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Nucleoside Diphosphate Kinase from *Escherichia coli*

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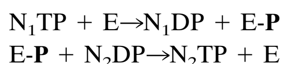
Nucleoside diphosphate (NDP) kinase from *Escherichia coli* was purified to homogeneity and was crystallized. Gel filtration analysis of the purified enzyme indicated that it forms a tetramer. The enzyme was phosphorylated with [γ-^{32}P]ATP, and the pH stability profile of the phosphoenzyme indicated that two different amino acid residues were phosphorylated. Both a histidine residue and serine residues, including Ser-119 and Ser-121, appear to be phosphorylated. A Ser119Ala/Ser121Ala double mutant (i.e., with a Ser-to-Ala double mutation at positions 119 and 121), as well as Ser119Ala and Ser121Ala mutants, was isolated. All of these retained NDP kinase activity; also, both the Ser119Ala and Ser121Ala mutants could still be autophosphorylated. In the case of the double mutant, a slight autophosphorylation activity, which was resistant to acid treatment, was still detected, indicating that an additional minor autophosphorylation site besides His-117 exists. These results are discussed in light of the recent report of N. J. MacDonald et al. on the autophosphorylation of human NDP kinase (J. Biol. Chem. 268:25780–25789, 1993).

Nucleoside diphosphate (NDP) kinase catalyzes the following transphosphorylation reaction, via the formation of a phosphoenzyme intermediate:



$$N_1TP + E \rightarrow N_1DP + E\text{-}\mathbf{P}$$
$$E\text{-}\mathbf{P} + N_2DP \rightarrow N_2TP + E$$

The primary role of NDP kinase in the cell was considered the maintenance of a pool of nucleotide triphosphates for the synthesis of DNA and RNA (22). NDP kinase was reported to be the only enzyme responsible for the synthesis of nucleotides other than ATP in *Salmonella typhimurium* (7). Also, the *ndk* gene appears to be essential in the gram-negative bacterium *Myxococcus xanthus* (20). Recently the enzyme has been implicated as a tumor suppressor candidate in studies of highly metastatic human cells (14, 26, 27) and has been associated with the development of *Drosophila* larvae (3, 25).

NDP kinase genes (*ndk*) from prokaryotic and eukaryotic organisms have been cloned and sequenced (8, 19). The deduced amino acid sequence of the enzyme from *Escherichia coli* indicated that NDP kinases are highly conserved throughout evolution; the *E. coli* and human (Nm23-H1) enzymes are 43% identical (8). The three-dimensional structures of NDP kinases from *Dictyostelium discoideum* (4), *M. xanthus* (33), and *Drosophila melanogaster* (2) have been determined. These studies again showed that NDP kinases from different sources share highly conserved three-dimensional structures.

NDP kinases are known to function by a ping-pong mechanism which involves the formation of a high-energy phosphoenzyme intermediate. Most of the intermediates have been shown to be alkali stable and acid labile, suggesting the formation of phosphohistidine, which has been identified in NDP kinases from various sources (22). Recently, histidine 118 has been identified as the phosphorylation site in one of the human NDP kinases, Nm23 (6). This histidine residue is invariant among all the NDP kinases sequenced so far (8). However phospho-NDP kinases with drastically different stability characteristics have been reported (22). Phosphoserine formation has been reported to occur in NDP kinase from rat mucosal mast cells (11) and in NDP kinase from *M. xanthus*, in which the formation of phosphohistidine has also been reported to occur (19). A role for NDP kinase as a novel protein kinase without sequence similarities to the classical protein serine-threonine kinases (10) was proposed. Differential phosphorylation implies diverse roles for NDP kinase in cellular metabolism. The recent report of the occurrence of serine phosphorylation in human NDP kinase Nm23-H1 lends further support to this idea (16).

In this study, *E. coli* NDP kinase was purified to homogeneity and was crystallized. We found that in addition to histidine, serine residues at positions 119 and 121 were phosphorylated. Substitution of these serine residues with alanine did not affect enzymatic activity. Phosphorylation of these serine residues may, therefore, play a role in controlling the interaction of NDP kinase with other cellular components. To our surprise, the *E. coli ndk* gene was found to be dispensable under normal growth conditions, indicating that *E. coli* contains another enzyme which functions as NDP kinase.

MATERIALS AND METHODS

Bacterial strains and media. Plasmid pKT8P3 (8), a pUC9-derived (32) plasmid, was used for overexpression of the *ndk* gene.

E. coli JM83 (*ara* Δ*lac-proAB rpsL* φ80 *lacZ*ΔM15) (34) and DH5α [*supE44* Δ*lac*U169 (φ80*lacZ*ΔM15) *hsdR17 recA1 endA1 gyrA96 thi-1 relA1*] (9) were used for harboring plasmid pKT8P3. Strain BL21 (DE3) [*hsdS gal* (λ*c*I*ts*857 *ind1 S*am7 *nin5 lac*UV-5-T7 gene1)] was used for overexpression of the mutant and wild-type *ndk* genes cloned in a pET11a-derived expression vector (28). Strain TG1 [*supE hsd*Δ*5thi* Δ(*lac-proAB*) F′(*traD36 proAB*$^+$ *lacI*^{q} *Z*ΔM15)] (5) was used for preparation of single-stranded DNA for mutagenesis (Amersham Corp.), and double-stranded DNA was used for subcloning from M13 into a pET-derived vector and for sequencing reactions. Strain SB221 (*lpp hsdR* Δ*trpE5 leuB6 recA1*/F′ *lacI*^{q} *lac*$^+$ *pro*$^+$) was used for the selection of recombinant pET11a constructs containing the wild-type and mutant *ndk* genes.

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L broth (18) with 50 μg of ampicillin per ml or 2× TY medium (Amersham Corp.) was used, as appropriate.

Materials. The following materials were used in this study: [γ-^{32}P]ATP (5,000 Ci/mmol; Amersham); nonradioactive nucleotides, Ponceau Red stain, polyethyleneimine-cellulose plates for manual Edman degradation, triethylamine, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide, and sequencing-grade trifluoroacetic acid (TFA) (all from Sigma); phenyl isothiocyanate and acrylamine Sequelon membranes (Milligen Bioresearch); Immobilon-P membranes (Millipore); and the enzymes trypsin and V8 protease (Boehringer Mannheim).

Purification of NDP kinase. *E. coli* JM83 cells harboring plasmid pKT8P3 overproduced NDP kinase to approximately 20% of the total protein. For the growth of large cultures, freshly transformed cells (approximately 200 colonies per plate) were used as an inoculum. Cells were grown at 37°C to stationary phase (10 to 11 h) and harvested by centrifugation at 2,300 × *g* for 10 min at 4°C, and the pellets were washed in 20 ml of 50 mM potassium phosphate buffer (pH 7.5) containing 1 mM EDTA and were frozen at −70°C until further use.

Thawed cell pellets (wet weight, 2 g) were resuspended in 20 ml of buffer and subjected to French press treatment at 12,000 lb/in^2. Lysed cells were centrifuged at 2,300 × *g* for 10 min at 4°C to remove cell debris, and the supernatant was centrifuged at 140,000 × *g* for 90 min to separate membrane and cytoplasmic fractions. A precipitate obtained after fractionation of the cytoplasm with 40 to 60% ammonium sulfate was found to contain the major portion of NDP kinase, which was resuspended in 2.0 ml of 50 mM potassium phosphate buffer (pH 7.5) containing 1 mM EDTA and dialyzed twice in 1,000 ml of the same buffer. The protein of the dialyzed solution was diluted to a concentration of less than 5 mg/ml, and the diluted solution was then loaded onto a preequilibrated DE-52 column (1.8 by 38 cm). After washes with 50 mM potassium phosphate buffer (pH 7.5) containing 1 mM EDTA and with buffer containing 50 mM KCl, a gradient of 50 to 150 mM KCl was applied to the column. Fractions containing NDP kinase (the enzyme eluted from the column at 100 mM KCl) were pooled and diluted to a final concentration of 50 mM KCl. The pooled fractions were then loaded onto a preequilibrated fast protein liquid chromatography (FPLC) MonoQ ion-exchange column washed with buffer containing 50 mM KCl, and a gradient of 50 to 400 mM KCl was applied. Fractions containing pure NDP kinase, as judged with a Coomassie blue-stained sodium dodecyl sulfate (SDS)-polyacrylamide gel, were either further concentrated for crystallization or stored at −70°C in 20% glycerol (see Fig. 1).

Crystallization of NDP kinase. Crystals of NDP kinase were obtained by the hanging-drop method with concentrated NDP kinase (30 mg/ml) and 0.1 M acetate (pH 4.88), with 2.0 M ammonium sulfate as a precipitant.

Oligomeric structure of NDP kinase. Approximately 1 mg of purified NDP kinase was mixed with 1 mg of each protein bovine serum albumin (BSA) (68 kDa), ovalbumin (45 kDa), chymotrypsinogen (24 kDa), and cytochrome *c* (12.4 kDa) in 1 ml and loaded onto a Sephacryl S-200 column (1.77 by 70 cm) preequilibrated with 50 mM potassium phosphate buffer (pH 7.5) containing 1 mM EDTA. The fractions collected were analyzed by SDS-polyacrylamide gel electrophoresis (PAGE). The elution volume of each protein was estimated, and the void volume of the column was determined with dextran blue.

pH stability profile of the phosphoenzyme and identification of phosphoserine. Determination of the stability profile of phospho-NDP kinase and identification of phosphoserine were carried out as previously described (13, 19). The reaction mixture contained purified NDP kinase (8 μg) which was incubated with [γ-^{32}P]ATP (10 μCi) in 20 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid) buffer (pH 8.0) containing 1 mM EDTA, 100 mM NaCl, and 2 mM ATP.

Autophosphorylation of NDP kinase and isolation of radiolabelled peptide. Purified protein (600 μg) was autophosphorylated in a reaction mixture containing 20 mM HEPES (pH 8.0), 100 mM NaCl, 1 mM EDTA, 0.1% Lubrol, and 200 μCi of [γ-^{32}P]ATP. The reaction mixture was incubated on ice for 5 min, ATP was added to a final concentration of 1 mM, and the reaction mixture was incubated on ice for a further 10 min. The reaction mixture was then dialyzed against 50 mM Tris HCl (pH 8.0) containing 0.1 mM $CaCl_2$ for 20 h and with several changes of buffer to remove any free radioactivity. Two microliters of the dialyzed solution was run on an SDS-polyacrylamide gel and stained with Coomassie blue to estimate the amount of phosphorylated protein, trypsin was added in a 1:20 (wt/wt) ratio, and the mixture was incubated at 37°C for 18 h. The resultant peptides were separated by reverse-phase high-pressure liquid chromatography (HPLC). The tryptic digest was loaded onto an HPLC C_8 column (4.6 by 22.5 mm; Brownlee) equilibrated in 0.1% TFA, and peptides were eluted with a linear gradient of 0 to 70% acetonitrile in 90 min. The flow rate was 0.2 ml/min, and A_{215}s were determined. Fractions were collected manually at each peak and radioactivity was determined.

The radiolabelled peptide obtained from the tryptic digestion was dried in a Speed-Vac (Savant) and dissolved in 25 mM sodium phosphate buffer (pH 7.8). Twenty micrograms of V8 protease was added to the solution, and the reaction mixture was incubated at 25°C for 18 h. The digest was loaded onto the C_8 column, and the peptides were eluted by the same method as programmed for the separation of tryptic peptides.

Amino acid analysis. The dried peptides were resuspended in 50 μl of 6 N HCl, sealed in a microcapillary tube, and hydrolyzed at 110°C for 2 h (15). The 6 N HCl was diluted and evaporated in a Speed-Vac and the analyses were performed as described by Meltzer et al. (17).

Automated and manual sequencing of peptides. Automated sequence analysis of peptides was performed with an Applied Biosystems 475A/900A/120A gas-phase sequencer. For manual Edman degradation the peptides were resuspended in 50% acetonitrile–0.1% TFA and sequenced according to the method described by Sullivan and Wong (29).

Cloning of the *ndk* gene into pET11a. The *ndk* gene was cloned by PCR amplifying pKT8P3 with 5′ and 3′ primers containing *Nde*I and *Bam*HI sites, making the fragment blunt, and ligating the fragment to *Sma*I-cut M13mp19. The complete sequence of the *ndk* clone was verified, and it was subcloned as an *Nde*I-*Bam*HI fragment into pET11Zc (a derivative of pET11a [28]). Site-directed mutagenesis of the M13mp18 clone was carried out with an Amersham kit (version 2), the mutations were verified by sequencing, and the mutated *ndk* genes were cloned into pET11Zc.

Overexpression and purification of wild-type and mutant NDP kinase proteins. The pET11a-derived plasmids containing the wild-type and mutant *ndk* genes were used to transform *E. coli* BL21 (DE3) cells. Overnight inoculum (20 ml) in L broth with 50 μg of ampicillin per ml was added to 500 ml of fresh medium, and cultures were then incubated on a shaker at 37°C for 2 to 3 h (approximately 60 to 70 Klett units) before the addition of 1 mM IPTG (isopropyl-β-D-thiogalactopyranoside). Following induction, the cells were cultured for 4 to 5 h longer. The cells were harvested by centrifugation at 2,300 × *g* for 10 min at 4°C, washed once in chilled 50 mM Tris HCl (pH 7.5) buffer, and frozen at −70°C until further use. Once thawed, the cells were resuspended in 50 mM Tris HCl (pH 7.5) containing 1 mM EDTA, and extracts were prepared by French press treatment at 12,000 lb/in^2. Cell debris and unbroken cells were removed by centrifugation at 2,300 × *g* for 10 min. The supernatant was then used to separate membrane and cytoplasmic fractions by centrifugation at 140,000 × *g* for 45 min. As found previously, wild-type and mutant NDP kinases precipitated mostly in a 40 to 60% ammonium sulfate fraction. Each precipitate was resuspended in 50 mM Tris HCl buffer (pH 7.5) containing 1 mM EDTA (about 2 ml) and dialyzed overnight at 4°C against 1,000 ml of the same buffer to remove ammonium sulfate. Approximately 1 ml of the dialyzed solution was then loaded onto an FPLC polyethyleneimine anion-exchange ion-exchange column (2-ml column volume), and NDP kinase was eluted with a gradient of 0 to 1 M KCl for 40 min. NDP kinase eluting from this column was approximately 90% pure, as determined by Coomassie blue staining.

Coupled-enzyme assay for NDP kinase. The coupled-enzyme assay was performed as described by Ratliff et al. (23). The reaction mixture (1 ml) contained 100 mM Tris HCl (pH 7.5), 20 mM $MgCl_2$, 20 mM β-mercaptoethanol, 200 mM KCl, 2 mM phosphoenolpyruvate, 0.9 mM ATP, 0.2 mM NADH, 1.8 mM dTDP, 5 U of lactate dehydrogenase, and 5 U of pyruvate kinase. The reaction was started by the addition of crude cell extracts or pure protein, and the decrease in A_{340} was measured for 2 min.

Phosphorylation of wild-type and mutant proteins. Equal amounts (approximately 1 μg), as judged by the A_{280}, of pure wild-type and mutant NDP kinase were incubated with 0.5 μCi of [γ-^{32}P]ATP on ice for 10 min as described above. The reaction was stopped by adding 5× SDS loading buffer and boiling the samples for 2 min. Phosphorylated proteins were separated by SDS-PAGE. The gel was stained with Coomassie blue, destained extensively in 10% acetic acid–20% methanol, dried, and exposed to X-ray film for 3 to 4 h at −70°C.

RESULTS

Purification and crystallization of NDP kinase. Purification of NDP kinase was carried out as described in Materials and Methods. NDP kinase of approximately 85% purity, as judged by Coomassie blue staining of the SDS-polyacrylamide gel, was obtained after elution from the DE-52 column (Fig. 1, lane 4). In contrast to the enzyme from *M. xanthus*, which eluted in the wash fractions from a DE-52 column (21), NDP kinase from *E. coli* eluted at a low salt concentration (100 mM KCl). This is as expected from the pI value deduced from the primary structure of the *E. coli* enzyme, which is more acidic than NDP kinase from *M. xanthus*. For further purification an FPLC MonoQ ion-exchange column was included, and the use of this stronger anion exchanger resulted in the separation of minor impurities to obtain NDP kinase for crystallization. The purified enzyme was highly soluble, and a solution with a concentration as high as 30 mg/ml was made. Rhombic-shaped crystals approximately 0.04 mm long (Fig. 2) were formed in a precipitant containing 0.1 M acetate (pH 4.88) and 2.0 M ammonium sulfate.

Oligomeric structure of *E. coli* NDP kinase. NDP kinase was eluted from the Sephacryl S-200 column in fractions 5 and 6

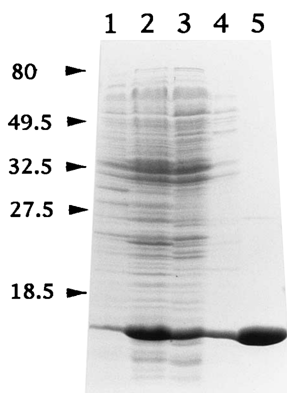

FIG. 1. Purification of *E. coli* NDP kinase. NDP kinase was purified as described in Materials and Methods. SDS-PAGE was carried out at different steps of purification. Lane 1, cell extract obtained after French press treatment of *E. coli* JM83 cells harboring plasmid pKT8P3; lane 2, soluble fraction obtained after centrifugation; lane 3, precipitate obtained after fractionation with 40 to 60% ammonium sulfate; lane 4, pool of fractions containing NDP kinase after elution from the DE-52 column; lane 5, NDP kinase obtained after elution from the MonoQ column. Note that the faint band migrating at approximately 30 kDa corresponds to the molecular mass expected of a dimer of NDP kinase. The amount of this band (intensity of staining α amount of protein) was increased in the absence of β-mercaptoethanol. Positions of molecular mass markers (in kilodaltons) are indicated in the left margin.

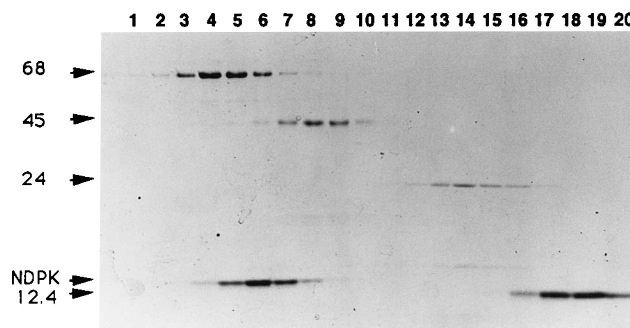

FIG. 3. Formation of a tetramer of NDP kinase. Purified NDP kinase (NDPK) (1 mg) was applied with BSA (68 kDa), ovalbumin (45 kDa), chymotrypsinogen (24 kDa), and cytochrome *c* (12.4 kDa) to a Sephacryl S-200 column (1.77 by 70 cm). An aliquot (10 μl) from each fraction of 7 ml was applied to an SDS-polyacrylamide gel. The order of elution of fractions is from lane 1 (earlier fractions) through lane 20 (later fractions). Positions of molecular mass markers (in kilodaltons) are indicated in the left margin.

after BSA (68 kDa) and before ovalbumin (45 kDa). This indicates that the apparent molecular mass of the enzyme was higher than 45 kDa (Fig. 3), which is much higher than the deduced molecular mass of 16 kDa (8). K_{av} values were calculated for each protein and plotted against the log of the molecular mass for the standard proteins. The molecular mass of NDP kinase was thus estimated to be 60 kDa. These results indicate that *E. coli* NDP kinase exists as a tetramer.

pH stability profile of the phosphoenzyme and identification of phosphoserine. The pH stability of the ^{32}P-labelled phosphoenzyme indicated that more than one amino acid residue is phosphorylated; 32% of the total counts on the membrane were released by acid treatment and 76% of the counts were released by alkaline treatment. The former is characteristic of the formation of phosphoramidates (likely due to phosphohistidine), and the latter is characteristic of the formation of O phosphomonoesters (likely due to the phosphorylation of serine, threonine, or tyrosine).

Phosphoamino acid analysis of the acid hydrolysate of NDP kinase labelled with [γ-^{32}P]ATP demonstrated that NDP kinase from *E. coli* autophosphorylates at serine but at not tyrosine or threonine, as shown in Fig. 4.

Identification of the phosphoserine residues. To identify the serine residue(s) that is phosphorylated, phospho-NDP kinase was digested with trypsin and a radiolabelled peptide was isolated by reverse-phase HPLC separation of the digest as described in Materials and Methods (data not shown). The radiolabelled purified peptide was further digested with V8 protease. This digest was again subjected to HPLC (Fig. 5A), and a single new radioactive peak was detected after V8 digestion (Fig. 5B).

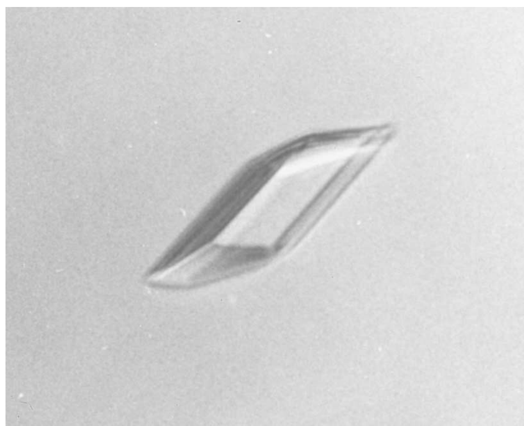

FIG. 2. NDP kinase crystal. Crystals of NDP kinase were obtained in 0.1 M acetate (pH 4.88)–2.0 M ammonium sulfate. The crystal shown is approximately 40 μm in length.

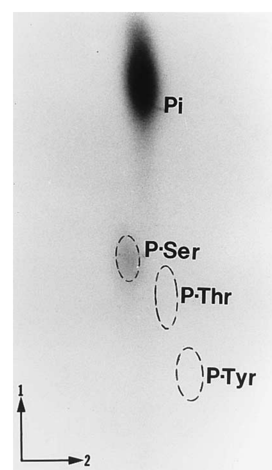

FIG. 4. Phosphoamino acid analysis of phosphorylated *E. coli* NDP kinase. An aliquot of 5 μl of the acid hydrolysate of the phosphorylated protein prepared as described previously (19) was spotted onto a cellulose plate together with nonradioactive phosphoserine (P-Ser), phosphothreonine (P-Thr), and phosphotyrosine (P-Tyr). Electrophoresis was carried out at pH 3.5 for 40 min at 1,000 V in glacial acetic acid-pyrimidine-water (50:5:45) in the first dimension and by ascending chromatography with isobutyric acid–0.5 M ammonia (5:3) in the second dimension. The plate was dried and exposed to X-ray film for 5 days, and the positions of standard phosphoamino acids were detected with ninhydrin. The origin is indicated by the intersection of the arrows labelled 1 (direction of electrophoresis in the first dimension) and 2 (chromatography in the second dimension). Pi, free radioactive phosphate.

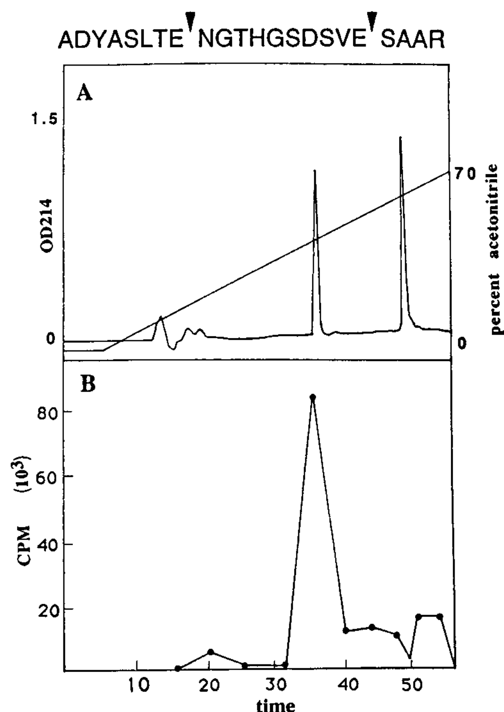

FIG. 5. Reverse-phase HPLC separation of peptides resulting from digestion with V8 protease. The isolated radiolabelled tryptic peptide from reverse-phase HPLC was dissolved in 25 mM sodium phosphate buffer (pH 7.8) and digested with 20 μg of V8 protease at 25°C for 18 h. The digest was applied to a Brownlee C_8 column (4.6 mm by 22 cm) with a linear gradient of 0 to 70% acetonitrile and with 0.1% TFA over 90 min at a flow rate of 0.2 ml/min. (A) Absorption profile at 214 nm (OD214) of peptides eluting from the column. (B) Radioactive profile of the peptides. The sequence of the tryptic peptide deduced from amino acid analysis is indicated at the top, and the sequence of the peptide obtained after V8 digestion (Fig. 6) is indicated by arrowheads. CPM, counts per minute.

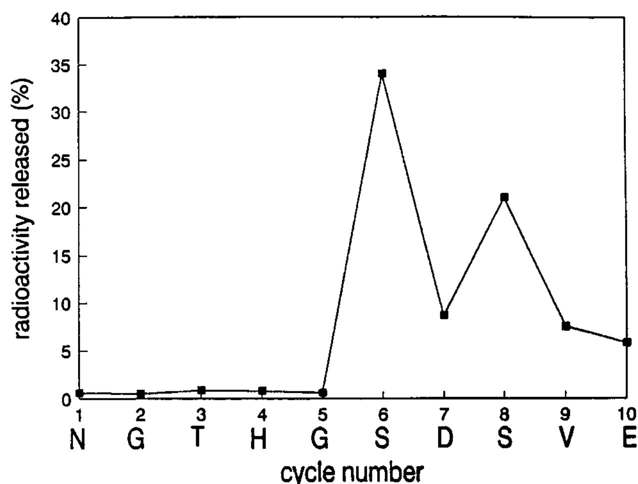

FIG. 6. Manual Edman degradation of the V8 peptide. The peptide which was eluted at 35 min (Fig. 5) was subjected to Edman degradation as described in Materials and Methods. The wash was collected after each cycle of degradation, and the counts which were recovered in the wash and retained on the membrane after each cycle were quantitated. The percentage of total counts released into the wash after each cycle was plotted against the cycle number. The sequence of this peptide had been confirmed by automated gas-phase sequencing.

Both the radioactive tryptic and V8 peptides were used for amino acid analyses. The amino acid analysis of the tryptic peptide revealed that it corresponds to the sequence spanning residues 104 to 127 and includes the highly conserved HGSD sequence, which is invariant among all NDP kinases sequenced so far (8). Amino acid analysis and automated gas-phase sequencing confirmed that the peptide eluting from the C_8 column at approximately 35 min after V8 digestion comprises the sequence NGTHGSDSVE (amino acids 114 to 123). Manual sequencing of this peptide resulted in release of 34% of the total ^{32}P radioactivity from the membrane in the sixth cycle of sequencing and 21% of the radioactivity in the eighth cycle of sequencing, indicating that both Ser-119 and Ser-121 were phosphorylated (Fig. 6).

Overexpression and purification of wild-type and mutant NDP kinases. Wild-type and mutant proteins were overexpressed by using a pET11a-derived vector (28). All the NDP kinase proteins were overexpressed to approximately 25% of the total cellular proteins after induction with IPTG; at least 50% of the NDP kinase proteins were localized in the cytoplasmic fraction. The purification was therefore facilitated, and a two-step purification scheme, ammonium sulfate precipitation followed by FPLC with a polyethyleneimine anion-exchange ion-exchange column, was sufficient to obtain 90% pure protein.

Effects of mutations at the autophosphorylation sites. In order to examine the role of serine residues at positions 119 and 121, these residues were substituted with alanine (mutants thus formed are designated Ser119Ala and Ser121Ala, respectively). Both Ser119Ala and Ser121Ala mutant NDP kinases were still autophosphorylated, as shown in Fig. 7A, lanes 2 and 3, respectively. In comparison with the level of acid-stable phosphorylation of the wild-type (lane 1) and Ser121Ala (lane 3) enzymes, that of the Ser119Ala mutant (lane 2) was significantly reduced, indicating that Ser-119 is the major phosphorylation site. This is consistent with the result from Edman degradation shown in Fig. 6A. Note that the same amount of protein was applied to each lane, as judged by Coomassie blue staining of the gel (Fig. 7B).

The double mutant Ser119Ala/Ser121Ala was also con-

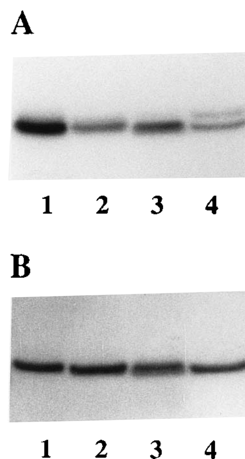

FIG. 7. Phosphorylation of wild-type and mutant proteins. Approximately 1 μg of each pure protein was incubated with 0.5 μCi of [γ-^{32}P]ATP as described in Materials and Methods. The reaction was stopped by the addition of 5× SDS loading buffer, and the reaction mixture was then subjected to SDS-PAGE. The gel was stained with Coomassie blue. (A) Autoradiogram of the dried gel. Lane 1, wild-type NDP kinase; lane 2, Ser119Ala NDP kinase; lane 3, Ser121Ala NDP kinase; lane 4, Ser119Ala/Ser121Ala NDP kinase. (B) SDS-polyacrylamide gel stained with Coomassie blue. Lanes are same as in panel A.

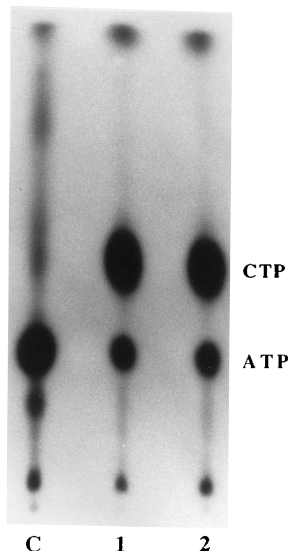

FIG. 8. NDP kinase activity of the Ser119Ala/Ser121Ala double mutant. Purified enzymes (1 μg each of wild-type and mutant enzymes) were incubated in a reaction mixture (10 μl) containing [γ-^{32}P]ATP (1 μCi), 5 mM $MgCl_2$, and 1 mM CDP in 20 mM HEPES (pH 8.0). The reaction was carried out as described previously (19). The plate was exposed to X-ray film for 1 h. Lane C, buffer control; lane 1, wild-type NDP kinase; lane 2, Ser119Ala/Ser121Ala NDP kinase.

structed. As shown in Fig. 7A, lane 4, acid-stable phosphorylation dropped to approximately 8% of that of the wild-type enzyme. This residual phosphorylation indicates that there is another autophosphorylation site(s) besides Ser-119 and Ser-121. When the phosphorylated Ser119Ala/Ser121Ala protein was digested with Lys-C endopeptidase, the largest product from the protease digestion corresponding to the sequence from proline 58 to the C-terminal residue was not labelled (data not shown), indicating that the phosphorylation site(s) resided in the N-terminal region of NDP kinase between methionine 1 and lysine 57. Interestingly, the double mutant retains almost full NDP kinase activity, as shown in Fig. 8. The enzymes with single mutations also have NDP kinase activity comparable to that of the wild-type enzyme (data not shown).

DISCUSSION

In this study, we purified *E. coli* NDP kinase to homogeneity, and the purified enzyme was crystallized. The protein was found to be autophosphorylated not only at a histidine residue but also at Ser-119 and Ser-121. As described in a recent report, MacDonald et al. (16) have identified the serine residue at position 44 as the major acid-labile phosphorylation site in Nm23-H1, a human NDP kinase. This serine residue is replaced by threonine 43 in *E. coli* NDP kinase, which is not phosphorylated (Fig. 4). MacDonald et al. (16) reported substantial ^{32}P radioactivity in a tryptic peptide comprising amino acids NIIHGSDSVESAEK (residues 117 to 128) in addition to ^{32}P radioactivity in the tryptic peptide containing Ser-44. They attributed the ^{32}P incorporation in the second peptide to His-118, the primary phosphorylation site of all NDP kinases. However, since the isolation of tryptic peptides was carried out in 0.1% TFA (pH 2.3), it is highly possible that there was no or little phosphohistidine in the purified peptide and that most of the radioactivity was due to phosphorylation at Ser-120.

The invariant residues HGSD (residues 117 to 120), which include Ser-119, are present in all NDP kinases. Histidine 118 has been identified by peptide mapping and amino acid composition analysis as the residue that is phosphorylated in human NDP kinase (6). This highly conserved histidine residue is also involved in nucleotide binding, as indicated by the absence of nucleotide binding activity and autophosphorylation in the mutant *M. xanthus* enzyme in which His-117 has been replaced with glutamine (19). The corresponding mutant *E. coli* enzyme also lacks autophosphorylation activity (data not shown). This implies that phosphorylation of serine residues is dependent on phosphorylation of His-117. In contrast, MacDonald et al. (16) have reported that mutant murine NDP kinase in which the active-site histidine has been replaced with glycine retains acid-stable autophosphorylation activity.

A molecular model of the *E. coli* enzyme was generated on the basis of the crystal structure of the enzyme from *M. xanthus* (33). In the three-dimensional structure, His-117, Ser-119, and Ser-121 were found to be in close proximity to each other, the latter two residues occupying space around the nucleotide binding pocket into which the side chain of His-117 protrudes. This arrangement may facilitate the transfer of phosphate groups among these residues. The structure of the cocrystal of *M. xanthus* NDP kinase with ADP (33) indicates that the phosphate groups of a bound nucleotide are not directly accessible to either Ser-119 or Ser-121, further supporting the argument for transphosphorylation of serine residues rather than direct autophosphorylation by ATP.

In the tetrameric structure of the enzyme from *M. xanthus* (33), the monomers associated to form dimers involving primarily hydrophobic or aromatic residues, whereas dimer-dimer contacts involve mainly salt links between residues 38 to 46 and 130 to 137 of each monomer to form a tetramer. These regions include Ser-43 of *M. xanthus* and Thr-43 of *E. coli* (the residues corresponding to Ser-44 of human NDP kinase, which has been reported to be phosphorylated), possibly making them unavailable for phosphorylation. In contrast, the enzymes from *D. discoideum* (4), *D. melanogaster* (2), and human NDP kinase (6) form hexamers. Although the same overall monomeric structure is maintained, the interactions between monomers that form oligomers involve different regions of the molecule. This difference in the quaternary structural arrangement has been proposed to account for regulatory functions of NDP kinase that are distinct from their function as phosphate transfer enzymes (2) and could also explain the different patterns of phosphorylation observed for Nm23-H1 and NDP kinase from *E. coli.*

Although MacDonald et al. (16) have shown an inverse correlation between the acid-resistant (serine) phosphorylation of Nm23-H1 and the suppression of metastatic ability and signal responsiveness, the in vivo functional significance of serine phosphorylation of *E. coli* NDP kinase remains uncertain. It has been demonstrated that in vitro the activity of NDP kinase from bovine liver (as measured by the transfer of the high-energy phosphate from ATP to GDP) responds to the energy charge of the adenylate pool, i.e., (ATP + 1/2 ADP)/(ATP + ADP + AMP) (30). A decrease in the energy charge results in a sharp decrease in the activity of NDP kinase and thereby a decrease in the control of the transfer of phosphate from ATP to other nucleotide pools. The regulatory response of NDP kinase may be significant in the allocation of a cell's growth resources (1). Serine phosphorylation of *E. coli* NDP kinase may be associated with such regulation. A regulatory role for serine phosphorylation has been described for the HPr protein from streptococcal cells (24) and isocitrate dehydrogenase (12, 31); both proteins are also phosphorylated at histidine residues. A similar mechanism may be involved in the regulation of NDP kinase activity.

Recently we have found that *ndk* is dispensable in *E. coli*, indicating that there is another enzyme functioning as NDP kinase (15a).

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