Characterization of a Phosphoprotein Phosphatase for the Phosphorylated Form of Nucleoside-diphosphate Kinase from Pseudomonas aeruginosa*

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Nucleoside-diphosphate kinase (ATP:nucleoside-diphosphate phosphotransferase, EC 2.7.4.6; NDP kinase) is an important enzyme for the maintenance of the correct cellular levels of nucleoside triphosphates (NTPs) and their deoxy derivatives (dNTPs) and is involved in the regulation of cellular development. The enzyme is under the dual control of algR2 and algH in Pseudomonas aeruginosa. We report here the purification and characterization of a protein that dephosphorylates the phosphorylated intermediate form of the P. aeruginosa NDP kinase (Ndk). Dephosphorylation of Ndk phosphate leads to loss of its enzymatic activity. The 10.1-kDa polypeptide shows 77% homology at the N terminus with the Spo0E phosphatase, identified as a negative regulator of sporulation in Bacillus subtilis and 66% with the human Bax protein, identified as an effector of programmed cell death. The phosphatase termed Npp showed varied specificity toward phosphorylated Ndk from different sources including human erythrocyte Ndk phosphate. Its activity toward other histidine phosphates such as CheA or the α-subunit of succinyl-CoA synthetase or phosphoesters such as p-nitrophenyl phosphate was quite limited. Npp was stable at room temperature up to 2 h and required Mg** for activity. The presence of a phosphatase capable of dephosphorylating the phosphorylated form of P. aeruginosa Ndk represents an interesting and efficient mode of post-translational modification of an enzyme crucial to cellular development.

Nucleoside-diphosphate kinase (ATP:nucleoside-diphosphate phosphotransferase, EC 2.7.4.6; Ndk) is an important enzyme that catalyzes the reversible transfer of the 5'-terminal phosphate from NTPs to NDPs (or dNTPs to dNDPs) by a ping-pong enzyme mechanism (1). The enzyme utilizes an autophsophorylated reaction intermediate (2) and catalyzes the final step in NTP and dNTP synthesis, converting γ-phosphate bond energy (in the form of ATP) from oxidative phosphorylation into synthesis of DNA and RNA precursors and appears to be essential for growth of most types of cells under aerobic conditions (3). Ndk has been implicated in regulating or effecting developmental changes in eukaryotic cells. Reduced transcript levels for the human Ndk gene called nm23 were found to be associated with higher metastatic potential in tumor cells (4, 5). Expression of nm23 from a constitutive promoter in highly metastatic murine tumor cell was found to suppress tumor metastasis (6), defining its role as a suppresser of cancer metastasis. Null mutations in the ndk gene of Drosophila, termed awd, cause abnormalities in development of the larvae leading to tissue necrosis and death at the prepupal stage (5, 7). In the slime mold Dictyostelium discoideum, the ndk gene is developmentally regulated with a sharp decrease in ndk transcript levels coinciding with the onset of the starvation-induced developmental cycle (8, 9). A gene encoding a DNA-binding protein, PuF, which is required for the expression of c-myc in vitro, is highly homologous to the human ndk gene nm23-H2 (10). This implies that an alternate form of Ndk, nm23-H2, may be involved in the regulation of c-myc. The intermediate in NTP synthesis by Ndk is a phosphorylated form of the enzyme where an active site histidine is involved (2), although it is now known that the enzyme undergoes phosphorylation at internal serine residues as well, albeit at a lower level than histidine phosphorylation (11, 12).

Very little is known about how Ndk formation or its activity is regulated in eukaryotic and prokaryotic cells. We have recently described the purification and characterization of Ndk from P. aeruginosa that forms a complex with succinyl-CoA synthetase (Scs) (13). Both Ndk and the α-subunit of Scs require phosphorylation for their enzymatic activity (13). We have also shown that formation of Ndk and Scs is positively regulated by two separate genes, algR2 and algH in P. aeruginosa (14). In the algR2 algH double mutant, which has extremely low Ndk levels, NTP formation is mediated by an alternative kinase, which is sensitive to Tween 20. Thus while the wild type cells grow readily in the presence of Tween 20, the algR2 algH double mutant cannot grow in its presence, suggesting that either Ndk or the alternate kinase is essential for NTP synthesis and therefore for cellular growth (14). In this paper, we report the presence of a phosphatase that is highly active on the phosphorylated form of P. aeruginosa Ndk and may regulate intracellular Ndk activity through a phosphorylation/dephosphorylation mechanism.

EXPERIMENTAL PROCEDURES

Labeling of Nucleoside-diphosphate Kinase—Ndk was autophosphorylated using a technique previously described (13). 980 ng of pure protein was incubated with 10 μCi of [γ-32P]ATP in a final reaction volume of 50 μl of 50 mM Tris-HCl containing 10 mM MgCl2 and 1 mM dithiothreitol. The labeling of P. aeruginosa Ndk was quantitated on an Ambis densitometric scanner, and the efficiency of phosphorylation was evaluated as a function of the pmol of phosphate group added per pmol of Ndk. Before use in any reaction, the unreacted [γ-32P]ATP and residual ADP were removed by Biospin column chromatography, and the stability of the phosphorylated Ndk was evaluated by assessing the loss of radioactivity from the substrate by incubation at room temperature for 1 h at pH 8.0 in the absence of any acceptor NDPs.

Assaying for Ndk-P Phosphatase (Npp) Activity—The phosphatase
was assayed by incubating the stably phosphorylated Ndk (Ndk-P) in a 20-μl final reaction volume in the labeling buffer with nanomolar amounts of the phosphatase enzyme preparation. The loss of 32P radioactivity from the Ndk band was analyzed for correlation with the corresponding release of free radioactive inorganic phosphate by thin layer chromatography on polyethyleneimine plates in 1 M LiCl-1 M HCOOH solvent and was found to agree within 0.2% error. For the analysis of the effect of various inhibitors on the phosphatase activity, the labeled Ndk was preincubated with defined concentrations of the inhibitors for 30 min followed by the addition of the phosphatase. As these compounds were used as dimethyl sulfoxide solutions, suitable controls were run to analyze the effect of solvent on the enzymatic activity of the phosphatase in the range of the added volumes and was found to be noninterfering.

Purification of the Npp from *P. aeruginosa* 8830—Cells were grown in batches of four, 1 liter each in LB with vigorous aeration at 37°C to an *A*~max~ of 1.2. The cells were harvested by centrifugation at 4500g in a refrigerated RCSC centrifuge for 10 min. The cell pellet was resuspended in 3 volumes (w/v) of buffer A (50 mM Tris-HCl, pH 7.6, 10 mM MgCl₂, and 1 mM dithiothreitol) and sonicated through 13 cycles of 13-s duration with a gap of 13 s between cycles. The sonicated suspension was centrifuged at 10,000g for 30 min, and the supernatant was transferred to a fresh tube. Ammonium sulfate was added to a final saturation of 45%, and the suspension was kept chilled for 1 h. The pellet was collected by centrifugation, resuspended in 3 volumes of buffer A, and dialyzed against two changes of 50 volumes of buffer A through 10 h at 4°C. Ammonium sulfate was added to a final concentration of 1.0 M, and the suspension was loaded onto a phenyl-Sepharose hydrophobic interaction column at a flow rate of 1.0–0.0 M ammonium sulfate fractionated (80% saturated) fraction. This appeared to suggest that there was an inhibitor of the Ndk activity that was removed at 45% ammonium sulfate saturation. This inhibitory activity later turned out to be a phosphatase. Fig. 1A shows a Coomassie-stained gel demonstrating the purity of the preparation. The -fold purification we normally achieved was in the range of 95–110 (Table I). The yield of the protein was low, the maximum being 50 μg of the homogeneous polypeptide from 400 mg (wet weight) of harvested cells. The activity was stable for over 3 months stored at −10°C in 30% glycerol. The molecular mass of the polypeptide as determined by SDS-polyacrylamide gel electrophoresis followed by Coomassie Blue R 250 staining.

**N-terminal Amino Acid Sequence Determination**—The N terminus sequence analysis of the phosphatase was carried out by Dr. Ka-Leun Ngai at the Noyes Laboratory Genetic Engineering Facility at Urbana-Champaign. Modified Edman degradation method was used on an Applied Biosystems, Inc., model 477A protein sequencer. The analysis limits were 1–13 min, the integration limits were 0–35 min, and the base-line limits were set between 0.1 and 1.00 min. The search length was 0.20 min, and the set sensitivity was 0.10 min. The RT factor was tuned to 1.00 min, and the detector scale was set at 0.010 A.U.S. The sampling rate frequency was 2 Hz. Approximately 10 pmol of protein was injected for analysis.

Preparation of the 12-kDa Ndk from the 16-kDa Form by Proteolytic Cleavage—For the proteolytic reaction, 1 μg of the 16-kDa Ndk was incubated with 10 μl (1 μl was defined as that amount of the protease that could completely convert 1 μg of the 16-kDa Ndk to the 12-kDa form in 5 min at 37°C) of the protease. The protease was separated from the reaction mixture by Superose-12 gel filtration chromatography, and the fractions containing the active 12-kDa form were also analyzed for protease contamination by the reactivity of the Ndk pool on BSA. Additionally, the protease was found not to have any effect on the activity of the phosphatase, precluding any type of inactivation of the phosphatase by any contaminating protease in the 12-kDa fraction.

**RESULTS**

Purification and Activity Characterization of the 10.1-kDa Phosphatase—During initial stages of purification of Ndk from *P. aeruginosa* extracts, we consistently observed that the Ndk activity went up substantially after ammonium sulfate fractionation and removal of the 45% saturated fraction. This appeared to suggest that there was an inhibitor of the Ndk activity that was removed at 45% ammonium sulfate saturation. This inhibitory activity later turned out to be a phosphatase. Fig. 1A shows a Coomassie-stained gel demonstrating the purity of the preparation. The -fold purification we normally achieved was in the range of 95–110 (Table I). The yield of the protein was low, the maximum being 50 μg of the homogeneous polypeptide from 400 mg (wet weight) of harvested cells. The activity was stable for over 3 months stored at −10°C in 30% glycerol. The molecular mass of the polypeptide as determined by SDS-polyacrylamide gel electrophoresis was also estimated by Superose-12 gel filtration column chromatography. The molecular masses determined were in agreement with each other, suggesting the phosphatase to be a monomer of 10.1 kDa. The N-terminal sequence of the purified phosphatase was determined for 9 amino acids. Fig. 1B shows the results of the homology search of the N terminus region using the LFASTA and the TFASTA programs. Substantial homology (77% identity) was observed with the SpoOE phosphatase of *B. subtilis* involved in sporulation (15) and with the Bax protein (66% identity) implicated in its involvement in accelerating cell death (16). The activity of the purified phosphatase was tested on phosphorylated Ndk, and the specific activity units were defined as the amount of phosphatase required to completely dephosphorylate 1 pmol of *P. aeruginosa* Ndk-phosphate (Ndk-P). The ability of various concentrations of Npp to dephosphorylate Ndk-P (Fig. 2A) and a diagrammatic representation of such dephosphorylation activity with release of *P* is shown in Fig. 2B.

Specificity of the Phosphatase on the 16- and 12-kDa Forms of Ndk—We have recently isolated and characterized an 80-kDa protease from *P. aeruginosa* strain 8830 that degrades the 16-kDa Ndk specifically to a 12-kDa form and not any further even on prolonged incubation up to 2 h. The 12-kDa form of Ndk has been shown to be membrane-associated in *P. aeruginosa* while the 16-kDa form has been found to be cytoplasmic. The 12-kDa form was found to undergo autophosphorylation to the same extent as the 16-kDa form. As Fig. 3 demonstrates,
TABLE I
Purification of Ndk phosphate phosphatase (Npp)

| Purification step | Protein | Specific activity | -fold purification achieved |
|-------------------|---------|------------------|-----------------------------|
| Crude extract     | 160     | 71               | 1                           |
| 0–45% Am2SO4      | 95      | 212              | 3                           |
| Phenyl-Sepharose  | 42      | 1699             | 24                          |
| Q-Sepharose       | 13      | 4436             | 63                          |
| Superose-12       | 0.8     | 7125             | 101                         |

Fig. 2. Titration of the activity of the purified Npp. A, dephosphorylation activity of Npp on Ndk-P from P. aeruginosa. Lane 1, phosphorylated Ndk incubated without Npp for 5 min; lanes 2–9 represent incubation of the phosphorylated Ndk with 2, 4, 6, 8, 10, 12, 14, and 16 ng of Npp, respectively, for 5 min. B, plot of the dephosphorylation of P. aeruginosa Ndk-P with increasing concentrations of Npp, demonstrating release of inorganic phosphate (P) from the protein within the 5-min period (Fig. 5A–C) in 5 min. In the case of phosphorylated CheA (Fig. 5A, A–C), complete dephosphorylation required a 7–8-fold higher amount of Npp. For the dephosphorylation of the α-subunit of succinyl-CoA synthetase, even a 10-fold higher concentration of Npp was not able to completely dephosphorylate the protein within the 5-min period (Fig. 5B). Npp had no effect on p-nitrophenyl phosphatase, while the reaction with the E. coli alkaline phosphatase Type III (Sigma) was linear with respect to time and substrate under the experimental conditions (Fig. 5C).

Stability, pH Optima, and Metal Ion Requirement—We were also interested in analyzing some of the general properties of Npp, and the results are summarized in Fig. 6, A–D. The phosphatase was stable at room temperature for about 2 h, after which it lost activity rapidly (Fig. 6A). The phosphatase was optimally active at a pH of 7.6 (Fig. 6B). Npp could be inactivated completely by the inclusion of EDTA at a final concentration of 100 μM in the assay (Fig. 6C), indicating that the enzyme required a metal ion for activity. Reactivation using Mg2+, Mn2+, or Na+ showed that Mg2+ was the cation of choice for Npp (Fig. 6D), the order of effectiveness being Mg2+ > Mn2+ > Na+. A number of other metal ions such as Li+, K+, Cu2+, and Co2+ had little or no effect.

DISCUSSION

Nucleoside-diphosphate kinase has been implicated in a variety of physiological and developmental effects in both prokaryotes as well as eukaryotes. However, the regulation of this enzyme in various systems is still far from understood. We have previously shown that the effect of mutations on two disparate genes, algR2 and algH, is also manifested in the form of a drastically reduced level of nucleoside-diphosphate kinase (Ndk) in P. aeruginosa (14). We have recently reported that in the case of E. coli, the insertional inactivation of a gene rnk results in a drastic reduction of Ndk activity (18) and further that E. coli Ndk is primarily involved in GTP formation at low concentrations of NDPs (19). The implication of Ndk in the preferential synthesis of GTP over other nucleotides is not entirely surprising. GTP plays a crucial role in regulating numerous cellular events such as signal transduction, elongation steps in protein biosynthesis, tubulin polymerization, and malignant transformation (20). This nucleotide has also been proposed as having a general role in regulating anabolic processes involved in growth and cell proliferation (21).

The fact that the levels of an enzyme of such importance to
the cell need to be finely regulated need not be overemphasized. Moreover, for the type of reaction that this enzyme catalyzes, a remarkable degree of control and a highly stringent regulation can be achieved by regulating the ratio of phosphorylated enzyme to its non-phosphorylated counterpart. Recently, autophosphorylation on residues different from the active site histidine (notably serine) was reported for both the human (22) and the Myxococcus xanthus (2) enzymes. However, the manner in which phosphorylation/dephosphorylation activities modulate Ndk activity and consequently the ATP/ADP or the NTP/NDP ratios within the cell is unknown at present. The levels of any or all of these NTPs may vary at any given time of cell growth depending on a variety of factors including the oxygen tension and/or nutrient deprivation. The levels of enzymes that are constantly in demand by the cell can be more efficiently managed when placed under an efficient but fully reversible post-translational signal.

An intriguing but interesting aspect of the characterization of the P. aeruginosa Npp is its high level of identity at the N-terminus with the human Bax protein, an effector of mammalian programmed cell death (16). It has been suggested that both effector and repressor genes exist within each mammalian cell death pathway. One such mammalian gene has been identified, bcl-2, that functions as a repressor of programmed cell death (24). Bcl-2 blocks cell death following a variety of stimuli. Bcl-2 conferred a death-sparing effect to certain hematopoietic cell lines following growth factor withdrawal (25–27). Bcl-2 has also been shown to protect primary neuronal cell cultures from nerve growth factor withdrawal cell death (28). Thus Bcl-2 may...
Fig. 6. A, stability of Npp at room temperature. 10 ng of Npp was aliquoted out into the standard assay buffer for Npp and preincubated at room temperatures for periods ranging from 1 to 8 h. 12.5 pmol of phosphorylated P. aeruginosa Ndk was added at the end of each incubation time point, and the reaction continued for another 30 s. Reaction products were analyzed as described previously. Lane 1, 12.5 pmol of Ndk-P with no Npp; lane 2, + 0 s preincubated Npp; lane 3, + 2 h preincubated Npp; lane 4, + 4 h preincubated Npp; lane 5, + 6 h preincubated Npp; lane 6, + 8 h preincubated Npp. B, activity of Npp at various pH values. The Npp reactions were reconstituted as described previously in 25 mM HEPES-KOH buffers of pH values 6.5, 7.0, 7.6, 8.2, 9.0, and 10.0. MgCl2 was added to a final concentration of 10 mM. Npp activity is shown at assay buffer pH values of 6.5 (lane 1), 7.0 (lane 2), 7.6 (lane 3), 8.2 (lane 4), 9.0 (lane 5), and 10.0 (lane 6). Lane 7 shows control, 25 pmol Ndk-P without any Npp. C, effect of EDTA on the dephosphorylating activity of Npp. EDTA was added to final concentrations ranging from 0 to 100 μM and preincubated with Ndk-P prior to the addition of Npp. Reaction products were analyzed as described previously. Lane 1, 25 pmol Ndk-P; lane 2, + 14 ng of Npp; lane 3, + 25 μM EDTA + 14 ng of Npp; lane 4, + 50 μM EDTA + 14 ng of Npp; lane 5, + 100 μM EDTA + 14 ng of Npp. D, reactivation of Npp by Na+, Mn2+, or Mg2+ after inactivation by 100 μM EDTA. The assay system containing Ndk-P, 100 μM EDTA and Npp that was apparently inactive, Na+, Mn2+, or Mg2+ were added to final concentrations of 5–75 μM in the assay. Lanes 1, 7, and 13, 25 pmol of control Ndk-P + 100 μM EDTA + 14 ng Npp; lanes 2–6, 5, 10, 25, 50, and 75 μM Na+; lanes 8–12, 5, 10, 25, 50, and 75 μM Mn2+; lanes 14–18, 5, 10, 25, 50, and 75 μM Mg2+, respectively.

be needed to save the progenitor and long lived cells in a variety of cell lineages. Despite the progress in defining the physiological roles of Bd-2, the biochemical basis of its actions remains largely unknown. Recent reports, however, have shown that another protein Bax can complex with Bd-2 and that under conditions where Bax predominates, cell death is accelerated (16). Nothing is known about the mode of action of Bd-2 or Bax. It is tempting to speculate that the interaction of Bax and Bcl-2 is mediated by a phosphorylation/dephosphorylation cycle not very much unlike that seen in the case of the dephosphorylation of Ndk by the phosphatase.

The strong homology of N terminus amino acid sequence of Npp with the SpoOE phosphatase is also quite significant. The initiation of sporulation in Bacillus subtilis is under the control of the SpoOA transcription factor; this protein is a member of the response regulator class of the two component systems and is inactive unless phosphorylated (23). SpoOA-P acts both as a repressor of certain vegetative genes and as an activator of certain genes required for the initiation of sporulation. The SpoOE protein, long known as a negative inhibitor of sporulation, was recently characterized as a phosphatase specific for SpoOA (23). Overproduction of the SpoOE protein was known to severely inhibit sporulation, whereas deletion of this locus caused premature sporulation and accumulation of mutations in the phosphorylray. Given the central role that Ndk plays in the maintenance of NTP levels of the cell and the accumulating evidence that the enzyme is also crucial to development as well as differentiation, it is not entirely surprising that Npp shows significant N terminus sequence homology with a phosphatase that plays a pivotal role in the sporulation process in B. subtilis.

Isolation and characterization of the Npp gene is now ongoing in our laboratory, and it would be interesting to see if a similar gene might be characterized in the human cDNA library and also if there exists any type of functional identity between Npp and SpoOE. Given the highly conserved nature of NdkPs from various sources (13) and the paucity of information on its regulation, further studies on the role of the Npp in energy metabolism are worth pursuing as well.

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