Adenylate kinase, which catalyzes the reversible ATP-dependent phosphorylation of AMP to ADP and dAMP to dADP, can also catalyze the conversion of nucleoside diphosphates to the corresponding triphosphates. Lu and Inouye (Lu, Q., and Inouye, M. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 5720–5725) showed that an *Escherichia coli* ndk mutant, lacking nucleoside diphosphate kinase, can use adenylate kinase as an alternative source of nucleoside triphosphates. Bacteriophage T4 can reproduce in an *Escherichia coli ndk* mutant, implying that adenylate kinase can meet a demand for deoxyribonucleoside triphosphates that increases by up to 10-fold as a result of T4 infection. In terms of kinetic linkage and specific protein-protein associations, NDP kinase is an integral component of T4 dNTP synthetase, a multienzyme complex containing phage-coded enzymes, which facilitates the synthesis of dNTPs and their flow into DNA. Here we asked whether, by similar criteria, adenylate kinase of the host cell is also a specific component of the complex. Experiments involving protein affinity chromatography, immunoprecipitation, optical biosensor measurements, and glutathione S-transferase pulldowns demonstrated direct interactions between adenylate kinase and several phage-coded enzymes, as well as *E. coli* nucleoside diphosphate kinase. These results identify adenylate kinase as a specific component of the complex. The rate of DNA synthesis after infection of an *ndk* mutant was found to be about 40% of the rate seen in wild-type infection, implying that complementation of the missing NDP kinase function by adenylate kinase is fairly efficient, but that adenylate kinase becomes rate-limiting for DNA synthesis when it is the sole source of dNTPs.

Adenylate kinase catalyzes the reversible ATP-dependent phosphorylation of AMP to ADP. The reaction is involved in the *de novo* biosynthesis of adenine nucleotides, and it is also thought to participate in adjusting adenine nucleotide levels to meet the energy demands of a cell (1). In addition, there is evidence that the enzyme in *Escherichia coli* participates in phospholipid biosynthesis, although the specific involvement has not been defined (2). Temperature-sensitive mutations in *adk*, the structural gene for adenylate kinase, cause defective phospholipid synthesis when bacteria are grown at a nonpermissive temperature (3).

A novel capability for *E. coli* adenylate kinase was described when Lu and Inouye (4) found that the wild-type *adk* gene could complement a site-specific disruption of *ndk*, the structural gene for nucleoside diphosphate kinase. Lu and Inouye (5) showed that adenylate kinase could catalyze the conversion of nucleoside diphosphates to triphosphates. Experiments in our laboratory (5) showed that the phosphate donor for at least some of these reactions was ADP. So, instead of catalyzing the well known reaction, 2ADP ⇌ AMP + ATP, the enzyme was evidently substituting a different nucleoside diphosphate for one of the two ADPs: (d)NDP + ADP ⇌ AMP + (d)NTP.

Both NDP¹ kinase and adenylate kinase were shown, some years ago, to participate in the synthesis of dNTPs after T4 infection of *E. coli* (6). Most reactions in T4 dNTP and DNA synthesis are catalyzed by phage-coded enzymes, consistent with the large increase in DNA accumulation rate per cell that occurs as a result of infection. An important exception is NDP kinase, an active enzyme of low specificity, which was found capable of phosphorylating the phage-modified nucleotide 5-hydroxymethyl-dCDP (7). There is no phage-specific form of NDP kinase, and the bacterial enzyme carries out synthesis of all four dNTPs from the respective deoxyribonucleoside diphosphates. At the monophosphate level, however, a phage-coded enzyme is required, because no *E. coli* enzyme can phosphorylate 5-hydroxymethyl-dCMP to the diphosphate. This reaction is carried out by an unusual dNMP kinase, the product of gene 1, which also acts upon dGMP and dTMP (7, 8). This trifunctional enzyme, however, does not act upon dAMP, and it was assumed that the conversion of dAMP to dADP after infection is catalyzed by adenylate kinase; dAMP, like the other dNMPs, is derived from the breakdown of *E. coli* DNA, a process catalyzed by phage-coded nucleases. This is a quantitatively minor pathway, however, because nucleotides derived from host-cell DNA degradation represent only about 10% of the total nucleotides used for phage DNA synthesis (9).

The enzymes of dNTP synthesis in T4 infection interact to form a multienzyme complex, which we call the T4 dNTP synthetase complex (10), and which facilitates the flow of metabolites *en route* to dNTPs, and their subsequent flow into DNA. *E. coli* NDP kinase interacts directly or indirectly with several T4 enzymes of dNTP synthetase and DNA replication, and we consider it an integral component of the dNTP synthetase complex (11). In an early study (12) our laboratory found about 5% of the total adenylate kinase activity in the cell to cosediment with enzymes of the dNTP synthetase complex; NDP kinase behaved similarly. However, we did not inquire at

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¹ The abbreviations used are: NDP, nucleoside diphosphate; IPTG, isopropyl-β-D-thiogalactoside; DTT, dithiothreitol; ADK, adenylate kinase; BSA, bovine serum albumin; GST, glutathione S-transferase; dNMP, dNDP, and dNTP, deoxyribonucleoside mono-, di-, and triphosphate, respectively; AMP-PNP, 5′-adenylyl-β,γ-imidodiphosphate.
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the time whether the association of adenylate kinase with the T4 dNTP synthetase complex was functional, i.e. whether adenylate kinase is a specific component of the complex. This paper deals primarily with that issue.

EXPERIMENTAL PROCEDURES

Plasmaids, Phage, and Bacterial Strains—Bacteriophage T4D, a wild-type strain, has been maintained in this laboratory for many years. E. coli strains used included KA786 (ara, thi, proA, lac, ndk wild-type) and PB11814 (KA786Δndk::kan), an ndk null mutant; both of these strains were obtained from Dr. Roel M. Schaaper, NIEHS National Institutes of Health. E. coli CV2 (fhuA22, rpsA8, adk2::Tn5, ompF627(T.o), relA1, glpR2(gpI), glpD3, pit-10, spoT1, rnb2::B, mcrB1, creC510), used because of its ts mutation in the adk gene (13), was obtained from the E. coli Genetic Stock Center, Yale University. E. coli Origami(DE3) was purchased from Novagen (Madison, WI).

E. coli strains overexpressing T4 enzymes as recombinant proteins were as follows. E. coli HB101/pBK5, expressing gene 1 (dNMP kinase), was obtained from Dr. Maurice J. Bessman, Johns Hopkins University (14). E. coli RR1/pSP104, expressing gene fd (dihydrofolate reductase), and E. coli BL21(DE3)/pET7T2, expressing dCMP hydroxymethylase, were developed in this laboratory (15, 16). Plasmid pmdAB, expressing a crude extract of the reductase, was a gift from Dr. G. Greenberg, University of Michigan (17), and used to transform E. coli Origami(DE3). E. coli MB901, expressing T4 thymidylate synthase, was obtained from Dr. Marlene Belfort, New York State Department of Health (18), and E. coli BL21(DE3)/pET3c-CD5, expressing T4 deoxyadenosine kinase, was obtained from Dr. Frank Maley, also of the New York State Department of Health (19).

Cloning and Expression of Adenylate Kinase—The adk gene, encoding E. coli adenylate kinase, was amplified from E. coli B genomic DNA by PCR using primers containing NdeI and BamHI restriction sites.

Following ligation of the NdeI-BamHI fragment into the corresponding site of pET-9a (Novagen), the pET9a-adk construct was transformed into competent DH5α cells. After PCR analysis of the transformants, the plasmid was then sequenced and subsequently transformed into BL21(DE3)pLysS cells. For expression, 1-liter cultures of cells were grown in LB medium supplemented with 25 μg/ml kanamycin or 50 μg/ml chloramphenicol at 37 °C. When the cell density reached an optical density of 0.6 to 0.8, the entire run was 5.0 ml/min. The major peak was concentrated in a Centriprep-10 (Millipore) at 4 °C and further purified on a Superdex 200 column (GE HealthCare). For some experiments the adk gene was cloned in pGEX and expressed in E. coli Origami(DE3), was induced with 0.5 mM IPTG (1 mM IPTG for GST-γ-glutamylcysteine adduct), and XhoI, the fragment was gel purified and ligated into plasmid using T4 ligase.

Following centrifugation (4 °C, 20,000 × g for 30 min), the supernatant was transferred to another tube to which streptomycin sulfate was slowly added to a final concentration of 1%. After induction, bacteria were centrifuged and resuspended in 10 volumes of buffer containing 10 mM Na2HPO4, 1.8 mM KH2PO4 (pH 7.4), 2.7 mM KCl, 140 mM NaCl, 1.8 mM phenylmethylsulfonyl fluoride, and 5 mM DTT, plus 0.1 volume of egg white lysozyme, 10 mg/ml in 10 mM Tris-HCl, pH 8.0. The resuspended cells were incubated on ice for 30 min, then brieﬂy sonicated. DNase I was added (10 μg/ml), and incubation on ice was continued for 30 min more. Lysates were cleared by centrifugation at 4 °C at 15,000 rpm for 30 min, then ﬁltered through a 0.45-μm syringe ﬁlter. The supernatant was applied to a His-select_HC nickel affi-gel column (Sigma). After washing off unbound proteins with buffer A, bound proteins were eluted with buffer A plus 250 mM imidazole.

Glutathione S-Transferase Pulldown Assays—GST fusion proteins were prepared, and GST pulldown experiments carried out, as described in previous publications from this laboratory (11, 20). Fusion proteins made for this study included gpl (deoxyribonucleoside monophosphate kinase), gpg2 (dCMP hydroxymethylase),(gpp(6 dihydrofolate reductase), and E. coli nucleoside diphosphate kinase. Each fusion protein gene, cloned in pGEX and expressed in E. coli Origami(DE3), was induced with 0.5 mM IPTG (1 mM IPTG for GST-γ-glutamylcysteine adduct), and XhoI, the fragment was gel purified and ligated into plasmid using T4 ligase.

Fluoride was mixed with TiterMax gold adjuvant (Sigma) in a 1:1 ratio, and the solution was injected subcutaneously into two New Zealand White rabbits from which preimmune sera had been collected before injection. After 1 month, the rabbits were boosted intramuscularly with ~250 μg of His-tagged ADK mixed 1:1 with TiterMax gold adjuvant. Subsequent bleeding of the antisera has allowed us to purify antibodies in their quality was tested by immunoblotting for endogenous ADK in a bacterial extract, and purified His-ADK was separated by 12% SDS-PAGE. To purify E. coli ADK antibodies, 3 mg of purified His-ADK was immobilized on Affi-Gel 10 (Bio-Rad) as described previously (21, 22). Antibodies in the antiserum were precipitated by adding ammonium sulfate to 40% saturation, and the precipitate was dissolved in 10 mM Na2HPO4, 1.8 mM KH2PO4 (pH 7.4) with 10% glycerol and 0.5% Nonidet P-40. Each mixture was treated with Protein A-Sepharose beads, and the beads were washed and centrifuged as described (20). Each pellet was placed in a boiling water bath for 5 min, then subjected to one-dimensional gel electrophoresis. Approximately 500 μg of purified enzyme, 1 mg/ml, was mixed with TiterMax gold adjuvant (Sigma) in a 1:1 ratio, and the solution was injected subcutaneously into two New Zealand White rabbits from which preimmune sera had been collected before injection. After 1 month, the rabbits were boosted intramuscularly with ~250 μg of His-tagged ADK mixed 1:1 with TiterMax gold adjuvant. Subsequent bleeding of the antisera has allowed us to purify antibodies in their quality was tested by immunoblotting for endogenous ADK in a bacterial extract, and purified His-ADK was separated by 12% SDS-PAGE.

Antisera and Coimmunoprecipitation Experiments—Purity of the histidine-tagged adenylate kinase was assayed by SDS-polyacrylamide gel electrophoresis. Approximately 500 μg of purified enzyme, 1 mg/ml, was mixed with TiterMax gold adjuvant (Sigma) in a 1:1 ratio, and the solution was injected subcutaneously into two New Zealand White rabbits from which preimmune sera had been collected before injection. After 1 month, the rabbits were boosted intramuscularly with ~250 μg of His-tagged ADK mixed 1:1 with TiterMax gold adjuvant. Subsequent bleeding of the antisera has allowed us to purify antibodies in their quality was tested by immunoblotting for endogenous ADK in a bacterial extract, and purified His-ADK was separated by 12% SDS-PAGE.

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**Adenylate Kinase in the dNTP Synthetase Complex**

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### RESULTS

**Effect of ndk Mutation on Rate of T4 DNA Synthesis—**Although adenylate kinase complements an ndk mutation for growth of *E. coli* (4) and T4 can productively infect an ndk mutant host strain (26), the quantitative effect of an ndk mutation on T4 reproduction has not been reported. Therefore, we determined rates of radiolabeled thymidine incorporation into DNA after T4 infection at 30 °C of an isogenic pair of hosts, the latter infection, to 36 for the mutant. Thus, complementation of the ndk function was not complete. Similar findings have been made in our laboratory in infection of a different pair of host strains (27).

Although complementation by adk was incomplete, its apparent efficiency was surprising. Lu and Inouye (4) reported the NDP kinase specific activity of adenylate kinase to be only 1–5% that of NDP kinase itself, depending upon the specific substrates used. This observation, to which we return under “Discussion,” raises the question whether adenylate kinase is specifically associated with the dNTP synthetase complex.

**Affinity Chromatography with Adenylate Kinase as the Ligand—**Each of the known enzymes in the dNTP synthetase complex, when immobilized on Affi-Gel™, has been shown to retain a specific ensemble of 6–12 early T4 proteins of dNTP synthesis and DNA metabolism (21, 22). If adenylate kinase is a specific component of the complex, we expect it to behave similarly. Accordingly, an extract of T4 proteins, labeled with [35S]methionine from 3 to 8 min after infection, was applied to an ADK column, and the moderately strongly bound proteins (retained at 0.2 M NaCl, eluted at 0.6 M NaCl) were displayed by two-dimensional gel electrophoresis and autoradiography. Results of this experiment are shown in Fig. 2, panel A. Panel B shows a control experiment carried out with immobilized bovine serum albumin. Proteins retained by the ADK column, but not by BSA, are assumed to be specifically associated, either directly or indirectly, with adenylate kinase. Prominent among these were gpfrd (dihydrofolate reductase), gp42 (dCMP hydroxymethylase), and several proteins of DNA replication and recombination, gp32 (single-strand DNA-binding protein), gpuv (recombination and recombination-dependent DNA initiation), and gpw (DNA 3'-phosphatase, 5'-kinase). An earlier analysis, using immobilized His-tagged ADK, showed the same proteins bound (data not shown), indicating that with this protein at least, the histidine tag does not interfere with protein-protein associations.

**Coinmunoprecipitation—**Associations involving adenylate kinase were also investigated by an immunoprecipitation experiment. Antiserum was generated to purified His-tagged adenylate kinase. Fig. 3, panel A, shows that this antiserum strongly and specifically recognizes adenylate kinase in an extract of total T4 proteins. Purified antibodies from both ADK antiserum and preimmune serum were used to precipitate proteins from a [35S]methionine-labeled extract of T4-infected bacteria. Two-dimensional gel electrophoresis and autoradiography (Fig. 3, panel B) showed several phage proteins precipitated by anti-ADK, but not by preimmune serum, the most prominent of which we identified as dihydrofolate reductase. Smaller amounts of proteins identified as thymidine kinase, gpw (T4 RecA counterpart), and gp60 (topoisomerase subunit) were also seen. An immunoblotting experiment (bottom of panel B) showed that the ADK antibodies specifically precipitated nucleoside diphosphate kinase from this extract, suggesting that the two bacterial enzymes, NDP kinase and adenylate kinase, interact with each other.

**Direct Protein-Protein Interactions, as Shown by GST Pull-down—**Because the analyses involving affinity chromatography and immunoprecipitation cannot distinguish between direct protein-protein interactions and indirect associations mediated by a common ligand, it was of interest to determine whether *E. coli* adenylate kinase can interact directly with T4 enzymes in the dNTP synthetase complex. GST pulldown experiments can generate this kind of information provided one analyzes either purified proteins or extracts when only limited numbers of phage proteins are present. The latter
condition is readily met by using extracts of *E. coli* in which only one T4 enzyme is expressed, namely, as a recombinant protein. Deoxyribonucleoside monophosphates are converted in T4 infection to diphosphates primarily through the action of gp1 (dNMP kinase). Hence, this enzyme acts sequentially with nucleoside diphosphate kinase and/or the NDP kinase activity of adenylate kinase. If the dNTP synthetase complex involves interactions between sequentially acting enzymes, then we expect gp1 to interact with NDP kinase, and possibly also with adenylate kinase. Accordingly, we prepared a fusion protein linking GST to T4 gp1 and tested the ability of this GST-gp1 fusion to pull down either ADK or NDP kinase. In the experiment of Fig. 4, panel A, the GST-gp1 fusion protein was mixed with an extract of *E. coli* carrying plasmid pnrdAB, which expresses both subunits of T4 aerobic ribonucleotide reductase. Analysis of the pulldown products by immunoblotting showed that both adenylate kinase and NDP kinase were pulled down by GST-gp1. So also was the small subunit of ribonucleotide reductase (gp nrdB), but not the large subunit (gp nrdA). This experiment seems to imply that T4 dNMP kinase interacts directly with ribonucleotide reductase small subunit (R2 pro-
tein), adenylate kinase, and NDP kinase. However, because *E. coli* NDP kinase is known to interact with T4 ribonucleotide reductase (11), we had to consider the possibility that the NDP kinase was linked directly to ribonucleotide reductase in the GST pulldown product and, hence, did not interact directly with dNMP kinase. Therefore, we carried out an experiment using an *E. coli* extract containing no T4 proteins. As shown in Fig. 4, panel B, adenylate kinase was pulled down by GST-gp1 in this extract, but NDP kinase was not. This result indicates, perhaps surprisingly, that dNMP kinase (gp1), one of the enzymes that synthesize deoxyribonucleoside diphosphates, does not interact specifically with NDP kinase, the major enzyme that converts those diphosphates to dNTPs. However, aerobic ribonucleotide reductase, the other major source of deoxyribonucleoside diphosphates, is linked to NDP kinase (11), which in turn is linked to adenylate kinase. Moreover, because dNMP kinase is also linked to both ribonucleotide reductase (Fig. 4, panel A) and adenylate kinase, it is possible that the shared affinity of the dNMP kinase and *E. coli* NDP kinase for ribonucleotide reductase and adenylate kinase brings these two sequentially acting enzymes, dNMP kinase and NDP kinase, close enough for facile transfer of dNDPs between these two enzymes, even if they are not directly linked. Whether or not these interactions do bring dNMP kinase and NDP kinase into functional juxtaposition, we believe the two enzymes to be closely aligned within infected cells, because we have found the two enzymes to be kinetically linked in assays of multistep reaction pathways in *vitro* (12, 25).

Note from Fig. 4 that adenylate kinase is linked directly to dNMP kinase. This can be taken as evidence that ADK plays a significant role in accepting dNDPs from dNMP kinase (gp1) and converting them to dNTPs. Alternatively, this may involve joint positioning of two enzymes that play parallel roles in phosphorylating deoxyribonucleoside monophosphates to diphosphates. Because gp1 acts upon three dNMPs, but not on dAMP, the action of ADK in converting dAMP to dADP is comparable, and the association of ADK with gp1 may allow for coordination among the four dNMP phosphorylation activities.

Fig. 5 describes GST pulldown experiments with purified proteins. In the experiment of panel A, purified adenylate kinase in varying amounts was mixed with purified GST fusion protein. This experiment confirms the direct interaction of adenylate kinase with dNMP kinase (gp1) and NDP kinase, and it also presents evidence for direct interactions of adenylate kinase with gp42 (dCMP hydroxymethylase) and gp42 (dihydrofolate reductase). The pulldown experiment with GST-gp42 shows ADK to have been pulled down in a negative control, in which no ADK was added to the incubation mixture. This may be because of a high affinity between dihydrofolate reductase and adenylate kinase, such that during purification of GST-gp42 from an *E. coli* extract some endogenous ADK is carried along. The same may be true for dCMP hydroxymethylase.

Fig. 5, panel B, shows a comparable experiment in which purified NDP kinase in varying amounts was mixed with GST fusion proteins. At the highest NDP kinase concentration used (+ + +, corresponding to 10 μM), the results are unreliable, because GST itself, with no fusion partner, pulled down NDP kinase. However, the results of experiments at the two lower activities are reliable, and they confirm our finding (Fig. 4B) that NDP kinase does not interact directly with dNMP kinase (gp1). This experiment supports our previously reported finding (10) of direct interactions between *E. coli* NDP kinase and T4 dihydrofolate reductase, and in addition it shows that NDP kinase does not interact with dCMP hydroxymethylase (gp42).

Fig. 6 depicts another pulldown experiment with purified proteins. Panel A shows that purified adenylate kinase is itself pulled down by GST-gp1 (dCMP hydroxymethylase) and GST-gp42 (dihydrofolate reductase) and shows the same input panels, as those used in a contemporaneous experiment that was previously reported (11).

![Figure 4](image-url) Interactions involving T4 dNMP kinase, as shown by analysis of GST pulldowns. A, three *E. coli* strains, Origami(DE3) (lane 1), Origami(DE3)/pGEX (lane 2), and Origami(DE3)/pGEX-gp1 (lane 3), were cultured in superbroth at 37 °C, and expression of GST-gp1 was induced with 1 mM IPTG. Cell lysates were prepared and mixed with a cell lysate of *E. coli* strain Origami(DE3)/pGexAB, which expresses T4 aerobically ribonucleotide reductase (RNR, gpordAR). GST pulldown was carried out with pre-equilibrated glutathione-Sepharose 4B, and proteins pulled down were separated in 12% SDS-PAGE, followed by staining with Coomassie Blue (top panel) or by immunoblotting with antisera to the respective enzymes. B, an identical analysis, except that the pulldown involved an extract of *E. coli* Origami(DE3), which expressed no phage proteins. Top panels in both A and B represent Coomassie Blue-stained 12% SDS-PAGE gels. All of the other gels involved immunoblotting with antisera to the respective enzymes. Total protein in each reaction (input) is shown below each pulldown reaction. This experiment used the same dNDP reductase R1 and R2 proteins, and show the same input panels, as those used in a contemporaneous experiment that was previously reported (11).
proteins, this one carried out to assess the effects of nucleotides upon binding affinity among the interacting pairs we are exploring, because we have found certain nucleotides, principally ATP, to have strong effects upon protein-protein interactions involving NDP kinase (11) and ribonucleotide reductase (20). In the experiment of Fig. 6A, purified NDP kinase was incubated with purified GST fusion proteins involving dCMP hydroxymethylase (gp42) and dihydrofolate reductase (gp42d). NDP kinase was seen to interact with both dCMP hydroxymethylase, as we have reported previously (11), and dihydrofolate reductase. What was striking in this experiment was the pronounced and specific effect of dADP, among the nucleotides tested, upon the affinity of NDP kinase for dihydrofolate reductase, and to a lesser extent, for dCMP hydroxymethylase. The direct interaction between NDP kinase and dCMP hydroxymethylase was almost completely dependent upon substrate nucleotides for NDP kinase (dADP in this experiment). Panel B shows a similar experiment involving purified adenylate kinase and several purified GST fusion proteins. This experiment suggests a much smaller effect of the nucleotides tested on these interactions, although dADP showed a small effect in stimulating the binding of ADK to dNMP kinase (gp1).

Interactions among Purified Proteins, Shown in an Optical Biosensor—Although the experiments described so far identify several specific interactions involving E. coli adenylate kinase and phage enzymes, the approaches are largely qualitative and do not permit one to determine whether the same interactions occur within phage-infected bacteria. Optical biosensors such as the BIAcore (BIAcore AB, Uppsala, Sweden) and the IAsys (Affinity Sensors, Cambridge, UK) permit quantitative analysis of protein-protein interactions in real time, based upon minute refractive index changes at the interface between a protein immobilized on a solid surface and an interacting protein in solution. Fig. 7 depicts the time course of a single association reaction and the effect of ATP upon that association. In this experiment T4 dCMP deaminase in solution was seen to bind to immobilized ADK, and ATP at 1 mM was seen to stimulate this association. This experiment demonstrates an additional interaction between E. coli adenylate kinase and a T4 enzyme constituent of the dNTP synthetase complex. Moreover, it represents another significant effect of nucleotides upon protein-protein interactions in the complex.

DNA Synthesis in Permeabilized Infected Bacteria—The experiment of Fig. 1 shows the effect upon T4 DNA synthesis of knocking out the expression of the host cell ndk gene. It would be desirable to explore the function of adenylate kinase by carrying out a similar knockdown of adk gene expression. However, even though adk expression can be ablated with a temperature-sensitive adk mutation, the results would be difficult to interpret because of the known functions of adenylate kinase in adenine ribonucleotide metabolism. Accordingly, we designed experiments using bacteria that had been permeabilized by suroce plasmolysis (24), so that we could bypass these latter functions by providing ribonucleotides, including both ADP and ATP. In the experiment of Fig. 9, panel A, we asked whether adenylate kinase plays an essential role in T4 DNA synthesis when the precursors are supplied as deoxyribonucleoside monophosphates. E. coli CV2, which contains a ts adk mutation, was infected, and the infected cells were permeabilized and incubated with the indicated nucleotides at either 25 or 42 °C. Incorporation of radiolabeled deoxyadenosine was inhibited at 42 °C, confirming the necessity of adenylate kinase for converting dAMP to ADP under these conditions. Comparable inhibition was not seen when bacteria with a wild-type adk gene were treated similarly. Panel B shows a similar experiment, in which DNA synthesis was made dependent upon added deoxyribonucleoside diphosphates. Under these conditions, DNA synthesis was not dependent upon a functional ADK protein. Both in the ts adk mutant and the wild-type host, incorporation of radiolabeled deoxyguanosine into
DNA was actually stimulated at 42 °C relative to what was seen at 25 °C. These experiments confirm the role of adenylate kinase in converting dAMP to dADP, but they suggest that the enzyme does not play an additional essential function, so long as nucleoside diphosphate kinase is available and active.

**DISCUSSION**

Most of the nucleotides used for T4 phage DNA synthesis come from ribonucleotide metabolism, via ribonucleotide reductase. These nucleotides come both from continued de novo nucleotide synthesis after infection and from messenger RNA turnover (28). Because T4 infection blocks host-cell RNA synthesis and increases the rate of DNA accumulation, the bulk of deoxyribonucleotides used for DNA synthesis, the fourth dNMP, dAMP, come from ribonucleotide metabolism, via ribonucleotide reductase and into dNTP metabolism. The phage-evoked breakdown of the bacterial chromosome provides another source of DNA precursors, as deoxyribonucleoside monophosphates (9). Because the dNMP kinase encoded by gene 1 acts on three of the four dNDPs, at least, in the absence of NDP kinase. The GST pulldown experiments show direct contacts between adenylate kinase and T4 dNMP kinase (gene 1), dCMP deaminase, thymidylate synthase and dCMP hydroxymethylase (gene 42), and dihydrofolate reductase (gene frd), as well as E. coli NDP kinase. The immunoprecipitation and affinity chromatography experiments show associations, either direct or indirect, with T4 dihydrofolate reductase and thymidylate kinase. The biosensor experiments show associations with T4 thymidylate synthase and dCMP deaminase. Thus, we conclude that adenylate kinase is a specific component of the T4 dNTP synthetase complex, just as is NDP kinase, and adenylate kinase is definitely capable of synthesizing dNTPs from dNDPs, at least, in the absence of NDP kinase. The GST pulldown experiments show direct contacts between adenylate kinase and T4 dNMP kinase (gene 1), dCMP hydroxymethylase (gene 42), and dihydrofolate reductase (gene frd), as well as E. coli NDP kinase. The immunoprecipitation and affinity chromatography experiments show associations, either direct or indirect, with T4 dihydrofolate reductase and thymidylate kinase. The biosensor experiments show associations with T4 thymidylate synthase and dCMP deaminase. Thus, we conclude that adenylate kinase is a specific, possibly integral, component of the complex, held in place by multiple protein-protein interactions. Fig. 10 is a schematic diagram, showing all of those direct protein-protein interactions identified in our work to date. Note that a significant number of those interactions do not involve enzymes that act sequentially. However, all of the enzymes are functionally related, and the interactions with the gene 32 single-strand DNA-binding protein, as we have discussed elsewhere (20), suggest a functional relationship with the replisome as well.

Our finding that the two known host-cell enzymes in the complex, NDP kinase and adenylate kinase, interact with each other may be significant with respect to the physiology of the uninfected bacterium, because both enzymes participate in the redistribution of high-energy phosphate that arises initially as ATP. More to the point of this paper, the interaction may be
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FIG. 10. Protein-protein interactions in the T4 dNTP synthetase complex. In this schematic diagram, adapted from Ref. 20, each solid line depicts a direct interaction shown either in this article or in previous papers from this laboratory (5, 10, 11, 20, and 22). Interactions involving adenylate kinase, which have not previously been reported, are depicted with double-headed arrows. 1, gene 32 single-stranded DNA-binding protein; 2, E. coli NDP kinase; 3, aerobic ribonucleotide reductase; 4, thymidylate synthase; 5, dihydrofolate reductase; 6, dCMP hydroxymethylase; 7, dCMP deaminase; 8, dNMP kinase; 9, dCTPase/dUTPase; 10, E. coli adenylate kinase.

significant to the operation of a “replication hyperstructure” in uninfected E. coli (29, 30), a supramolecular complex linking dNTP synthesis and DNA replication, comparable with what we see in the T4 system.

Two interesting questions emerge from the work reported here. First, what is the basis for the high efficiency with which ADK complements an ndk defect, when the NDP kinase activity of ADK is so low? Lu and Inouye (4) reported that the activity of ADK acting as an NDP kinase is 20–100-fold lower than that of NDP kinase itself, acting on the same substrate. If the T4 dNTP synthetase complex contains just one molecule each of ADK and NDP kinase, as seems likely from the molecular weight of the complex and the number of proteins present (10), and if ADK is driving DNA synthesis at 40% of its normal rate, then NDP kinase would seem to be present in considerable excess over its quantitative requirement. Alternatively, the NDP kinase activity of ADK may be increased as a consequence of the interactions that draw it into the complex. Another possibility is that in infection of an Escherichia coli ndk mutant host, most of the deoxyribonucleoside diphosphates formed by the complex diffuse away and are converted to dNTPs by that large majority of adenylate kinase molecules not associated with the complex (12).

Under these “unnatural” conditions, most of the dNTPs used by the replisome would not have been synthesized in situ at replication sites, and the replisome would be driving from non-compartmentalized dNTP pools, distributed throughout the cell. Even though all of the ADK molecules might be contributing to dNTP synthesis under these conditions, its low catalytic efficiency as an NDP kinase would limit the total rate of DNA synthesis.

The other issue pertains to the effects of nucleotides. Numerous observations in our laboratory (11, 22, 31) point to the effects of nucleotides, often, but not always, ATP, in stabilizing associations within the dNTP synthetase complex. The associations involving NDP kinase may be particularly significant (Ref. 11 and Fig. 6A). Bacterial NDP kinases are tetrameric proteins. Using nondenaturing gel electrophoresis, we have found that ATP promotes tetramerization of the enzyme in vitro, although we do not yet know whether this effect stimulates other activities of the complex. Comparable observations on promotion of oligomerization of NDP kinase have been made with the hexameric Dictyostelium enzyme (32), where ADP has been found to stabilize the enzyme, evidently by stimulating subunit interactions within the enzyme. More recently, Shen et al. (33) have described effects of nucleotides upon interactions between Arabidopsis thymidylate synthase and one of the plant isozymes of NDP kinase. DCDP was found to stimulate the binding of hexameric NDPK2 to the Pf enzyme form of thymidylate synthase, and thymidylate synthase was found to stimulate binding of substrate nucleoside diphosphates by NDPK2, evidently by accelerating formation of the phosphorylated enzyme intermediate. All of these observations with other NDP kinases, plus our own findings in the T4/E. coli system suggest that the effects of nucleotides upon the dNTP synthetase complex may involve oligomerization of NDP kinase, as well as its interaction with other enzymes. Some of the results shown in Fig. 6 show elements of specificity that suggest physiological significance to the effects of nucleotides. The pronounced stimulation by dADP of the binding of NDP kinase to both dCMP hydroxymethylase and dihydrofolate reductase is particularly intriguing. Also of interest is the significant stimulatory effect of the nonhydrolyzable ATP analog, AMP-PNP, whereas ATP has no such effect. This result may indicate that the phosphorylation state of the enzyme influences both its homotypic and heterotypic interactions in a way that might affect activities of the complex. In any event, these preliminary observations raise the intriguing possibility that intracellular flux rates for dNTP synthesis are controlled in part by the composition of the small-molecule pool. The pronounced effects of nucleotides, shown both here and in our previous articles (11, 22, 31), suggest that we can use this information to stabilize the dNTP synthetase complex in vitro, perhaps allowing systematic analysis of the effects of small molecules upon activities and substrate specificity of the complex.

Acknowledgment—We thank Linda Wheeler for outstanding technical assistance.

REFERENCES

1. Noda, L. (1973) in The Enzymes (Boyer, P. D., ed) Vol. 8, pp. 279–305, Academic Press, New York.
2. Goelz, S. E., and Cronan, J. E., Jr. (1982) Biochemistry 21, 189–195.
3. Haase, G. H. W., Brune, M., Reinstein, J., Pai, E. F., Pingoud, A., and Wittmann-Liebold, B. (1989) J. Mol. Biol. 207, 151–162.
4. Lu, Q., and Inouye, M. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 5740–5745.
5. Bernard, M. A., Ray, N. B., Olcott, M. C., Hendricks, S. P., and Mathews, C. K. (2000) J. Bioenerg. Biomembr. 32, 259–267.
6. Bello, L. J., and Bessman, M. J. (1963) Biochim. Biophys. Acta 72, 647–652.
7. Bello, L. J., and Bessman, M. J. (1963) J. Biol. Chem. 238, 1777–1787.
8. Duckworth, D. H., and Bessman, M. J. (1967) J. Biol. Chem. 242, 2877–2885.
9. Mathews, C. K. (1966) Biochemistry 5, 2092–2100.
10. Mathews, C. K. (1973) J. Biol. Chem. 248, 1567–1579.
11. Mathews, C. K. (1999) J. Biol. Chem. 274, 2935–2939.
12. Brush, G. S., Bhatnagar, S. K., and Bessman, J. M. (1996) J. Bacteriol. 178, 5740–5745.
13. Cronan, J. E., Jr., Ray, T. K., and Vagelos, P. R. (1970) Mol. Gen. Genet. 116, 199–210.
14. Mathews, C. K. (1993) J. Biol. Chem. 268, 2286–2291.
15. Young, J. P., and Mathews, C. K. (1992) J. Biol. Chem. 267, 10796–10799.
16. Tseng, M.-J., Hilfinger, J. M., He, P., and Greenberg, G. R. (1992) J. Bacteriol. 174, 5740–5744.
17. Belfort, M., Muckleken, A., Maley, F. G., and Maley, F. (1983) J. Biol. Chem. 258, 2045–2051.
18. Moore, M. J., Silverstein, R. E., Maley, F. G., and Maley, F. (1993) J. Biol. Chem. 268, 2286–2291.
19. Kim, J., Wheeler, L. J., Shen, R., and Mathews, C. K. (2005) Mol. Microbiol. 55, 1502–1514.
20. Wheeler, L. J., Wang, Y., and Mathews, C. K. (1992) J. Biol. Chem. 267, 7664–7670.
21. Wheeler, L. J., Ray, N. B., Ungermark, C., Hendricks, S. P., Bernard, M., 2 J.-H. Kim, unpublished observations.
Hanson, E., and Mathews, C. K. (1996) *J. Biol. Chem.* **271**, 11156–11162
23. Mathews, C. K., and Sinha, N. K. (1982) *Proc. Natl. Acad. Sci. U. S. A.* **79**, 302–306
24. Collinsworth, W. L., and Mathews, C. K. (1974) *J. Virol.* **13**, 908–915
25. Reddy, G. P., and Mathews, C. K. (1978) *J. Biol. Chem.* **253**, 3461–3467
26. Zhang, X., Lu, Q., Inouye, M., and Mathews, C. K. (1996) *J. Bacteriol.* **178**, 4115–4121
27. Bernard, M. A. (1999) *Functions of Nucleotide Kinases of the Host Cell in the Bacteriophage dNTP Synthetase Complex*. Ph.D. thesis, Oregon State University
28. Cohen, S. S., and Barner, H. D. (1961) *J. Biol. Chem.* **237**, 1376–1378
29. Guzmán, E. C., Caballero, J. L., and Jiménez-Sánchez, A. (2002) *Mol. Microbiol.* **43**, 487–495
30. Molina, F., and Skarstad, K. (2004) *Mol. Microbiol.* **52**, 1597–1612
31. Hanson, E. S., and Mathews, C. K. (1994) *J. Biol. Chem.* **269**, 30999–31005
32. Giartosio, A., Erent, M., Cervoni, L., Moreira, S., Janin, J., Konrad, M., and Lascu, I. (1996) *J. Biol. Chem.* **271**, 17845–17851
33. Shen, Y., Kim, J-L., and Song, P-S. (2005) *J. Biol. Chem.* **280**, 5740–5749
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JuHyun Kim, Rongkun Shen, Michael C. Olcott, Indira Rajagopal and Christopher K. Mathews

J. Biol. Chem. 2005, 280:28221-28229.
doi: 10.1074/jbc.M502201200 originally published online June 7, 2005

Access the most updated version of this article at doi: 10.1074/jbc.M502201200

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