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Adenylate kinase complements nucleoside diphosphate kinase deficiency in nucleotide metabolism

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ABSTRACT **Nucleoside diphosphate (NDP) kinase is a ubiquitous nonspecific enzyme that evidently is designed to catalyze *in vivo* ATP-dependent synthesis of ribo- and deoxyribonucleoside triphosphates from the corresponding diphosphates. Because *Escherichia coli* contains only one copy of *ndk*, the structural gene for this enzyme, we were surprised to find that *ndk* disruption yields bacteria that are still viable. These mutant cells contain a protein with a small amount NDP kinase activity. The protein responsible for this activity was purified and identified as adenylate kinase. This enzyme, also called myokinase, catalyzes the reversible ATP-dependent synthesis of ADP from AMP. We found that this enzyme from *E. coli* as well as from higher eukaryotes has a broad substrate specificity displaying dual enzymatic functions. Among the nucleoside monophosphate kinases tested, only adenylate kinase was found to have NDP kinase activity. To our knowledge, this is the first report of NDP kinase activity associated with adenylate kinase.**

Nucleoside diphosphate (NDP) kinase is a ubiquitous enzyme to catalyze the ATP-dependent synthesis of ribo- and deoxyribonucleoside triphosphates from the corresponding diphosphates via a phosphoenzyme intermediate. It has a broad specificity for phosphoryl donors and acceptors to maintain the balanced levels of nucleoside triphosphates in the cell (1, 2). The structures of NDP kinases are highly conserved from *Escherichia coli* to human (43% identity), and they are believed to be a housekeeping enzyme essential for DNA and RNA synthesis (2–4). In addition, NDP kinases have been shown to have additional regulatory functions for growth and developmental control (5–9), signal transduction (10, 11), and tumor metastasis suppression (12, 13). Human and mouse NDP kinases/Nm23 have been considered to play an important role in tumor metastasis suppression. High metastatic potential of low nm23-expressing murine melanoma and human breast carcinoma cell line was inhibited with transfection of nm23 cDNA (4, 14). A human DNA-binding protein, PuF, identified as NDP kinase Nm23-H2, was shown to bind to the promoter region of c-*myc in vitro* and to activate the c-*myc* transcription, which suggested its regulatory role in oncogene expression (15). The cytoplasmic membrane of *Dictyostelium discoideum* possesses a cAMP receptor-stimulated NDP kinase activity that may contribute to mediation of hormone action for the activation of G proteins (10). A null mutation of the *awd* gene, a *Drosophila* homologue of NDP kinase, results in abnormalities in the larvae development (6). A point mutation of Awd leads to a lethal phenotype when it is put together with a mutation in the *prune* gene (16). Studies of NDP kinase from *Myxococccus xanthus*, a gram-negative bacterium, have suggested that the enzyme may be essential for cell growth (5). Surprisingly, however, it was found that the *ndk* gene, the only structural gene for this enzyme in *E. coli*, could be disrupted without affecting cell viability except for a mutator phenotype (17), which suggests the existence of an interesting unidentified enzyme(s) that complements the NDP kinase activity for (d)NTPs synthesis in the cell.

In this study, we attempted to identify the enzyme that complements the NDP kinase function in the *ndk*-disrupted strains of *E. coli*. NDP kinase activity of the *ndk* cell extract was ≈10–15% of that of the wild-type cell extract (17). Because there is only one *ndk* gene on the *E. coli* chromosome (18), the residual NDP kinase activity may be attributed to a novel NDP kinase. There are two possible enzymes that may complement NDP kinase activity in *E. coli*: pyruvate kinases (PykA and PykF; ref. 19) and succinyl-CoA synthetase (Scs), that may form a complex with NDP kinase (20). However, we have constructed an *ndk pyk*A *pyk*F triple mutant as well as an *ndk* Scs double mutant and demonstrated that they still grew normally without apparent morphological changes (17). Residual NDP kinase activities in these triple and double mutants were comparable to the activity in the single *ndk*-disrupted cells, indicating that neither pyruvate kinases nor Scs is the complementing NDP kinase in the *ndk* cells. We now demonstrate that this complementing enzyme is adenylate kinase, a monophosphate kinase known to be essential for the generation of ADP from AMP and normal cell growth.

MATERIALS AND METHODS

Bacterial Strains and Plasmids. *E. coli* strain QL7623, an *ndk*-disrupted strain derived from strain JC7623 (17) and strain BL21(DE3) were used. The latter strain was obtained from New England Biolabs. Cells were grown in Luria–Bertani (LB) medium (for strain JC7623 and QL7623) or in M9 minimal medium [for strain BL21(DE3)] at 37°C. When necessary, chloramphenicol (25 μg/ml) and ampicillin (50 μg/ml) were added.

Plasmid pKX110 and plasmid pKX7 were obtained from K. Yamanaka (21). Plasmid pKX110 carries the wild-type *smb*A (*umk*) gene and plasmid pKX7 carries the wild-type *mss*A (*cmk*) gene.

Detection of the Residual NDP Kinase Activity in the *ndk* Cells. Cell extracts obtained by sonication from a 2-ml overnight culture of strain QL7623 were incubated at 55°C for 5 min in the sample loading buffer (80 mM Tris·HCl, pH 6.8/1% SDS/10% glycerol/4% 2-mercaptoethanol/0.005% bromophenol blue). SDS/PAGE was carried out with use of a 15% polyacrylamide gel at 4°C with constant current of 7 mA. After electrophoresis the gels were sliced into 3 mm (see Fig. 1*A*) or 4 mm (see Fig. 1*B*) segments. Each segment was then separately extracted with 200 μl renaturation buffer {100 mM Hepes, pH 8.0/2% glycerol/0.1% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate] (CHAPS)/1 mM dithiothreitol (DTT)} for 48 hr at 4°C. Ten microliters of the extract was used for the NDP kinase assay as described (22), using 1 mM dCDP as substrate and 5 μCi [γ-^{32}P]ATP (6000 Ci/mmol;

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Abbreviations: NDP, nucleoside diphosphate; Scs, succinyl-CoA-synthetase; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate.

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1 Ci = 37 GBq) as phosphate donor in a 20-μl reaction mixture containing 50 mM Hepes (pH 8.0), 50 mM NaCl, 10 mM $MgCl_2$, and 1 mM DTT. After 2 hr incubation, 1 μl of the reaction mixture was loaded on a polyethyleneimine-cellulose plate. Synthesis of [γ-^{32}P]dCTP was analyzed by ascending chromatography with 0.75 M KH_2PO_4 (pH 3.5) and quantitated by a Molecular Imager GS-250 (Bio-Rad).

Purification of the Complementary NDP Kinase (Sdk) from *ndk* Cells. Whole cell extracts were prepared from 6 liters of the *ndk*-disrupted strain QL7623 (*ndk::cm*^{r}) grown in LB medium overnight. Cells were harvested and washed once with buffer A (50 mM Tris·HCl, pH 8.0/50 mM NaCl/1 mM DTT) and lysed in 40 ml of buffer A with a French press (at 12000 psi, three times). Cell debris and the membrane fraction were removed by centrifugation at 45,000 rpm for 90 min in a Beckman Ti50 rotor. To the supernatant fraction $(NH_4)_2SO_4$ was added to 65% saturation. The resulted precipitates were collected by centrifugation and dissolved in 10 ml of buffer A. The solution was extensively dialyzed with buffer A, then loaded onto a Q-sepharose column (Pharmacia), and equilibrated in buffer A. The column was then washed with buffer A containing 100 mM KCl. Fractions eluted with 200 mM KCl in buffer A were found to contain the major NDP kinase activity. NDP kinase activity was assayed as described (22). The active fractions were pooled and concentrated to 4 ml with a YM-10 concentrator (Amicon) that was then loaded onto a polyethyleneimine anion exchange-1000 column (Amicon). Proteins were eluted with a salt gradient of 100 to 500 mM KCl in buffer A. Fractions containing NDP kinase activity were pooled and CHAPS was added to a final concentration of 0.1%. This sample was concentrated to 0.3 ml which was then loaded at 0.3 ml/min onto an fast protein liquid chromatography (FPLC) superose 12 column. Chromatography was carried out with buffer A containing 100 mM KCl and 0.1% CHAPS. Peaks containing NDP kinase activity were pooled and further loaded onto an FPLC Mono-Q HR 5/5 column. The active fraction was eluted with a salt gradient of 50–300 mM in buffer A containing 0.1% CHAPS.

HPLC Separation of the Partially Purified Sdk. Partially purified Sdk obtained above was further purified by chromatography with on a C8-column [Brownlee Labs model RP-300, Aquapore octyl (C8), 4.6 mm × 10 cm]. Proteins were eluted with a linear gradient from 30 to 70% acetonitrile containing 0.1% trifluoroacetic acid. Each peak fraction monitored at 280 nm was collected manually, lyophilized, and dissolved in 50 mM Hepes (pH 8.0), 1% glycerol, 0.1% CHAPS, and 1 mM DTT. NDP kinase was assayed for the activity converting CDP to [γ-^{32}P]CTP by using [γ-^{32}P]ATP.

Adenylate Kinase from Other Sources. Adenylate kinase from porcine and chicken muscle were obtained from Sigma. They were further purified to remove contaminated proteins by HPLC as above except that a C4-column was used [Brownlee Labs model RP-300, Aquapore Butyl (C4), 4.6 mm × 3 cm] to homogeneity as judged by SDS/PAGE.

Overexpression and Purification of *E. coli* Adenylate Kinase and Other Nucleoside Monophosphate Kinases. To purify *E. coli* adenylate kinase without any contamination of NDP kinase, we first constructed BL21(DE3) containing the *ndk::cm*^{r} mutation from strain QL7623. BL21(DE3) strain contains the gene for T7 polymerase for high expression of a gene under a T7 promotor. *ndk::cm*^{r} locus was transduced into BL21(DE3) cells by P1 transduction as described (17). The transduced gene was confirmed by Southern blot analysis. The *E. coli* gene for adenylate kinase was isolated from Kohara genomic lambda library (DNA fragment from phage 152) (23) by PCR using oligonucleotide 6501 (ttccatatgcgtatcattctgctt) as an upstream primer and oligonucleotide 6502 (gtgggatccttcgaattagccgaggat) as a downstream primer. The PCR product was gel-purified, digested with *Nde*I and *Bam*HI, and cloned into pET11a vector (New England Biolabs) digested with *Nde*I and *Bam*HI to generate plasmid pETadk. The entire region of *adk* insert in pETadk was sequenced to confirm that no errors were generated by the PCR process. The BL21(DE3) *ndk::cm*^{r} strain was transformed with pET-adk. The maximal induction of adenylate kinase was obtained in M9 minimal medium at 37°C for 4 hr in the presence of 2 mM isopropyl β-D-thiogalactoside. Adenylate kinase was produced at a level of ≈60% total cellular protein. The enzyme was then purified basically by the same methods as described (24) with some modifications. Briefly, a cell extract from a 500-ml culture was subjected to ammonium sulfate precipitation. The 30–65% precipitate fraction was resuspended in 2 ml buffer B (50 mM Tris·HCl, pH 8.0/1 mM DTT/10% glycerol) and loaded to a 200-ml Sephacryl S200 column equilibrated with the same buffer with a flow rate 0.4 ml/min. Fractions containing adenylate kinase were pooled and loaded directly onto a 30-ml Q-Sepharose column equilibrated with buffer B and eluted with a 0–0.3 M KCl gradient. Adenylate kinase was eluted at a salt range between 50 and 70 mM. Active fractions were pooled and dialyzed briefly against buffer B. The dialyzed solution was then subjected to an FPLC polyethyleneimine anion exchange-1000 column with a gradient of 0–0.3 M KCl. Adenylate kinase eluting from this column was ≈90% pure, as determined with SDS/PAGE followed by staining with Coomassie brilliant blue staining.

Constructions of plasmid pETcmk and pETumk for overexpression of cytidylate (CMP) kinase and uridylate (UMP) kinase, respectively, are similar to that of pETadk. Plasmid pETcmk was constructed by PCR amplification of the *cmk* gene from plasmid pKX7 using upstream primer oligonucleotide 6676 (gagatacatatgacggcaattgcc) and downstream oligonucleotide 6677 (tgcggatccggtcgcttatgcgag) followed by cloning the PCR fragment into pET11a vector. The *cmk* gene was amplified from plasmid pKX110 by PCR with oligo 6674 (gaaagacatatggctaccaatgca) as an upstream primer and oligo 6675 (tatggatcccgggaattattccgt) as a downstream primer. Thus, plasmid pETumk was generated, which contained the *umk* gene under a T7 promotor. Plasmid pETcmk or pETumk was introduced into strain BL21(DE3) *ndk::cm*^{r} for overproduction of CMP kinase or UMP kinase. The purification procedures are essentially the same as that of adenylate kinase above.

RESULTS

Identification of the Complementary NDP Kinase. We attempted to identify the complementing NDP kinase in the *ndk*-disrupted cells. Total cell extracts from wild-type and *ndk* cells were applied to SDS/PAGE, and after electrophoresis the gels were sliced. Proteins in gel slices were renatured, and the NDP kinase activity was assayed for each slice as described in *Materials and Methods*. In wild-type cells (Fig. 1*A*), there was a minor peak at slice 16 in addition to the major peak near 16 kDa (slice 23–28), which corresponds to the wild-type *ndk* gene product. When the *ndk* mutant cell extract was analyzed (Fig. 1*B*), a single peak at slice 11 exhibited the activity, which corresponds to slice 16 at ≈25 kDa in Fig. 1*A*. This 25-kDa protein (designated Sdk for the second NDP kinase) from the *ndk* mutant cells was purified by ion exchange and gel filtration chromatography as described in *Materials and Methods*. Partially purified Sdk was then subjected to SDS/PAGE, and proteins in the bands between 21 and 35 kDa (Fig. 2, lane 1) were extracted from the gel and renatured to analyze their NDP kinase activity. One of the bands at about 25 kDa (indicated by an arrow) had NDP kinase activity. This fraction was further subjected to reverse-phase HPLC chromatography. Sdk was then purified to homogeneity as shown by silver staining in Fig. 2, lane 2. Protein microsequencing of Sdk revealed that the N-terminal 18-residue sequence of this protein was M R I I L L G A P G A G K G T Q A Q, which was identical to that of *E. coli* adenylate kinase (25, 26), the *adk* product, which indicated that Sdk is adenylate kinase.

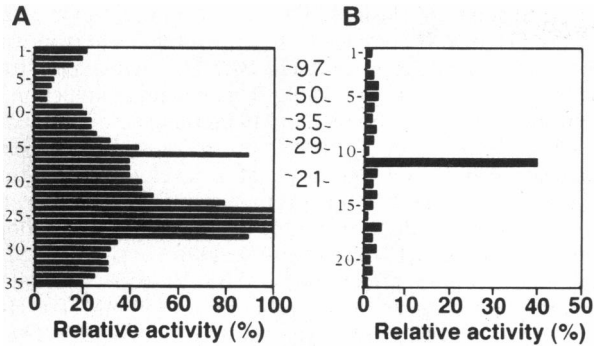

FIG. 1. Separation of NDP kinases by SDS/PAGE. Cell extracts from wild-type (JC7623) and *ndk*-disrupted strain (QL7623) were subjected to SDS/PAGE. The gels were sliced and the proteins in each slice were renatured as described. NDP kinase assay for each slice was carried out as described (22). Purified *E. coli* NDP kinase (10 ng) (24) was used as a control for determining relative activities. Positions of molecular weight markers are shown between *A* and *B* (in kDa).

Adenylate kinase was originally identified in 1943 as myokinase in animal muscle that was required for energy metabolism by converting two ADP molecules to AMP and ATP (27). Subsequently, it was found to function as adenosine monophosphate kinase to generate ADP from AMP with the use of ATP (28). Its three-dimensional structure has been also determined by both x-ray crystallography and NMR (29–34), revealing two nucleotide binding sites, one for ATP or ADP and the other for AMP or ADP. It undergoes the induced-fit movement with dramatic conformation change during nucleotide binding (30, 31). ATP can be substituted with 2′-dATP, GTP, CTP, UTP, or ITP to some extent, while there is a stringent specificity for AMP (28). Human adenylate kinase deficiency is associated with hemolytic anemia (35, 36). Adenylate kinase has been shown to be essential for cell growth in *E. coli* (25). Both adenylate kinase and NDP kinase are highly active in *E. coli* when compared to other nucleoside monophosphate kinases (2, 28).

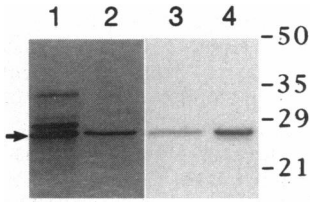

FIG. 2. Purification of the second NDP kinase (Sdk). The different preparations of Sdk were analyzed by SDS/PAGE (17.5% gel). Partially purified Sdk was obtained after ion exchange column chromatography and gel filtration as described, which was applied to lane 1. The partially purified Sdk was further purified by HPLC and applied to lanes 2 and 3. Lane 4 shows the purified adenylate kinase from the T7 expression system as described. Lanes 1 and 2 were stained by silver staining, and lanes 3 and 4 were stained by Coomassie brilliant blue R-250. Arrow indicates the position of Sdk. Positions of molecular weight markers (in kDa) are shown at right.

Adenylate Kinase Functions as NDP Kinase. To unambiguously demonstrate that adenylate kinase indeed functions as NDP kinase, we purified adenylate kinase to homogeneity (Fig. 2, lane 4) from *ndk*-disrupted cells overproducing adenylate kinase under a T7 promoter as described in *Materials and Methods*. In this fashion, we were able to avoid any contaminated NDP kinase in the adenylate kinase preparation. Using the purified adenylate kinase of *E. coli*, the substrate specificity as NDP kinase was next examined. As shown in Fig. 3*A*, like NDP kinase (lanes 2–4 and 9–11), adenylate kinase can convert ribonucleoside diphosphates (lanes 5–7) and deoxyribonucleoside diphosphates (lanes 12–14) tested to the respective nucleoside triphosphates using ATP as a phosphate donor. Note that with dTDP, ADP was also formed as shown by an arrow in lane 14. Since adenylate kinase is known to efficiently convert ADP plus dTDP to dTTP plus AMP (37), AMP thus formed appears to be further converted by the enzyme to ADP using ATP. We also compared the adenosine monophosphate kinase activity of adenylate kinase with that of NDP kinase preparation from *E. coli* (Fig. 3*B*). In contrast to a high activity to convert AMP to ADP by adenylate kinase (lane 3), a small amount of ADP was

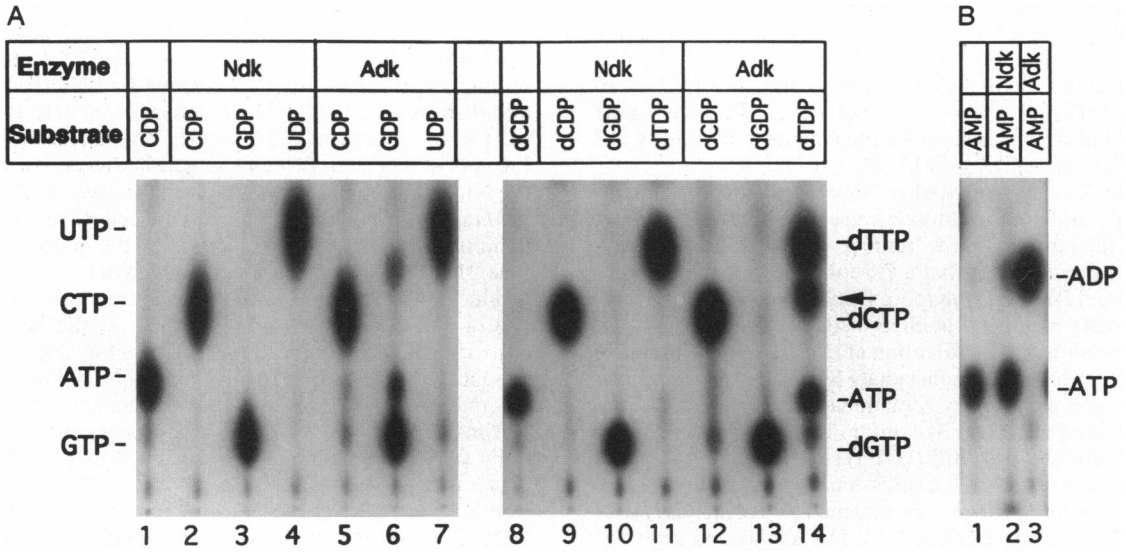

FIG. 3. Substrate specificity of NDP kinase (Ndk) and adenylate kinase (Adk). (*A*) Purified Ndk (10 ng) (lanes 2–4 and 9–11) and 100 ng purified Adk (lanes 5–7 and 12–14) were assayed for NDP kinase activity as described (22). The reactions were carried out with 1 μCi [γ-^{32}P]ATP (6000 Ci/mmol), 1 mM ribonucleoside diphosphate (CDP, GDP, or UDP), or deoxyribonucleoside diphosphate (dCDP, dGDP, or dTDP) as indicated. Reactions were terminated after 30 min by the addition of EDTA to a final concentration of 10 mM. Samples were then analyzed by thin-layer chromatography followed by autoradiography to show the formation of [γ-^{32}P]CTP (lanes 2 and 5), GTP (lanes 3 and 6), UTP (lanes 4 and 7), dCTP (lanes 9 and 12), dGTP (lanes 10 and 13), and dTTP (lanes 11 and 14). Lanes 1 and 8 are controls with [γ-^{32}P]ATP plus CDP, and [γ-^{32}P]ATP plus dCDP in the absence of the enzyme, respectively. (*B*) Assay for adenylate kinase activity of Ndk and Adk using AMP (1 mM) and [γ-^{32}P]ATP (1 μCi, 6000 Ci/mmol). Ndk (100 ng) (lane 2) and 100 ng Adk (lane 3) were used for the assay. Lane 1, control with [γ-^{32}P]ATP plus AMP in the absence of the enzymes.

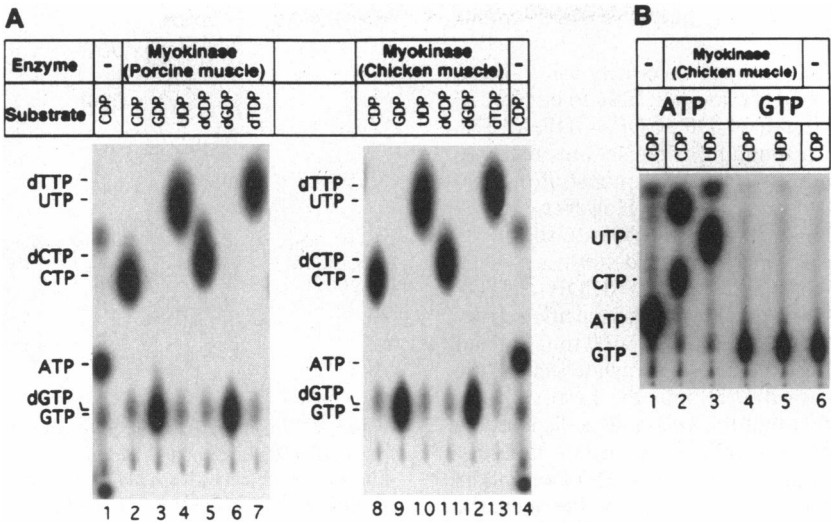

FIG. 4. NDP kinase activity of eukaryotic adenylate kinases. (*A*) Myokinases or adenylate kinases from porcine muscle (100 ng) (lanes 2–7) and chicken muscle (100 ng) (lanes 8–13) were assayed for NDP kinase activity as shown in Fig. 3*A* by using different nucleoside diphosphate substrates. Both enzymes were obtained from Sigma and further purified by HPLC as described. (*B*) Purified myokinase (100 ng) from chicken was assayed for NDP kinase activity using [γ-^{32}P]ATP (1 μCi, 6000 Ci/mmol) (lanes 2 and 3) and [γ-^{32}P]GTP (1 μCi, 5000 Ci/mmol) (lanes 4 and 5) as phosphate donors to convert substrate NDPs to corresponding NTPs as indicated in the figure. No NTPs formation was detected with [γ-^{32}P]GTP as phosphate donor. Lanes 1 and 6 are controls with [γ-^{32}P]ATP plus CDP and [γ-^{32}P]GTP plus CDP in the absence of the enzymes, respectively.

produced with the NDP kinase preparation used. At present, it is not known whether the NDP kinase preparation was contaminated with a trace amount of adenylate kinase or whether NDP kinase has a weak adenylate kinase activity. It should be noted, however, that the temperature-sensitive phenotype of an *adk*^{ts} mutant (strain CV2; ref. 25) was not suppressed even by the overexpression of NDP kinase (data not shown). Adenylate kinases (also called myokinase) from higher eukaryotic sources such as porcine and chicken muscle were also found to have NDP kinase activity similar to *E. coli* adenylate kinase activity (Fig. 4*A*).

NDP Kinase Specificity of Adenylate Kinase. NDP kinase is known to use any nucleoside triphosphate as a phosphate donor. Indeed, NDP kinase can utilize both ATP (Fig. 5*A*, lanes 2 and 3) and GTP (lanes 4 and 5). In contrast, adenylate kinase can utilize only ATP (lanes 6, 7 and 12) but not GTP (lanes 8, 9 and 13) as a phosphate donor for nucleoside diphosphates. Similarly, muscle adenylate kinase from chicken can utilize ATP (Fig. 4*B*, lanes 2 and 3) but not GTP (lanes 4 and 5) for its NDP kinase activity. It is interesting to note, however, that adenylate kinase can efficiently use GTP as a phosphate donor in the reaction which converts AMP to ADP (Fig. 5*A*, lane 11, compare with lane 10). We found that AMP was a potent inhibitor for the NDP kinase function of adenylate kinase (Fig. 5*B*). When AMP and UDP were added together with [γ-^{32}P]ATP, ADP formation from AMP was not significantly affected, while UTP production from UDP was severely blocked (lane 3, compare with lane 4). These results indicate that (d)NDPs bind to the AMP binding site of adenylate kinase, while GTP binds to the ATP binding site. It

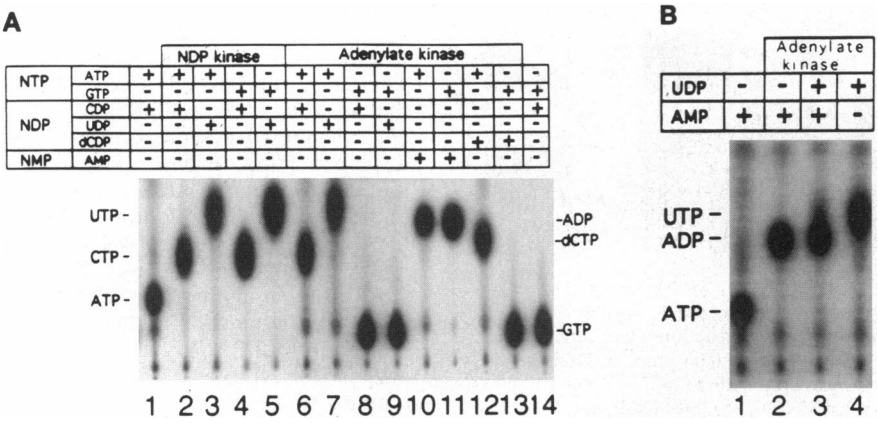

FIG. 5. Comparison of ATP and GTP used as phosphate donors for NDP kinase and adenylate kinase when assayed for NDP kinase activity. (*A*) In the presence of Ndk (100 ng), [γ-^{32}P]ATP (1 μCi, 6000 Ci/mmol) (lanes 2 and 3) and [γ-^{32}P]GTP (1 μCi, 5000 Ci/mmol) (lanes 4 and 5) were used as phosphate donors to convert nucleoside diphosphate substrates to corresponding NTPs as indicated in the figure. Adk (100 ng), [γ-^{32}P]ATP (lanes 6, 7, and 12), and [γ-^{32}P]GTP (lanes 8, 9, and 13) were used as phosphate donors for the NDP kinase assay as above. Substrates and products are indicated in the figure. Adenylate kinase activity of Adk was also assayed using [γ-^{32}P]ATP (lane 10) and [γ-^{32}P]GTP (lane 11) as phosphate donors with AMP as substrate. The formations of [γ-^{32}P]CTP (lanes 2, 4, and 6), [γ-^{32}P]UTP (lanes 3, 5, and 7) and [γ-^{32}P]-dCTP (lane 12) are shown. Lanes 1 and 14 are controls with [γ-^{32}P]ATP plus CDP, and [γ-^{32}P]GTP plus dCDP in the absence of the enzyme, respectively. (*B*) AMP inhibits the NDP kinase activity of adenylate kinase. AMP (1 mM) was added into the NDP kinase reaction mixture using 1 mM UDP as substrate (lane 3) with 1 μCi [γ-^{32}P]ATP (6000 Ci/mmol). Formation of [γ-^{32}P]UTP was severely inhibited in the presence of AMP (lane 3). Lanes 2 and 4 are controls with AMP and UDP as substrates for adenylate kinase, respectively. Lane 1 contained [γ-^{32}P]ATP and AMP in the absence of the enzyme.

appears that GTP binding to this site blocks (d)NDP binding to the AMP binding site.

We further examined the substrate specificity using *E. coli* adenylate kinase. The purified enzyme was able to convert all nucleoside diphosphates tested (UDP, CDP, GDP, dCDP, dGDP, and dTDP) to corresponding triphosphates required for DNA and RNA synthesis, clearly demonstrating that adenylate kinase has NDP kinase activity. However, in contrast to NDP kinase with relative lack of substrate specificity (Fig. 6*A*), adenylate kinase displayed biased substrate specificity as UDP > CDP > GDP and dGDP > dCDP > dTDP (Fig. 6*B c, a, b, e, d,* and *f*, respectively) and the specific activity of NDP kinase was estimated to be about 20–100 times higher than that of the NDP kinase function of adenylate kinase; the highest specific activity toward UDP and the lowest toward dTDP. Nevertheless, the fact that the *ndk* gene is dispensable indicates that the NDP kinase activity of adenylate kinase is sufficient enough to maintain appropriate (d)NTPs pools in the *ndk*-disrupted cells. However, the balance between the nucleotide triphosphate pools appear to be somewhat distorted as evident from the mutator phenotype of the *ndk*-disrupted cells (17).

NDP Kinase Activity in Other Nucleoside Monophosphate Kinases. Since adenylate kinase was shown having NDP kinase activity, it is interesting to know whether other nucleoside monophosphate kinases have the similar activity or not. It has been reported that purified thymidylate (TMP) kinase from yeast does not contain NDP kinase activity (38). *E. coli* guanylate (GMP) kinase was also not found to have the NDP kinase activity (39). In the present paper, we purified CMP kinase and UMP kinase, both of which were overexpressed from pET11a-derived plasmid pETcmk and pETumk, respectively, to ≈50% of the total cellular proteins after induction of isopropyl β-D-thiogalactoside. Both CMP and UMP kinases were purified to 90% purity as described in *Materials and Methods*. Purified adenylate kinase and CMP kinase and UMP kinase were then examined for NMP kinase activity and NDP kinase activity. As shown in Fig. 7*A*, the purified Adk, CMP kinase, and UMP kinase can convert AMP, CMP, and UMP to the corresponding nucleoside diphosphates, ADP, CDP, and UDP, respectively (lanes 2–4). However, only the Adk was shown to have NDP kinase activity (Fig. 7*B*, lane 2); both CMP kinase and UMP kinase were unable to convert UDP to UTP (lane 3 and 4, respectively). These results indicate that adenylate kinase is the only enzyme possessing NDP kinase activity among all NMP kinases.

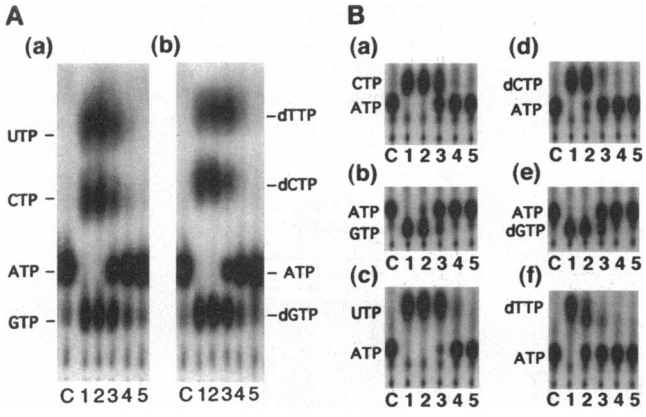

FIG. 6. Different substrate specificity of NDP kinase and adenylate kinase when assayed for their NDP kinase activity as described (22). (*A*) NDP kinase was serially diluted. The reactions were carried out in the presence of 15.6, 3.1, 0.78, 0.15, and 0.03 pmol of NDP kinase, respectively (lanes 1–5) and incubated with 10 μCi [γ-^{32}P]ATP (6000 Ci/mmol) and a mixture of 1 mM each of CDP, GDP, and UDP (*a*), and 1 mM each of dCDP, dGDP, and dTDP (*b*) for 30 min in a 20-μl reaction mixture. One microliter of reaction mixture was taken to 10 μl of 5 mM EDTA, and 1 μl of the final mixture was applied to a PEI-cellulose plate. The formation of the corresponding [γ-^{32}P](d)NTPs was shown as indicated. (*B*) NDP kinase activity of *E. coli* adenylate kinase. The reactions were carried out in the presence of 19, 9.5, 4.8, 2.4, and 1.2 pmol of adenylate kinase (lanes 1–5, respectively) with 1 mM nucleoside diphosphate to be tested and 10 μCi [γ-^{32}P]ATP (6000 Ci/mmol) for 30 min in a 20-μl reaction mixture as in *A*. The formations of [γ-^{32}P]CTP (*a*), -GTP (*b*), -UTP (*c*), -dCTP (*d*), -dGTP (*e*), and -dTTP (*f*) are indicated. Lane C is a control with [γ-^{32}P]ATP in the absence of the enzymes.

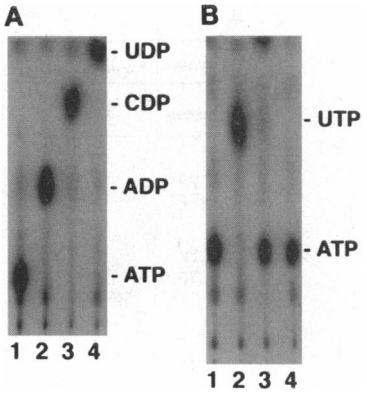

FIG. 7. NMP kinase activity and NDP kinase activity of the purified NMP kinases. (*A*) Adenylate kinase, CMP kinase, and UMP kinase (100 ng each) were assayed for NMP kinase activity to convert 1 mM each AMP (lane 2), CMP (lane 3), and UMP (lane 4), respectively, to the corresponding [γ-^{32}P]ADP, -CDP, and -UDP by using 1 μCi [γ-^{32}p]ATP (6000 Ci/mmol) in 10-μl reaction solution for 10 min at 30°C. (*B*) Substrate UDP (1 mM) was used for NDP kinase assay for all NMP kinases. Only adenylate kinase (lane 2) is shown to be able to convert UDP to [γ-^{32}p]UTP by using [γ-^{32}p]ATP (1 μCi, 6000 Ci/mmol) as a phosphate donor, while there is no [γ-^{32}p]UTP formation with UMP kinase (lane 3) and CMP kinase (lane 4), respectively. The reactions were performed at 30°C for 30 min in a 10 μl reaction mixture. Lane 1 is a control with [γ-^{32}P]ATP in the absence of the enzymes.

DISCUSSION

For a long time, it has been believed that NDP kinase is the key enzyme that is responsible for the maintenance of the pools of all nucleoside triphosphates, since it is the only enzyme known to effectively convert all nucleoside diphosphates to corresponding triphosphates. However, our recent findings show that *E. coli ndk*-knockout mutants are still viable without apparent growth defect except for the fact that the mutant cells display a mutator phenotype (17). Because the pyruvate kinases have been suggested to function as NDP kinase activity under an anaerobic condition in *E. coli* (19), a triple-disruption (*ndk pykA pykF*) mutant was constructed. However, it also grew normally and displayed a similar level of NDP kinase activity to that of the single *ndk* mutant cell (17). We have also excluded the possibility that Scs, which can catalyze the synthesis of ATP, GTP, and ITP, may be the complementary enzyme for NDP kinase by constructing an *ndk scs* double mutant, which again grew normally (17). Thus, nucleotide metabolism and its control appear not to be nearly as simple as suggested by the dogma of the indispensable role of NDP kinase in nucleoside triphosphate synthesis.

The present data clearly demonstrated that the complementing enzyme for NDP kinase is adenylate kinase. We also demonstrated that muscle adenylate kinase (myokinase) from higher eukaryotic sources possesses NDP kinase activity. Interestingly, a dTTP-synthesizing enzyme was purified from porcine skeletal muscle to homogeneity and was identified as cytosolic adenylate kinase (40). These results suggest that not only NDP kinase but also adenylate kinase play important

roles in nucleotide metabolism in cells. Adenylate kinase may therefore play dual roles in the cells functioning as NDP kinase as well as a nucleoside monophosphate kinase converting AMP to ADP with the use of ATP. The latter function of adenylate kinase is known to be essential for cell growth by properly maintaining the energy state of the cell (28). The different preference for various NDPs exhibited by NDP kinase and adenylate kinase is interesting. *E. coli* NDP kinase was shown to have a high affinity toward GDP for GTP synthesis (Fig. 6*A*) since NDP kinase was originally identified as a GTP-binding protein by using 8-azido GTP in *M. xanthus* (5). However, *E. coli* adenylate kinase when tested for its NDP kinase activity exhibits the highest preference toward UDP for UTP synthesis (Fig. 6*B*). Similar observation has been noticed with an alternative NDP kinase in *Pseudomonas aeruginosa* (41). The yeast gene for NDP kinase (YNK) has been shown to be dispensable (42), suggesting that adenylate kinase may be also complementing NDP kinase deficiency in yeast.

Adenylate kinase is a highly flexible protein that displays substantial changes upon nucleotide binding (30, 31). Two nucleotide binding domains for ATP and AMP are converted to two ADP binding domains during the catalytic reaction, which results in relatively more open conformation (31). Our present results suggest that there may exist an intermediate state with a broader specificity for binding of nucleoside diphosphates, which can receive a γ-phosphate group from ATP producing the corresponding nucleoside triphosphates. Interestingly, studies of other nucleoside monophosphate kinases such as thymidylate kinase (38) and guanylate kinase (39) have been shown to have no NDP kinase activity. The purified CMP kinase and UMP kinase were also not found to contain NDP kinase activity (Fig. 7*B*). Apparently, adenylate kinase is the only NMP kinase possessing NDP kinase activity among all those NMP kinases.

Because *E. coli* adenylate kinase has been shown to be one of the components in the multienzyme complex for T4 phage DNA synthesis together with NDP kinase (2, 43, 44), it is tempting to speculate that adenylate kinase in the complex plays an important role as NDP kinase to facilitate the synthesis of DNA precursors and maintain high and balanced levels of dNTP at the site of DNA synthesis.

The dual roles of adenylate kinase acting as not only nucleoside mono- but also diphosphate kinase might have had an evolutionary significance before an independent NDP kinase had evolved. The present finding of the NDP kinase activity of adenylate kinase provides an intriguing new insight into intracellular nucleotide metabolism as well as the regulation of RNA and DNA biosynthesis.

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