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ORIGINAL PAPER

Molecular interactions involving *Escherichia coli* nucleoside diphosphate kinase

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Abstract Nucleoside diphosphate kinase plays a distinctive metabolic role as the enzyme poised between the last reaction of deoxyribonucleoside triphosphate (dNTP) biosynthesis and the DNA polymerization apparatus. In bacteriophage T4 infection, NDP kinase is one of very few enzymes of host cell origin to participate in either dNTP synthesis or DNA replication. Yet NDP kinase forms specific contacts with phage-coded proteins of dNTP and DNA synthesis. This article summarizes work from our laboratory that identifies and characterizes these interactions. Despite these specific interactions, the enzyme appears to be dispensable, both for T4 replication and for growth of the host, *Escherichia coli*, because site-specific disruption of *ndk*, the structural gene for NDP kinase, does not interfere with growth of the host cell and only partly inhibits phage replication. However, *ndk* disruption unbalances the dNTP pools and stimulates mutagenesis. We discuss our attempts to understand the basis for this enhanced mutagenesis.

Keywords Nucleoside diphosphate kinase · Multienzyme complex · Protein-protein interactions · Nucleotide metabolism · Deoxyribonucleotides · Mutator phenotype · Mutagenesis · dNTP pools

Introduction

Our laboratory's interest in nucleoside diphosphate (NDP) kinase arose through our investigations of relationships between DNA replication in T4 phage-infected *Escherichia coli* and synthesis of the deoxyribonucleotide precursors. The rate of DNA accumulation increases up to tenfold after T4 infection, creating a corresponding demand for dNTPs. This demand is met largely through the action of phage-coded enzymes, which catalyze nearly all the reactions leading from ribonucleoside diphosphates to dNTPs, including enzymes responsible for the replacement of cytosine in phage DNA by 5-hydroxymethylcytosine (Mathews & Allen, 1983). An exception is NDP kinase; Bello and Bessman (1963) showed that the activity of this enzyme, as measured in extracts, did not change after infection, indicating that T4 uses a host-cell enzyme to catalyze a reaction crucial to DNA replication.

The rate of DNA chain growth, either in infected or uninfected *E. coli*, is fast—about 800 nucleotides per second at 37°C. To explain how T4 DNA polymerase remains saturated with dNTPs in the face of this rapid turnover, we proposed (Reddy et al., 1977) that the enzymes of dNTP synthesis form a multienzyme complex that is juxtaposed with the replisome and facilitates the synthesis of precursors and their flow into DNA. Fractionation of T4-infected cell extracts, either by gradient centrifugation or gel filtration, revealed that the phage enzymes of dNTP synthesis formed a large aggregate, which we called the "T4 dNTP synthetase complex" (Reddy et al., 1977). Associated with this complex was host-cell NDP kinase activity. Not only was this enzyme physically associated with phage-coded enzymes, it was kinetically linked as well, as shown by analysis of multistep reaction pathways *in vitro*, such as dUMP→dTMP→dTDP→dTTP. Kinetic coupling was abolished if one of the other enzymes in the complex was defective, suggesting that physical integrity of the entire complex is required for substrate channeling to occur (Reddy & Mathews, 1978; Thylén & Mathews, 1989).

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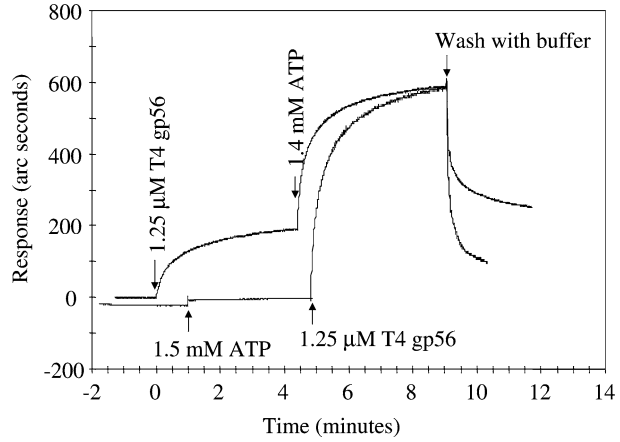

Fig. 1 Enhancement by ATP of the association of T4 dCTPase-dUTPase (gp56, gene 56 product) with immobilized *E. coli* NDP kinase. Approximately 12.6 ng of NDP kinase was immobilized on an IAsys carboxylate cuvette (Affinity Sensors, Inc.) as described in Kim et al. (2005a). Baseline was reached with KGMT (0.15 potassium glutamate, 4 mM magnesium acetate, and 20 mM Tris-HCl, pH 7.4). As shown on the figure, either gp56 was added first, followed by ATP, or else ATP was added first, followed by gp56

Materials and methods

Purified *E. coli* NDP kinase and T4 phage-coded dCTPase/dUTPase were isolated as recombinant proteins, as described in Shen et al. (2004). Experiments on protein-protein interactions were carried out in an IAsys optical biosensor (Affinity Systems) as decribed in Kim et al. (2005a,b). Further details are given in the legend to Fig. 1.

Sedimentation equilibrium measurements were carried out in a Beckman Optima XL-A analytical ultracentrifuge. Buffer densities and viscosity corrections were made according to Laue et al. (1992). Partial specific volume, estimated from the protein amino acid sequence (Perkins, 1986), was 0.733 cm^3/g. Buffer was KGMT (see Fig. 1). Experiments were conducted at 20°C according to Ausio et al. (1992). Samples of 120 μl each at the protein concentrations indicated in Results were sedimented to equilibrium at rotor speeds from 15,000 rpm to 24,000 rpm. Scans used absorbance optics at wavelengths between 230 and 290 nm, chosen so that no points had absorbance exceeding 1. Radial step size was 0.001 cm, and each *c vs. r* data point was the average of 15 independent measurements. Equilibrium data spanning the concentration range were examined by global fitting using UltraScan software (Demeler, 2005). Equilibrium data were fit to multiple models by global fitting, and the best model was chosen based on best statistics and on visual inspection of the residual run patterns.

Deoxyribonucleotide pools and mutation frequencies were measured as described in previous papers from this laboratory (Zhang et al., 1996; Wheeler et al., 2005).

Results and discussion

Protein-protein associations involving NDP kinase

By catalyzing the last reaction before precursors are incorporated into DNA, NDP kinase is poised between small-molecule and macromolecular metabolism. Association of this host-cell enzyme with enzymes encoded by a virus that will destroy the cell was sufficiently unexpected that we have explored the specific protein-protein interactions involved, by several approaches, beginning with protein affinity chromatography (Wheeler et al., 1996). Purified *E. coli* NDP kinase, immobilized on a chromatographic column, was found to retain several phage proteins involved both in dNTP synthesis and in DNA replication and repair. Retention was specific, in that immobilized control proteins showed no such effects. These findings supported the idea that the dNTP synthetase complex is linked to the replication apparatus. However, the approach used could not readily distinguish between direct protein-protein interactions and indirect linkages, involving shared affinity for a single protein. Therefore, we have turned to other approaches, including optical biosensor analysis and other techniques, as shown in Table 1, which lists a remarkable number of interactions involving T4 proteins and NDP kinase. Quantitative biosensor studies (Shen et al., 2004; Kim et al., 2005a; Shen, 2006) show dissociation constants for these interactions to be in the low micromolar range. Because estimated intracellular concentrations of the proteins involved fall within this range, it is likely that the direct interactions shown *in vitro* exist *in vivo* as well.

Effects of nucleotides on protein interactions

Several of the protein-protein interactions we have discovered are strengthened by nucleotides. Table 1 lists five T4 proteins whose affinity for NDP kinase is significantly strengthened by 1 to 1.5 mM ATP, and Fig. 1 shows data illustrating the effect of ATP on one of these interactions. There is some specificity to the effects we have observed; for example, 1 mM dADP strengthens the association between NDP kinase and T4 dihydrofolate reductase, while ATP has no evident effect upon this interaction (Kim et al., 2005a). These findings raised the possibility that intracellular nucleotides help to stabilize the dNTP synthetase complex *in vivo*, or to regulate its stability and hence, its ability to channel precursors to DNA. However, preliminary experiments involving sedimentation analysis of enzyme activities have not yet yielded evidence supporting this hypothesis.

We wondered, however, whether nucleotides might be exerting their effects through influence upon the quaternary structure of NDP kinase. The *E. coli* enzyme, like other microbial NDP kinases, is a homotetramer with a

Table 1 Interactions involving *E. coli* NDP kinase and T4-encoded proteins

Interacting phage protein	ATP stimulation	Interaction shown by	Reference
DNA polymerase		NDE	Bernard et al., 2000
Aerobic ribonucleotide reductase	Yes	NDE, FLU, BIO, GST, IP	Bernard et al., 2000; Shen et al., 2004
Anaerobic ribonucleotide reductase	Yes	BIO, FLU	Shen et al., 2004; Shen, 2006
Thymidylate synthase	No	FLU, BIO	Shen et al., 2004
Dihydrofolate reductase	No	GST	Kim et al., 2005a
dCTPase-dUTPase	Yes	BIO, FLU	Shen et al., 2004
dCMP deaminase	Yes	BIO	Shen, 2006
dCMP hydroxymethylase		GST, IP	Shen et al., 2004
Single-strand DNA-binding protein (gp32)	Yes	BIO, GST	Shen et al., 2004; Kim et al., 2005b

Note. Techniques used to demonstrate interactions include native gel electrophoresis (NDE), optical biosensor analysis (BIO), glutathione-S-transferase pulldown (GST), immunoprecipitation (IP), and fluorescence spectroscopy (FLU). Two interactions have been found not to be stimulated by ATP.

subunit molecular mass of 15,332 Da, while the eukaryotic enzymes, with considerable sequence conservation, form homohexamers. However, subunit association among the hexameric NDP kinases is stronger than in the *E. coli* enzyme (Lascu et al., 2000; Janin et al., 2000). In studies on the *Dictyostelium discoideum* enzyme, Mesnildrey et al. (1998) reported that a mutant form of this enzyme is a homodimer in the absence of ATP, but that 0.5 mM ATP drives formation of the familiar homohexamer. We have used two approaches to determine whether ATP similarly promotes oligomerization of the *E. coli* enzyme. First, we used the IAsys optical biosensor to follow the association of NDP kinase in solution with immobilized NDP kinase. These experiments revealed that 1 mM ATP causes an increase of about tenfold in the affinity of immobilized NDP kinase for the same enzyme in solution (Shen, 2006).

A quantitative analysis was carried out by sedimentation equilibrium measurements. When 25 μM NDP kinase was centrifuged to equilibrium at 16,500 rpm in the absence of added nucleotides, the profile showed an average molecular mass of 52,000 Da, suggesting a mixture of dimers (30,664 Da) and tetramers (61,328 Da). When the run was carried out in the presence of 1 mM ATP or 1 mM ADP, the evident molecular weights were 62,800 and 62,300, respectively, suggesting that in the presence of nucleotide the enzyme was entirely in the tetrameric state.

To determine the dependence of protein oligomerization upon protein concentration, we carried out sedimentation equilibrium measurements at three different concentrations—0.8 μM, 4.65 μM, and 32.6 μM, based upon the tetramer—and at three different rotor speeds. The data were analyzed with UltraScan software (Demeler, 2005) to determine the percent of total protein in dimeric and tetrameric forms at each concentration and rotor speed (Fig. 2). This figure reveals the protein to be half dimer and half tetramer at 0.8 μM, which therefore represents the dissociation constant for tetramerization. As noted above, either ADP or ATP at 1 mM—within the physiological concentration range—drives the enzyme entirely into the tetrameric state. From the specific activity of NDP kinase in a crude *E. coli* extract and that of the highly purified enzyme (0.4 and 2400 μmol/min/mg protein, respectively, Ray, 1992), we can estimate that an *E. coli* cell contains 800 molecules of the enzyme. From the volume of an *E. coli* cell (Mathews, 1972), we can estimate the intracellular enzyme concentration at 1.5–2 μM. Thus, it is likely that the nucleotide concentration within the cell is a determinant of the enzyme's quaternary structure *in vivo*. Whether this parameter in turn regulates the intracellular interactions of the enzyme with other proteins remains to be seen.

The mutator phenotype of *ndk* mutants

In view of its central role in metabolism, both of deoxyribonucleotides and ribonucleotides, it was surprising to learn that NDP kinase is evidently dispensable for growth under laboratory conditions, as shown by the fact that the *ndk* gene

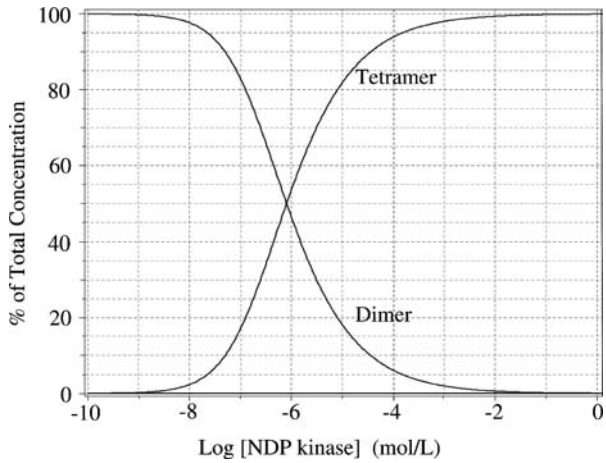

Fig. 2 NDP kinase quaternary structure as a function of protein concentration. Details are presented in materials and methods

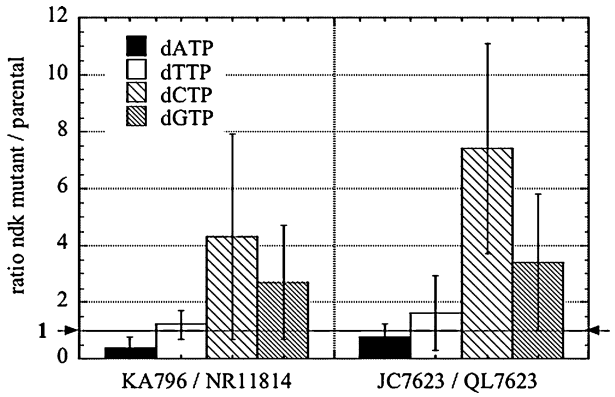

Fig. 3 Effect of *ndk* mutations on dNTP pools in two different strain backgrounds. The ordinate shows the ratio of the pool size in the mutant cell to that in the wild-type parent. Pools were measured in extracts of logarithmically growing cells (Zhang et al., 1996)

could undergo site-specific disruption without loss of viability (Lu et al., 1995). Subsequently Lu and Inouye (1996) found that adenylate kinase has the ability to convert nucleoside diphosphates to the respective triphosphates, and that this enzyme can complement the missing *ndk* gene function.

Of interest to us was the mutator phenotype of an *ndk* mutant (Lu et al., 1995). We found that dNTP pools were grossly abnormal in *E. coli* QL7623, the *ndk* disruptant isolated by Lu et al. dCTP pools were elevated up to twentyfold, and CTP and dGTP pools expanded as well. (Zhang et al., 1996; Bernard et al., 2000). We have tested several hypotheses to explain how the substitution of adenylate kinase activity for NDP kinase could lead to these pool imbalances, but so far none of our models have been supported.

Also unexpected were the results of preliminary genetic analysis of *E. coli* QL7623 (R. Schaaper, personal communication). The simplest explanation for the mutator phenotype would be enhanced substitutions, such as AT→GC, transitions caused by insertion errors resulting from excess dGTP or dCTP. However, Schaaper found AT→TA transversions to predominate, a mutational event not readily explicable strictly from dCTP and dGTP accumulation, unless the accumulations led to inhibition of proofreading past certain AT base pairs. Part of the mystery evaporated when Miller et al. (2002) found that AT→GC substitution rates were enhanced when the analysis was conducted in a mismatch repair-defective background.

The original disruption of the *ndk* gene (Lu et al., 1995) was carried out in a rather complex genetic background, which might have affected nucleotide pools independently of the *ndk* mutation. For his continuing genetic analysis Schaaper, working in collaboration with our laboratory, moved the disrupted *ndk* gene into a simpler background. We have compared the effects of the *ndk* mutation upon dNTP pools in both backgrounds, as shown in Fig. 3. In both strains we see the dCTP and dGTP accumulations that we noted earlier, although our more recent assays show these accumulations to be less extreme than what we originally reported. Of interest is the dATP pool drop caused by the *ndk* mutation in the simpler background (KA796/NR11814. This could help to explain insertion errors that would result in AT→TA transversions.

In explaining the mutator phenotype of *ndk* mutations it is essential to establish whether enhanced mutagenesis is, in fact, caused by dNTP pool imbalances. Because of the complex metabolic functions of NDP kinase, discussed elsewhere in this volume, one could imagine that the NDK protein interacts with other proteins involved in maintaining genomic stability, and it is loss of these interactions that stimulates mutagenesis. We tested this idea by expressing a structurally intact, but catalytically inactive form of NDP kinase in an *ndk* mutant strain and testing the effect of this expression upon dNTP pools and mutagenesis. As shown in Table 2, this strain behaves identically to the untransformed *ndk* mutant, establishing that the loss of NDK catalytic function is responsible both for the pool imbalance and the mutator phenotype. Note that overexpression of wild-type *ndk* in a mutant background gives results similar to those seen with the wild-type parent, suggesting that NDP kinase activity in a wild strain is not limiting for the maintenance of dNTP pools.

Table 2 Effects of active and inactive NDP kinase proteins upon dNTP pools and mutagenesis

	NDP kinase activity	dNTP pool, pmol/10^8 cells				Mutants per 10^8 cells	
E. coli strain	μmol/min/mg protein	dCTP	dATP	dTTP	dGTP	Rif^R	Nal^R
KA796 (wt)	1.1	39	7.2	9.4	3.5	0.3	<0.1
NR11814 (*ndk*)	<0.1	105	1.4	16.1	5.0	17.9	112.6
NR11814/pKT	8.9	12	6.7	13.3	5.3	0.2	1.0
NR11814/ipKT	0.35	152	1.8	19.0	6.0	23.0	101.7

Note. E. coli KA796 is the wild-type parent to NR11814, which contains a disrupted *ndk* gene. pKT is a plasmid expressing wild-type NDP kinase, and ipKT expresses a catalytically inactive but structurally intact (H117G) mutant form of the enzyme (Shen et al., 2004). Both rifampicin-resistant and nalidixic acid-resistant mutants were quantitated.

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