Autophosphorylation of *Arabidopsis* Nucleoside Diphosphate Kinase 2 Occurs Only on Its Active Histidine Residue†

Yu Shen,‡ Jeong-Il Kim,§,|| and Pill-Soon Song*,‡,§,||,⊥

Department of Chemistry, University of Nebraska−Lincoln, Lincoln, Nebraska 68588-0304, Environmental Biotechnology Research Center, Gyeongsang National University, Jinju 660-701, Korea, Kumho Life and Environmental Science Laboratory, 1 Oryong-Dong, Gwangju 500-712, Korea, and Cheju National University, Jeju 690-756, Korea

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ABSTRACT: *Arabidopsis* nucleoside diphosphate kinase 2 (NDPK2) is a component in the phytochrome-mediated light signaling. In the present study, its autophosphorylation was investigated. Acid-stable and alkali-stable phosphorylated residues were analyzed under two different conditions. Results revealed that NDPK2 is phosphorylated only on its active histidine residue His197 and the presence of serine/threonine phosphorylation is an experimental artifact due to the harsh condition applied in the treatment of the phosphorylated protein sample. To resolve the controversy of whether serine/threonine phosphorylation of NDPK occurs as has been suggested by other NDPK studies, NDPK2 putative phosphorylation site mutants were generated and examined. No serine/threonine phosphorylation was identified in NDPK2 or implicated in its enzymatic activity. Further studies indicated that the low enzymatic activity and autophosphorylation level of NDPK2 mutant S199A are shown to be due to a damaged H-bonding with the active histidine residue His197 in the nucleotide-binding pocket. In addition, NDPK2 Kpn loop mutant T182A was found to possess an extremely low enzymatic activity and almost no autophosphorylation, suggesting the importance of the oligomeric states of NDPK2 in NDPK2 functioning.

Nucleoside diphosphate kinase (EC 2.7.4.6, NDPK) catalyzes the transfer of the γ-phosphate from nucleoside triphosphate to nucleoside diphosphate via a ping-pong mechanism (*1*). Autophosphorylation of NDPK on its active histidine residue has been widely accepted as part of its catalytic mechanism (*2, 3*). However, serine phosphorylation was also reported in NDPKs from various species, including human NDPKA (nm23-H1) (*4*), *Myxococcus xanthus* NDPK (*5*), sugar cane NDPK (*6*), spinach NDPK II (*7*), and *Pisum sativum* mitochondrial NDPK (*8*). Although the significance of serine phosphorylation is still unclear, the hypothesis suggests that it plays a regulatory role in multiple cellular functions identified for NDPKs. In human NDPK, residue Ser120 has been suggested as a possible serine phosphorylation site. Human NDPKA was identified to have a Ser120Gly (S120G) mutation in aggressive human neuroblastoma (*9*), and this mutant appeared more effective in promoting neuroblastoma metastasis (*10*). Mutant S120G also abrogated the motility-suppressive phenotype in a tumor cell motility assay, and phosphorylation on residue Ser120 was suggested to contribute to the inhibitory function of NDPKA (*11, 12*). In addition, S120G mutation abolished the interaction between NDPKA and human PRUNE (*13*). On the other hand, several reports suggested that phosphorylation on the serine residues in NDPK occurs (*14*−*16*). The observed serine phosphorylation might be due to the nonenzymatical phospho transfer from the active histidine residue to its nearby serine during the analysis of phosphorylated NDPK. Since the putative serine phosphorylation site Ser120 is located closely to the active histidine residue in the nucleotide-binding pocket of NDPK, this possibility could not be excluded.

We recently elucidated the three-dimensional structures of *Arabidopsis* NDPKs (*17*). Similar to mammalian NDPKs, *Arabidopsis* NDPK2 has been suggested as a potential multifunctional protein as well (*18*−*22*). As a phytochrome-signaling partner, NDPK2 was shown to share the same subcellular space with phytochrome and function as a positive signaling component (*18*). NDPK2 was also found to be able to interact with phytochrome in yeast two-hybrid screening (*18*) and in vitro (*23*). To better understand the mechanism of NDPK2 functioning and to settle the serine phosphorylation controversy in NDPK, NDPK2 autophosphorylation was investigated.

MATERIALS AND METHODS

NDPK2 Protein Preparation. NDPK2, the N-terminal deleted NDPK2 (Δ79 NK2), and NDPK2 site-specific mutants were prepared as previously described (*18*, *23*).

NDPK2 Autophosphorylation and Analysis. NDPK2 autophosphorylation was performed as described (*23*). The phosphorylated NDPK2 were either denatured for 5 min at

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* To whom correspondence should be addressed. Tel: +82-62-971-2263. Fax: +82-62-972-5085. E-mail: pssong@kkpc.com.

‡ University of Nebraska−Lincoln.

§ Gyeongsang National University.

|| Kumho Life and Environmental Science Laboratory.

⊥ Cheju National University.

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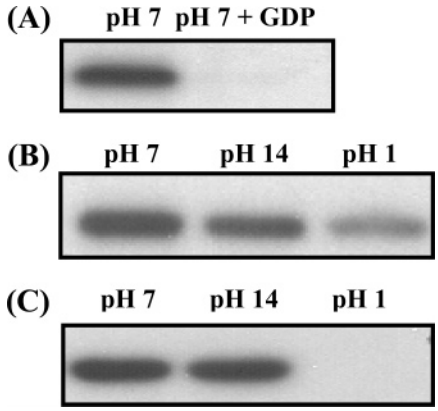

FIGURE 1: Investigation of NDPK2 autophosphorylation. The protein denaturation method is critical in the discrimination of the phosphorylated residues in NDPK2. (A) The phosphorylated NDPK2 is not stable and transfers the phosphate group to the GDP molecules in pH 7 buffer: pH 7, pH 7 buffer; pH 7 + GDP, pH 7 buffer with the addition of GDP. (B) NDPK2 autophosphorylation with protein samples treated under harsh conditions. The remaining phosphate groups in NDPK2 were measured after incubation (2 h at 45 °C) in buffer with the indicated pH values. A small amount of acid-stable phosphorylation is evident (lane 3). (C) NDPK2 autophosphorylation with protein samples treated under mild conditions. No acid-stable phosphorylation is evident (pH 1, lane 3).

100 °C (the harsh condition) or denatured for 1 h at room temperature (the mild condition). The denatured samples were separated on a SDS−PAGE gel and then transferred to a PVDF membrane. The radioactive signals were collected on X-ray films. The exact amount of the phosphate group bound by NDPK2 in the autophosphorylation reaction was obtained by measuring the radioactivity of the specific phosphorylated NDPK2 protein band with a liquid scintillation counter. NDPK2 γ-phosphate exchange activity, the pH dependence, size exclusion chromatography, and the thermal stabilities of NDPK2 wild-type and mutant proteins were analyzed as previously described (*23*).

Discrimination between Acid-Stable and Alkali-Stable Phosphorylated Residues. The discrimination between acid-stable and alkali-stable phosphorylated residues was performed as described (*5*, *16*). NDPK2 was autophosphorylated, separated on a SDS−PAGE gel, and then transferred to a PVDF membrane. The portion of the membrane containing NDPK2 was cut into several pieces. Each piece was incubated separately in each of the following buffers (200 μL): 50 mM KCl−HCl (pH 1), 0.1 M Tris-HCl (pH 7), and 1 M KOH (pH 14). After 2 h incubation at 45 °C, the radioactivity released into buffer and the radioactivity remaining on the membrane were measured with a liquid scintillation counter.

RESULTS

The Protein Denaturation Method Applied to the Phosphorylated NDPK2 Is Critical in the Discrimination of the Phosphorylated Residues in NDPK2. NDPK2 was autophosphorylated and transferred to a PVDF membrane, followed by incubation in buffer at pH 7 with or without the addition of GDP. Results indicated that the phosphorylated NDPK2 was not stable and transferred the bound phosphate group from NDPK2 to GDP during the incubation (Figure 1A). To investigate the possibility of serine phosphorylation, a discrimination analysis between acid-stable and alkali-stable phosphorylated residues was conducted. Results revealed a dominant presence of alkali-stable phosphorylation (Figure 1B), indicating that the active histidine residue plays a critical role in NDPK2 autophosphorylation. However, a small but evident amount of acid-stable phosphorylation was also observed (Figure 1B), suggesting that NDPK2 might also possess a capacity for serine/threonine phosphorylation. To investigate this possibility, the protein denaturation method applied to the phosphorylated NDPK2 was closely examined. In a harsh condition, the phosphorylated NDPK2 was boiled in the SDS sample buffer (acidic) prior to the separation by SDS−PAGE. Under this condition, the phosphohistidine (alkali-stable) would be unstable, and a loss of the phosphate group attached to the active histidine residue would be inevitable. To avoid this situation, a mild condition (1 h incubation in the SDS sample buffer at room temperature) was applied. Results indicated that no evident acid-stable phosphorylation was observed (Figure 1C). Further analysis revealed a very small amount of acid-stable phosphorylation (~3%) under the mild condition compared to 30% under the harsh condition. Therefore, serine/threonine phosphorylation in NDPK2 may be an artifact of chemical treatment of the phosphorylated protein.

Residue His197 Is the Only Site Responsible for NDPK2 Autophosphorylation. To further examine serine/threonine phosphorylation in NDPK2, the putative phosphorylation site mutants were made and analyzed, including the N-terminal deletion mutant NK2 (1−79 aa deleted, Δ79 NK2) and the site-specific mutants His197Cys (H197C), Ser199Ala (S199A), Ser201Ala (S201A), and Thr182Ala (T182A). The N-terminal deletion did not affect NDPK2 autophosphorylation (Figure 2A) and its enzymatic activity (Table 1). The mutation on the active histidine residue His197 (H197) completely abolished the autophosphorylation capability (Figure 2A) as well as the enzymatic activity (Table 1), indicating the pivotal role of residue H197 in the NDPK2-catalyzed enzymatic reaction and NDPK2 autophosphorylation. Residue Ser199 (S199) is the corresponding serine residue in NDPK2 to residue Ser120 in human NDPK. Residue Ser201 (S201) is another serine residue in close proximity to the active histidine residue H197 besides residue S199 and is the most likely serine residue in NDPK2 for phosphorylation (*24*). Additionally, residue Thr182 (T182) is the highest predicted threonine residue possibly to be phosphorylated. Results showed that mutant S201A possessed the same autophosphorylation level as the wild type (Figure 2B). In contrast, the autophosphorylation level of mutant S199A was much lower, and the autophosphorylation of mutant T182A was virtually undetectable during the same time period (Figure 2B). Mutant S201A also possessed the same enzymatic activity as the wild type, whereas the activities of mutants S199A and T182A were much lower (Table 1).

To confirm that residues S199 and T182 are not responsible for NDPK2 autophosphorylation, the discrimination analysis between acid-stable and alkali-stable phosphorylations for NDPK2 mutants was carried out under mild conditions. A predominant histidine phosphorylation (alkali-stable) was observed in all NDPK2 mutants (Figure 2C), which is the same pattern as the wild type, indicating the absence of serine/threonine phosphorylation in NDPK2.

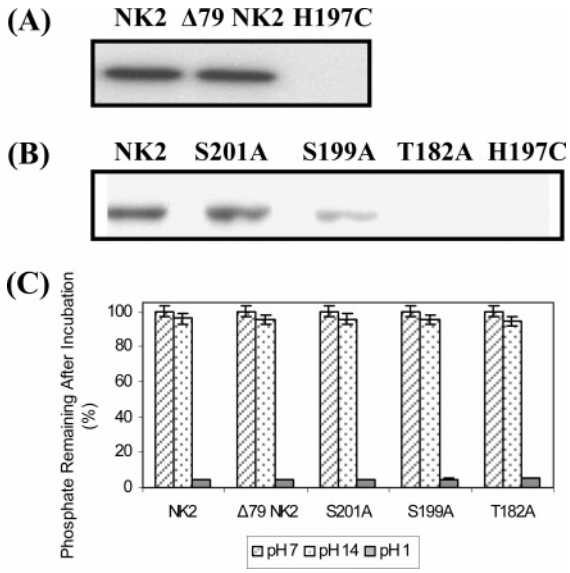

FIGURE 2: Investigation of NDPK2 putative autophosphorylation site mutants. (A) Autophosphorylation of the NDPK2 N-terminal deletion mutant Δ79 NK2 and the site-specific mutant H197C. The N-terminal deletion does not change NDPK2 autophosphorylation. Residue His197 is essential for NDPK2 autophosphorylation. (B) Autophosphorylation of NDPK2 putative autophosphorylation site mutants. Mutant S201A showed no difference in the autophosphorylation experiment compared to the wild type. Mutant S199A showed a lower autophosphorylation level. The autophosphorylation of mutant T182A was not detected within the same time period. (C) The amount of alkali-stable and acid-stable phosphorylation of NDPK2 was analyzed under mild sample treatment conditions. No evident acid-stable phosphorylation was observed. NDPK2 putative autophosphorylation site mutants showed the same autophosphorylation pattern as that of the wild type. pH 7 (lane 1), pH 14 (lane 2), and pH 1 (lane 3) were shown for the NDPK2 wild type, mutant Δ79 NK2, mutant S201A, mutant S199A, and mutant T182A, respectively.

Table 1: Enzymatic Activities of NDPK2 Putative Phosphorylation Site Mutants

NDPKs	enzymatic activity (%)	NDPKs	enzymatic activity (%)
wild type	100	S201A	100
H197C	0	S199A	68.3
Δ79 NK2	100	T182A	23.7

DISCUSSION

The serine phosphorylation of NDPK has been hypothesized as the phosphate group phospho-relayed from the active histidine. However, other reports suggested that the observed serine phosphorylation could be the phosphate group hydrolyzed from the active histidine. Our first result demonstrating NDPK2 autophosphorylation indicated that the phosphorylated NDPK2 is able to function as a phospho intermediate, transferring the phosphate group to its substrate, such as GDP (Figure 1A). This result supports both mechanisms, including phospho relay and nonenzymatic phospho transfer, since both mechanisms require an unstable phosphorylated NDPK2. To investigate the serine phosphorylation in NDPK2, two conditions of protein sample treatment were tested to discriminate alkali-stable and acid-stable phosphorylations. The harsh condition had been applied in previous reports that demonstrated serine phosphorylation and phosphoserine residue in NDPKs (*4*−*8*). Similar results were observed in NDPK2. However, the harsh condition is disadvantageous to the phosphohistidine (alkali-stable), which is vulnerable when denatured at high temperature in acidic buffer (*16*). The phosphate group could be hydrolyzed from the active histidine and captured by the nearby serine residue(s) in the NDPK nucleotide-binding pocket. In this case, the decreasing amount of phosphohistidine versus the increasing amount of phosphoserine would lead to an impression of serine phosphorylation in NDPK, albeit a false impression. Compared to the harsh condition, the mild condition is more reasonable since it reduces the loss of the phosphate group attached to the active histidine and thus minimizes the chance of experimental artifacts. A negligible amount of acid-stable phosphorylation was identified under the mild condition, suggesting that previously observed serine phosphorylation was an artifact. Further studies of NDPK2 putative serine phosphorylation site mutants confirmed our speculation. Results showed that residue H197 is essential and residue S201, residue S199, residue T182, and the serine residues located in the N-terminal fragment are not the serine phosphorylation sites (Figure 2). We concluded that the active histidine residue is the only site responsible for NDPK2 autophosphorylation.

Although residue S199 has been excluded as the possible phosphorylation site, the investigation of this mutant may be helpful to understand the mechanism of why human NDPKA is dysfunctional after mutation on residue S120. Recent studies have reported that the H-bonding with the active histidine residue inside the nucleotide-binding pocket is critical for NDPK-catalyzed enzymatic activity (*23*, *25*). In NDPK2, this H-bonding change has been demonstrated as the variation of the pK_a value of residue H197 (*23*). We found that the lower the pK_a value of residue H197, the higher the enzymatic activity of NDPK2. Therefore, we speculate that the low autophosphorylation and enzymatic activity of mutant S199A could be due to a damaged H-bonding inside the nucleotide-binding pocket of NDPK2. A pH dependence profile of mutant S199A was obtained and compared to that of NDPK2 wild type. A pK_a value of 6.55 of residue H197 was observed in mutant S199A, higher than 6.35 observed in the wild type. This pK_a value increase could slow the phospho transfer from residue H197 to its substrate and thus lower the NDPK2 autophosphorylation level and enzymatic activity. Meanwhile, the optimal pH for the enzymatic activity was also changed in mutant S199A (pH 8.15−8.80) compared to that of the wild type (pH 8.00−8.95). The thermal stability assay of NDPK2 confirmed this speculation. Mutant S199A possessed a lower T_m value (56 °C) than the wild type (60 °C), suggesting a H-bonding change inside the nucleotide-binding pocket after the mutation on residue S199.

The nature of low enzymatic activity and autophosphorylation ability of mutant T182A is complicated since residue T182 is located in the Kpn (Killer of prune) loop of NDPK2. The investigation of mutant T182A revealed the importance of the oligomeric states in NDPK2 autophosphorylation. Results from the thermal stability assay showed that mutant T182A possessed a very low T_m value, 39 °C. Further experiments using size exclusion chromatography indicated that mutant T182A is unstable and contains a significant amount of the dimeric form of NDPK2 (41%), whereas the hexameric form is still predominant (100%) in mutant S199A. The hexameric form of NDPK2 has been shown to

be essential for the interaction with phytochrome (*23*). Our study found that it is also critical for the interaction with G-proteins and its GAP (GTPase activating protein) function (Shen and Song, unpublished data). The damage of the oligomeric states could be the major reason contributing to the observed low activity of mutant T182A and its minimal autophosphorylation.

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