Molecular Conversion of NAD Kinase to NADH Kinase through Single Amino Acid Residue Substitution*

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NAD kinase phosphorylates NAD\(^+\) to form NADP\(^+\) and is strictly specific to NAD\(^+\), whereas NADH kinase phosphorylates both NAD\(^+\) and NADH, thereby showing relaxed substrate specificity. Based on their primary and tertiary structures, the difference in the substrate specificities between NAD and NADH kinases was proposed to be caused by one aligned residue: Gly or polar amino acid (Gln or Thr) in five NADH kinases and a charged amino acid (Arg) in two NAD kinases. The substitution of Arg with Gly in the two NADH kinases relaxed the substrate specificity, whereas substitution with charged and hydrophobic amino acids did not show a similar result. In contrast, the substitution of Gly with Arg in one NAD kinase failed to convert it to NAD kinase. These results suggest that a charged or hydrophobic amino acid residue in the position of interest is crucial for strict specificity of NAD kinases to NAD\(^+\), whereas Gly or polar amino acid residue is not the sole determinant for the relaxed substrate specificity of NADH kinases. The significance of the conservation of the residue at the position in 207 NAD kinase homologues is also discussed.

NAD\(^+\) and NADH are almost exclusively involved in catalytic reactions, whereas NADP\(^+\) and NADPH primarily participate in anabolic reactions and cellular defense against oxidative stress (1, 2). A recent study has revealed that in addition to these functions, NAD\(^+\) is also used as a substrate for mono- and poly-ADP ribosylations and for the formation of cyclic ADP-ribose, dimeric ADP-ribose, and nicotinic acid adenine nucleotide (3). Thus, the regulation of the cellular concentrations of NAD\(^+\), NADP\(^+\), and their respective reduced equivalents (NADH and NADPH) is considered to govern numerous cellular biological processes.

NAD kinase (EC 2.7.1.23) catalyzes the formation of NADP\(^+\) through the phosphorylation of NAD\(^+\) and is a key enzyme required for the regulation of cellular concentrations of NAD\(^+\) and NADP\(^+\) (4). Based on the phosphoryl donor specificity, NAD kinase is divided into two types: inorganic polyphosphate (poly(P))\(^1\)/ATP-NAD kinase and ATP-NAD kinase (5, 6). Poly(P) is a linear polymer of orthophosphate residues linked by phosphoanhydride bonds (7). Poly(P)/ATP-NAD kinase utilizes both poly(P) and ATP as phosphoryl donors, whereas ATP-NAD kinase is specific for ATP. Poly(P)/ATP-NAD kinase has been identified in the prokaryotes of the actinobacteria such as *Mycobacterium tuberculosis* and *Micrococcus flavus*, and is designated as Ppnk (*M. tuberculosis*) and Mfnk (*M. flavus*) (5). ATP-NAD kinase has been identified in the prokaryotes of the proteobacteria such as *Escherichia coli* and *Sphingomonas sp.* A1 and in eukaryotes such as the yeast *Saccharomyces cerevisiae* and are designated as YjB (*E. coli*); NadK (*Sphingomonas sp.* A1); and Utr1p, Yef1p, and Pos5p (*S. cerevisiae*) (2, 6, 8–10). Among the NAD kinases, the tertiary structures of Ppnk (11, 12) and those of Ppnk complexed with NAD\(^+\) (Ppnk-NAD) (11) have been determined.

NADH kinase (EC 2.7.1.23) catalyzes the formation of NADPH through the phosphorylation of NADH and is involved in the regulation of cellular concentrations of NADH and NADPH (2). At least three ATP-NAD kinases of *S. cerevisiae* (Utr1p, Yef1p, and Pos5p) exhibit NADH kinase activity (2, 10) in addition to NAD kinase activity (i.e. they show relaxed substrate specificities to NAD\(^+\) and NADH and have been referred to as “ATP-NAD kinase”). Unlike the yeast enzymes, the NadK of proteobacterium *Sphingomonas sp.* A1 does not exhibit NADH kinase activity and shows strict substrate specificity to NAD\(^+\); it is referred to as ATP-NAD kinase and not as ATP-NAD kinase (8). The NADH kinase activities of prokaryotic Ppnk, Mfnk, and YjB have not been reported.

Since the primary structures, particularly the amino acid residues constituting the probable NAD\(^+\)-binding sites, of NAD and NADH kinases are highly conserved (11), we believed that a few amino acid residues would discriminate between NAD and NADH kinases. However, due to the lack of information regarding NADH kinases, the identification of these amino acid residues is difficult. This study was aimed at identifying these residues. Initially, we revealed Ppnk and Mfnk to be “poly(P)/ATP-NADH kinases” and YjB to be an ATP-NAD kinase. Based on the structural information, we succeeded in converting the NADH kinases to NADH kinases by substituting a single Arg residue in NADH kinases. The Arg residue as well as the charged and hydrophobic amino acid residue were proposed to confer strict specificity to NAD\(^+\), at least in NADH kinases.

**MATERIALS AND METHODS**

*Assays—Poly(P)- and ATP-dependent NAD kinase activities were assayed spectrophotometrically at $A_{440}$ as follows (5). The reaction*
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**Results and Discussion**

**NADH Kinase Activities of Prokaryotic and Eukaryotic NAD Kinases**—The prokaryotic (actinobacterial) poly(P)/ATP-NAD kinases of *M. flavus* (Mnk) and *M. tuberculosis* (Ppnk) and the prokaryotic (proteobacterial) ATP-NAD kinase of *E. coli* (YbB) were purified from the *E. coli*, in which they were expressed, and the NADH kinase activities of the purified enzymes were determined. Mnk exhibited poly(P)- and ATP-dependent NADH kinase activities (3.04 units/mg and 8.66 units/mg, respectively), which constituted ~45% of poly(P)- and ATP-dependent NADH kinase activities (7.35 units/mg and 18.26 units/mg, respectively) of Mnk (Table I). Ppnk also exhibited poly(P)- and ATP-dependent NADH kinase activities (0.22 units/mg and 0.65 units/mg, respectively), which constituted ~15% of poly(P)- and ATP-dependent NADH kinase activities (1.70 units/mg and 4.33 units/mg, respectively) of Ppnk (Table I). NADH kinase activity has also been determined in the case of eukaryotic ATP-NAD kinases of *S. cerevisiae* (Utr1p, Yef1p, and Pos5p). In Utr1p and Yef1p, the NADH kinase activity was ~15% of the ATP-dependent NAD kinase activities (2, 10). However, in the case of Pos5p, the ATP-dependent NADH kinase activity was ~170% of ATP-NAD kinase activity of the enzyme (Table I) (10). On the other hand, YbB did not exhibit ATP-dependent NADH kinase activities (see Table I and Fig. 4B). Similarly, the prokaryotic (proteobacterial) ATP-NAD kinase of *Sphingomonas* sp. A1 (NadK) also did not exhibit NADH kinase activities (Table II) (8). Therefore, it was revealed that actinobacterial enzymes (Ppnk and Mnk) as well as eukaryotic *S. cerevisiae* enzymes (Utr1p, Yef1p, and Pos5p) are NADH kinases showing relaxed substrate specificities, whereas proteobacterial enzymes (YbB and NadK) are NAD kinases exhibiting strict specificity to NAD*. Further, the ratio of NADH kinase activity to NAD kinase activity is ~15–45%, except for Pos5p. Although the detailed results are not presented in this paper, no marked differences were observed in the $K_m$ values (Table II) and other properties (phosphoryl donor specificities, requirement for bivalent metal ions, optimum pH, and thermal stability) between poly(P)/ATP-dependent NADH and NAD kinase activities of Mnk. On the other hand, $V_{max}$ values for NAD kinase activities were higher than those for NADH kinase activities (Table II).

**Proposition of Amino Acid Residues Discriminating between NAD and NADH Kinases**—Based on the primary structures of various NAD and NADH kinases and the crystal structure of...
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The NADH kinase (Ppnk) complexed with NAD$^+$ (Ppnk-NAD) (11), we attempted to propose the amino acid residues that discriminated between NAD kinases (YfjB and NadK) and NADH kinases (Mfnk, Ppnk, Utr1p, Yef1p, and Pos5p) (Table I). The crystal structure of Ppnk-NAD (Fig. 1A) revealed Ppnk to be a homotetramer (Fig. 1B) and indicated the amino acid residues involved in the NAD$^+$-binding site (Fig. 1C). In particular, Asp-189 that contributes the NAD$^+$-binding site of Ppnk (Fig. 1C) is highly conserved among NAD and NADH kinases (Fig. 1D) and (ii) NAD and NADH kinases exhibit homo-oligomeric structures (i.e. dimeric (Mfnk and NadK) (5, 8), tetrameric (Ppnk) (5), hexameric (YfjB and Utr1p) (6, 9), and octameric (Yef1p)) (5).

Thus, we succeeded in relaxing the strict substrate specific-
ity of the NAD kinases and in converting the NAD kinases to NADH kinases through the substitution of a single amino acid. These results indicated that Arg-175 in YfjB or Arg-185 in NadK would be one of the crucial residues responsible for conferring strict specificity to NAD⁺ and hindering the NADH kinase activity in proteobacterial NAD kinases (YfjB and NadK).

Further enzymatic properties of purified YfjB R175G were determined and compared with those of purified YfjB (6) (Table III). The $K_{m}$ values of YfjB R175G for NAD⁺/H₁₁₀₀₁ and ATP in NAD kinase activity and for NADH and ATP in NADH kinase activity were almost equal to those of NAD⁺ and ATP of YfjB in NAD kinase activity (Table III and Fig. 5). However, the $V_{max}$...
values of YfjB R175G in NAD kinase activity were significantly lower than those of YfjB (Table III and Fig. 5). The \( K_m \) and \( V_{max} \) values of YfjB R175G and YfjB indicated that Arg-175 is also significant for high expression of NAD kinase activity of YfjB but not for binding to NAD. No significant differences were observed in the other properties (phosphoryl donor specificities, requirement for bivalent metal ions, optimum pH, and thermal stability) among NAD and NADH kinase activities of YfjB R175G and the NAD kinase activities of YfjB.

NADH Kinase Activities of YfjB Mutants—Since the residues corresponding to Arg-175 in YfjB were polar amino acids (22) in the other eukaryotic yeast NADH kinases (Utr1p, Yef1p, and Pos5p) (Fig. 1D), it was considered that YfjB mutants (YfjB R175Q and YfjB R175T, in which Arg-175 was converted to Gln and Thr, respectively) also show ATP-dependent NADH kinase activity. These YfjB mutants were expressed in E. coli and assayed for NADH kinase activity by using crude cell extracts containing the YfjB mutants. As expected, YfjB R175Q and YfjB R175T showed ATP-dependent NADH kinase activities (0.013 units/mg and 0.016 units/mg, respectively) (Fig. 2A), thereby suggesting the importance of the polar amino acid residues in the position of Arg-175 in YfjB for the expression of NADH kinase activity (i.e. relaxing the substrate specificity).

Further, to examine the effects of the other polar, charged, and hydrophobic amino acid residues (22) in this position on NADH kinase activity, we constructed YfjB mutants (YfjB R175H, YfjB R175K, YfjB R175E, and YfjB R175I, in which Arg-175 was converted to His (polar amino acid), Lys (positively charged amino acid), Glu (negatively charged amino acid), and Ile (hydrophobic amino acid), respectively). These mutants were expressed in E. coli and assayed for NADH kinase activity by using crude cell extracts containing the YfjB mutants. YfjB R175H exhibited ATP-dependent NADH kinase activity (0.010 units/mg), whereas YfjB R175K, YfjB R175E, and YfjB R175I did not produce the same result (Fig. 2A).
Collectively, these results indicated that the polar amino acid or Gly residue, and not the positively and negatively charged or hydrophobic amino acid residue in the position corresponding to the Arg-175, is a prerequisite for the expression of NADH kinase activity in YfjB (i.e. for conferring the relaxed substrate specificities in YfjB). In other words, these results also indicated that the charged and hydrophobic amino acid residue could confer strict specificity to NAD. As mentioned above, NADH is formed through the addition of a hydride ion (H\(^{-}\)) to the C4 position of the nicotinamide ring (Fig. 1C) in NAD\(^{+}\) (21). The side chains of the charged and hydrophobic amino acid residues in the focused position were proposed to be arranged around the “nicotinamide ring” of NAD\(^{+}\) (Fig. 1C). Hence, we believed that the charge and hydrophobicity of the side chains of these residues prevent NADH from binding to NAD\(^{+}\)-binding sites in YfjB and possibly in NadK, thereby hindering the expression of NADH kinase activity of YfjB and NadK. Accordingly, it was considered that NADH did not bind to the NAD\(^{+}\)-binding site in YfjB (i.e. it did not compete with NAD\(^{+}\) for the site, since competitive inhibition by NADH for NAD kinase activity was not observed in YfjB) (6). Thus, we assumed that, at least in the case of YfjB and NadK, NADH may bind to NAD\(^{+}\)-binding sites through the substitution of the charged and hydrophobic amino acid residues with Gly or polar amino acid residues.

**NADH Kinase Activities of Mfnk G183R**—The Gly-183 in Mfnk occupied the position corresponding to Arg-175 in YfjB and Arg-183 in NadK. Catalytic sites and intersubunit contacts between homodimeric subunits (A and A’) are illustrated. The positive and negative effects of the residues on the \(V_{\text{max}}\) values for NAD and NADH kinase activities are represented by dotted arrows with plus and minus signs, respectively. A. Arg-175 and Gly-183 come from the other adjacent subunit. The substitution conferred NADH kinase activity to YfjB and decreased the \(V_{\text{max}}\) value for NADH kinase activity; however, it had little effect on the \(K_{\text{m}}\) value for NAD\(^{+}\). B. Gly-183 and Arg-183 come from the other adjacent subunit. The substitution increased the \(K_{\text{m}}\) value for NAD\(^{-}\) and decreased the \(V_{\text{max}}\) values for NAD and NADH kinase activities; however, it had little effect on the \(K_{\text{m}}\) value for NADH.
G183R, in which Gly-183 was conversely converted to Arg, it was expected that NADH kinase activity would be lowered or lost, whereas NAD kinase activity would be retained. Mfnk G183R was expressed in E. coli and purified. The purified Mfnk G183R was a homodimer similar to Mfnk (Fig. 3, C and D).

Several properties (phosphoryl donor specificities, requirement for bivalent metal ions, optimum pH, and thermal stability) related to NADH and NAD kinase activities of Mfnk G183R were also similar to those of Mfnk (data not shown).

Both NAD and NADH kinase activities of Mfnk G183R were decreased when compared with those of Mfnk, and NADH kinase activity was partially retained in Mfnk G183R (Table IV). Thus, NADH kinase was not converted to NAD kinase through single amino acid substitution of Gly-183 in Mfnk, thereby indicating that Gly-183 is not the sole determinant for relaxed substrate specificities of Mfnk (NADH kinase) and that other unidentified determinants definitely exist.

The $V_{\text{max}}$ values of Mfnk G183R for NAD and NADH kinase activities were lower than those of Mfnk, and the $K_m$ values of Mfnk G183R for NAD$^+$ were higher than those of Mfnk (Table IV and Fig. 5). The $K_m$ values of Mfnk G183R for NADH and for phosphoryl donors were approximately the same as those of Mfnk (Table IV and Fig. 5). The $K_m$ and $V_{\text{max}}$ values of Mfnk G183R and Mfnk indicated that Gly-183 is important for high expression of NADH and NAD kinase activities of Mfnk and for binding to NAD$^+$.

Conservation of the Residues Corresponding to Arg-175 in YfjB in the Primary Structures of 207 NAD Kinase Homologs—In one of the proteobacterial NAD kinases (YfjB), Arg-175 as well as the charged and hydrophobic amino acid residues in this position were shown to confer specificity to NAD$^+$ (Fig. 2A and Table III). In another proteobacterial NAD kinase (NadK), the Arg-180 corresponding to the Arg-175 in YfjB was also demonstrated to confer the specificity (Fig. 2B). The Gly residues occupy the focused positions in actinobacterial NADH kinases (Mfnk and Ppnk), whereas the polar amino acid residues (Gln and Thr) occupy the positions in eukaryotic yeast NADH kinases (Fig. 1D).

In order to determine whether the residues in the focused position were conserved in the available primary structures of NAD kinase homologs, the primary structures of 207 NAD kinase homologs, including YfjB, NadK, Mfnk, Ppnk, Utr1p, Yef1p, and Pos5p, from various organisms were aligned. Of 207 homologs, the residues in the focused position, which corresponded to Arg-175 in YfjB, are mainly conserved as a charged amino acid (Arg, Lys, Glu, and Asp) (68%; 140 residues in 207 homologs), a hydrophobic amino acid (Ile, Ala, Val, Leu, and Phe) (15%; 31 residues), a polar amino acid (Ser, Gln, Thr, His, and Trp) (11%; 23 residues), and Gly (6%; 13 residues) (Table V). Furthermore, the categorization of the organisms containing the homologs indicates that the charged amino acid residues are mainly found in 195 prokaryotic homologs (71%; 138 charged amino acid residues in 195 homologs), whereas the charged amino acid residues are rarely found in 12 eukaryotic homologs (17%; merely 2 residues in 12 homologs). In particular, the Arg residue is found most frequently in 138 prokaryotic homologs containing the charged amino acid residues in the focused position (84%; 116 Arg residues in 138 homologs including YfjB and NadK) (Table V). The hydrophobic amino acid residues are conserved in 28 prokaryotic homologs, particularly in γ-proteobacterial homologs and in three eukaryotic homologs (Table V). In conclusion, the charged and hydrophobic amino acid residues are conserved in 166 (138 plus 28) prokaryotic homologs of total 195 prokaryotic ones (85%) and thus are characteristic residues in the focused position of almost all the prokaryotic homologs (Table V). However, in actinobacterial homologs, including Mfnk and Ppnk, Gly and polar amino acid residues are conserved (Table V). The Gly residue is found only in the actinobacterial homologs (Table V). In the case of eukaryotic homologs, including Utr1p, Yef1p, and Pos5p, the polar amino acid residues are major residues (58%; 7 polar amino acid residues in 12 homologs), whereas in the case of prokaryotic homologs, the polar amino acid residues are not major residues (8%; 16 residues in 195 homologs) (Table V).

**Significance of the Residue in the Focused Position and of Intersubunit Contact**—Although the charged and hydrophobic amino acid residues are conserved in the focused positions in 166 prokaryotic homologs and five (2 plus 3) eukaryotic homologs, we were unable to define the 171 (166 plus 5) homologs as NAD kinases, because Mfnk G183R is not an NAD kinase (Table IV). However, we believe that the charged and hydrophobic amino acid residue in the focused position might determine the specificity to NAD$^+$ in some “NAD kinases” among the 171 homologs. Furthermore, the conservation of the residues in the focused position might determine the specificity to NAD$^+$ in some “NAD kinases” among the 171 homologs. Furthermore, the conservation of the residues in the focused position might indicate for the expressions of activities in all NAD and NADH kinases.

Finally, the residue in the focused position (e.g. Arg-175 in YfjB and Gly-183 in Mfnk), possibly arranged around the nicotinamide ring of NAD$^+$ bound to one subunit, is provided by the other subunit (Fig. 1 and 5). Hence, the possible significance of the residue in the focused position of NAD kinase homologs and at least the evident significance of Arg-175 in YfjB and Gly-183 in Mfnk for the activities (Tables III and IV and Fig. 5) supported our previous structure-based proposal that intersubunit contact is crucial for constituting the NAD$^+$-binding site in NAD kinase (11) and also suggested that the contact is important for the NADH-binding site in NADH kinase. Furthermore, the significance of Arg-175 in YfjB and Arg-180 in NadK in conferring strict specificity to NAD$^+$ also

**Table IV: Kinetic values for NAD and NADH kinase activities of Mfnk G183R**

| Enzyme | P$_i$ donor | NAD kinase activity | | NADH kinase activity |
|--------|-------------|---------------------|---|---------------------|
|        |             | $V_{\text{max}}$/mg | $K_m$/mM | $V_{\text{max}}$/mg | $K_m$/mM |
| Mfnk   | ATP         | 18.26 0.53 ± 0.024 | 0.23 ± 0.008 | 18.67 0.693 | 20.12 ± 0.643 |
| Mfnk G183R | ATP       | 1.86 1.43 ± 0.078 | 0.16 ± 0.003 | 1.78 0.069 | 2.12 ± 0.097 |
| Mfnk G183R | Poly(P)$_i$ | 7.35 0.26 ± 0.013 | 0.33 ± 0.010 | 8.63 0.282 | 8.92 ± 0.535 |
| Mfnk G183R | Poly(P)$_i$ | 1.40 1.86 ± 0.043 | 0.21 ± 0.004 | 1.40 0.067 | 2.34 ± 0.099 |

**Notes:**
- $V_{\text{max}}$ (μmol/mg/min).
- $K_m$ (mM).
- $V_{\text{max}}$ for NAD and NADH kinase activities of Mfnk G183R were determined in the presence of 5.0 mM ATP or 5.0 mM poly(P)$_4$ by using purified enzyme.
- $V_{\text{max}}$ for NAD and NADH kinase activities of Mfnk G183R were determined in the presence of 5.0 mM ATP or 5.0 mM poly(P)$_4$ by using purified enzyme.
- $V_{\text{max}}$ for NAD and NADH kinase activities of Mfnk G183R were determined in the presence of 5.0 mM ATP or 5.0 mM poly(P)$_4$ by using purified enzyme.
conveys another important aspect of the intersubunit contact, at least in NAD kinases (Fig. 5).

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