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Metabolic Functions of Microbial Nucleoside Diphosphate Kinases

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This article summarizes research from our laboratory on two aspects of the biochemistry of nucleoside diphosphate kinase from *Escherichia coli*—first, its interactions with several T4 bacteriophage-coded enzymes, as part of a multienzyme complex for deoxyribonucleoside triphosphate biosynthesis. We identify some of the specific interactions and discuss whether the complex is linked physically or functionally with the T4 DNA replication machinery, or replisome. Second, we discuss phenotypes of an *E. coli* mutant strain carrying a targeted deletion of *ndk*, the structural gene for nucleoside diphosphate kinase. How do bacteria lacking this essential housekeeping enzyme synthesize nucleoside triphosphates? In view of the specific interactions of nucleoside diphosphate kinase with T4 enzymes of DNA metabolism, how does T4 multiply after infection of this host? Finally, the *ndk* disruption strain has highly biased nucleoside triphosphate pools, including elevations of the CTP and dCTP pools of 7- and 23-fold, respectively. Accompanied by these biased nucleotide pools is a strong mutator phenotype. What is the biochemical basis for the pool abnormalities and what are the mutagenic mechanisms? We conclude with brief references to related work in other laboratories.

KEY WORDS: NDP kinase; adenylate kinase; (dNTP:AMP) phosphotransferase; CTP synthetase; nucleotide pools; mutator phenotype; bacteriophage T4; dNTP synthetase complex; anti-idiotypic antibody.

Research in the past decade has spotlighted a bewildering variety of metabolic and regulatory roles for nucleoside diphosphate kinases of eukaryotic origin, as detailed elsewhere in this issue of the journal. Our experience over the past two decades with NDP[6] kinase of *Escherichia coli* suggests similar complexity in functions of this bacterial enzyme. Studies from our laboratory and others, focused upon NDP kinase from both *E. coli* and other microorganisms, confirm that this microbial protein does more than simply interconvert nucleoside di- and triphosphates. In this article we discuss two particular features of the biology of *E. coli* NDP kinase: (1) its specific interactions with T4 bacteriophage-coded proteins as part of a multienzyme complex for deoxyribonucleotide biosynthesis; and (2) unusual phenotypes of *E. coli* mutants containing targeted deletions in *ndk*, the structural gene for NDP kinase.

FUNCTIONS OF *Escherichia coli* NDP KINASE IN BACTERIOPHAGE T4 DNA METABOLISM

Among the earliest investigations of *E. coli* NDP kinase were experiments in the 1960s from M. J. Bess-

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[6] Key to abbreviations: NDP, nucleoside diphosphate; dNMP, dNDP, and dNTP, deoxyribonucleoside 5′-monophosphate, diphosphate, and triphosphate, respectively; PVDF, polyvinylidene fluoride.

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man's laboratory (reviewed in Mathews, 1971). The findings that T-even phage infection increases DNA synthesis rates up to tenfold and that DNA from these phages contains a novel pyrimidine base, 5-hydroxymethylcytosine, led investigators to seek and find new enzyme activities in extracts of infected bacteria. These included a unique deoxyribonucleoside monophosphate kinase with specificity toward 5-hydroxymethyl-dCMP, -dGMP, and -dTMP. However, when Bello and Bessman (1963) analyzed T4-infected cell extracts for nucleoside diphosphate kinase, they found no increase after infection; the specific activity in either uninfected or infected cell extracts was 15- to 20-fold higher than activity of the phage-coded dNMP kinase in infected cell extracts. In retrospect, this should not have been a surprising finding, because like all known NDP kinases, the *E. coli* enzyme participates in synthesis of both the deoxyribonucleoside triphosphates and the far more abundant ribonucleoside triphosphates. Because specificity of the enzyme is low, the *E. coli* enzyme could synthesize the phage-specific dNTP, 5-hydroxymethyl-dCTP, once the phage-coded dNMP kinase had formed the corresponding diphosphate. It soon became evident that after T4 infection nearly all of the reactions in dNTP synthesis and DNA replication are catalyzed by phage-coded enzymes. NDP kinase was one of very few exceptions; another was adenylate kinase, an enzyme known to catalyze the ATP-dependent phosphorylation of either AMP or dAMP.

A decade later, several lines of evidence suggested that the T4 phage-coded enzymes of dNTP synthesis interact to form a multienzyme complex, which could facilitate both the synthesis of DNA precursors and their flow into DNA (reviewed by Mathews *et al.,* 1979). The role of NDP kinase was of particular interest, because it catalyzes the last reaction in DNA precursor synthesis. Thus, if the putative "dNTP synthetase" complex somehow directly passed dNTPs to the T4 replication complex, or replisome, a host cell enzyme, NDP kinase, occupied a strategic position as the functional link between two multienzyme complexes comprising phage-coded proteins almost exclusively (Ray and Mathews, 1992). This idea was confirmed by our earliest experiments demonstrating the existence of a dNTP synthetase complex (Mathews *et al.,* 1979). When we sedimented extracts of T4-infected *E. coli* through sucrose gradients, about 5% of the total NDP kinase activity in the cell cosedimented with an aggregate of phage-coded enzymes. The activity was both physically and kinetically linked to the viral enzymes with which it was associated. Thus, when we assayed these enzyme aggregates for the ability to catalyze multistep reaction sequences, we found that reaction sequences involving NDP kinase showed immeasurably short transient times and low accumulation of intermediates, as expected from the operation of an organized enzyme complex. Later, after the 1300-kDa complex had been highly purified, we could still readily detect kinetic coupling of bacterial NDP kinase to the several phage-coded enzymes in the complex (Moen *et al.,* 1988). Moreover, mutations in several T4 genes abolished both the physical and kinetic integrity of this complex, suggesting that its formation did not involve a simple self-assembly process, but was the result of a number of highly specific protein–protein interactions.

What are those interactions? We have pursued this question by several approaches, including protein affinity chromatography (Ray, 1992; Wheeler *et al.,* 1996). This became possible when Hama *et al.* (1991) cloned and expressed *ndk,* the *E. coli* structural gene for NDP kinase. We immobilized the purified recombinant enzyme and used two-dimensional gel electrophoresis and radioautography to identify radiolabeled T4 proteins specifically retained by this column. Several phage proteins not retained by control columns of immobilized bovine serum albumin were found to bind strongly to the NDP kinase column (retained at 0.2 *M* NaCl, eluted by 0.6 *M* NaCl). These included dihydrofolate reductase, the large subunit of ribonucleotide reductase, and several proteins involved in DNA replication and repair. Surprisingly, when the same analysis was carried out with radiolabeled *E. coli* proteins, much smaller amounts of protein were bound, and were found exclusively among the weakly bound proteins, eluted by 0.2 *M* NaCl. Thus, by this criterion, *E. coli* encodes an NDP kinase that interacts much more strongly with proteins of a virus destined to kill it than with its own functionally related proteins.

The important question of whether the T4 dNTP synthetase complex is physically linked to the replication complex, or replisome, is still unresolved. To be sure, the immobilized NDP kinase affinity column retained several T4 proteins of DNA replication, including gene 32 single-strand DNA-binding protein, gene 45 polymerase-accessory protein, and gene 61 DNA primase. However, these interactions might be indirect. Therefore, we have carried out analyses of direct protein–protein interactions, using electrophoretic mobility-shift assays (Bernard, 1999). In the experiments of Fig. 1, purified proteins or protein mix-

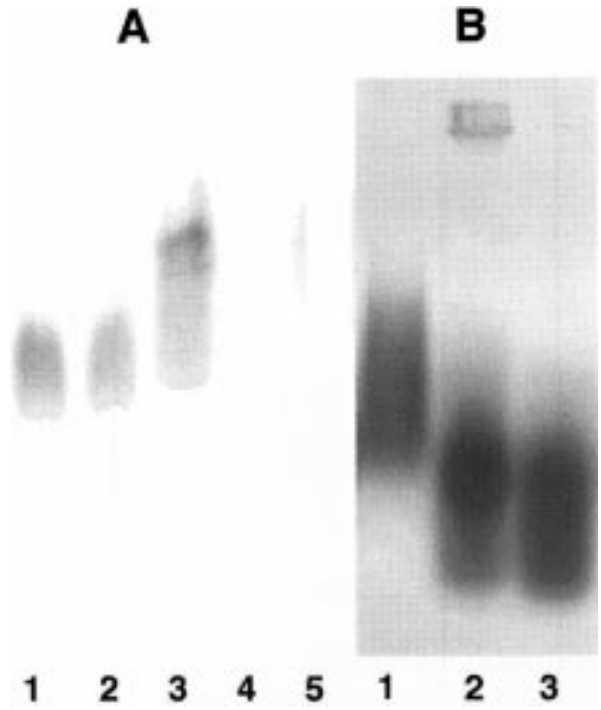

Fig. 1. Protein–protein interactions involving *E. coli* NDP kinase demonstrated by electrophoretic mobility shift assay. Purified recombinant proteins were incubated at the indicated concentrations for 30 min at 37°C in the presence of 1 m*M* ATP and 7.5% polyethylene glycol (PEG 3000). The mixtures were then subjected to nondenaturing electrophoresis on 0.7% agarose gels for 1 h at 100 V. Proteins were blotted from the gels onto PVDF membranes, which were probed with polyclonal antisera as indicated. (A) Probing with antibody to T4 gene 43 DNA polymerase. Lane 1, analysis of 0.6 μ*M* T4 gene 43 DNA polymerase plus 1.7μ*M E. coli* NDP kinase; lane 2, 0.6 μ*M* T4 DNA polymerase plus 1.6 μ*M*T4 gene 32 single-strand DNA-binding protein; lane 3, 0.6 μ*M* T4 DNA polymerase; lane 4, NDP kinase alone; lane 5, gene 32 protein alone. (B) Probing with antibody to *E. coli* NDP kinase. Lane 1, analysis of 0.09 μ*M* NDP kinase; lane 2, 0.09 μ*M* NDP kinase plus 0.33 μ*M* T4 ribonucleotide reductase; lane 3, 0.09 μ*M* NDP kinase plus 0.52 μ*M* T4 gene 43 protein. For complete details see Bernard (1999).

tures were resolved by nondenaturing gel electrophoresis in 0.7% agarose and then transferred to PVDF membranes, which were probed with antibodies. Panel A shows that purified *E. coli* NDP kinase and T4 gene 43 DNA polymerase formed a complex that migrated more rapidly than did purified DNA polymerase alone. Panel B confirms the polymerase–kinase interaction and shows that purified NDP kinase similarly formed a specific complex with purified T4 ribonucleotide reductase. Thus, protein–protein interactions thought to stabilize intracellular complexes can be demonstrated *in vitro*.

The affinity chromatography and gel-shift experiments represent essentially *in vitro* approaches. Accordingly, we have sought more physiological approaches to determine whether *E. coli* NDP kinase interacts specifically with T4 proteins *in vivo*. One such strategy uses anti-idiotypic antibodies as probes for interacting proteins (Young and Mathews, 1992). The rationale is that a polyclonal antiserum to a protein might include antibodies specific to a domain involved in a protein–protein interaction and antibodies prepared against that antibody, in turn, could form an immune complex with proteins interactive with the original protein of interest. Accordingly, we purified antibodies to *E. coli* NDP kinase by affinity chromatography on NDP kinase itself and immunized rabbits against these purified antibodies (Ray, 1992). As shown in Fig. 2, one of the two anti-idiotypic sera immunoprecipitated a T4 protein of approximately 43

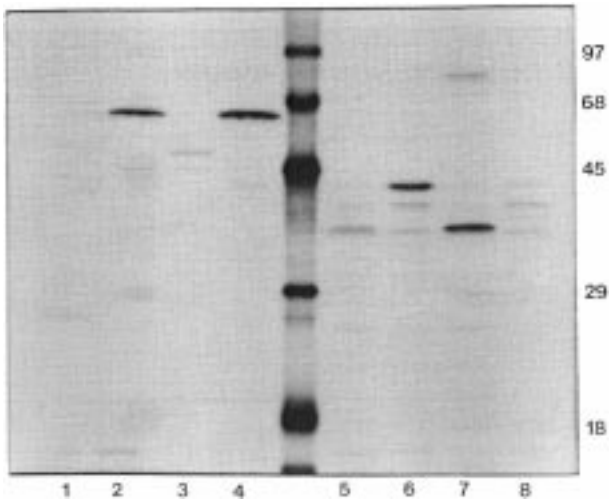

Fig. 2. Interaction of *E. coli* NDP kinase with a 43-kDa T4 protein, as revealed by immunoprecipitation analysis of anti-idiotypic sera prepared against *E. coli* NDP kinase. Extracts of [^{35}S]methionine-labeled *E. coli* or T4 proteins were incubated either with preimmune sera or with anti-anti-NDP kinase (antisera prepared against affinity-purified antibodies in two different polyclonal antisera prepared against recombinant *E. coli* NDP kinase). After incubation, immune complexes were precipitated with goat antirabbit antiserum and analyzed by SDS-polyacrylamide gel electrophoresis and radioautography. Complete details are given in Ray (1992). Lanes 1 and 2, analysis of *E. coli* proteins immunoreactive with anti-anti-NDP kinase from rabbits 1 and 2, respectively; 3 and 4, *E. coli* proteins immunoreactive with preimmune sera from rabbits 1 and 2; 5 and 6, analysis of T4 proteins immunoreactive with anti-anti-NDP kinase from rabbits 1 and 2; 7 and 8, T4 proteins immunoreactive with preimmune sera from rabbits 1 and 2. Unnumbered lane, molecular weight markers, with molecular weight values indicated at the right.

kDa, while preimmune serum from the same animal showed no such reaction. That protein evidently is a binding partner to NDP kinase *in vivo*. Although the protein has not yet been definitively identified, analysis of the peptides after cyanogen bromide cleavage, plus inspection of the T4 genomic sequence, suggest that the protein is a truncated form of the gene 39 product, one of three subunits of T4 topoisomerase (Bernard, 1999). If confirmed by isolation and analysis of that specific protein, now under way, this would indicate a novel dual function for the gene 39 product, as well as a novel mode of expression of the gene.

Escherichia coli NUCLEOSIDE DIPHOSPHATE KINASE: A NONESSENTIAL ENZYME?

Nucleoside diphosphate kinase would appear to be the quintessential housekeeping enzyme, a catalyst that plays an essential but ordinary function in redistributing to other nucleotides the γ-phosphate of ATP, placed there in turn by substrate-level and oxidative phosphorylation. That the enzyme might not play an essential function was implied by findings of Saeki et al. (1974, 1975), who found that *E. coli* cultures grown anaerobically became resistant to the growth-inhibitory effects of desdanine, an antibiotic that acts by specifically inhibiting NDP kinase. These investigators found pyruvate kinase levels to be greatly increased in anerobiosis and reported that pyruvate kinase has a broad specificity for phosphate acceptors, suggesting that phosphoenolpyruvate could transfer its phosphate to all of the common ribo- and deoxyribonucleoside diphosphates. Because our work, even in the 1970s, suggested a special relationship between NDP kinase and the T4 phage DNA replication machinery, we asked whether desdanine could inhibit T4 DNA synthesis, when the host cells were grown and infected anaerobically (Reddy and Mathews, 1978). The answer was "yes;" desdanine, at about 50 μ*M*, was a potent inhibitor of anerobic T4 DNA synthesis, an observation reinforcing our idea that NDP kinase, through its associations with phage proteins, plays an indispensable role in T4 DNA replication.

Accordingly, we were surprised many years later, when M. Inouye told us that he and Q. Lu had engineered a site-specific deletion of the *E. coli ndk* gene. Not only were these cells viable, but they supported T4 phage growth. What prompted them to contact us, however, was their finding that the *ndk* mutant bacteria possessed a mutator phenotype. Could this result from abnormalities of nucleotide metabolism? The answer is, evidently, "yes." In a collaborative study (Lu *et al.*, 1995), we found the *ndk* deletion mutant cells to contain steady-state pools of dCTP up to 23-fold higher than those in the parent and other wild-type strains. The dGTP pool was also expanded, but to a lesser extent—about sevenfold. Later analyses of the ribonucleoside triphosphate pools showed CTP to be elevated some sevenfold and dTTP about twofold above wild-type levels (Bernard, 1999). We confirmed that the *ndk* disruption did not abolish the capacity of the cells to support T4 growth, but rates of phage DNA synthesis were reduced severalfold when compared to the wild-type parental host (Bernard, 1999).

THE MUTATOR PHENOTYPE OF *Escherichia coli ndk* MUTANTS

The pool imbalances observed in *E. coli ndk* mutants are considerably higher than those previously shown to stimulate mutagenesis in cultured mammalian cells and in bacteriophage T4 (reviewed in Kunz *et al.*, 1994). Generally, pool imbalances stimulate mutagenesis by either of two mechanisms: (1) as a result of insertion errors, where, for example, a dNTP present in excess can stimulate formation of non-Watson–Crick base pairs as replication intermediates, or (2) from proofreading effects where, for example, a dNTP in excess can extend from a mismatched 3′ terminal nucleotide before the mismatched nucleotide can be edited out by the polymerase-associated 3′ exonuclease. In our first paper describing the mutator phenotype of *ndk* mutants (Lu *et al.*, 1995), mutations were scored simply in terms of drug resistance, with no efforts to identify sequence changes accompanying mutagenesis. Since that time, R. Schaaper (personal communication) has identified base sequence changes in a number of mutations driven by the pool imbalance in an *ndk* deletion strain and has found that most of the induced mutations are A·T→TA transversions. Conventional pathways leading to mutations of this type are not expected to be affected by excesses of either dCTP or dGTP. Accordingly, analysis of the sequence context within which these mutations arise is important if we are to understand the relationship between abnormal nucleotide metabolism and mutagenesis in these mutant bacteria.

SUBSTITUTE ACTIVITY FOR SYNTHESIS OF NUCLEOSIDE TRIPHOSPHATES IN AN *ndk* MUTANT

If *E. coli* cells contain only one NDP kinase, how do bacteria lacking this enzyme carry out the essential synthesis of nucleoside triphosphates? Lu and Inouye (1996) detected, in their *ndk* targeted deletion strain, a vestigial activity for synthesis of ribo-and deoxyribonucleoside triphosphates. Purification and amino acid sequence analysis of this protein revealed it, unexpectedly, to be adenylate kinase. This enzyme is well known to interconvert adenine nucleotides as follows: 2 ADP ⇋ ATP + AMP. The enzyme also acts upon deoxyadenosine nucleotides; its role in the T4 dNTP synthetase complex is probably to phosphorylate dAMP released from the phage-induced breakdown of the host–cell chromosome, so that the nucleotides can be reused for phage DNA synthesis.

How can this enzyme account for the synthesis of nucleoside triphosphates? A hint came from inspection of the crystal structure of *E. coli* adenylate kinase (Müller and Schulz, 1992). One of the two adenine nucleotide-binding sites on the enzyme was found to have only one base-specific contact with the enzyme, a hydrogen bond to the purine ring involving the backbone carboxyl of Lys200 of the enzyme. This suggested that almost any ribo- or deoxyribonucleoside diphosphate could bind at this site, with ADP bound at the adjacent site, which has far more contacts between the enzyme and the base of the bound nucleotide (Bernard, 1999). By this scheme, the phosphate donor for nucleoside triphosphate synthesis would be ADP and the reaction would proceed in the same direction as that shown above, e.g., ADP + dTDP ⇋ AMP + dTTP. We have obtained evidence for this activity of purified recombinant *E. coli* adenylate kinase, using the spectrophotometric assay for nucleotide kinases, in which the production of ADP is coupled to the activities of pyruvate kinase and lactate dehydrogenase (Bernard, 1999). Because the assay monitors the production of ADP, it must be run in the reverse of the direction shown above (i.e., AMP + dTTP ⇋ ADP + dTDP). When this was done, the hypothesized activity was readily detected (Fig. 3B, closed circles), but the reaction is far slower than the transfer of phosphate from ADP to ADP (Fig. 3A). In contrast, the reaction of ATP with dTMP was undetectable (Fig. 3B, open circles), as expected if the thymidine nucleotide can bind at only one of the two nucleotide-binding sites on adenylate kinase. More recently, we have devised a direct assay

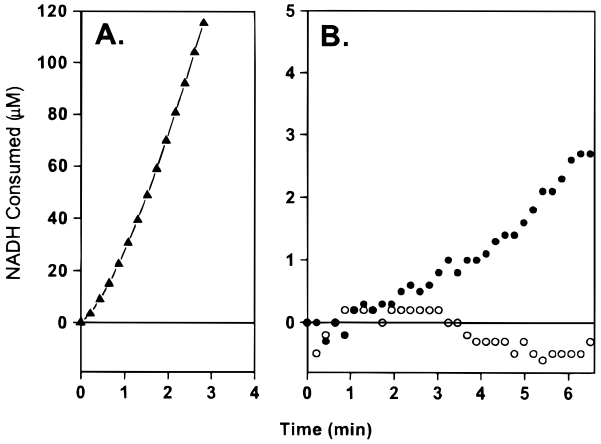

Fig. 3. Activities of recombinant *E. coli* adenylate kinase. Assays monitored ADP production by coupling the reactions to pyruvate kinase and lactate dehydrogenase, both of which were added in excess. Reaction mixtures, incubated at 25°C contained 80 m*M* Tris-HCl, pH 7.4, 80 m*M* KCl, 8 m*M* $MgCl_2$, 1.5 units/ml pyruvate kinase, 5 units/ml lactate dehydrogenase, 2 m*M* phosphoenolpyruvate, 0.2 m*M* NADH, 1 m*M* β-mercaptoethanol, 5 m*M* phosphate donor (ATP or dTTP, as indicated), and 1 m*M* phosphate acceptor (AMP or dTMP, as indicated). The reaction of dTTP and AMP (closed circles, panel B) is the reverse of the proposed phosphotransferase reaction, in which ADP serves as phosphate donor for the conversion of dTDP to dTTP. Closed triangles (panel A) represent the adenylate kinase reaction, ATP + AMP ⇌ 2 ADP). The open circles (panel B) denote a reaction run in the presence of ATP and dTMP; the negligible reaction rate suggests that for the reaction of dTDP and ADP, ADP can transfer its β-phosphate to dTDP, but the converse reaction (phosphorylation of ADP by dTDP) does not occur.

for nucleoside triphosphate synthesis in the "forward" direction, using HPLC to monitor substrate disappearance and product formation. For these experiments it was essential that all nucleotide substrates be chromatographically purified, to remove (for example) ADP as a significant contaminant of commercial ATP preparations. Preliminary experiments, as shown in Fig. 4, indicate that ADP is the phosphate donor for conversion of other nucleoside diphosphates to triphosphates and that ATP is used for this conversion extremely poorly.

Alternative enzymes that phosphorylate (d)NDP's have potential clinical significance. Chain-terminating antiviral drugs such as 3′-azidothymidine (AZT) or dideoxyinosine (ddI) are phosphorylated by cellular enzymes to the triphosphate level before they are incorporated by retroviral reverse transcriptase. Nucleoside diphosphate substrates lacking the 3′-OH are poor substrates for NDP kinase, largely because the 3′-OH group of (d)NDP substrates participates in the reaction

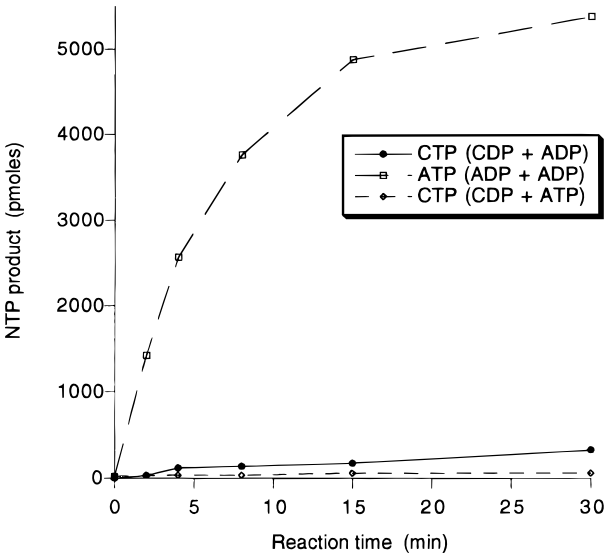

Fig. 4. ADP as the phosphate donor for nucleoside triphosphate synthesis by adenylate kinase. Two nanograms of recombinant *E. coli* adenylate kinase was incubated with 2.0 m*M* CDP and either 0.1 m*M* ADP or 0.1 m*M* ATP in a 200-μl reaction mixture containing 20 m*M* Tris/100 m*M* potassium glutamate, pH 7.4, and 5 m*M* magnesium acetate. Reactions were carried out at 22°C and terminated by adding phosphoric acid to aliquots removed at appropriate time intervals. The aliquots were frozen in liquid N_2 and subsequently analyzed by anion-exchange HPLC. The inset identifies in parentheses the two nucleotide substrates and the dNTP product measured in each reaction. As shown, ADP was a better phosphate donor than ATP. The phosphotransferase activity seen (ADP + CDP ⇌ AMP + CTP) was determined to be 5 μmol/min/mg protein. The competing adenylate kinase reaction (2 ADP ⇌ AMP + ATP) was calaculated to be 350 μmol/min/mg protein. Formation of CTP from CDP plus ATP was undetectable.

mechanism of NDP kinase (Bourdais *et al.*, 1996). Instead of NDP kinase, there must be other alternative kinase(s) or phosphotransferase(s) that activate these important 2′,3′-dideoxy or 3′-azido drugs to the triphosphate level. Based upon results presented here, as well as earlier studies by Lu and Inouye (1996), investigators should consider adenylate kinase as a candidate alternative enzyme catalyzing the final step in metabolic activation of these important nucleoside analogs.

PHENOTYPES OF AN *Escherichia coli* MUTANT CONTAINING A SITE-DIRECTED *Ndk* DELETION

Confirming the observations of Lu and Inouye, we found that *E. coli* QL7623, the strain carrying an *ndk* deletion, supports T4 infection, although phage yields are somewhat reduced relative to wild-type infections (Zhang et al., 1996). It was of particular interest to study the effect of T4 infection on dNTP pools in this strain. On one hand, because T4 encodes almost all of its own enzymes for deoxyribonucleotide and DNA synthesis, we might expect the pool imbalance to disappear. On the other hand, if the nucleotide pool imbalance was found to persist after infection, then using the genetics of T4 could provide a powerful route to understanding the mechanism of mutagenesis induced by dNTP pool bias. As it turned out, the dNTP pools after T4 infection of the *ndk* deletion strain were close to normal and the phage itself did not show increased spontaneous mutagenesis after infection of this strain (Zhang *et al.*, 1996). Among the enzymes encoded by T4 is a powerful nucleotidase which, by cleaving dCTP to dCMP, helps to exclude cytosine from phage DNA and this enzyme efficiently destroys the dCTP pool even when it is expanded by more than twentyfold as a result of the *ndk* deletion.

Because of early reports that pyruvate kinase can replace nucleoside diphosphate kinase in synthesizing dNTPs under anerobic conditions (Saeki *et al.*, 1974), it was of interest to examine T4 reproduction and dNTP pools during anaerobic growth and infection. For these experiments we used a triple mutant developed by Lu and Inouye—an *E. coli* strain, QL1387, defective in *ndk* and the two *pyk* genes (*pykA* and *pykF*), which encode the two isoforms of pyruvate kinase found in *E. coli.* The viability of these bacteria supports the finding that adenylate kinase can substitute for NDP kinase in driving NTP biosynthesis and it calls into question the significance of the pyruvate kinase reaction as a biosynthetic source of nucleoside triphosphates. We found that T4 could grow abundantly on all four strains tested—(1) JC7623, the wild-type parent to the *ndk* mutant; (2) QL7623, the *ndk* deletion-bearing strain; (3) HW1387, the *pykA pykF* double mutant; and (4) QL1387, the *ndk pykA pykF* triple mutant (Zhang *et al.*, 1996). In no case could we detect mutagenesis above background levels in the infecting phage. Moreover, the *ndk* deletion strain, JC7623, was the only one to show a significant mutator effect. Finally, we examined phage growth and mutation frequencies in all four strains during anaerobic growth. In all cases studied—comparing mutation frequencies of T4 with its *E. coli* hosts, comparing the *E. coli* cultures grown aerobically with those grown anaerobically, and comparing the four strains with each other—the dNTP pool imbalance and the mutator phenotype

Table I. Kinetic Properties of the NDP Kinase Activity of *E. coli* Adenylate Kinase[a]

Phosphoryl acceptor	K_m (μM)	V_{max} (μmol/min/mg)	k_{cat}(s^{-1})	Catalytic efficiency k_{cat}/K_m ($M^{-1}\ s^{-1} \times 10^{-3}$)
dADP	920	16.6	6.5	7.11
dCDP	2108	12.6	4.9	2.34
dGDP	497	10.5	4.1	8.28
dTDP	458	9.8	3.9	8.40
ADP	92	834	328	3560

[a] Purified recombinant *E. coli* adenylate kinase was assayed spectrophotometrically, by measuring ADP production via its coupling to the pyruvate kinase and lactate dehydrogenase reactions, with the two coupling enzymes added in excess as purified proteins. Assay reaction mixtures contained 5 m*M* ATP, 1.5 units/ml of pyruvate kinase, 5.0 units/ml of lactate dehydrogenase, 2 m*M* phosphoenolpyruvate, 0.2 m*M* NADH, 80 m*M* KCl, 8 m*M* $MgCl_2$, 1 m*M* β-mercaptoethanol, 80 m*M* potassium phosphate buffer, pH 7.4, and variable concentrations of the dNDP substrate. Reactions were monitored at 340 nm. Because deoxyribonucleoside diphosphates can serve to varying extents as phosphate acceptors in the pyruvate kinase reaction, all data shown here are corrected for control reactions run in the absence of adenylate kinase. For comparison, the bottom row records data for the "natural" phosphoryl acceptor, ADP, reported by Rose *et al.* (1991).

went hand-in-hand. In fact, a strong mutator effect was seen only in the *ndk* deletion strain, QL7623, when grown aerobically. The finding that the mutator phenotype and the dNTP pool abnormalities were jointly alleviated under all conditions analyzed strongly supports the idea that the nucleotide pool imbalance in the *ndk* deletion strain is directly responsible for the elevated mutation rates.

Two surprising results emerged from these experiments (Zhang *et al.*, 1996). First, uninfected *E. coli* cells were found to have great asymmetry in their dNTP pools, with dGTP pools at almost undetectably low levels, only about 2000 molecules per cell. This result was seen in all four *E. coli* strains tested. Even in aerobic bacteria, dGTP is the least abundant of the four dNTPs and the asymmetry becomes far more pronounced, for still unknown reasons, under anaerobiosis. Second, HW 1387, the *pykA pykF* double mutant, was found to be incapable of growth under anaerobic conditions. This, despite the fact that QL1387, the *pykA pykF ndk* triple mutant, grew anaerobically at almost the rate of the $pykA^+$ $pykF^+$ ndk^+ parent. One implication of this latter observation is that the *ndk* gene product exerts an inhibitory effect upon some metabolic function that becomes essential for anaerobic growth in the absence of a functional pyruvate kinase.

BASIS FOR THE NUCLEOTIDE IMBALANCE CAUSED BY A TARGETED *ndk* DELETION

What might be the mechanism by which an *ndk* disruption causes the CTP, dCTP, and dGTP pools to expand, with dCTP accumulating more than 20-fold above wild-type levels? We first considered the possibility that these effects result from substrate specificity of adenylate kinase acting as a nucleoside diphosphate kinase, in particular, the idea that CDP, dCDP, and/or dGDP are favored substrates for conversion to the respective triphosphates. However, when we compared the four common dNDPs for their conversion by adenylate kinase to dNTPs, we found dCDP to be the least efficiently used substrate, with a catalytic efficiency (V_{max}/K_m) at least threefold lower than those for the other three substrates (Table I; Bernard, 1999). Nor does dGDP appear to be a favored substrate.

The accumulation of CTP and dCTP could be explained if the *ndk* mutant has an elevated activity of CTP synthetase, the enzyme that converts UTP to CTP. Accordingly, we assayed crude bacterial extracts for CTP synthetase activity and, as shown in Table II, found that *E. coli* QL7623, the *ndk* deletion strain, shows a specific activity 3.2-fold higher than that of wild-type strains (Bernard, 1999). Although this observation can explain the accumulations of CTP, dCTP,

Table II. CTP Synthetase Activities[a]

E. coli strain	Relevant genotype	CTP synthetase-specific activity (nmol/min/mg protein)
B	Wild-type	1.15
JC7623	Parent of *ndk* strains	1.36
QL7623	*ndk::cm*^{R}	4.35
HW760	Parent of *pyk* strains	0.53
HW1387	*pykA pykF*	0.89
QL1387	*pykA pykF ndk::cm*^{R}	0.61

[a] *Escherichia coli* cultures in SM9 glycerol-casamino acids medium plus 0.5 μg/ml thiamin were grown at 37° C to midlogarithmic phase. After harvesting, cell-free extracts were prepared by sonic oscillation and extracts assayed for CTP synthetase activity by the method of Van Kuilenberg *et al.* (1994). Complete details are reported in Bernard (1999).

and dTTP, the elevated pool size of dGTP is not so easily explained. Further investigation of the factors controlling CTP synthetase activity is under way.

One possibility we are considering is that the NDP kinase protein plays a regulatory role in addition to its catalytic function, possibly as a repressor. As such, the *ndk* deletion would activate CTP synthetase by relieving a partial repression. This model could explain why we found a pyruvate kinase-negative *E. coli* strain to be incapable of anaerobic growth, while the triple mutant, with a *pykA pykF ndk* genotype, is able to grow anaerobically. Perhaps the NDP kinase protein represses a function essential for anaerobic growth in the absence of pyruvate kinase activity. Although speculative, the model can be tested, and such a test is under way.

OTHER INVESTIGATIONS OF BACTERIAL NUCLEOSIDE DIPHOSPHATE KINASES

As shown from the above survey of our research, the complexities of the metabolic roles of *E. coli* NDP kinase rival those revealed by studies on eukaryotic forms of the enzyme, as detailed elsewhere in this issue of the Journal. Additional interactions and metabolic roles involving this enzyme are suggested by investigations in the laboratories of M. Inouye and A. M. Chakrabarty. Like all known NDP kinases, the *E. coli* enzyme functions via a phosphoenzyme intermediate, with His117 being the site of phosphorylation. Working with NDP kinase from *Myxococcus xanthus*, Muñoz-Dorado *et al.* (1993) showed that the enzyme is phosphorylated at two sites—His118 (the equivalent of *E. coli* His117) and a serine residue. Replacement of His118 by glutamine abolished phosphorylation at the serine site, suggesting that NDP kinase contains an intramolecular protein kinase activity. Later work by Lu *et al.* (1996) indicated that *E. coli* NDP kinase also contains a protein kinase activity and that it can function intermolecularly. These investigators found that NDP kinase could phosphorylate the EnvZ and CheA proteins, both of which are protein histidine kinase proteins in two-component regulatory systems. Two-component systems involve first, phosphorylation of a "sensor kinase," which is a protein histidine kinase, and then activation of a physiological response by transfer of the phosphate from the sensor kinase to an aspartate residue in the second protein component, the "response regulator." Of particular interest in the study of Lu *et al.* (1996) was their finding that GTP, which can phosphorylate His117 of *E. coli* NDPK, efficiently labels either EnvZ or CheA *in vitro*, whereas the autophosphorylation of these proteins shows a strict dependence upon ATP. The data suggested a role for NDP kinase in signal transduction, as an upstream element controlling two-component systems.

Working primarily with NDP kinase from *Pseudomonas aeruginosa*, Chakrabarty and his colleagues have generated new insights into regulation of NDP kinase and to some of its intermolecular interactions. Regulation of NDP kinase is somehow related to the development of mucoid colony growth in these bacteria. Two genes, *algR2* and *algH*, that regulate mucoidy, also positively regulate levels of NDP kinase (Shankar *et al.*, 1995a). In an *algR2 algH* double mutant, where NDP kinase levels are low and cells cannot synthesize the alginic acid needed for mucoid growth, Shankar *et al.* detected a second nucleoside triphosphate-synthesizing activity, which they suggested might be pyruvate kinase, by extension of the work of Saeki *et al.* (1974, 1975). Shankar *et al.* also described a specific protein phosphatase in *P. aeruginosa*, an enzyme capable of dephosphorylating, and hence, inactivating, the phosphorylated form of NDP kinase. These workers have also identified two genes, *rnk* and *sspA*, that positively regulate expression of the *E. coli* NDP kinase (Shankar *et al.*, 1995b). The *rnk* gene had not been previously described and its role is unknown, while *sspA* is one of the genes controlling the stringent response to limiting nutrients.

Chakrabarty's laboratory has also reported that *P. aeruginosa* NDP kinase interacts specifically with succinyl-CoA synthetase and with pyruvate kinase under different conditions (Kavanaugh-Black *et al.*, 1994; Sundin *et al.*, 1996). These workers have also reported that the enzyme is subject to proteolytic modification, with a 12-kDa truncated form specifically associated with membranes (Shankar *et al.*, 1996) and which, surprisingly, is active. The truncated form was also reported specifically to bind to Pra, a Ras-like protein that is a G-protein homolog (Chopade *et al.*, 1997). Binding of Pra, either to NDP kinase or to pyruvate kinase, may inhibit its intrinsic GTPase activity. Finally, *Mycobacterium smegmatis* and *Mycobacterium tuberculosis* have been reported to contain membrane-associate G-protein homologs that bind NDP kinase (Shankar *et al.*, 1997a, b). All of these observations suggest complexities of regulation and intracellular metabolic interactions that remain to be elucidated.

Finally, with respect to our own work, we can identify the following objectives of our near-term research to better understand the metabolic roles of *E. coli* NDP kinase: (1) partial or complete reconstitution of the T4 dNTP synthetase complex using NDP kinase, adenylate kinase, and the purified phage proteins known to be present in the complex; (2) further analysis of the role of *E. coli* adenylate kinase as a substitute for NDP kinase, both as a constituent of the multienzyme complex and as a supplier of nucleoside triphosphates; (3) Identification of the biochemical changes in the *ndk* disruption strain that lead to dramatic pool elevations of dCTP and dGTP; (4) identification of mechanisms by which these pool biases stimulate mutagenesis; (5) understanding the mechanism by which an *ndk* deletion mutation relieves the inhibition of anaerobic growth seen in an *E. coli* strain lacking pyruvate kinase.

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