mM Tris-HCl (pH8.0) containing 0.30 M NaCl. Human NADK was eluted

22 with 10 mM Tris-HCl (pH8.0) containing 0.30 M NaCl and 150 mM imidazole.

23 Fractions were combined, dialyzed against 10 mM Tris-HCl (pH 8.0), and used as

24 purified human NADK. Alternatively, the crude extract prepared from MK2802 cells

1   was treated as mentioned above and was directly applied to the TALON column

2   without heat treatment. The column was washed with 2 l of 10 mM Tris-HCl (pH8.0)

3   containing 0.30 M NaCl and 30 mM imidazole, and eluted as above.

4

5   Other analytical methods

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7   Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was

8   performed using a 12.5% polyacrylamide gel [25]. Proteins in the gel were visualized

9   with Coomassie brilliant blue R-250. The molecular mass of the enzyme was

10   estimated by gel filtration chromatography as described [26]; however, 10 mM

11   Tris-HCl (pH 8.0) containing 0.15 M NaCl was used as an elution buffer. For the

12   N-terminal amino acid sequence, purified and unpurified enzymes were transferred

13   to a polyvinylidene difluoride membrane and analyzed using a Procise 492 protein

14   sequencing system (Applied Biosystems Division of Perkin-Elmer, Foster City, CA,

15   USA).

16

17   **Results and discussion**

18

19   Improved methods to express and purify human NADK

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21   According to the method described by Lerner *et al.* [22], we expressed the

22   full-length human NADK cDNA cloned into pQE-30 (pMK2071) using an *E. coli*

23   M15 (pREP4) host cell and obtained 0.23 U/mg NADK activity in the crude extract

24   (Table 1). To improve expression, effects of the host cells (*E. coli* SG13009 (pREP4)

9

1 or Rosetta-gami), concentrations of isopropyl-β-D-thiogalactopyranoside (0.025 or

2 1.0 mM), and induction conditions (16 or 37 °C and 4, 8, 12, or 24 h) were examined.

3 Maximum expression (1.02 U/mg) was obtained in the crude extract when

4 expression was induced at 37 °C for 8 h with 1.0 mM

5 isopropyl-β-D-thiogalactopyranoside using *E. coli* Rosetta-gami (Table 1). The

6 expressed human NADK was treated according to the purification procedure

7 described by Lerner *et al*. [22]. However, this procedure yielded an unpurified

8 enzyme. The unpurified enzyme was further purified by Mono Q chromatography as

9 described in Materials and methods. The purified enzyme (0.39 mg; 7.21 U/mg) was

10 obtained from a 2 l culture. SDS-PAGE analysis showed that the subunit molecular

11 mass of the purified enzyme was 43 kDa, which was smaller than that predicted (49

12 kDa) from the primary structure (data not shown). Analysis of the N-terminal amino

13 acid sequence of the purified enzyme confirmed that the enzyme lacked the

14 N-terminal sequences consisting of 60, 63, and 74 amino acid residues of human

15 NADK, indicating the heterogeneity of the purified enzyme.

16   To circumvent this problem, the DNA region that encoded the N-terminal

17 sequence consisting of 87 amino acid residues of human NADK was excised from

18 the full-length human NADK cDNA as described in Materials and methods. The

19 deleted human NADK was expressed in *E. coli* Rosetta-gami(DE3), and it showed a

20 specific activity of 0.79 U/mg (Table 1). Further optimization of the expression

21 condition revealed that induction at 37 °C for 8–12 h in the presence of 1.0 mM

22 isopropyl-β-D-thiogalactopyranoside using *E. coli* Rosetta-gami(DE3)pLysS as a

23 host cell gave the maximum specific activity (4.29 U/mg). This activity was

24 improved by approximately 18.7-fold compared with our initial attempt (0.23 U/mg;

Table 1). Moreover, we found that the heat treatment, followed by TALON column chromatography was sufficient to obtain the purified enzyme (9.34 U/mg, Fig. 1a). The subunit molecular mass of the purified enzyme (43 kDa) was in good agreement with the predicted mass (41 kDa). The N-terminal amino acid sequence of the enzyme was RGSHH, which corresponded to that of the His-tag, confirming the homogeneity of the purified enzyme. In all purification experiments, the purified enzyme (15 mg) was routinely obtained from a 13.5 l culture. Thus, we improved the methods for expression and purification of human NADK (Table 2, Fig. 1a).

We also found that the enzyme could be purified only through TALON column chromatography without heat treatment (Fig. 1b). However, the purified NADK without heat treatment yielded three peaks (representing 210, 400, and 650 kDa) on gel filtration chromatography; each peak showed NADK activity. NADK in each peak was not divided into three peaks on repeated gel filtration chromatography (data not shown), thus indicating the heterogeneity in the quaternary structure of this human NADK. In contrast, the purified enzyme obtained by heat treatment, which was followed by TALON column chromatography (Fig. 1a), yielded a single peak representing 172 kDa (data not shown). Thus, the procedure using heat treatment was considered preferable for enzyme purification, and the properties of human NADK purified by this procedure were determined.

Properties of human NADK

Although the properties of purified human NADK were similar to those reported [22] (Table 3), we found that human NADK exhibits sigmoidal kinetic behavior

1   toward ATP, while saturation curve toward NAD^+ (Fig. 2). The sigmoidal kinetic

2   behavior was emphasized by a Hanes–Woolf plot (Fig. 2b). The sigmoidal kinetic

3   behavior toward ATP was also observed in NADKs from *B. subtilis* [16] and *M.*

4   *tuberculosis* [18]. With regard to NAD^+, *M. tuberculosis* NADK shows sigmoidal

5   kinetic behavior [18], whereas *B. subtilis* NADK exhibits a saturation curve [16].

6   NADH-phosphorylating (NADH kinase) activity of recombinant human NADK

7   was qualitatively, but not quantitatively, demonstrated by Pollak *et al.* [27]. We

8   showed that human NADK exhibited NADH kinase activity (0.6 U/mg), accounting

9   for only 5.0% of NADK activity (11.9 U/mg) determined in the presence of 2.0 mM

10   NAD^+. Some bacterial NADKs, such as those from *M. tuberculosis*, utilize inorganic

11   polyphosphate as a phosphoryl donor [17, 18]. Inorganic polyphosphate is a polymer

12   of inorganic orthophosphate residues linked by a high-energy phosphoanhydride

13   bond [28], and is available as metaphosphate, hexametaphosphate, and

14   tetrapolyphosphate. The relative activities of purified human NADK in the presence

15   of 1 mg/ml metaphosphate, 1 mg/ml hexametaphosphate, and 5 mM

16   tetrapolyphosphate were 2.3, 1.9, and 0.5%, respectively, compared with NADK

17   activity assayed in the presence of 5.0 mM ATP.

18

19   Inhibitory effects of NADPH and NADH, but not of $NADP^+$

20

21   Determining how human NADK is regulated by $NADP^+$, NADPH, and NADH

22   is a crucial step in understanding how human NADK influences the intracellular

23   redox balance. Although $NADP^+$ exhibited no inhibitory effect on NADK activity of

24   human NADK, NADPH and NADH showed competitive inhibitions with K_i values

1 of 0.13 mM for NADPH and 0.34 mM for NADH (Table 4, Fig. 3, Online Resource

2 Fig. S1). This agrees with a previous report, which shows that NADK activity of

3 partially purified pigeon liver NADK is competitively inhibited by NADPH and

4 NADH, while the effect of $NADP^+$ on NADK activity of the enzyme was not

5 investigated [29]. Although the information regarding the effect of these nucleotides

6 on NADK activity has been limited, *M. tuberculosis* NADK has been reported to be

7 inhibited by NADH, $NADP^+$, and NADPH, wherein $NADP^+$ is the most potent

8 inhibitor [18]. The residual NADK activity of *M. tuberculosis* NADK was 22, 43,

9 and 50% in the presence of 0.40 mM $NADP^+$, NADPH, and NADH, respectively,

10 although the inhibitory mechanism has not been investigated [18]. The fact that

11 $NADP^+$ inhibits *M. tuberculosis* NADK but not human NADK is a clue to design a

12 novel antimicrobial drug, i.e., an analog of $NADP^+$, which would possibly inhibit

13 only *M. tuberculosis* NADK, thereby being lethal to the bacterium and not the host.

14 Recently, Pollak *et al.* [27] established human cell lines overexpressing human

15 NADK (NADK(+) cells). In NADK(+) cells that showed approximately 180-fold

16 higher NADK activity than control cells, the NADPH level increased by 4- to 5-fold,

17 and not 180-fold, while the $NADP^+$ level was not prominently altered. The authors

18 suggested that human NADK may be inhibited by NADPH. Our data demonstrating

19 the inhibition of human NADK by NADPH convincingly support their suggestion.

20 Taken together the low NADH kinase activity of human NADK, NADPH would be

21 produced by $NADP^+$-dependent dehydrogenases through reduction of $NADP^+$. The

22 fact that human NADK is localized in the cytosol [27] and that NADPH, but not

23 $NADP^+$, is significant for the regulation of human NADK would indicate that the

24 function of cytosolic $NADP^+$-dependent dehydrogenases is important for the

13

1   regulation of $NADP^+$ synthesis (NADK activity), although the physiological role of

2   inhibition of human NADK by NADH remains unclear.

3   NADPH is required to protect cells against oxidative stress, and cytosolic

4    $NADP^+$-dependent glucose-6-phosphate dehydrogenase in human cells is induced

5   when exposed to oxidative stress [30]. When cells survive against oxidative stress,

6   intracellular NADPH is consumed and resynthesized quickly, while human NADK

7   synthesizes $NADP^+$ to guarantee the intracellular requirement for NADPH. When

8   oxidative stress is removed, the abundant NADPH inhibits $NADP^+$ synthesis to

9   prevent accumulation of excess $NADP^+$ and NADPH, thus resulting in the

10   maintenance of homeostasis of intracellular $NADP^+$ and NADPH.

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1 **Figure legends**

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3 **Fig. 1.** SDS-PAGE of purified human NADK. (a) Purified human NADK (110 μg

4 protein). (b) Purified human NADK obtained without heat treatment (60 μg protein).

5 Arrowheads indicate the positions of the purified human NADK.

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7 **Fig. 2.** Kinetic analysis of human NADK. (a) The effect of substrate concentration

8 ([ATP] or [NAD^+], mM) on the initial velocity (V, U/mg) of the reaction was assayed

9 in the presence of 5 mM NAD^+ and various concentrations of ATP (closed squares)

10 or 5 mM ATP and various concentrations of NAD^+ (open squares). Data represent the

11 means of three measurements. (b) Hanes–Woolf plot of the data in (a).

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13 **Fig. 3.** Inhibitory effects of NADH (a, b) and NADPH (c, d) on human NADK. The

14 initial velocity (V, U/mg) of the reaction was assayed in the presence of various

15 concentrations of NAD^+ ([NAD^+], mM). (a) Saturation curve for NAD^+ determined

16 in the presence of 0 mM (closed squares), 0.3 mM (open circles), and 0.5 mM (closed

17 circles) NADH. (b) Lineweaver–Burk plot of the data in (a). (c) Saturation curve for

18 NAD^+ determined in the presence of 0 mM (closed squares) and 0.5 mM (open

19 squares) NADPH. (d) Lineweaver–Burk plot of the data in (c).

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21 **Online Resource figure legend**

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23 **Online Resource Fig. S1.** Inhibitory effects of NADH (a, b) and NADPH (c, d) on

24 human NADK. (a) Hanes–Woolf plot of the data in Fig. 3a. (b) Eadie plot of the data

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1   in Fig. 3a. The plot was determined in the presence of 0 mM (closed squares), 0.3

2   mM (open circles), and 0.5 mM (closed circles) NADH. (c) Hanes–Woolf plot of the

3   data in Fig. 3c. (d) Eadie plot of the data in Fig. 3c. The plot was determined in the

4   presence of 0 mM (closed squares) and 0.5 mM (open squares) NADPH.

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Table 1

Expression of human NADK.

Host cell	Specific activity (U/mg)[a]	Relative activity (fold)
M15 (pREP4)	0.23	1
Rosetta-gami	1.02	4.4
Rosetta-gami(DE3)	0.79	3.4
Rosetta-gami(DE3)pLysS	4.29	18.7

[a]Specific activity in crude extract.

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Table 2

Improvement in the methods for expression and purification of human NADK.

	Previous study [22]	Present study
Specific activity of expressed human NADK in crude extract	n.r.[a] (0.23 U/mg)[b]	4.29 U/mg
Purification procedure	Three steps (Heat treatment and DEAE as well as nickel–nitrilotriacetic acid column chromatography)	Two steps (Heat treatment and TALON column chromatography)
Yield of purified human NADK	n.r.[a]	15 mg[c]
Specific activity of purified human NADK	n.r.[a]	9.34 U/mg
Homogeneity of purified human NADK	n.r.[a, d]	Homogeneity[d]

[a]n.r.; not reported.

[b]The value obtained in this study (Table 1).

[c]Purified human NADK (15 mg) was obtained from a 13.5 l culture.

[d]N-terminal amino acid sequence of the purified human NADK was not determined

in the previous report [22], while homogeneity of this enzyme was confirmed by

analysis of the N-terminal amino acid sequence in this study.

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Table 3

Properties of human NADK.

	Previous study [22]	Present study
Subunit molecular mass	49 kDa	43 kDa
Subunit composition (Molecular mass)	Homotetramer (200 kDa)	Homotetramer (172 kDa)
K_m (mM) for NAD^+	0.54	1.07
K_m (mM) for ATP	3.3	-
$S_{0.5}$ (mM) for ATP	-	3.6
n_H	-	1.6
V_{max} (U/mg) for NAD^+	6.7	18.5
V_{max} (U/mg) for ATP	-	18.5
Optimum temperature (°C)	55	50
Optimum pH	7.5	8.0

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Table 4

Inhibitory effects of NADP$^+$, NADPH, and NADH on human NADK[a].

Compound	Concentration (mM)	Specific activity (U/mg)[b]	Relative activity (%)
None	0	8.75 ± 0.66	100
NADP$^+$	0.5	8.92 ± 2.26	102
NADPH	0.5	5.83 ± 0.49	66
NADPH	0.3	7.08 ± 0.42	81
NADH[c]	0.5	3.54 ± 0.48	40
NADH[c]	0.3	5.03 ± 0.61	58
NADH[c]	0.1	6.30 ± 0.36	72

[a]NADK activity was assayed in the presence of 1.0 mM NAD$^+$ and indicated compound.

[b]Means and standard deviations of three independent assays are indicated.

[c]Inhibitory effect of NADH was not attributed to NADH kinase activity of human NADK since the activity assayed in the presence of 0.3 or 0.5 mM NADH was only 10% of NADK activity assayed in the presence of 1.0 mM NAD$^+$ (data not shown).

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2   Fig. 1

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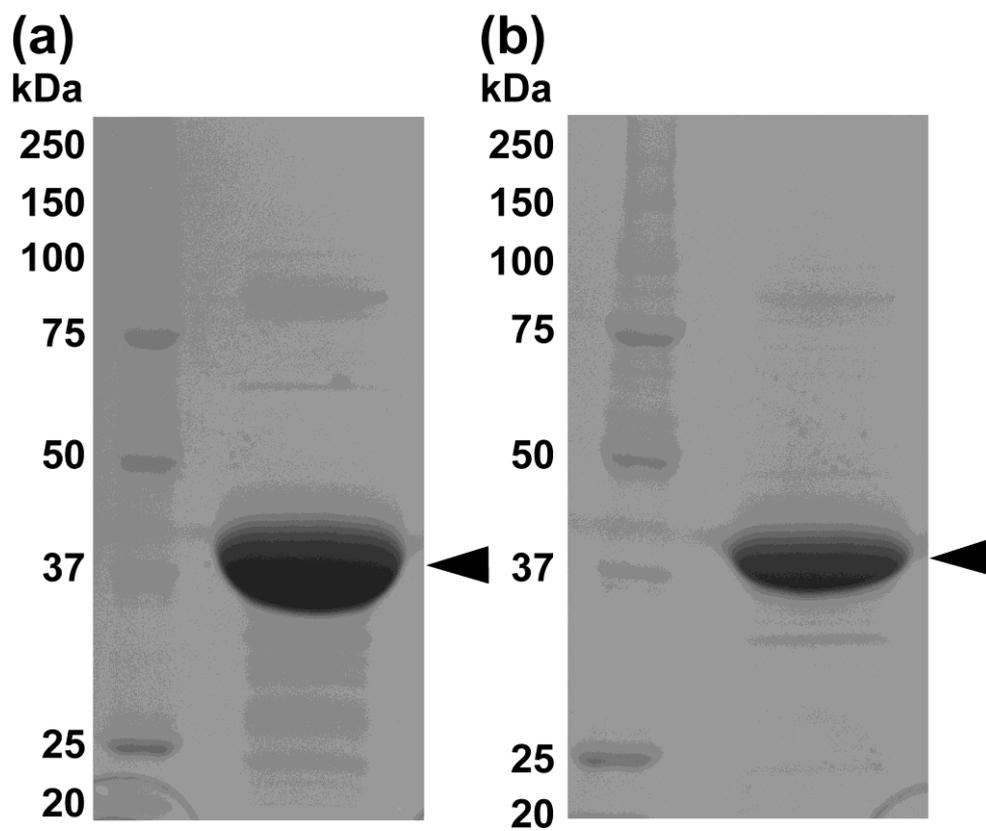
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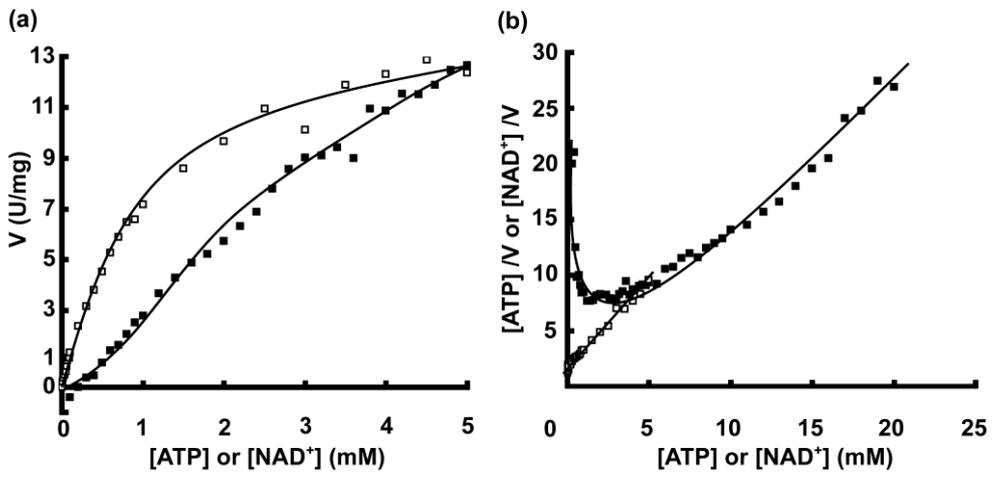
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3   Fig. 2

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(a)

(b)

(c)

(d)
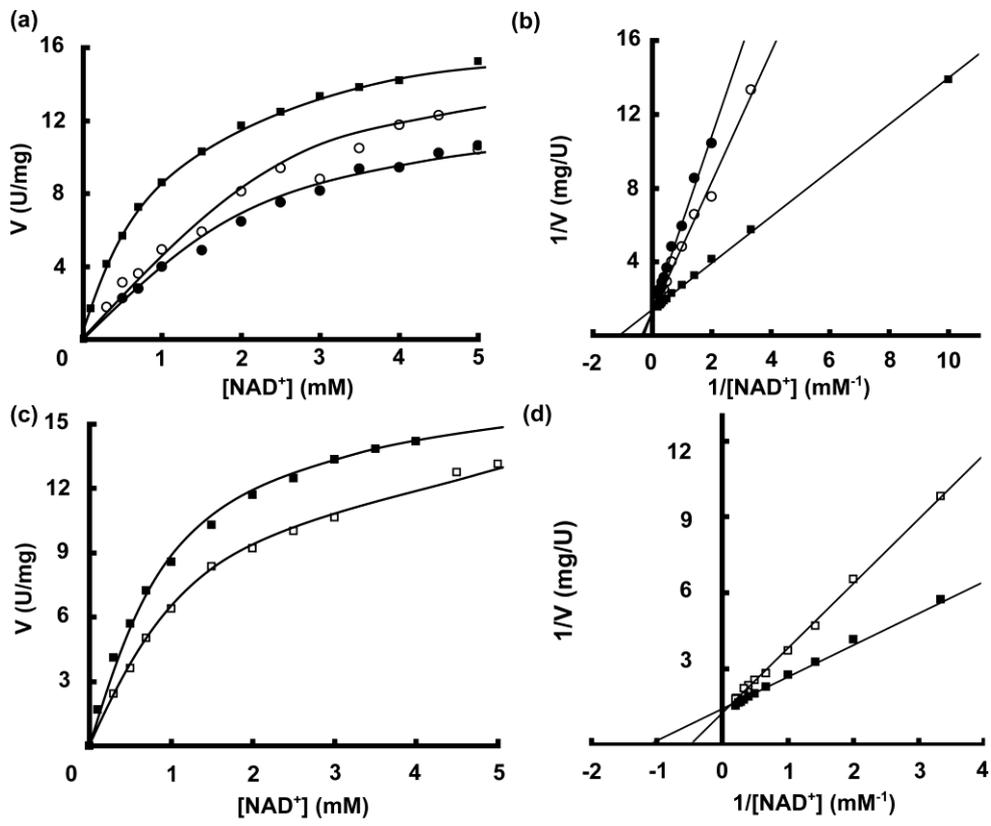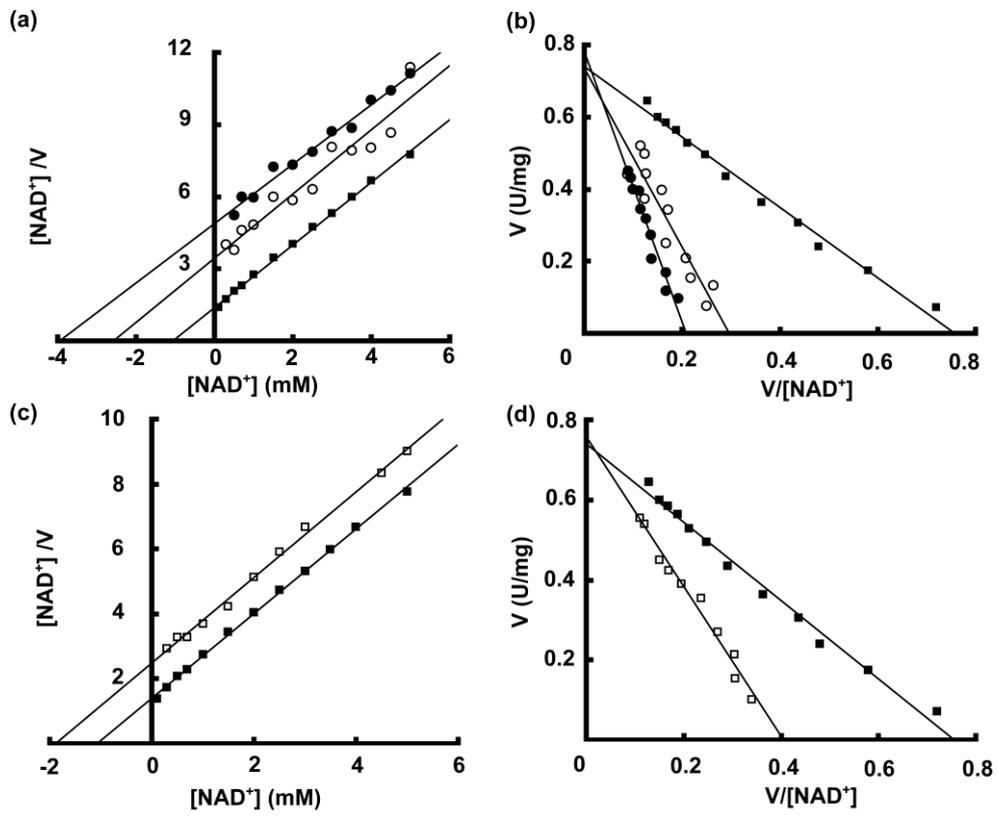
Supplementary Fig. S1 Ohashi et al.

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