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Conferring the ability to utilize inorganic polyphosphate on ATP-specific NAD kinase

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NAD kinase (NADK) is a crucial enzyme for production of NADP⁺. ATP-specific NADK prefers ATP to inorganic polyphosphate [poly(P)] as a phosphoryl donor, whereas poly(P)/ATP-NADK utilizes both ATP and poly(P), and is employed in industrial mass production of NADP⁺. Poly(P)/ATP-NADKs are distributed throughout Gram-positive bacteria and Archaea, whereas ATP-specific NADKs are found in Gram-negative α - and γ -proteobacteria and eukaryotes. In this study, we succeeded in conferring the ability to utilize poly(P) on γ -proteobacterial ATP-specific NADKs through a single amino-acid substitution; the substituted amino-acid residue is therefore important in determining the phosphoryl-donor specificity of γ -proteobacterial NADKs. We also demonstrate that a poly(P)/ATP-NADK created through this method is suitable for the poly(P)-dependent mass production of NADP⁺. Moreover, based on our results, we provide insight into the evolution of bacterial NADKs, in particular, how NADKs evolved from poly(P)/ATP-NADKs into ATP-specific NADKs.

Inorganic polyphosphate [poly(P)], a polymer of orthophosphate (Pi) residues linked by high-energy phosphoanhydride bonds, is found in every organism from bacteria to animals¹. Poly(P) can be formed from Pi by dehydration at elevated temperature and can also be produced by volcanic activity, implicating poly(P) as a plausible prebiotic source of nucleoside triphosphate^{2,3}.

From the perspective of industrial applications, poly(P) is a promising phosphoryl donor, with several advantages relative to ATP in the context of enzymatic production of valuable phosphoryl compounds: poly(P) is cheaper than ATP, and can be obtained more easily and economically at higher purity⁴. Large-scale industrial production of NADP⁺ has successfully utilized poly(P)/ATP-NAD kinase [poly(P)/ATP-NADK], which can phosphorylate NAD⁺ using poly(P) or ATP⁴. One practical limitation of poly(P)-dependent production, however, is that the capacity to use poly(P) is restricted to very few enzymes, including NADK, glucokinase, poly(P) kinase, and poly(P):AMP phosphotransferase⁵. Consequently, the aforementioned poly(P)-dependent NADP⁺-production system is, to our knowledge, the only successful case of industrial use of poly(P); other valuable compounds than NADP⁺ have not been industrially produced due to the limited poly(P)-dependent enzymes. If the ability to utilize poly(P) could be conferred on various ATP-specific enzymes, the enzymes will be industrially useful to produce various valuable compounds. Therefore, it will be necessary to explore methods for conferring the ability to utilize poly(P) on a more diverse set of ATP-specific enzymes. To this end, the structural determinants of poly(P) utilization must be identified.

From the standpoint of evolution, poly(P) is a plausible source of nucleoside triphosphate, i.e., an ancient energy carrier, as mentioned above. We have assumed that NADKs evolved from poly(P)/ATP-NADKs into ATP-specific NADKs, which prefer ATP to poly(P), and that recently diverged organisms obtained ATP-specific NADKs during their evolution, based on the following: (i) it has been deduced that bacteria evolved from Gram-positive bacteria or Archaea into Gram-negative γ -proteobacteria in the following order: Gram-positive bacteria (low G + C) (\Leftarrow Archaea) \Rightarrow Gram-positive bacteria (high G + C) \Rightarrow others (Gram-negative bacteria except for proteobacteria) \Rightarrow Gram-negative ϵ - and δ -proteobacteria \Rightarrow Gram-negative α -proteobacteria \Rightarrow Gram-negative β -proteobacteria \Rightarrow Gram-negative γ -proteobacteria^{6,7}; (ii) poly(P)/ATP-NADKs are distributed throughout Gram-positive bacteria (e.g. *Mycobacterium tuberculosis* and *Bacillus subtilis*) and Archaea (*Methanococcus jannaschii* and *Pyrococcus horikoshii*), whereas ATP-specific NADKs are found in Gram-negative α -proteobacterium (*Sphingomonas* sp. A1), Gram-negative γ -proteobacteria (*Escherichia coli* and *Salmonella enterica*), and eukaryotes including fungi, plants, and human^{8–19}. However, it remains unclear how NADKs



evolved from poly(P)/ATP-NADKs into ATP-specific NADKs. Identification of the structural determinants of poly(P) utilization, i.e. of phosphoryl-donor specificity, would give insights into the evolution of NADKs.

In order to elucidate the structural determinants of poly(P) utilization by NADKs, we focused on ATP-specific NADKs of Gram-negative γ -proteobacteria (e.g. *E. coli* and *Vibrio cholerae*), the most recently diverged group⁶. In this study, we successfully conferred the ability to utilize poly(P) on ATP-specific NADKs of *E. coli* and *V. cholerae* (ecoNADK and vchNADK) via a single substitution of a specific conserved amino-acid residue. We thereby identified the structural determinant of poly(P) utilization by γ -proteobacterial NADKs, and gained insight into how bacterial NADKs evolved from poly(P)/ATP-NADKs into γ -proteobacterial ATP-specific NADKs.

Results

Initial failure to confer the ability to utilize poly(P) on ATP-specific NADK. ATP-specific NADKs are present in Gram-negative α - and γ -proteobacteria and eukaryotes, whereas poly(P)/ATP-NADKs are found in Archaea and Gram-positive bacteria^{8–19}. BLASTP analysis revealed that the primary structures of the two types of NADKs are similar. The structure of human ATP-specific NADK is similar to those of the ATP-specific NADKs of Gram-negative γ -proteobacteria (*E. coli* ecoNADK: 49% similarity over 259 residues, $E = 3e-22$; *S. enterica* styNADK: 49% over 259 residues, $E = 9e-23$; *V. cholerae* vchNADK: 48% over 330 residues, $E = 1e-28$) and to those of the poly(P)/ATP-NADKs of an archaeon (*P. horikoshii* PH1074: 46% similarity over 277 residues, $E = 4e-18$) and a Gram-positive bacterium (*M. tuberculosis* Ppnk: 49% similarity over 228 residues, $E = 1e-18$). Despite these similarities, it is difficult to identify the key conserved amino-acid residues specific to all ATP-specific NADKs or to all poly(P)/ATP-NADKs (Fig. S1).

In order to confer the ability to utilize poly(P) on ATP-specific ecoNADK, we constructed mutants of ecoNADK (ecoNADK E36A, E163V, R232D, L259E, E36A/E136V, R232D/L259E, E36A/E136V/R232D/L259E, R95L, N97R, and R95L/N97R) (Fig. 1). We targeted Arg-95 and Asn-97 of ecoNADK because these residues correspond to Leu-107 and Arg-109 of Ppnk, which are probably located near the 2'-hydroxyl group of NAD⁺ in the crystal structure of Ppnk complexed with NAD⁺ (PDB ID: 1Y3I). Other amino-acid residues were selected based on comparisons of the primary structures of ATP-specific NADKs with those of poly(P)/ATP-NADKs (Fig. S1). However, the poly(P)-dependent NADK activity of ecoNADK was not increased by any of these substitution; thus, in these experiments, we failed to confer the ability to utilize poly(P) on ATP-specific NADK.

The highly conserved GGDGN motif in the primary structures of γ -proteobacterial NADKs. The GGDG motif is highly conserved in the primary structures of NADKs (Fig. S1), probably participates in catalysis, and is also conserved in diacylglycerol kinases and 6-phosphofructokinases^{20,21}. We noticed that the longer motif GGDGN (GGDG plus N) is conserved in the primary structures of the γ -proteobacterial NADK homologs ecoNADK, styNADK, and vchNADK (Fig. S1), in which the Asn residues in the GGDGN motif are Asn-75, Asn-75, and Asn-76, respectively. Moreover, the GGDGN motif is found in 292 NADK homologs, among which 291 are restricted to γ -proteobacteria, the most recently diverged bacteria (Table 1)⁶; the remaining one is from an ϵ -proteobacterium (*Campylobacter fetus*; Table 1). By contrast, the GGDGT motif is much more prevalent in NADK homologs, including both ATP-specific NADKs and poly(P)/ATP-NADKs: it is found in 254 of 286 eukaryotic NADK homologs, 1,100 of 1,836 bacterial NADK homologs, and 124 of 127 archaeal NADK homologs (Table 1). Due to the high conservation of the GGDGN motif in the most

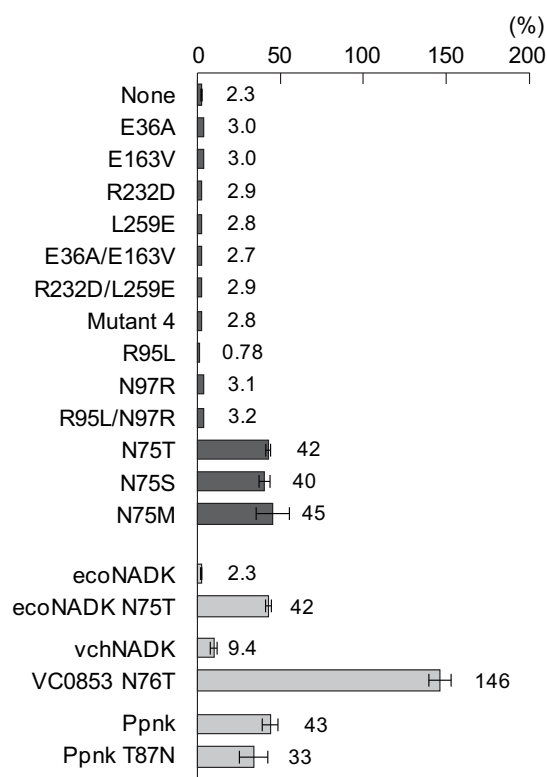


Figure 1 | Changes of the poly(P)-dependent NADK activity via mutations. The relative poly(P)-dependent NADK activity compared to ATP-dependent NADK activity of NADKs are depicted as bars and given numerically to the right of the bars. ATP-dependent NADK activity was relatively taken as 100%. Meta(P)_n was used as poly(P). Dark-gray bars indicate the activity of crude ecoNADK mutants, whose activity was assayed using crude extracts of *E. coli* expressing these mutants; light-gray bars indicate the activity of purified NADKs. Mutant 4, ecoNADK E36A/E163V/R232D/L259E. Means and standard deviations of three independent experiments for crude ecoNADKs and purified NADKs are shown, except in the case of crude NADK, which exhibited no effect upon substitution.

recently diverged γ -proteobacterial NADK homologs, which are presumably ATP-specific NADKs, we decided to clarify the role of this residue by converting the Asn residues of ecoNADK (Asn-75) and vchNADK (Asn-76) to Thr residues.

Success in conferring the ability to utilize poly(P) on γ -proteobacterial ATP-specific NADK. Replacement of ecoNADK Asn-75 with Thr conferred the ability to utilize poly(P) on this enzyme. As a result of the substitution, the specific activity (U/mg) of ecoNADK for ATP decreased from 27 ± 2 to 6.4 ± 0.3 , whereas its specific activities for poly(P)s increased: from 0.28 ± 0.004 to 1.6 ± 0.1 for tetrapolyphosphate [poly(P)₄], 0.65 ± 0.02 to 2.7 ± 0.04 for hexametaphosphate [hexameta(P)_n], and 0.63 ± 0.06 to 2.7 ± 0.1 for metaphosphate [meta(P)_n]; however, the specific activity for triphosphate [poly(P)₃] decreased from 0.24 ± 0.01 to 0.22 ± 0.002 (Fig. 2). The specific activities (U/mg) of vchNADK exhibited similar behavior, decreasing from 11 ± 1 to 4.4 ± 0.1 for ATP, but increasing from 0.091 ± 0.008 to 0.37 ± 0.10 for poly(P)₃, 0.50 ± 0.04 to 2.8 ± 0.05 for poly(P)₄, 0.75 ± 0.07 to 6.1 ± 0.1 for hexameta(P)_n, and 1.0 ± 0.1 to 6.5 ± 0.4 for meta(P)_n. Neither pyrophosphate nor trimetaphosphate was utilized as a phosphoryl donor by either NADK (data not shown). These N => T amino-acid substitutions increased the relative meta(P)_n-dependent NADK activity compared to ATP-dependent NADK activity, from 2.3 ± 0.4 to 42 ± 1 (%) (ecoNADK) and from 9.4 ± 1.4 to 146 ± 7 (%)

Table 1 | Amino-acid residue corresponding to Asn-75 of ecoNADK^a

| | Amino-acid residue | | | | | | | | | | Total |
|--|--------------------|------|----|-----|----|-----|----|----|----|----------------|-------|
| | N | T | M | S | A | F | L | V | E | X ^b | |
| Eukaryotic NADKs | - | 254 | - | 1 | - | - | 3 | 19 | - | 9 | 286 |
| Archaeal NADKs | - | 124 | - | 3 | - | - | - | - | - | - | 127 |
| Bacterial NADKs ^c | 292 | 1100 | 80 | 120 | 35 | 115 | 45 | 3 | 38 | 8 | 1836 |
| Gram-positive bacteria (Low G + C) | - | 324 | 79 | 7 | 26 | - | - | - | 1 | - | 437 |
| Gram-positive bacteria (High G + C) | - | 204 | - | 17 | - | - | - | - | - | 1 | 222 |
| Others | - | 283 | - | 17 | 4 | 4 | 2 | - | - | - | 310 |
| δ-proteobacteria | - | 49 | - | - | - | 3 | - | - | - | - | 52 |
| ε-proteobacteria | 1 | 71 | - | - | - | - | - | - | - | - | 72 |
| α-proteobacteria | - | 31 | 1 | - | 5 | 85 | 43 | 3 | 37 | 3 | 208 |
| β-proteobacteria | - | 117 | - | - | - | - | - | - | - | - | 117 |
| γ-proteobacteria ^d | 291 | 21 | - | 79 | - | 23 | - | - | - | 4 | 418 |
| Others | 8 | - | - | 6 | - | - | - | - | - | 6 | 20 |
| <i>Thiotrichales</i> | 15 | 4 | - | - | - | - | - | - | - | - | 19 |
| <i>Xanthomonadales</i> | - | - | - | - | - | 23 | - | - | - | - | 23 |
| <i>Methylococcales</i> and <i>Chromatiales</i> | - | 12 | - | - | - | - | - | - | - | - | 12 |
| <i>Legionellales</i> | - | - | - | 12 | - | - | - | - | - | - | 12 |
| <i>Pseudomonadales</i> | - | - | - | 46 | - | - | - | - | - | - | 46 |
| <i>Oceanospirillales</i> | - | - | - | 7 | - | - | - | - | - | - | 7 |
| <i>Alteromonadales</i> | 30 | - | - | 6 | - | - | - | - | - | 3 | 39 |
| <i>Aeromonadales</i> | 3 | - | - | - | - | - | - | - | - | - | 3 |
| <i>Vibrionales</i> | 40 | - | - | - | - | - | - | - | - | - | 40 |
| <i>Pasteurellales</i> | 36 | - | - | 2 | - | - | - | - | - | - | 38 |
| <i>Enterobacteriales</i> | 159 | - | - | - | - | - | - | - | - | - | 159 |

^aNumber of NADKs that possess the indicated amino-acid residue at the position aligned with Asn-75 of ecoNADK, i.e., the position corresponding to the Asn residue in GGDGN. Almost all NADKs (254 of 286 eukaryotic NADKs, 1,100 of 1,836 bacterial NADKs, and 124 of 127 archaeal NADKs) have a Thr residue at this position.

^bX; Y, H, G, I, or W.

^cThe number of NADKs of each bacterial class is shown under the line below "Bacterial NADKs". The bacterial classes are listed in branching order; γ-proteobacteria are the most recently diverged group⁸.

^dThe number of NADKs of each γ-proteobacterial order is shown under the line below "γ-proteobacteria". The γ-proteobacterial orders are listed in branching order; *Enterobacteriales* are the most recently diverged group²². We also inspected the numbers and distribution of the critical amino-acid residues of γ-proteobacterial NADKs (Table S1).

(vchNADK) (Fig. 1). TLC analysis confirmed that ecoNADK N75T produced a larger quantity of NADP⁺ than ecoNADK in the presence of meta(P)_n as a phosphoryl donor (Fig. 3).

Accordingly, this single substitution increased the k_{cat} values of each enzyme for poly(P)₄ while decreasing the k_{cat} value of ecoNADK for ATP (Table 2). The K_m values of ecoNADK and in particular

vchNADK for poly(P)₄ were decreased, whereas the K_m values of each enzyme for ATP were either approximately the same (ecoNADK) or slightly higher (vchNADK) (Table 2). Consequently, ecoNADK N75T and vchNADK N76T lost their specificity for ATP and acquired the ability to utilize poly(P) as a phosphoryl donor, at levels comparable to or higher than that of Ppnk (Fig. 2, Table 2). Furthermore, replacing

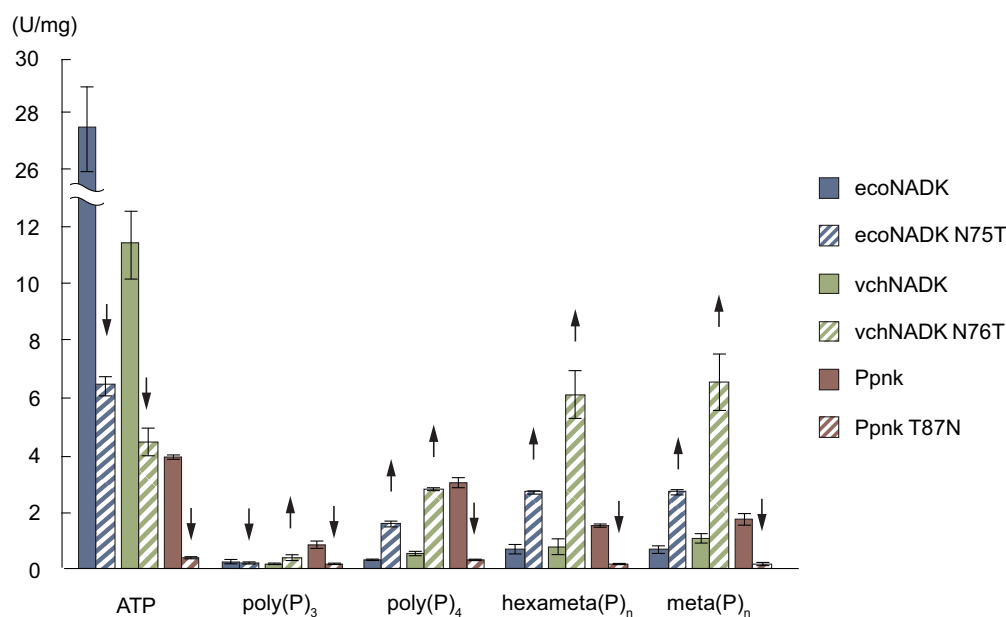


Figure 2 | Biotechnological manipulation to confer the ability to utilize poly(P) on an ATP-specific NADK. Specific activity of purified NADKs is shown. Phosphoryl donors were used at 5 mM, except for hexameta(P)_n and meta(P)_n, which were used at 1.0 mg/ml. Up and down arrows respectively indicate increased and decreased NADK activities upon each substitution. Means and standard deviations of three independent experiments are shown.

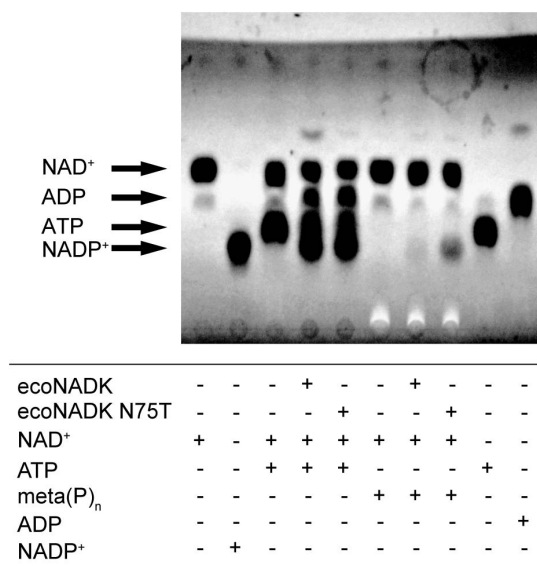


Figure 3 | ecoNADK N75T produces NADP⁺ from NAD⁺ and poly(P). NADK reaction mixtures (5 μ l) were incubated at 37°C for 10 h and analyzed on TLC plates. The reaction mixture was as follows: 5 mM NAD⁺, 5 mM MgCl₂, 100 mM Tris-HCl (pH 7.5), 5 mM ATP or 1.0 mg/ml meta(P)_n, and 0.43 μ g/ml purified ecoNADK or ecoNADK N75T. Standard controls were 5 mM NAD⁺, 5 mM NADP⁺, 5 mM ATP, and 5 mM ADP (5 μ l each); their positions are shown by arrows.

Asn-75 with Ser or Met in ecoNADK, resulting in ecoNADK N75S or N75M, also increased the relative meta(P)_n-dependent NADK activity compared to ATP-dependent NADK activity, from 2.3 ± 0.4 to 40 ± 3 (N75S) or 45 ± 10 (%) (N75M) (Fig. 1). Collectively, these are the first successes in biotechnologically conferring the ability to utilize poly(P) on an ATP-specific kinase. We conclude that this Asn residue is important in determining the phosphoryl-donor specificity of γ -proteobacterial NADKs containing the GGDGN motif; the presence of a Thr, Ser, or Met residue at the Asn position is critical for poly(P) utilization by these NADKs.

Conversely, upon substituting Thr-87 to Asn in Ppnk, which can utilize ATP or poly(P), we predicted that the substituted Ppnk would lose poly(P)-dependent activity and acquire higher ATP-dependent activity. However, the purified Ppnk T87N did not acquire higher ATP-dependent NADK activity, but rather lost this activity, although poly(P)-dependent NADK activity was also decreased (Fig. 1 and 2, Table 2). The k_{cat} values for ATP and poly(P)₄ were decreased upon replacement, while the K_m values for ATP and poly(P)₄ were increased, suggesting that the Thr residue in the GGDGT motif in Ppnk (and probably those of other poly(P)/ATP-NADKs) is critical for not only poly(P)-dependent activity but also ATP-dependent NADK activity.

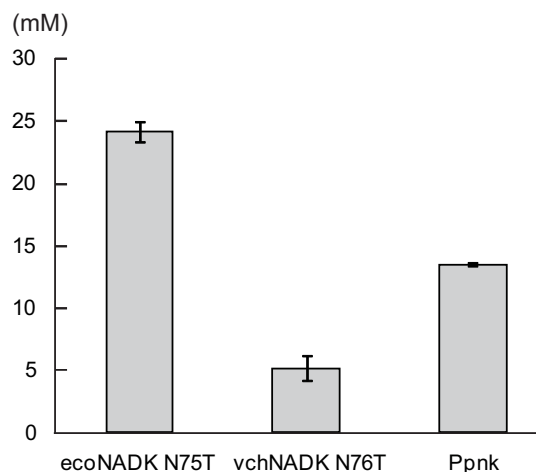


Figure 4 | Mass production of NADP⁺ by NADKs. The amounts of NADP⁺ produced by immobilized *E. coli* cells expressing ecoNADK N75T, vchNADK N76T, or Ppnk in the presence of 50 mM NAD⁺ and 100 mg/ml meta(P)_n are shown. Cell extracts of *E. coli* expressing ecoNADK N75T, vchNADK N76T, or Ppnk exhibited poly(P)-dependent NADK activities of 0.48, 0.94, or 0.089 (U/mg), respectively. Detailed reaction conditions are described in Methods. Means and standard deviations of three independent experiments are shown.

EcoNADK N75T is more suitable for mass production of NADP⁺ than Ppnk. A poly(P)-dependent industrial NADP⁺-production system using Ppnk has been established⁴. In order to determine whether ecoNADK N75T and vchNADK N76T are more suitable for mass production of NADP⁺ than Ppnk, we compared the efficiency of producing NADP⁺ using each of these three enzymes. *E. coli* cells overexpressing each NADK were immobilized and incubated for 16 h at 37°C in a reaction mixture containing 50 mM NAD⁺ and 100 mg/ml meta(P)_n. Cells expressing ecoNADK N75T, vchNADK N76T, and Ppnk produced 24 mM, 5.2 mM, and 13 mM NADP⁺, respectively (Fig. 4). ecoNADK N75T produced more NADP⁺ than Ppnk and is much more suitable for mass production of NADP⁺ than Ppnk. Therefore, our method for conferring the ability to use poly(P) on ATP-specific NADK is also applicable to practical and industrial mass production of NADP⁺. It should be noted that cells expressing vchNADK N76T produced less NADP⁺ than those expressing ecoNADK N75T, although the specific activity and expression level of vchNADK N76T were higher than those of ecoNADK N75T (Fig. 2 and Fig. S4). We confirmed that NADP⁺ strongly inhibits the poly(P)-dependent NADK activity of vchNADK N76T, but not that of ecoNADK N75T. In the presence of 0.20 mM NADP⁺, the residual poly(P)-dependent NADK activity of vchNADK N76T was 39%, but no inhibition of the activity of ecoNADK N75T was detected. Thus, the lower production of

Table 2 | Kinetic parameters of NADKs

| | ATP | | | poly(P) ₄ | | |
|--------------|-----------------|---------------|-------------------|----------------------|------------------|-------------------|
| | K_m | k_{cat} | k_{cat}/K_m | K_m | k_{cat} | k_{cat}/K_m |
| ecoNADK | 1.1 ± 0.1 | 3.7 ± 0.7 | 19 ± 1 | 3.7 ± 0.7 | 0.26 ± 0.03 | 0.070 ± 0.008 |
| ecoNADK N75T | 0.94 ± 0.01 | 2.5 ± 0.4 | 4.2 ± 0.4 | 2.5 ± 0.4 | 1.2 ± 0.1 | 0.50 ± 0.04 |
| vchNADK | 1.2 ± 0.3 | 2.9 ± 0.8 | 5.7 ± 0.5 | 2.9 ± 0.8 | 0.40 ± 0.05 | 0.14 ± 0.02 |
| vchNADK N76T | 2.2 ± 0.2 | 3.1 ± 0.1 | 1.4 ± 0.1 | 1.0 ± 0.2 | 1.8 ± 0.1 | 1.8 ± 0.1 |
| Ppnk | 3.2 ± 0.3 | 2.5 ± 0.5 | 1.0 ± 0.05 | 2.5 ± 0.5 | 2.4 ± 0.2 | 0.94 ± 0.09 |
| Ppnk T87N | 6.0 ± 2.1 | 2.5 ± 0.9 | 0.059 ± 0.016 | 4.2 ± 0.8 | 0.26 ± 0.003 | 0.062 ± 0.008 |

Data used to determine k_{cat} and K_m are included in the Supporting information (Fig. S2 and S3).



NADP⁺ by the cells expressing vchNADK N76T could be at least partially attributed to inhibition of the poly(P)-dependent NADK activity of vchNADK N76T by NADP⁺.

Discussion

NADKs with the GGDGN motif are restricted to γ -proteobacteria (Table 1). It has been deduced that γ -proteobacteria evolved in the following order: *Chromatiales*, *Methylococcales*, *Xanthomonadales*, *Thiotrichales* => *Oceanospirillales*, *Pseudomonadales* => *Alteromonadales* => *Vibrionales*, *Aeromonadales* => *Pasteurellales* => *Enterobacteriales* (Fig. 5)²². Remarkably, almost all of the γ -proteobacterial NADKs carrying the GGDGN motif are further concentrated within the most recently diverged group within γ -proteobacteria (*Alteromonadales*, *Vibrionales*, *Aeromonadales*, *Pasteurellales*, and *Enterobacteriales*; Fig. 5, Table 1). In addition, the γ -proteobacterial phylogenetic tree and the distribution of the critical amino-acid residues (e.g. Asn-75 of ecoNADK) among γ -proteobacterial NADKs imply that in γ -proteobacterial NADKs, the motif evolved from GGDGT via GGDGS into GGDGN, in which the underlined residue evolved in the order Thr => Ser => Asn (Fig. 5 and 6, Table 1). Because ecoNADK N75T and N75S are poly(P)/ATP-NADKs (Fig. 1) and ecoNADK is an ATP-specific NADK (Fig. 1)¹³, it can be deduced that γ -proteobacterial NADKs evolved from poly(P)/ATP-NADKs (carrying GGDGT) via poly(P)/ATP-NADKs (GGDGS) into ATP-specific NADKs (GGDGN) such as ecoNADK and vchNADK (Fig. 5 and 6). The evolution of the codons (corresponding to the critical amino-acid residues) to reach GGDGN, in which Asn is coded by ATT/C, may therefore have been as follows: ACT/C (Thr) => AGT/C (Ser) => AAT/C (Asn), in which the underlined nucleotide evolved in the order C => G => A (Fig. 5 and 6).

Among prokaryotic NADKs, many archaeal and bacterial NADKs contain the GGDGT motif (Table 1). Because NADKs containing the GGDGT motif in Archaea (*P. horikoshii* PH1074 and *M. jannaschii* MJ_0917), Gram-positive bacteria (*B. subtilis* bsu_11610 and *M. tuberculosis* Ppnk), and ecoNADK N75T are poly(P)/ATP-NADKs (Fig. 1)^{9–12}, we can deduce that all prokaryotic NADKs containing the GGDGT motif, except for some α -proteobacterial NADKs discussed below, are also poly(P)/ATP-NADKs. Moreover, the 124 NADKs containing the GGDGS motif are concentrated within Gram-positive bacteria (24 enzymes) and γ -proteobacteria (79 enzymes), but are not found in other proteobacteria (Table 1). Likewise, the 80 NADKs containing the GGDGM motif are almost entirely restricted to Gram-positive bacteria (79 enzymes), with the sole exception in a α -proteobacterium (Table 1). The observation that ecoNADK N75S and ecoNADK N75M are poly(P)/ATP-NADKs (Fig. 1) further supports the idea that bacterial NADKs with the GGDGS or GGDGM motif are poly(P)/ATP-NADKs. Taken together, these observations suggest that almost all NADKs of Archaea and bacteria that branched at deeper point than γ -proteobacteria^{6,7}, are poly(P)/ATP-NADKs. Therefore, we propose that NADKs evolved from poly(P)/ATP-NADKs (carrying GGDGT) via Gram-negative γ -proteobacterial poly(P)/ATP-NADKs (carrying GGDGS) into Gram-negative γ -proteobacterial ATP-specific NADKs (carrying GGDGN) over the course of bacterial evolution (Fig. 6).

To reach the GGDGN motif, in which Asn is encoded by AAT/C, the codon evolution could have proceeded as follows: ACT/C (Thr) => AGT/C (Ser) => AAT/C (Asn), in which the underlined nucleotide evolved in the order C => G => A (Fig. 5 and 6); i.e., a poly(P)/ATP-NADK carrying the GGDGT motif in which the Thr residue is encoded by ACT/C, but not by ACA/G, may be the direct ancestor of γ -proteobacterial ATP-specific NADKs carrying the GGDGN motif. In other words, a poly(P)/ATP-NADK carrying the GGDGM motif, in which the Met residue is encoded by ATG, was not the direct ancestor.

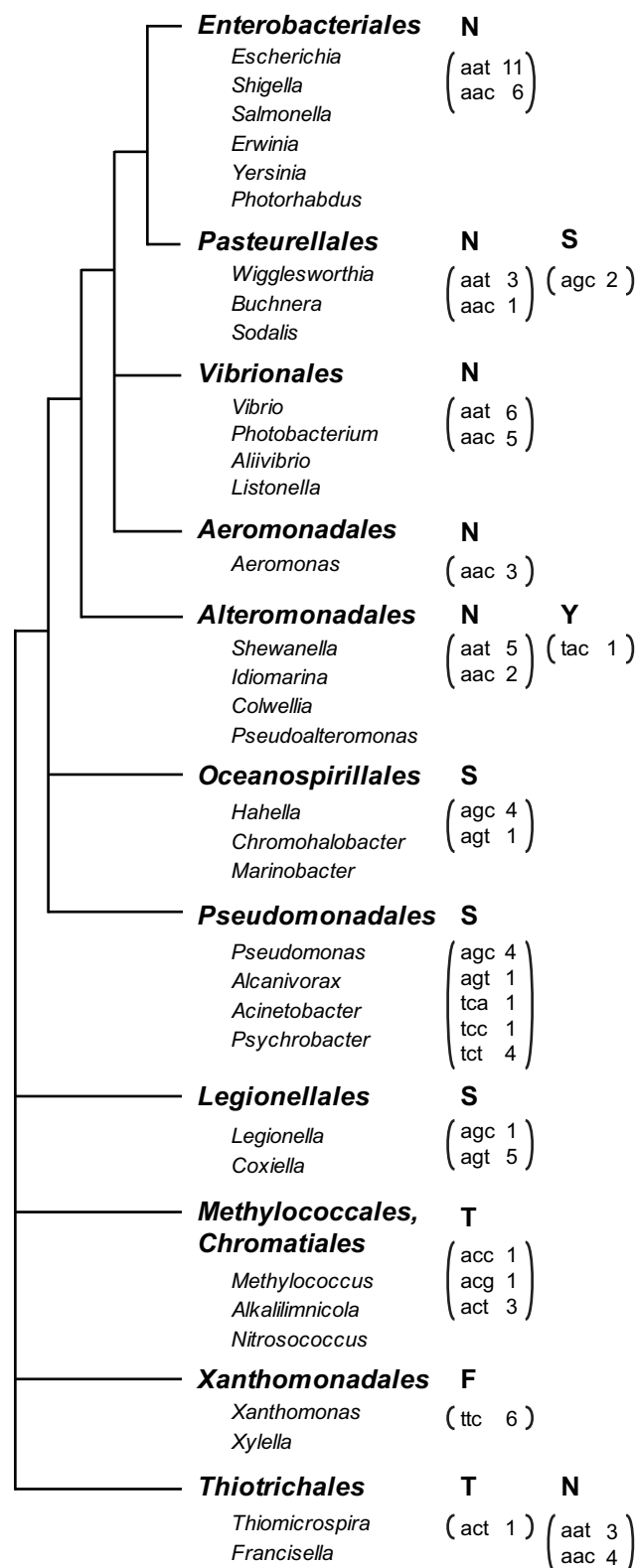


Figure 5 | The phylogenetic tree of γ -proteobacteria. The tree was constructed based on the original tree, reported previously²². Data regarding branch length and evolutionary distance are not shown. Under each order (boldface), genera are listed. The amino-acid residue corresponding to the Asn-75 of ecoNADK in NADKs of bacteria in each order is shown in boldface, and the codon encoding the residue is in parenthesis. The numbers of bacterial species that have these codons are also presented in parentheses. At most three species were randomly selected from each genus.

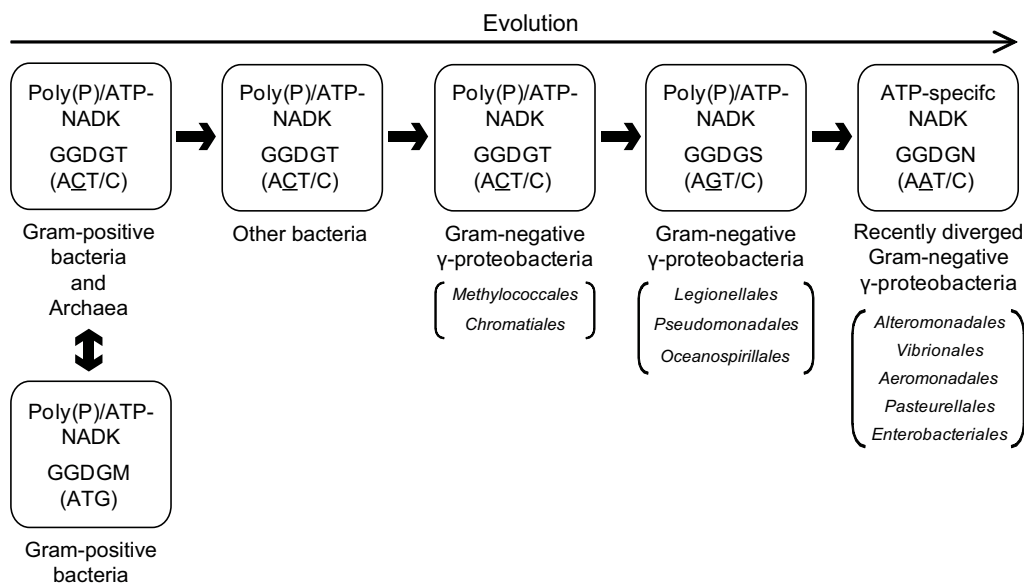


Figure 6 | The proposed evolution of bacterial NADK from poly(P)/ATP-NADK into γ -proteobacterial ATP-specific NADK. It has been deduced that bacteria evolved from Gram-positive bacteria or Archaea into Gram-negative γ -proteobacteria, within which the orders of *Alteromonadales*, *Vibrionales*, *Aeromonadales*, *Pasteurellales*, and *Enterobacteriales* are the most recently diverged group^{6,7,22}. As described in the text in detail, we propose that the motif evolved from GGDGT via GGDGS into GGDGN during bacterial evolution, and that the codon evolved from ACT/C (Thr) via AGT/C (Ser) into AAT/C (Asn), in which the underlined nucleotide evolved, to reach the GGDGN motif. Therefore, we suggest that poly(P)/ATP-NADKs carrying the GGDGT motif, in which the Thr residue is encoded by ACT/C, but not by ACA/G, are the direct ancestor of γ -proteobacterial ATP-specific NADKs carrying the GGDGN motif, in which Asn is encoded by AAT/C. We speculate that poly(P)/ATP-NADK carrying the GGDGM motif (in which the Met residue is encoded by ATG), which is concentrated within Gram-positive bacteria (Table 1), is not the direct ancestor of γ -proteobacterial ATP-specific NADKs.

α -Proteobacterial NADKs contain not only the GGDGT motif but also various other motifs such as GGDGF, GGDGL, and GGDGE; in other words, these motifs are characteristic of α -proteobacterial NADKs. Hence, α -proteobacterial NADKs may have evolved in a different manner than γ -proteobacterial NADKs. In this regard, it is noteworthy that NADK of α -proteobacterial *Sphingomonas* sp. A1 contains the GGDGT motif but is an ATP-specific NADK⁸. NADK of human mitochondria, believed to have originated from α -proteobacteria²³, carries the GGDGT motif and is a poly(P)/ATP-NADK²⁴.

By contrast, NADKs of eukaryotes (e.g., human and plants), with the exception of the human mitochondrial poly(P)/ATP-NADK noted above²⁴, act as ATP-specific NADKs despite the fact that they contain the GGDGT motif^{15–19}. By the same logic applied above, it could therefore be assumed that the eukaryotic NADKs have also evolved into ATP-specific NADKs in a different manner than the bacterial NADKs depicted in Fig. 6. Hence, the method used in this study for conferring the ability to utilize poly(P) on ATP-specific NADKs is probably not universally applicable to NADKs of all organisms.

Nonetheless, this study reports for the first time a method for successfully conferring the ability to utilize poly(P) on ATP-specific NADKs. Furthermore, our findings demonstrate that the poly(P)/ATP-NADK created through this method is much more suitable for poly(P)-dependent mass production of NADP⁺ than Ppnk, which exhibits inherent poly(P)-dependent NADK activity and has been employed for industrial mass production of NADP⁺ (Fig. 4). This study also provides a significant clue regarding how NADKs recognize and utilize poly(P), although the detailed mechanism remains to be determined. Determination and detailed analysis of the crystal structures of ecoNADK and its derivatives (ecoNADK N75T, N75S, and N75M) would contribute significantly to the elucidation of this mechanism.

Methods

Homology and sequence-alignment analysis. To find proteins homologous to NADKs from all species whose genomes have been sequenced, and to determine the

amino-acid residues corresponding to Asn-75 of ecoNADK, we used the BLAST²⁵ and CLUSTALW²⁶ programs. Both programs are available on the GenomeNet server (<http://www.genome.jp>).

Expression and purification of NADK. The vchNADK gene was synthesized with an *Nco*I site at the 5'-terminus and a *Bam*HI site at the 3'-terminus. The synthesized vchNADK gene was ligated into pET-14b digested with *Nco*I and *Bam*HI using Ligation High Ver.2 (Toyobo, Osaka, Japan). The resultant plasmid was designated pET-14b_vchNADK. The synthesized vchNADK gene encodes amino acids from Met-1 to Phe-294, but in order to add the *Nco*I site to 5'-terminus, Lys-2 (codon, AAA) of the native vchNADK was replaced with Glu-2 (codon, GAA). The plasmids used for overexpression of ecoNADK and *M. tuberculosis* NADK (Ppnk) were pET-14b_ecoNADK and pET-3a_Ppnk, described previously^{9,13}. NADK mutants were constructed with a KOD -Plus- Mutagenesis kit (Toyobo), but using KOD -Plus- Neo polymerase (Toyobo). The plasmid pET-14b_ecoNADK, pET-14b_vchNADK, or pET-3a_Ppnk was used as the PCR template. Nucleotide sequences of the resultant genes were confirmed by DNA sequencing. All wild-type or mutated NADKs were expressed without any tags in *E. coli* BL21(DE3)pLysS (Novagen) and purified to homogeneity as described^{9,13}, except that KNDE buffer [10 mM potassium phosphate (pH 7.0), 0.1 mM NAD⁺, 0.5 mM dithiothreitol, and 1 mM ethylenediaminetetraacetic acid] was used instead of KND buffer [10 mM potassium phosphate (pH 7.0), 0.1 mM NAD⁺, 0.5 mM dithiothreitol]. Protein concentration was determined by the Bradford method²⁷, and BSA (Sigma-Aldrich, St. Louis, MO, USA) was used for a standard.

Assay of NADK activity. ATP- or poly(P)-dependent NADK specific activities were continuously assayed at 37°C as described²⁴, in a reaction mixture containing 5 mM NAD⁺ (Oriental Yeast, Tokyo, Japan), 5 mM MgCl₂, 100 mM Tris-HCl (pH 7.5), phosphoryl donor, 5 mM glucose 6-phosphate (Nacalai Tesque, Kyoto, Japan), 0.5 U glucose 6-phosphate dehydrogenase (Sigma-Aldrich), and NADK. As phosphoryl donors, ATP (Oriental Yeast) and poly(P) [pyrophosphate (Nacalai Tesque), poly(P)₃ (Sigma-Aldrich), poly(P)₄ (Showa Chemical, Tokyo, Japan), and trimetaphosphate (Sigma-Aldrich)] were used at 5 mM; hexameta(P)_n (Wako, Osaka, Japan) and meta(P)_n (Wako) were used at 1 mg/ml. Hexameta(P)_n and meta(P)_n are mixtures of poly(P) with various chain lengths. Poly(P) [meta(P)_n]-dependent NADK activity was also assayed by a stop method²⁴ to determine the inhibitory effects of NADP⁺. Briefly, the amount of NADP⁺ formed was determined enzymatically with 0.5 U glucose-6-phosphate dehydrogenase after the reaction was terminated by immersing the test tube in boiling water for 5 min; control reactions without NADK were simultaneously performed in every assay. One unit of enzyme activity was defined as 1 μ mol NADP⁺ produced in 1 min at 37°C; specific activity was expressed in U/mg protein. TLC was performed using a solvent system of 5 : 3 (v/v) isobutyrate and 500 mM NH₄OH, and compounds were detected at 254 nm²⁸.



Kinetic parameters were calculated by fitting the data to the appropriate Michaelis-Menten equations using KaleidaGraph software (Synergy Software).

Immobilization of cells in polyacrylamide gel. *E. coli* cells expressing NADKs were immobilized to produce NADP⁺ as previously reported⁴. Cells of *E. coli* BL21(DE3)pLysS containing pET-14b_ecoNADK N75T, pET-14b_vchNADK N76T, or pET-3a_Ppnk cultured as above were collected by centrifugation and washed twice with cold 20 mM Tris-HCl (pH 7.5). Cells (1.0 g wet weight) were suspended in 1.5 ml of 100 mM Tris-HCl (pH 7.5). The cell suspension was thoroughly mixed with 1.5 ml of an acrylamide solution (30% acrylamide, 0.6% N,N'-methylenebisacrylamide, 0.25% N,N,N',N'-tetramethylethylenediamine, and 0.25% ammonium persulfate), and then kept at 0°C for 30 min. The resulting gel was cut into cubes (3.0 mm × 3.0 mm × 3.0 mm), suspended in 30 ml of acetone, and then incubated at 0°C for 5 min with gentle stirring. The gel was washed three times with cold 5 mM Tris-HCl (pH 7.5), and then kept at 4°C in the same buffer supplemented with 0.1 mM NAD⁺ and 0.1 mM MgCl₂ until use.

Mass production of NADP⁺. The NADP⁺-production reaction was carried out at 37°C with shaking in 1.0 ml of a reaction mixture consisting of 50 mM NAD⁺, 100 mM MgCl₂, 200 mM HEPES (pH 8.0), 100 mg/ml meta(P)_n, and 0.1 g (wet weight) immobilized cells. After 16 h of reaction, 50 μl of the reaction mixture was withdrawn and boiled for 5 min to stop the enzymatic reaction. The amount of NADP⁺ in the reaction mixture was enzymatically determined as described²⁴.

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Author contributions

Y.N., A.Y. and S.K. designed and performed the experiments. Y.N., S.K. and K.M. wrote, reviewed, and edited the manuscript.

Additional information

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