

NDPK2 as a Signal Transducer in the Phytochrome-mediated Light Signaling*

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Nucleoside-diphosphate kinase (NDPK) 2 in *Arabidopsis* has been identified as a phytochrome-interacting protein by using the C-terminal domain of phytochrome A (PhyA) as the bait in yeast two-hybrid screening. The far-red light-absorbing form of phytochrome (Pfr) A stimulates NDPK2 γ -phosphate exchange activity *in vitro*. To better understand the multiple functions of NDPK and its role in phytochrome-mediated signaling, we characterized the interaction between phytochrome and NDPK2. Domain studies revealed that PER-ARNT-SIM domain A in the C-terminal domain of phytochrome is the binding site for NDPK2. Additionally, phytochrome recognizes both the NDPK2 C-terminal fragment and the NDPK2 hexameric structure to fulfill its binding. To illustrate the mechanism of how the Pfr form of phytochrome stimulates NDPK2, His-197-surrounding residue mutants were made and tested. Results suggested that the H-bonding with His-197 inside the nucleotide-binding pocket is critical for NDPK2 functioning. The pH dependence profiles of NDPK2 indicated that mutants with different activities from the wild type have different pK_a values of His-197 and that NDPK2 hyperactive mutants possess lower pK_a values. Because a lower pK_a value of His-197 accelerates NDPK2 autophosphorylation and the phospho-transfer between the phosphorylated NDPK2 and its kinase substrate, we concluded that the Pfr form of phytochrome stimulates NDPK2 by lowering the pK_a value of His-197.

Nucleoside-diphosphate kinase (NDPK,¹ EC 2.7.4.6) is a ubiquitous enzyme that catalyzes the transfer of the γ -phosphate from NTP to NDP, following a ping-pong mechanism (1). Since its first detection in 1953 (2, 3), NDPK has been considered as a housekeeping enzyme used to maintain nucleoside

triphosphate levels in organisms, but growing evidence has indicated that NDPK also participates in the regulation of growth, development, and signal transduction processes. In *Drosophila*, mutation of Awd (a NDPK homologue) results in abnormal cell morphology (4). In human, Nm23-H1, a human NDPK, functions as a tumor metastasis suppressor (5, 6). Additionally, Nm23-H2, an isoform of Nm23-H1, acts as a transcription factor that binds to the *c-myc* oncogene promoter and stimulates transcription (7). The ability of NDPK to supply GTP also implies a role in G-protein-mediated signaling. Recent reports (8–10) suggested that NDPK could serve as a guanine nucleotide exchange factor as well as a GTPase-activating protein. Therefore, the idea that NDPK is a multifunctional enzyme is well accepted.

In plants, however, little is known about the roles of NDPK (11). NDPK has been characterized in rice (12), oat (13), and pea (14–16). Three NDPK isoforms have been isolated from spinach leaves. Among them, spinach NDPK1 is localized in the cytosol, whereas spinach NDPK2 and NDPK3 are localized in the chloroplasts (17). Evidence suggested that plant NDPK plays an important role in signal transduction. Red light-dependent phosphorylation of NDPK in both rice and pea has been reported (18, 19) and has suggested the possible involvement of NDPK in phytochrome-mediated signaling. Furthermore, in *Arabidopsis*, NDPK2 was reported as the only NDPK among the three isoforms to interact with phytochrome in yeast two-hybrid screening (20). Fusion proteins of NDPK2 with green fluorescent protein are localized mainly in the nucleus and cytoplasm, sharing the same subcellular space with phytochromes. In addition, a tDNA insertion mutant of NDPK2 showed a partial defect in response to both red and far-red light, indicating NDPK2 is a positive signaling component of phytochrome-mediated signaling. NDPK2 has also been reported to be involved in UV response (21) and protection against reactive oxygen species stress (22). Despite scanty reports in the literature and unlike NDPKs in mammals, little is known about the functional roles of plant NDPKs. They remain to be elucidated.

Phytochromes are major photoreceptors that regulate a variety of photomorphogenic processes in plants (23). The reversible phototransformation between the red light-absorbing Pr form and the far-red light-absorbing Pfr form is the most striking characteristic of phytochrome signaling, in which the Pfr form is considered as the active form (24, 25). Although the mechanism of phytochrome signaling is still unclear, continuous progress has been made in elucidating phytochrome kinase activity (26, 27), nuclear localization ability (28–30), regulated protein degradation (31), and phytochrome-interacting proteins (20, 32–34). Although *Arabidopsis* NDPK2 has been identified as a positive signal transducer in the phytochrome signaling and its enzymatic activity is stimulated by physical

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¹ The abbreviations used are: NDPK, nucleoside-diphosphate kinase; NDPK2, nucleoside-diphosphate kinase 2; Pr, red-light absorbing form of phytochrome; Pfr, far-red-light absorbing form of phytochrome; PhyA, phytochrome A; PhyB, phytochrome B; aa, amino acid; MBP, myelin basic protein; Mes, 4-morpholineethanesulfonic acid; NTE, N-terminal extension; PAS, PER-ARNT-SIM.

binding with phytochromes, the biochemical mechanisms remain to be elucidated.

To gain further insights into the mechanism of both phytochrome-mediated signaling and NDPK functioning, we characterized the interaction between phytochrome and NDPK2 in detail. In doing so, we have shown *in vitro* binding between phytochrome and NDPK2, and we located the specific binding region in both proteins and demonstrated the importance of NDPK2 oligomeric states in the interaction with phytochrome. We also characterized His-197-surrounding residue mutants to investigate the mechanism of how phytochrome stimulates NDPK2 activity, because the H-bonding with the active histidine residue is essential for NDPK functioning (35, 36). The pH dependence profiles of NDPK2 wild type and mutants revealed the relationship between NDPK2 activity and the pK_a value of His-197. The similarities between the hyperactive NDPK2 mutants and the Pfr form of phytochrome-stimulated NDPK2 suggested that the stimulation of NDPK2 activity by phytochrome is because of the direct modulation of the H-bonding with His-197, following the phytochrome binding to the NDPK2 C-terminal fragment.

EXPERIMENTAL PROCEDURES

Purification of Full-length and Partial-length NDPK1 and NDPK2; Purification of Native Oat PhyA and Recombinant Phytochromes—Recombinant oat PhyA, including full-length (1–1129 aa), A957 (1–957 aa), A686 (1–686 aa), the N-terminal domain (1–505 aa), and the C-terminal domain (506–1129 aa); recombinant *Arabidopsis* PhyA; and recombinant *Arabidopsis* phytochrome B (PhyB) were prepared as described previously (27). With *Arabidopsis* *PHYA* and *PHYB* genes, the C-terminal domains of *Arabidopsis* PhyA (572–1122 aa) and PhyB (604–1172 aa) were also prepared. Full-length *Arabidopsis* NDPK1, NDPK2, and the N-terminal 79-aa deleted NDPK2 were also prepared as described previously (20, 27). The C-terminal deletion mutants of NDPK2 for the phytochrome binding study were prepared as follows (STOP codon is underlined): R230Stop (80–229 aa) with 3'-primer, 5'-GAGACCCGGGCTATAGCCATGTAGCTAGAGCCG-3' (SmaI); L225Stop (80–224 aa) with 3'-primer, 5'-GAGACCCGGGCTAAGCCG-AATCCCACTTGCATAGC-3' (SmaI); K214Stop (80–213 aa) with 3'-primer, 5'-GAGACCCGGGCTAGAACCACAGACCAATCTCACG-3' (SmaI); N204Stop (80–203 aa) with 3'-primer, 5'-GAGACCCGGGCT-ATTCAGGGCTGTCACTACCATG-3' (SmaI); and S199Stop (80–198 aa) with 3'-primer, 5'-GAGACCCGGGCTAACCATGCACAATGTTCC-TTCC-3' (SmaI). Native oat PhyA was purified as previously reported (37).

For the *in vitro* binding assay with ^{14}C -labeled NDPK2, NDPK2 proteins were prepared by using the same method as that of unlabeled NDPK2 protein purification, with addition of 100 μCi of L-[methyl- ^{14}C]methionine (Amersham Biosciences) into 250 ml of *Escherichia coli* culture. The specific radioactivity of the final ^{14}C -labeled NDPK2 was around 600 cpm/ μM . The radioactivity of the bound NDPK2 after immunoprecipitation with phytochromes was measured by using a liquid scintillation counter (Beckman).

Site-directed Mutagenesis—The *in vitro* mutagenesis of NDPK2 was performed using QuickChange™ site-directed mutagenesis protocol (Stratagene). The synthetic primers (sense) designed to produce the desired point mutations are listed as follows: Y87D, 5'-GTTGAGGAG-ACTGACATTATGGTGAACC-3'; M89D, 5'-GGAGACTTACATTGAC-GTGAAACCTGATGG-3'; H130E, 5'-GAATTGGCTGAGGAGGAATAT-AAGGAGCTTAG-3'; H130Q, 5'-GAATTGGCTGAGGAGCAATATAAG-GAGCTTAG-3'; P175S, 5'-TAGGGAAAACAGATTCCGCTTCAAGCTG-AACC-3'; H197C, 5'-GAAGGAACATGTGTGTGGTAGTGACAGC-3'; G198D, 5'-GAACATTGTGCATGATAGTGACAGCCCTG-3'; G198N, 5'-GAACATTGTGCATAAATAGTGACAGCCCTG-3'; S199D, 5'-CATTGTG-CATGGTGATGACAGCCCTGAAAAC-3'; S199N, 5'-CATTGTGCATG-GTAATGACAGCCCTGAAAAC-3'; S199T, 5'-CATTGTGCATGACTGACAGCCCTGAAAAC-3'; N204D, 5'-GACAGCCCTGAAGACGGCAA-GCGTGAG-3'; E208D, 5'-GAAACCGCAAGCGTGACATTGGTCTGT-GG-3'; W212D, 5'-GTG-AGATTGGTCTGGAAGTTCAAAGAGGGC-3'; and W212K, 5'-GTGAGATTGGTCTGAAGTTCAAAGAGGGC-3'. NDPK2 dimeric form mutants, including RP175S, LP175S, and KP175S, were prepared using the corresponding deletion mutants (R230Stop, L225Stop, and K214Stop) and the primers designed for

P175S site-directed mutagenesis. All primers were PAGE-purified. The mutations were verified by DNA sequencing.

Purification of Phytochromobilin Chromophore and Its Assembly with Recombinant Phytochromes—Phytochromobilin was purified by chromatography as described previously (38). Holophytochromes were prepared by adding phytochromobilin to phytochrome apoproteins at a final concentration of 20 μM , followed by a 1-h incubation on ice.

In Vitro Binding Assay—The *in vitro* binding assays of phytochrome and NDPK2 were performed by incubating 10 μg of phytochrome and 20 μg of NDPK2 in TBS buffer (50 mM Tris-HCl (pH 7.5), 150 mM NaCl), containing 5 mM MgCl_2 , 2 mM dCDP, 0.1% Nonidet P-40, and protease inhibitors, at 4 °C for 30 min. Either antibody Oat-22 against oat PhyA or the specific antibody against NDPK2 was added to the reaction mixture and incubated for 40 min. The antibody-protein complexes were recovered by incubation with 0.1 volumes of protein-A/G beads (Oncogene) for an additional 30 min and then collected by centrifugation. Beads were washed five times in TBS buffer. The attached proteins were solubilized in 1× SDS sample buffer at 100 °C for 5 min and then resolved on 12% (w/v) SDS-polyacrylamide gels, followed by transferring to polyvinylidene difluoride membranes for Western blotting analysis.

Size Exclusion Chromatography—NDPK2 (5 μg) was applied to a Biosillect® SEC 250–5 column (Bio-Rad) in a Shimadzu LC-10AT high pressure liquid chromatography system and eluted with 50 mM Tris-HCl buffer (pH 7.4), with 150 mM NaCl and 6 mM dithiothreitol. The flow rate was 0.5 ml/min.

NDPK2 γ -Phosphate Exchange Activity Assay—NDPK2 γ -phosphate exchange activity was measured as described previously (20) with minor modifications. The assay buffer contained 50 mM Tris-HCl (pH 7.5), 5 mM MgCl_2 , 3 mM phosphoenolpyruvate, 2 mM ATP, 0.3 mM NADH, 5 units of pyruvate kinase, 5 units of lactate dehydrogenase, and 1 mM dCDP. The reaction was initiated by adding 3 nM NDPK2. NDPK2 activity was measured by monitoring the lactate dehydrogenase-pyruvate kinase-coupled NADH decrease at 340 nm. The phytochrome effect was examined by incubating a mixture of native oat PhyA and NDPK2 under illumination of red light (660 nm, Pfr form) or far-red light (730 nm, Pr form) for 8 min and measuring the NDPK2 activity. The K_m values of NDPK2 with different nucleotides were measured in a similar manner with a fixed concentration of 60 nM of Pfr phytochrome.

NDPK2 Autophosphorylation and Kinase Activity—NDPK2 autophosphorylation was performed in a buffer containing 5 μg of NDPK2 protein, 50 mM triethanolamine hydrochloride (pH 7.4), 1 mM EDTA, 150 mM NaCl, 2 mM MgCl_2 , 1 mM dithiothreitol, 2 μM ATP or GTP, 5 μCi of γ - ^{32}P -labeled ATP or GTP for 15 min on ice. NDPK2 kinase activity was measured by using 0.2 μg of NDPK2 protein and 20 μg of myelin basic protein (MBP) protein in the same reaction buffer as autophosphorylation for 10 min at 30 °C. The phytochrome effect was examined by adding the indicated amount of oat PhyA to the reaction mixture under either autophosphorylation conditions or kinase activity conditions.

NDPK2 Activity Measurements; pH Dependence and Thermal Stability—The pH dependence of NDPK2 was measured as described previously (35). AMT isoionic buffer (50 mM acetic acid, 50 mM Mes, and 100 mM Tris-HCl) (39) was used at a pH range of 5.0–9.5. The thermal stabilities of NDPK2 wild type and mutant proteins were analyzed by measuring NDPK2 activity after 10 min of incubation at the indicated temperatures.

NDPK2 Binding with [^3H]GDP; Equilibrium Dialysis Experiment—The binding of [^3H]GDP (Amersham Biosciences) by NDPK2 was measured via equilibrium dialysis as described previously with minor modification (40). A 12–14-kDa cut-off dialysis membrane (Spectra/Por) was used to separate the buffer containing NDPK2 (bottom) and the buffer containing [^3H]GDP (top). The bottom portion containing NDPK2 was monitored at the indicated time points with a liquid scintillation counter. The phytochrome effect was examined by adding native oat PhyA to the NDPK2 portion and illuminating the reaction mixture every 8 h during the incubation in the dark.

RESULTS

NDPK2 Interacts with Phytochrome in Vitro—We have reported previously (20) that NDPK2 interacts with the C-terminal domain of *Arabidopsis* PhyA in yeast two-hybrid screening and can be catalytically stimulated by the Pfr form of native oat PhyA. To better understand the interaction between NDPK2 and phytochrome, an *in vitro* binding experiment was conducted. Purified native oat PhyA was tested in the immunoprecipitation reaction with NDPK2. Results indicated that NDPK2 possessed a weak interaction with both Pr and Pfr forms of phytochrome in

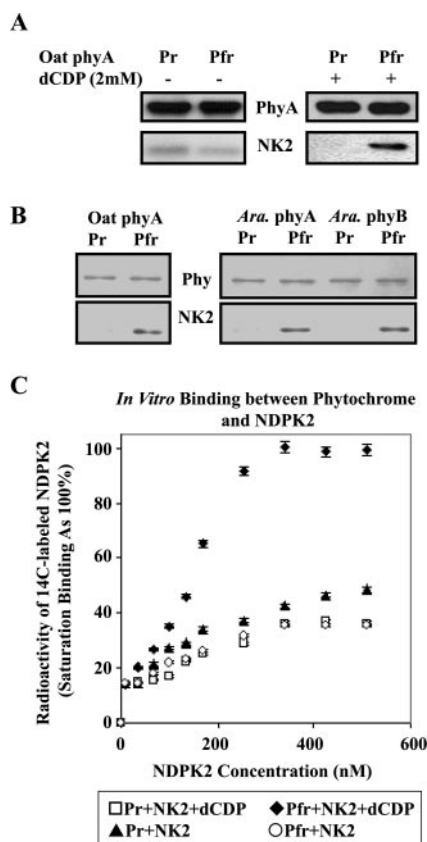


FIG. 1. *In vitro* binding between phytochrome and NDPK2. **A**, immunoprecipitation of NDPK2 and purified native oat PhyA with or without addition of dCDP (2 mM). Oat-22 antibody against phytochrome was used in this reaction. NDPK2 interacts with the Pfr form of phytochrome specifically in the presence of dCDP. **B**, immunoprecipitation of NDPK2 and recombinant phytochromes, including oat PhyA, *Arabidopsis* PhyA, and *Arabidopsis* PhyB. Oat-22 antibody against phytochrome was used in this reaction. 2 mM dCDP was added in the reaction buffer. NDPK2 interacts with the Pfr form of phytochromes. **C**, immunoprecipitation of purified native oat PhyA and NDPK2 incorporated with ¹⁴C-labeled methionine. 105 nM phytochrome was used in each reaction. The Pfr form of phytochrome (◆) binds NDPK2 preferentially. NDPK2 saturation binding was reached at ~330 nM, and 100% interaction was assumed. ■, Pr + NK2 + dCDP; ◆, Pfr + NK2 + dCDP; ▲, Pr + NK2; and ●, Pfr + NK2.

the absence of dCDP (Fig. 1A). However, NDPK2 was found to interact with the Pfr form of phytochrome preferentially in the presence of dCDP (Fig. 1A). This Pfr-specific interaction between phytochrome and NDPK2 with nucleotide addition is consistent with the current concept that the Pfr form of phytochrome is the active form in phytochrome-mediated signaling. Therefore, dCDP was applied in the future binding experiments between these two proteins. Recombinant phytochromes were also tested, including recombinant oat PhyA, *Arabidopsis* PhyA, and *Arabidopsis* PhyB. Results showed a Pfr preference in the binding as well (Fig. 1B). To investigate the binding kinetics between phytochrome and NDPK2, NDPK2 protein incorporated with ¹⁴C-labeled methionine was expressed and tested in a binding assay with native oat PhyA. Results confirmed the Pfr-preferred interaction between phytochrome and NDPK2 in the presence of dCDP and suggested that the interaction between these two proteins in the absence of dCDP is nonspecific (Fig. 1C). In addition, results also indicated that the saturation binding of NDPK2 occurred at ~330 nM when 105 nM phytochrome was tested. Thus, the binding ratio between phytochrome and NDPK2 is close to the ratio of phytochrome dimer (41) to NDPK2 hexamer (42).

To locate the NDPK2-binding site in the phytochrome molecule, partial-length phytochromes were expressed and tested. To be consistent with previous experiments, oat PhyA was selected as the phytochrome for the domain study. The N-terminal domain of oat PhyA (AN, 1–505 aa) showed no binding with NDPK2 when testing both Pr and Pfr forms, whereas the C-terminal domain (oat AC, 506–1129 aa) was sufficient to bind NDPK2 (Fig. 2A). Meanwhile, the C-terminal domains of both *Arabidopsis* PhyA and PhyB also interacted with NDPK2 in the *in vitro* binding assay (Fig. 2A). The γ -phosphate exchange activity assay confirmed that the phytochrome C-terminal domain alone is sufficient to bind and stimulate NDPK2 (Fig. 2B).

To determine the exact binding site of NDPK2 in the C-terminal domain, oat PhyA C-terminal deletion mutants, A957 and A686, were tested. A957 (1–957 aa) is composed of PAS domain A, the Quail box (Q box), and PAS domain B, with the histidine kinase-related domain (HKRD) removed, whereas A686 (1–686 aa) contains PAS domain A only (Fig. 2C). The zinc-blotting analysis confirmed the appropriate assembly between phytochrome apoproteins and chromophore phytychromobilin (Fig. 2D). Both A957 and A686 interacted with NDPK2, with A957 binding Pfr preferentially and A686 binding Pr and Pfr equally (Fig. 2D). Further experiments revealed that A686 undergoes a quick dark reversion from its Pfr to Pr forms (Fig. 2E). The structural difference between Pr and Pfr A686 could be compromised by this quick dark reversion, especially during a time range of 90 min in the immunoprecipitation reaction. Meanwhile, both A957 and A686 were able to stimulate NDPK2 γ -phosphate exchange activity (Fig. 2F). Because NDPK2 activity was measured within 10 min immediately after red light illumination, the quick dark reversion of A686 is not obvious in this enzymatic assay. Results indicated that Pfr A686 stimulated NDPK2 more than Pr A686. Because the N-terminal domain of oat PhyA (AN, 1–505 aa) did not bind to NDPK2 and PAS domain A is the only subdomain left in A686, PAS domain A is identified as the NDPK2-binding site.

Phytochrome Recognizes Both NDPK2 Hexameric Structure and Its C-terminal Fragment—To locate the phytochrome-binding site in NDPK2, NDPK2 mutants, including the C-terminal deletion mutants, kinase site mutant H197C, and Kpn loop mutant P175S, were made and tested in the binding assays with the Pfr form of native oat PhyA (Fig. 3A). Results indicated that mutants H197C and P175S interacted with Pfr phytochrome as well as the wild type, suggesting that the kinase activity and Kpn loop of NDPK2 are not involved in the binding with phytochrome directly. Furthermore, the C-terminal deletion mutants R230Stop and L225Stop interacted with Pfr phytochrome well, whereas mutant K214Stop showed almost no interaction with Pfr phytochrome. Moreover, no interactions between Pfr phytochrome and mutants N204Stop and S199Stop were observed. These results indicated that the NDPK2 C-terminal fragment, especially residues 214–224, is critical in the interaction with phytochrome. Results from the γ -phosphate exchange activity assay were consistent with the *in vitro* binding assay. Mutants R230Stop and L225Stop were stimulated by Pfr phytochrome significantly, whereas mutants K214Stop, N204Stop, and S199Stop were not stimulated under the identical conditions (Fig. 3B), suggesting the C-terminal fragment of NDPK2 as the phytochrome-binding site.

To investigate the role of NDPK2 oligomeric states in the interaction with phytochrome, the dimeric forms of NDPK2 mutants were made according to the method described previously (43), including mutants R230StopP175S (RP175S), L225StopP175S (LP175S), and K214StopP175S (KP175S). Among them, mutants LP175S and KP175S were confirmed by

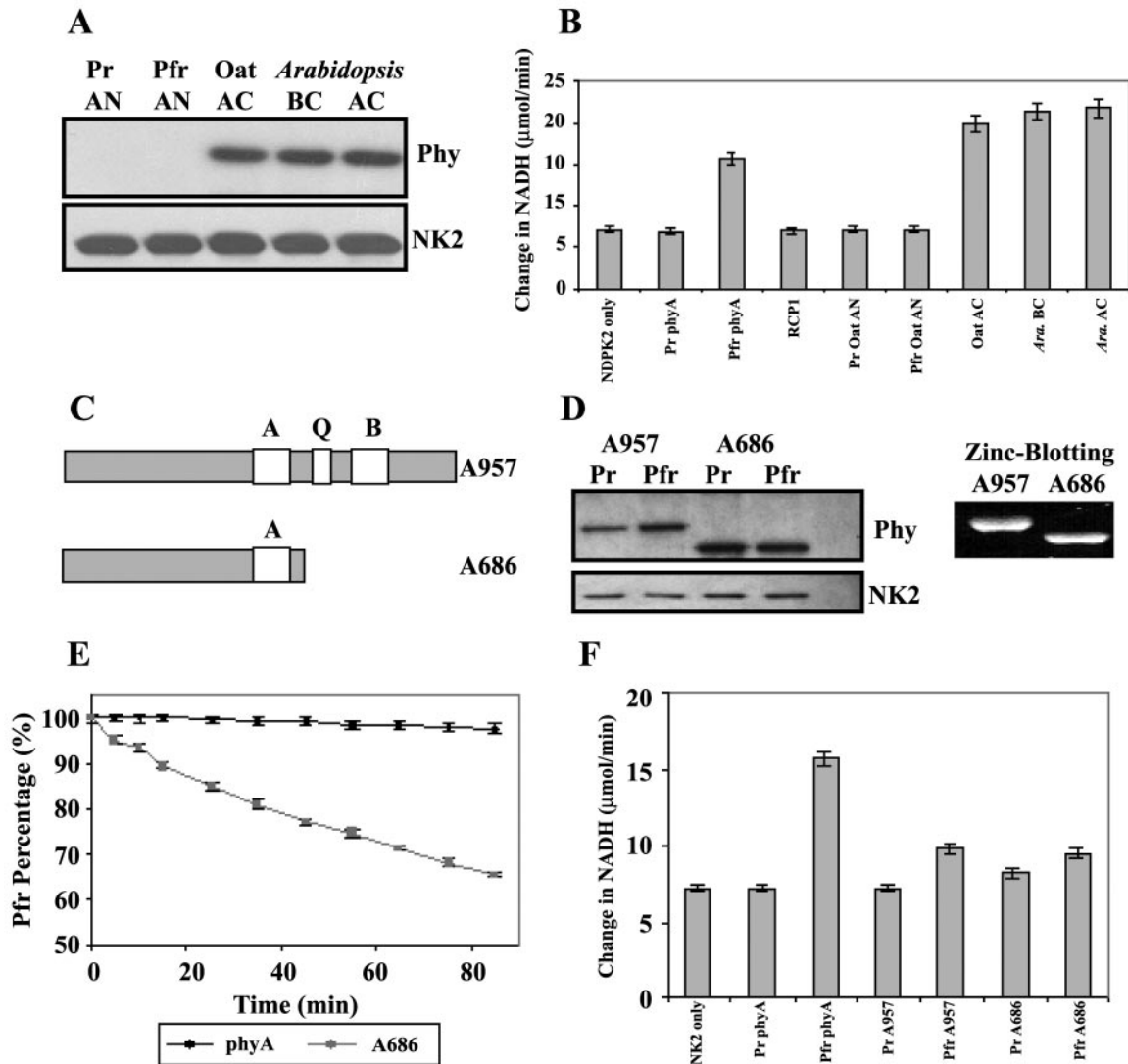


FIG. 2. Phytochrome domain study to locate the NDPK2-binding site. A, phytochrome C terminus binds NDPK2. Phytochrome N terminus, either Pr or Pfr, shows no binding. AN, oat PhyA N terminus; Oat AC, oat PhyA C terminus; Arabidopsis AC, Arabidopsis PhyA C terminus; Arabidopsis BC, Arabidopsis PhyB C terminus. NDPK2-specific antibody was used in this immunoprecipitation reaction. B, phytochrome C terminus stimulates NDPK2 γ -phosphate exchange activity. Phytochrome N terminus shows no effect, including both Pr and Pfr forms. NDPK2 activity was recorded as NADH change monitored at 340 nm. NDPK2 only and NDPK2 + RCP1 are controls. C, domain structures of C-terminal deleted oat PhyA mutants, including A957 (1–957 aa) and A686 (1–686 aa). A, PAS domain A; Q, the Quail box; B, PAS domain B. D, both A957 and A686 bind NDPK2. A957 shows Pfr-preferred binding with NDPK2. A686 shows Pr and Pfr equally binding. PAS domain A is the NDPK2-binding site. Left, immunoprecipitation of NDPK2 with A957 and A686; right, zinc-blotting analysis of A957 and A686 proteins after chromophore assembly. E, A686 shows a quick dark reversion between its Pfr and Pr forms. Phytochrome absorbance at 730 nm was recorded as Pfr components of the phytochrome mixture. \blacklozenge , full-length phytochrome A; \blacksquare , A686 only. F, the Pfr forms of both A957 and A686 stimulate NDPK2 activity, confirming the binding results in D. A957 shows Pfr preference. The Pfr form of A686 is also preferred within the reaction time, but the Pr form has minor stimulation as well.

size exclusion chromatography as the dimeric form of NDPK2, whereas the hexameric form was dominant in mutant RP175S (Table I). The *in vitro* binding assay showed that there is no interaction between Pfr phytochrome and the dimeric form of NDPK2 (Fig. 4A). The γ -phosphate exchange activity assay indicated a dramatic decrease in the activities of these NDPK2 mutants even though their corresponding deletion mutants showed relatively higher activities than the wild type (Fig. 4B). NDPK2 wild type and Kpn loop mutant P175S served as controls in this experiment. Although mutant P175S interacted well with Pfr phytochrome in the binding assay, its activity was affected due to the damage of the subunit interactions caused by the Kpn loop mutation. Meanwhile, the dimeric form of NDPK2 also lost the ability to be stimulated by the Pfr phytochrome (Fig. 4C). These results demonstrated the importance

of NDPK2 oligomeric states in the interaction between phytochrome and NDPK2.

Phytochrome Increases the Nucleotide Affinity of NDPK2 by Accelerating the Nucleotide Exchange during γ -Phosphate Exchange Reaction—To investigate the mechanism of phytochrome stimulation on NDPK2, the nucleotide affinity was the first issue addressed. By using two natural nucleotide substrates of NDPK2, ATP and GDP, the K_m values were measured (Table II). Addition of Pfr phytochrome lowers the ATP K_m value of NDPK2 from 0.286 to 0.183 mM and the GDP K_m value from 0.231 to 0.189 mM, respectively. To examine the relationship between the nucleotide affinity and NDPK activity, NDPK2 hyperactive mutants, including R230Stop and L225Stop, were also examined. Both mutants showed decreased K_m values as follows: 0.211 and 0.213 mM for ATP, and

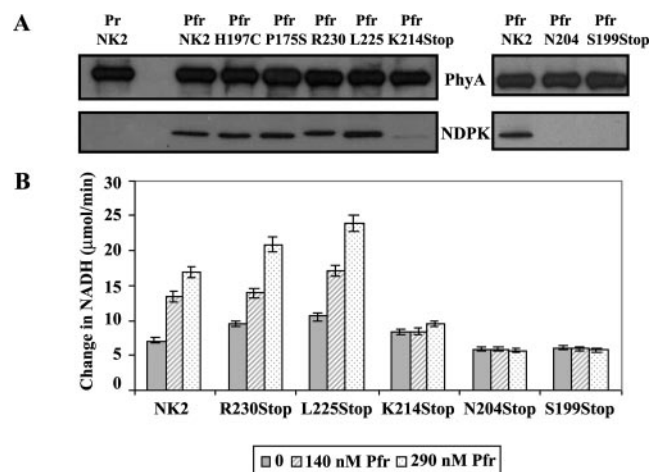


FIG. 3. **The extreme C-terminal fragment of NDPK2 is the phytochrome-binding site.** A, immunoprecipitation between native oat PhyA and NDPK2 C-terminal deletion mutants. L225Stop binds Pfr phytochrome well. K214Stop almost has no binding with Pfr phytochrome. NDPK2 kinase site mutant H197C and Kpn loop mutant P175S bind Pfr phytochrome equal to the wild type. B, the stimulation of Pfr phytochrome on NDPK2 C-terminal deletion mutants. 145 and 290 nM Pfr phytochrome were tested in this assay, respectively. Pfr phytochrome is not able to stimulate K214Stop in the γ -phosphate exchange activity assay.

TABLE I
Oligomeric states of NDPK2 and NDPK2 dimeric form mutants

Oligomeric states	Hexamer	Dimer
	%	
NK2 wild type	100	0
L225Stop	~95	~5
P175S	~71	~29
RP175S	~64	~36
LP175S	~25	~75
KP175S	~11	~89

0.203 and 0.191 mM for GDP. Therefore, it appears that a higher nucleotide affinity corresponds to a higher NDPK activity and that the Pfr phytochrome stimulates NDPK2 by increasing its nucleotide affinity.

There are two likely possibilities that could lead to a nucleotide affinity increase in the measurement of the γ -phosphate exchange activity, including an enhanced nucleotide binding ability of NDPK2 and an accelerated nucleotide exchange in and out of the nucleotide-binding pocket during the process of the γ -phosphate exchange reaction. To further understand the nucleotide affinity change caused by addition of phytochrome, the GDP binding ability of NDPK2 was investigated by using ^3H -labeled GDP in an equilibrium dialysis experiment with or without addition of the Pfr form of native oat PhyA. There are two reasons why GDP was selected as the nucleotide to study instead of ATP. One reason is that GDP is a relatively poor substrate of NDPK catalysis compared with ATP (44). A poor substrate would be an ideal target in the study of the nucleotide binding ability. Another reason is that ATP could interact with both NDPK2 and phytochrome. The result of the nucleotide binding experiment would be obscure if ATP were selected. NDPK2 wild type, NDPK2 kinase site mutant H197C, and NDPK2 C-terminal deletion mutants were examined in the equilibrium dialysis experiment. The control experiment indicated that NDPK2 binds GDP well, whereas the Pr and Pfr forms of phytochrome have no GDP binding ability (Fig. 5A). Most surprisingly, the presence of Pfr phytochrome did not enhance the GDP binding ability of NDPK2 (Fig. 5B). Instead, Pfr phytochrome inhibited NDPK2:GDP binding. Meanwhile, NDPK2 hyperactive mutants R230Stop and L225Stop also had

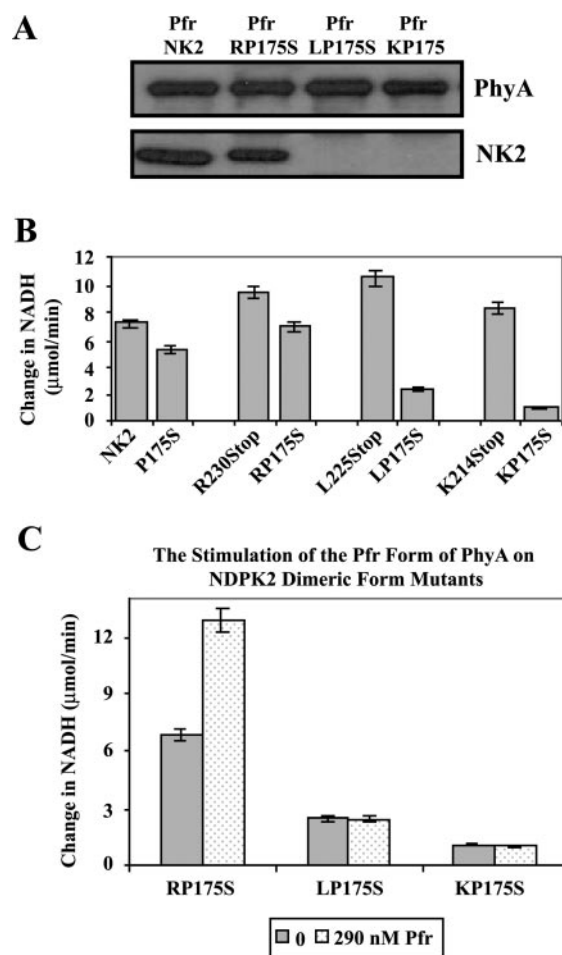


FIG. 4. **Phytochrome interacts with the hexameric form of NDPK2.** A, immunoprecipitation between the Pfr form of native oat PhyA and NDPK2 dimeric form mutants. LP175S and KP175S lose the ability to interact with phytochrome. B, the γ -phosphate exchange activities of NDPK2 mutants. R230Stop, L225Stop, and K214Stop are NDPK2 C-terminal deletion mutants. RP175S, LP175S, and KP175S are the corresponding mutants that have a P175S point mutation. C, Pfr phytochrome stimulates the hexameric form of NDPK2 but not the dimeric form. 290 nM Pfr phytochrome was used in this assay. NDPK2 mutant (lane 1) and NDPK mutant + Pfr phytochrome (lane 2) are shown in RP175S, LP175S, and KP175S, respectively.

TABLE II
The K_m values of NDPK2, Pfr phytochrome-stimulated NDPK2, and NDPK2 C-terminal deletion mutants determined by using ATP and GDP

K_m	ATP (dCDP as acceptor)	GDP (ATP as donor)
NDPK2	0.286 mM	0.231 mM
NDPK2 + Pfr PhyA (145 nM)	0.183 mM	0.189 mM
R230Stop	0.211 mM	0.203 mM
L225Stop	0.213 mM	0.191 mM

worse GDP binding than their wild type, similar to Pfr phytochrome-stimulated NDPK2. With addition of Pfr phytochrome, the GDP binding abilities of mutants R230Stop and L225Stop became even worse. Additionally, mutant K214Stop, another NDPK2 hyperactive mutant, also showed worse GDP binding than the wild type, but its GDP binding ability was not affected by Pfr phytochrome, which is consistent with the *in vitro* binding results. On the other hand, mutant H197C showed the same binding pattern as the wild type, confirming that NDPK2 kinase activity is not involved in the direct interaction with phytochrome. Because phytochrome does not enhance the nucleotide binding ability of NDPK2, it must affect the nucleotide

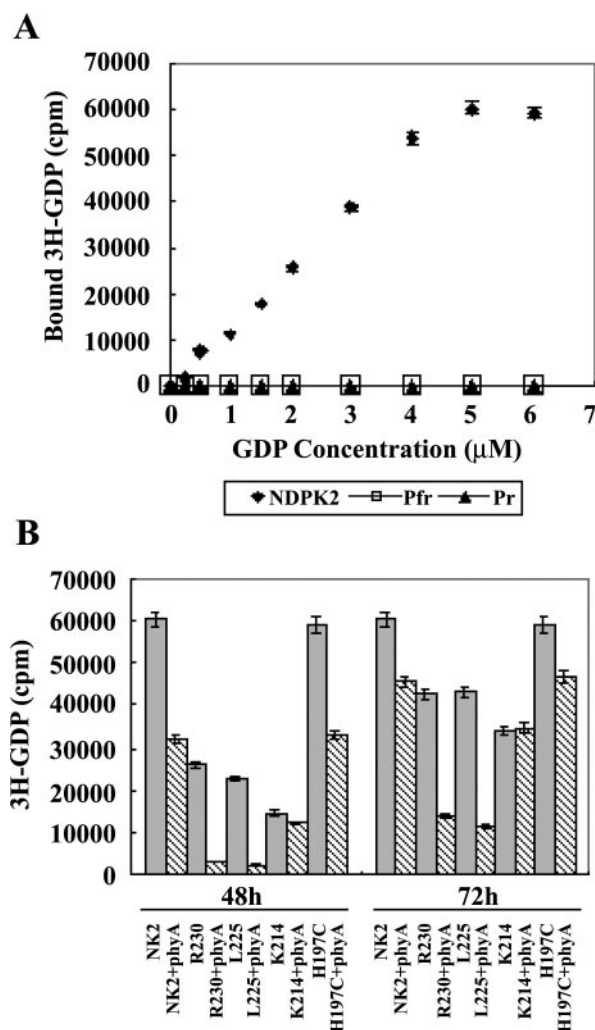


FIG. 5. Phytochrome addition interferes with NDPK2 GDP binding. A, NDPK2 binds ^3H -labeled GDP (\blacklozenge). The Pr (\blacktriangle) and Pfr (\blacksquare) forms of native oat PhyA show no binding. B, GDP binding of NDPK2 with or without Pfr phytochrome addition. R230Stop, L225Stop, and K214Stop are NDPK2 C-terminal deletion mutants, and H197C is NDPK2 kinase site mutant. Pfr phytochrome interferes with NDPK2 GDP binding within either a 48- or 72-h time range. H197C shows no difference in GDP binding compared with the wild type.

exchange during the process of the γ -phosphate exchange reaction. By doing so, the turnover of NDPK2 is accelerated.

Phytochrome Stimulates NDPK2 Nucleotide Exchange by Lowering the pK_a Value of His-197—The H-bonding with the active histidine residue in the nucleotide-binding pocket is very important for NDPK functioning. To investigate the mechanism of how NDPK2 nucleotide exchange as well as NDPK2 activity is modulated via the active His-197, His-197-surrounding residue mutants were designed according to the known NDPK crystal structures, in which the residues near His-197 were mutated to the charged residues (45–53). These selected residues include Tyr-87, Met-89, His-130, Gly-198, Ser-199, Asn-204, Glu-208, and Trp-212 that are very close to residue His-197 in terms of the three-dimensional structures. Results of the γ -phosphate exchange activity assay indicated that all mutations of selected residues affected NDPK2 activity, confirming that all the residues selected in the mutagenesis are structurally close to residue His-197. Among these mutants, only mutant Y87D possessed a higher activity than the wild type (Fig. 6A), whereas the other mutants showed a significant decrease in their activities. Some of these mutants were further characterized by investigating their protein thermal stabilities

(Fig. 6B). Because residues Ser-199 and Glu-208 are well known for their crucial roles in the H-bonding with His-197, mutants S199T and E208D were selected as examples. The decreased activities of these two mutants suggested a damaged H-bonding with His-197 inside their nucleotide-binding pockets (Fig. 6A). Furthermore, these two mutants also showed worse thermal stabilities compared with the wild type (Fig. 6B and Table III). The correlation between NDPK activity and protein thermal stability indicated the importance of the H-bonding with His-197 in NDPK2 functioning. This result is also in good agreement with the results of other NDPK2 hypoactive mutants and oligomeric state mutants, including P175S and L175S. These mutants showed decreased protein thermal stabilities and lower NDPK activities. Most surprisingly, the hyperactive NDPK2 mutants L225Stop and Y87D also showed a slight decrease in their protein thermal stabilities (Fig. 6B), suggesting another type of H-bonding change in these hyperactive mutants which is good for NDPK2 nucleotide exchange and its activity.

To understand further how these two types of H-bonding change affect NDPK2 nucleotide exchange and its activity, either to stimulate or inhibit, the pH dependence of NDPK2 wild type and mutants was studied. Fig. 6C shows the pH dependence profiles of NDPK2 with or without addition of Pfr phytochrome. pK_{a1} of 6.35 for NDPK2 (Table IV) is believed to be due mainly to the active histidine residue His-197. The pH range of 8.00–8.95 is the optimal pH condition for NDPK2-catalyzed γ -phosphate exchange reaction. pK_{a2} of 8.95 for NDPK2 is likely due to the other charged residues in the nucleotide-binding pocket, such as residues Lys-91 and Tyr-131 (corresponding to residues Lys-16 and Tyr-56 in *Dictyostelium* NDPK) (35). Addition of Pfr phytochrome changed the pH dependence profile of NDPK2 significantly, lowering its pK_{a1} to 5.65 and its pK_{a2} to 8.85, respectively. The pH dependence of NDPK2 mutants was also investigated. To be consistent with the previous experiment, mutants S199T and E208D were selected (Table IV). Additionally, mutant Y87D was also selected for investigation because it is the only hyperactive NDPK2 mutant of all the His-197-surrounding residue mutants (Table IV). The pK_{a1} values of the hypoactive mutants S199T and E208D are 6.50 and 7.00, respectively, which are higher than that of the wild type. In contrast, the hyperactive mutant Y87D has a lower pK_{a1} value of 6.20. The pK_a values of other related NDPK2 mutants were also determined. Results revealed that lower pK_a values, especially lower pK_{a1} values, correspond to higher NDPK activities (Table IV). Because the pK_{a1} value of NDPK2 represents either the deprotonation of N-8H on His-197 to allow phosphorylation or phospho-transfer from phosphorylated His-197 to its substrate, a lowered pK_{a1} means His-197 is easier to either become phosphorylated or to release the phosphate group to its substrate during the process of γ -phosphate exchange. Either scenario could lead to an acceleration of the nucleotide exchange and thereby contribute to the stimulation of NDPK2 activity.

This hypothesis was confirmed by the NDPK2 autophosphorylation experiment. Addition of Pfr phytochrome increased NDPK2 autophosphorylation significantly compared with the slight effect from the Pr phytochrome (Fig. 7A). Most interestingly, MBP, which serves as a NDPK2 kinase substrate, also slightly enhanced NDPK2 autophosphorylation, suggesting a mechanism of substrate-assisted autophosphorylation. Meanwhile, Pfr phytochrome was also found to stimulate NDPK2 phospho-transferring ability to MBP, whereas in the assays of NDPK2 alone or NDPK2 with addition of Pr phytochrome, the phospho-transferring from NDPK2 to MBP is too slow to detect (Fig. 7B). Therefore, by lowering the pK_a value of His-197,

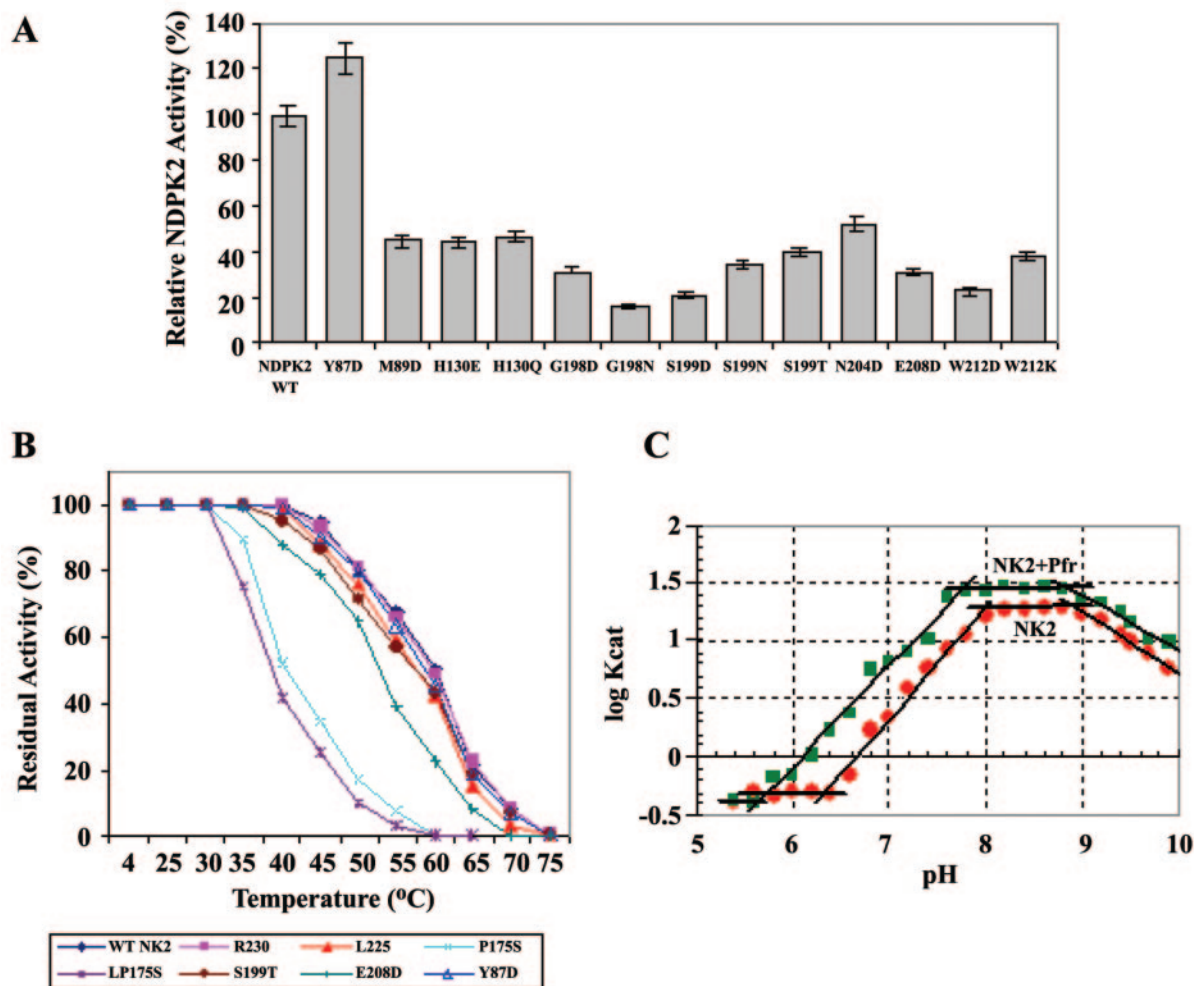


FIG. 6. Characterization of His-197-surrounding residue mutants. A, the γ -phosphate exchange activities of His-197-surrounding residue mutants. Y87D shows a higher activity than the wild type. Other mutants have a lower activity than the wild type. B, thermal stabilities of NDPK2 mutant proteins. NDPK2 activities were recorded as percentages of their maximal activities, respectively (wild type NDPK2 activity as 100%). The samples are labeled as shown in the graph. C, the pH dependence profiles of NDPK2 only (●) and NDPK2 stimulated by Pfr phytochrome (■).

TABLE III

Thermal stabilities of NDPK2 and NDPK2 mutant proteins

T_m values are measured at 50% of the optimal NDPK activities.

NDPKs	T_m
	°C
NK2 wild type	~60
R230Stop	~60
L225Stop	~57
P175S	~41
LP175S	~38
S199T	~57
E208D	~53
Y87D	~59

TABLE IV

The pK_a values observed in the pH dependence profiles of NDPK2, Pfr phytochrome-stimulated NDPK2, and NDPK2 mutants

NDPKs	pK_{a1}	Optimal pH	pK_{a2}
NK2 WT	6.35	8.00–8.95	8.95
NK2 + PhyA (Pfr)	5.65	7.80–8.85	8.85
L225Stop	6.10	7.85–8.90	8.90
L225Stop + PhyA (Pfr)	5.40	7.80–8.60	8.60
R230Stop	6.10	7.95–8.90	8.90
Y87D	6.20	7.95–8.60	8.60
N204D	6.35	8.10–8.85	8.85
S199T	6.50	8.20–8.80	8.80
E208D	7.00	8.00–9.10	9.10

phytochrome stimulates NDPK2 activity by accelerating both phosphorylation and dephosphorylation of His-197.

DISCUSSION

Phytochromes serve as molecular light switches in plants that regulate plant growth and development throughout their life cycle. Recent progress in the identification of phytochrome-interacting proteins (20, 32–34) revealed why phytochromes are so multifunctional. The C-terminal domain of phytochrome has been considered as the regulatory domain, containing PAS domain A, the Q box, PAS domain B, and an HKRD domain. The PAS domains are well known for protein-protein interactions and protein dimerization (54), and the histidine kinase

domain in cyanobacterial phytochrome possesses histidine kinase activity (55). On the other hand, a recent report (56) suggested that dimers of the N-terminal domain of *Arabidopsis* PhyB are functional in the nucleus, whereas the C-terminal domain attenuates the activity of PhyB. Because phytochrome-interacting proteins were all identified by using the phytochrome C terminus as the bait in yeast two-hybrid screenings, including PIF3, PIF4, PKS1, and NDPK2, it would be interesting to see if they share the same binding site. By studying the interaction between phytochrome and NDPK2, we hope to gain a deeper insight into the phytochrome-mediated signaling.

NDPK2 interacted with *Arabidopsis* PhyA and PhyB *in vitro*, as well as with oat PhyA (Fig. 1, A and B). This is not surprising

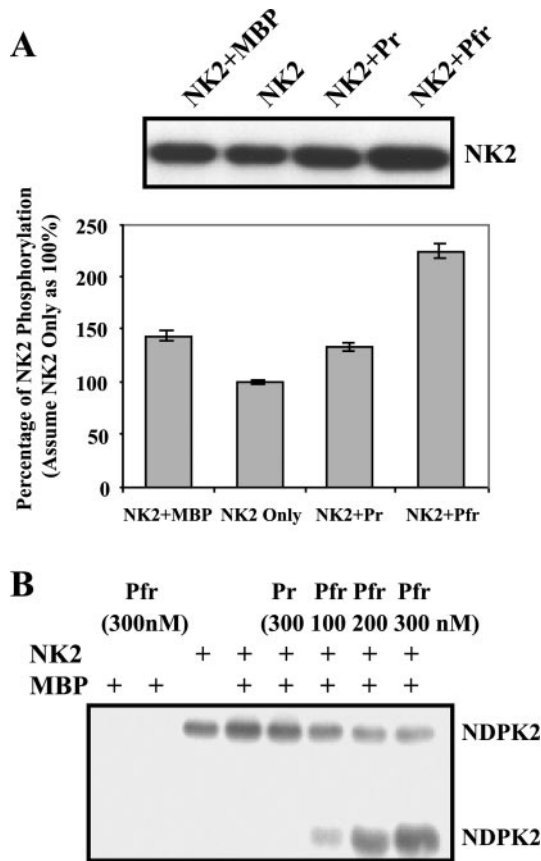


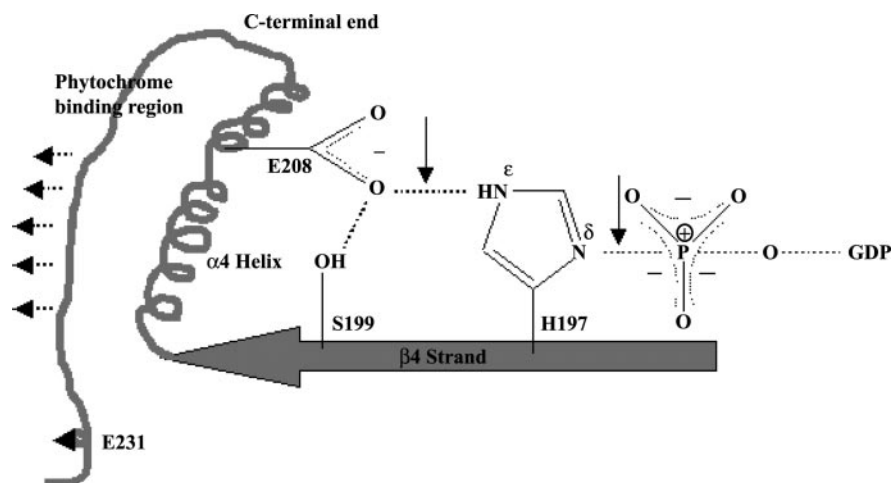
FIG. 7. The Pfr form of phytochrome stimulates NDPK2 autophosphorylation and kinase activity. A, NDPK2 autophosphorylation. Pfr phytochrome enhances NDPK2 autophosphorylation (4th lane). Pr phytochrome shows slight stimulation of NDPK2 autophosphorylation (3rd lane). Addition of NDPK2 kinase substrate (MBP) also shows slight enhancement of NDPK2 autophosphorylation (1st lane). B, NDPK2 phospho-transferring ability to MBP. Pfr phytochrome stimulates NDPK2 phospho-transferring to MBP (6th to 8th lanes). Pr phytochrome has no effect on NDPK2 phospho-transferring ability within the same period (5th lane). Phytochrome concentrations are indicated as Pfr 100 nM (6th lane), 200 nM (7th lane), 300 nM (8th lane), and Pr 300 nM (5th lane). MBP only (1st lane) and Pfr 300 nM + MBP (2nd lane) are controls.

because phytochromes share very high similarities in their C termini. Domain studies showed that the phytochrome C terminus alone is necessary to interact with NDPK2 and to stimulate its γ -phosphate exchange activity (Fig. 2, A and B). This result is different from PIF3, which binds to both the N-terminal and C-terminal domains and more strongly to full-length phytochrome (57). Meanwhile, in the *in vitro* binding assay between phytochrome and NDPK2, nucleotide addition was found to enhance the Pfr-preferred binding of phytochrome, including NDP and NTP. Previous reports (47, 48, 58) have shown that the bound nucleotide is located between the α_A - α_2 hairpin and the Kpn loop of NDPK, resulting in a nucleotide-binding pocket 2 Å narrower than the free form. The presence of nucleotide might stabilize the positions of both α_A - α_2 hairpin and C-terminal fragment and thereby stabilize the hexameric structure of NDPK2. By doing so, the mobile C-terminal fragment of NDPK2 could be fixed and be easier to recognize by the phytochrome. Nucleotide addition was also found to enhance the protein thermal stability of NDPK2, increasing the T_m value of NDPK2 Kpn loop mutant P175S by 2 °C (data not shown). The significance of nucleotide effect on the Pfr-preferred interaction between phytochrome and NDPK2 needs to be further examined.

By using partial-length phytochromes A957 and A686, the NDPK2-binding site was specified as PAS domain A (Fig. 2, C and D). Meanwhile, although A957 lacks the HKRD domain, its Pfr form still preferred to interact with NDPK2. Therefore, it can be concluded that the HKRD domain is not necessary for NDPK2 binding and that there is no relationship between the phytochrome HKRD domain and NDPK2 histidine autophosphorylation. Furthermore, A686 lost the Pfr preference in the NDPK2 binding, suggesting a conformational change in A686 compared with full-length phytochrome. The quick dark reversion of A686 (Pfr to Pr) may explain this Pr and Pfr indifference, which reduces the amount of Pfr in the phytochrome mixture and thus lowers the level of phytochrome binding and stimulation. When we performed the binding assay under continuous red light illumination to sustain a maximum for the Pfr form at the photostationary state, we obtained a Pfr-preferential binding of A686 to PhyA (data not shown). The rapid dark reversion might be due to the C-terminal deletion in A686, which removes part of the putative dimerization motif (59–62). On the other hand, Pr A686 also interacted with NDPK2 (Fig. 2D), which is consistent with the hypothesis of inter-domain signaling established previously by our group (25, 63). In this hypothesis, full-length phytochrome exists as a dimer. The extreme N-terminal extension (NTE) region of both monomers assumes a random coil conformation in the Pr form and obscures the C-terminal regulatory domains. Therefore, the C terminus of Pr phytochrome is not able to interact with its signaling partners and initiate signaling. Upon red-light illumination, the Pr form is transformed to the Pfr form. During this process, the NTE region forms an amphiphilic α -helix, exposing the C terminus for the interaction with its signaling partners, such as NDPK2. This structural difference between Pr and Pfr is significant in the full-length phytochrome. However, it might not be the case in A686 because of the deletion of the Q box, PAS domain B, and the HKRD domain. The NTE region of Pr A686 might not be able to contact or block the C-terminal domain completely like full-length phytochrome. Therefore, PAS domain A in Pr A686 might have chance to interact with NDPK2 like its Pfr form. Our results suggest that the NTE region might contact the deleted C-terminal region in A686, including the Q box, PAS domain B, and the HKRD domain. Meanwhile, the exact role of the Q box in the interaction between phytochrome and NDPK2 needs to be further examined because the Q box is not indispensable in this physical interaction.

Three NDPK isoforms in *Arabidopsis* possess very similar hexameric structures (42). The *in vitro* binding study using NDPK2 C-terminal deletion mutants suggested that the phytochrome-binding site in NDPK2 resides in its C-terminal fragment, consistent with the fact that the C-terminal fragment is the most variable and mobile part in the NDPK structures (64). One might question about the conformational integrity of these C-terminal truncated NDPK2 mutants and why these mutants failed to bind PhyA because of their folding problems. However, these mutants possessed NDPK activities close to that of the wild type. From the gel filtration experiments, we found that mutants R230Stop, L225Stop, K214Stop, and N204Stop all possessed a hexameric structure (>90%), although only L225Stop is shown in Table I. Furthermore, mutants R230Stop, L225Stop, K214Stop, and N204Stop showed similar thermal stabilities (R230Stop and L225Stop in Fig. 6B, and data not shown for K214Stop and N204Stop). Because the enzymatic activity of NDPK is associated with the hexameric structure and full enzymatic activity is indicative of correct quaternary structure (65, 66), we conclude that the inability of mutants K214Stop, N204Stop, and S199Stop to bind PhyA is not because of protein folding problem. Mean-

FIG. 8. A schematic transition state involving of His-197 in the NDPK2 nucleotide-binding pocket and the phytochrome-binding site in NDPK2 C-terminal fragment. GDP is the substrate shown. The active histidine residue His-197 forms H-bonding with Glu-208. The negative charge on Glu-208 is stabilized by the H-bonding with Ser-199. The interaction between phytochrome and NDPK2 C-terminal fragment may optimize the H-bonding between Glu-208 and His-197 and lower the energy barrier during the phospho-transfer. The phytochrome-binding region affected by the interaction with phytochrome is shown with arrows (modified from Ref. 36).



while, the oligomeric state of NDPK2 also plays a critical role in the interaction with phytochrome. We found that only the hexameric form of NDPK2 is able to bind phytochrome, whereas the dimeric form is not (Fig. 4A). Because each NDPK monomer of the dimeric enzyme is correctly folded (67), the loss of binding ability of dimeric mutants to phytochrome is not because of an incorrect folding of the monomer unit. Mutant LP175S serves as a good example to illustrate the importance of NDPK2 oligomeric states. Mutant L225Stop, the corresponding deletion mutant of LP175S, exists as a hexamer and possesses the phytochrome-binding site. L225Stop is able to interact with Pfr phytochrome and become stimulated, similar to NDPK2 wild type. After point mutation of residue Pro-175, mutant LP175S exists as a dimer and fails to interact with Pfr phytochrome (Fig. 4C), although it still contains the phytochrome-binding site. Therefore, we conclude that phytochrome recognizes the hexameric structure of NDPK2 and binds to its C-terminal fragment.

NDPK2 γ -phosphate exchange activity was stimulated by Pfr phytochrome. This stimulation is significant because it could not only fulfill its housekeeping duty by supplying the necessary amount of nucleoside triphosphate (mainly GTP) but also could provide GTP to G-proteins or simply transform the G-protein-bound GDP to GTP and activate G-proteins. Therefore, phytochrome-mediated signaling can be linked to G-protein-mediated signaling via NDPK2. Uncovering the mechanism of phytochrome stimulation on NDPK2 activity is also important for understanding the role of NDPK, because NDPK has been reported to possess multiple signaling partners. We first investigated NDPK2 nucleotide affinity with or without addition of Pfr phytochrome. The K_m values were found decreased in Pfr phytochrome-stimulated NDPK2 (Table II) and the C-terminal deletion mutants that are hyperactive (Fig. 4B). However, the GDP binding assay indicated that the GDP binding ability of NDPK2 was not enhanced by Pfr phytochrome (Fig. 5B). Meanwhile, the hyperactive mutants of NDPK2 also showed worse GDP binding compared with the wild type. Because of the fact that Pfr phytochrome increases NDPK2 nucleotide affinity in the measurement of the K_m values but fails to enhance the nucleotide binding ability of NDPK2, we propose that Pfr phytochrome may accelerate the nucleotide exchange during the process of the γ -phosphate exchange reaction. This acceleration of the nucleotide exchange implies an acceleration of NDPK2 turnover, during which NDPK2 either obtains a phosphate group from nucleoside triphosphate (ATP) easily or releases the phosphate group from phosphorylated NDPK2 to nucleoside diphosphate (GDP) quickly. In this scenario, the active histidine residue needs to be more efficient.

The H-bonding among residues His-197, Glu-208, and Ser-199 inside the nucleotide-binding pocket is critical for NDPK2

functioning (36). It stabilizes the N- ϵ H on His-197 and assists His-197 in maintaining the optimal orientation to lower the energy barrier of either becoming phosphorylated or transferring the phosphate group to the substrate. His-197-surrounding residue mutants were found to have decreased NDPK activities compared with the wild type, except for Y87D (Fig. 6A), suggesting that residue His-197 is very sensitive to its environment. The analysis of protein thermal stability showed lowered T_m values in the hypoactive His-197-surrounding residue mutants, including S199T and E208D, indicating a damaged H-bonding with His-197 (Fig. 6B). Most surprisingly, the protein thermal stabilities of the hyperactive mutants L225Stop and Y87D were also slightly lower than the wild type, suggesting another type of H-bonding change. But in this case, the H-bonding is optimized to accelerate the nucleotide exchange via His-197. Because the selected residues in His-197-surrounding residue mutants are located in or near the phytochrome-binding site in the C-terminal fragment of NDPK2, it is conceivable that the binding of phytochrome could affect the H-bonding with His-197 and its orientation in phospho-transfer (Fig. 8). The pH dependence studies of NDPK2 revealed that the pK_a value of His-197 decreased in Pfr phytochrome-stimulated NDPK2 compared with that of NDPK2 alone (Fig. 6C). A similar pattern of pK_a variations was also observed with the C-terminal deletion mutants and mutant Y87D that are hyperactive (Table IV), so the origin of the increased nucleotide affinity of these mutants is attributable to lower pK_a values of His-197 that accelerates the phospho-transfer process of NDPKs. In contrast, higher pK_a values of His-197 were observed in NDPK2 hypoactive mutants. A previous report (35) suggested that the pK_a value of His-197 represents the dissociation of N- δ on His-197. However, it is difficult to determine whether this pK_a value is because of the unphosphorylated or phosphorylated form of His-197 (35, 36). If it is due to the unphosphorylated form, a lowered pK_a value means His-197 is easier to deprotonate N- δ H and is ready for phosphorylation. If it is due to the phosphorylated form, a lowered pK_a value accelerates the phospho-transferring from His-197 to its substrate (nucleoside diphosphate). This acceleration could be significant because a nucleoside diphosphate is usually a poor substrate and phospho-transferring to a poor substrate is likely the rate-limiting step during the γ -phosphate exchange. Either case is possible in the current research. Results from NDPK2 autophosphorylation and NDPK2 kinase activity with MBP in the presence of Pfr phytochrome support both mechanisms (Fig. 7, A and B). We conclude that the pK_a value change of the active His-197 after phytochrome binding is the reason why NDPK2 is stimulated.

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