Molecular and Functional Interactions between Escherichia coli Nucleoside-diphosphate Kinase and the Uracil-DNA Glycosylase Ung*

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Samridhi C. Goswami1, Jung-Hoon Yoon1, Bozena M. Abramczyk§, Gerd P. Pfeifer‡, and Edith H. Postel‡2

From the 1Laboratory of Biochemistry and Molecular Biology, Department of Pediatrics, Robert Wood Johnson Medical School/University of Medicine and Dentistry of New Jersey, New Brunswick, New Jersey 08903-0019, 2Division of Biology, Beckman Research Institute of the City of Hope, Duarte, California 91010, and 3Department of Molecular Biology, Princeton University, Princeton, New Jersey 08546

Escherichia coli nucleoside-diphosphate kinase (Ndk) catalyzes nucleoside triphosphate synthesis and maintains intracellular triphosphate pools. Mutants of E. coli lacking Ndk exhibit normal growth rates but show a mutator phenotype that cannot be entirely attributed to the absence of Ndk catalytic activity or to an imbalance in cellular triphosphates. It has been suggested previously that Ndk, similar to its human counterparts, possesses nuclease and DNA repair activities, including the excision of uracil from DNA, an activity normally associated with the Ung and Mug uracil-DNA glycosylases (UDGs) in E. coli. Here we have demonstrated that recombinant Ndk purified from wild-type E. coli contains significant UDG activity that is not intrinsic, but rather, is a consequence of a direct physical and functional interaction between Ung and Ndk, although a residual amount of intrinsic UDG activity exists as well. Co-purification of Ung and Ndk through multicolor low pressure and nickel-nitritotriacetic acid affinity chromatography suggests that the interaction occurs in a cellular context, as was also suggested by co-immunoprecipitation of endogenous Ung and Ndk from cellular extracts. Glutathione S-transferase pulldown and far Western analyses demonstrate that the interaction also occurs at the level of purified protein, suggesting that it is specific and direct. Moreover, significant augmentation of Ung catalytic activity by Ndk was observed, suggesting that the interaction between the two enzymes is functionally relevant. These findings represent the first example of Ung interacting with another E. coli protein and also lend support to the recently discovered role of nucleoside-diphosphate kinases as regulatory components of multi-protein complexes.

Escherichia coli nucleoside-diphosphate (NDP)3 kinase (Ndk, EC 2.7.4.6) belongs to a large family of highly conserved oligomeric phosphate transfer enzymes consisting of 4–6 identically folded subunits of 16–20 kDa, each containing an active site. NDP kinases catalyze the reversible transfer of γ-phosphates between nucleoside diphosphates and triphosphates at very high efficiencies through an evolutionarily conserved active site histidine residue (1–3). E. coli NDK kinase is encoded by a single gene, ndk (4), whereas the genetically distinct forms of human NDP kinases are encoded by multiple genes termed NM23-H1 through H8 (5). The name NM (nonmetastatic)23 was initially accorded to the matriarch of the family, NM23-H1, on the basis of its reported action as a tissue-specific metastasis inhibitor (6). Moreover, there is evidence that NM23/NDK proteins promote tumor formation and play various roles in normal development and cellular proliferation (7, 8).

NM23/NDP kinases are also multifunctional in vitro. In addition to the phosphoryl transfer reactions, they can catalyze various types of DNA cleavage (9–12) and activate transcription (13–15). The involvement of NM23 in DNA repair was initially proposed on the basis of a covalent, lysine-mediated mechanism by which NM23-H2/NDK-B binds and cleaves DNA (9), a mechanism known as the signature of base excision repair enzymes (16). NM23-H1/NDK-A was subsequently identified as GADD, an apoptotic endonuclease (11), and as a 3′-5′-exonuclease (10, 12). A large body of research also indicates that NM23/NDKs can interact directly with other proteins to regulate or provide links between different cellular pathways (17, 18). Of note is the presence of NM23-H1/NDK-A/DNase I in a DNA repair complex, where it interacts with the exonuclease TREX1 to degrade DNA during granulocyte A-mediated cell death (11, 19) as well as the interactions of NM23-H2/NDK-B with integrin (20) and with a membrane receptor protein (21). Whether the developmental and cancer-related functions of NM23/NDP kinases are driven by the chemical reactions they catalyze and/or by their protein/protein regulatory interactions remains to be elucidated.

E. coli Ndk is also multifunctional; it can directly interact with a half dozen other proteins involved in DNA metabolism (22, 23) and has been found in a DNA replication complex (23),

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1 Present address: Sealy Ctr. for Molecular Science, University of Texas Medical Branch, Galveston, TX 77555.

2 To whom correspondence may be addressed: Laboratory of Biochemistry and Molecular Biology, Dept. of Pediatrics, Robert Wood Johnson Medical School/UMDNJ, One Robert Wood Johnson Pl., P. O. Box 19, New Brunswick, NJ 08903-0019. Tel.: 732-235-3318/3319; Fax: 732-235-6102; E-mail: posteleh@umdnj.edu.

3 The abbreviations used are: NDP, nucleoside diphosphate; NDK, NDP kinase; UDG, uracil-DNA glycosylase; GST, glutathione S-transferase; DTT, dithiothreitol.
and while providing nucleoside triphosphates for DNA and RNA synthesis, Ndk also plays a role in maintaining the pools of cellular nucleoside triphosphates (24, 25). Mutants of E. coli lacking Ndk exhibit normal growth rates but display a mutator phenotype that cannot be entirely attributed to the lack of phosphotransferase activity (26) or to an imbalance in the cellular concentrations of nucleoside triphosphates (24, 27). The possibility of a defective DNA repair function in the absence of Ndk as an additional or alternative explanation for the mutator phenotype has also been considered (27, 28).

E. coli Ndk, similar to its human counterparts, is capable of cleaving DNA (29), an activity that was described by Postel and Abramczyk (28) as related to uracil processing. Prior to that, all of the uracil-DNA glycosylase (UDG) activity in extracts of E. coli has been attributed to either Ung, the major uracil-DNA glycosylase, or to Mug, an auxiliary enzyme (30, 31). Furthermore, any association, intrinsic or extrinsic, of a uracil-DNA glycosylase activity with Ndk was ruled out by Bennett et al. (32) and Kumar et al. (33) on the bases that Ndk purified from Ung-deficient mutant bacteria did not possess UDG activity and that there was no evidence of a physical interaction between Ndk and Ung (32, 33). Our attempts to resolve these controversial findings led to the observations reported in this paper, whereby the great majority of the uracil excision activity associated with purified Ndk is not an inherent property but rather is a direct consequence of the physical and functional interaction between Ung and Ndk.

**Experimental Procedures**

**Bacteria**—Either E. coli strain BL21 (DE3) or BH217 (DE3) was used as a wild-type host. BH217, BH161 (ung-1 BH156 with DE3 prophage), and BH214 (ung<sup><mbox></mbox></sup> mug<sup><mbox></mbox></sup> BH158 (DE3) were gifts of Dr. Ashok Bhagwat (Wayne State University, Detroit, MI). BW504 (ung<sup><sup><mbox></mbox></sup></sup>) was obtained from Dr. Bernie Weiss (Emory University, Atlanta, GA). The DE3 derivative of BW504 was constructed using a kit from Novagen (Madison, WI). Although the *E. coli ung* gene was originally cloned as a 3.5-kb EcoRI fragment from [Emory University, Atlanta, GA]. The DE3 derivative of BW504 was constructed using a kit from Novagen (Madison, WI). Plasmids—Plasmid *ndkec* was a gift from Dr. M. Konrad, Max-Planck Institute (Goettingen, Germany). Site-directed mutants of *ndkec* were prepared using the unique site elimination method (34, 35). The sequences of the mutagenic primers to produce the K11Q and H117F mutants were: 5'-CGTACTTCATCCATGUGCAGCT-3' and 5'-CCGAAAACGTACCTCGTGTTGTAATTCCGTCT-3', respectively. Construction of histidine-tagged *ndk* (His-*ndk*) plasmid was as previously described (12).

Construction of GST-Ung Plasmid—A PCR fragment of the E. coli ung gene was prepared with E. coli genomic DNA, Pfu turbo DNA polymerase (Stratagene, La Jolla, CA), and two primers containing restriction enzyme sites (EcoRI and XhoI) and subcloned into pGEX-5X-1 (Amersham Biosciences). Oligonucleotide Substrate—5'-CCTGCCCGTUGACAGC-3' (21-mer top strand) was radiolabeled using [γ<sup>32</sup>P]ATP and T4 polynucleotide kinase. Double-stranded oligonucleotides were prepared by annealing the labeled top strand with a 1.5-fold molar excess of unlabeled complementary strand by heating for 10 min at 85 °C and slow cooling. The duplex oligonucleotide substrate was ethanol-precipitated and gel-purified.

**Purification of Native Recombinant Ndk**—Native Ndks were prepared using sequential chromatography on anion exchange, hydroxyapatite, and gel filtration in conjunction with a BioLogic LP (Bio-Rad) low pressure chromatography system equipped with the BioFrac fraction collector essentially as described previously (28), with the exception that only 100-ml cultures were processed. Following lysing (10 ml) and ammonium sulfate fractionation (40–60%), the precipitates were dissolved in 0.5 ml of 50 mM Tris, pH 8.0, and applied to a 30 × 5.5-cm DEAE-cellulose column equilibrated with 50 mM Tris, pH 8.0, from which the peak Ndk was eluted at ∼100 mM NaCl using a 200-ml 0–500 mM linear NaCl gradient. Peak Ndk fractions (2 ml) were pooled, concentrated in Amicon Ultra15 (MWCO 30 K) concentrators, equilibrated in 10 mM sodium phosphate, pH 7.1, and applied to a 5-ml hydroxyapatite (Bio-Rad) column. Proteins were eluted with a 50-ml 10–400 mM, pH 7.1, sodium phosphate gradient. Ndk, which was eluted in the flow-through fractions, was concentrated and applied to a 13 × 3.5-cm Sephadex G100 gel filtration column. Fractions from all three columns were assayed for both NDP kinase and UDG activities. All operations were performed at 4 °C. The final protein fractions were concentrated in Amicon Ultra15 (MWCO 30 K) concentrators, equilibrated in 0.1 M HM buffer (20 mM Hepes-KOH, pH 7.9, 100 mM KCl, 5 mM MgCl₂, 2 mM DTT, 20% glycerol, and protease inhibitors), and stored at −80 °C. The purity of each preparation was assayed by SDS-PAGE. The purification of His-Ndk was performed as previously described (12).

**Purification of GST and GST-tagged Ung Proteins**—Lysates of GST or GST-Ung were prepared from 1-liter cultures grown at 37 °C in LB medium and 50 µg/ml carbenicillin with vigorous shaking until A<sub>600</sub> was 0.6–0.7. Protein expression was induced by the addition of isopropyl 1-thio-D-galactopyranoside to 1 mM for 3 h. The pellet was resuspended in 10 ml of lysis buffer (50 mM sodium phosphate, pH 8.0, 0.2% Triton X-100, 300 mM NaCl) followed by treatment with 1 mg/ml lysozyme and 10 µg/ml DNase and RNase for 20 min at room temperature with stirring. Following brief sonication and centrifugation at 12,000 × g for 20 min, the lysate was mixed with 2 ml of buffer-equilibrated slurry, washed extensively with lysis buffer, and the GST proteins eluted with 50 mM Tris-HCl, pH 8, containing 20 mM reduced glutathione (Sigma) and 5 mM DTT. The proteins were concentrated in Amicon Ultra15 (MWCO 30 K) concentrators and then equilibrated in 0.1 M HM buffer and stored at −80 °C. The purity of each preparation was assayed by SDS-PAGE.

**NDP Kinase Assay**—NDP kinase activity was measured in a spectrophotometric assay using ATP as a phosphate donor and dTDP as an acceptor nucleotide in a coupled pyruvate kinase-lactate dehydrogenase reaction that measures ADP formation from ATP, as described previously (34, 35). The specific activities were ∼600 units/ml with the exception of His-Ndk, which was 350 units/ml, whereas the catalytic mutants H117F and K11Q had no detectable activity.

**UDG Assay**—A [γ<sup>32</sup>P]-radiolabeled duplex oligonucleotide containing a centrally located single uracil in the top strand was used as substrate, as described above and in Ref. 28. Purified Ung and Mug (Trevigen) served as positive controls. Reactions
were carried out in 5 µl of reaction buffer (20 mM Hepes-KOH, pH 7.4, and 5 mM EDTA) for 30 min at 37 °C. In assaying column fractions, 100 µg/ml bovine serum albumin was included in the assay buffer. One unit of the Ung inhibitor Ugi (PBS1 uracil glycosylase inhibitor protein, New England Biolabs) was added to the reactions as indicated. The DNA strands were subsequently cleaved with either Endonuclease IV (Trevigen) or 200 mM NaOH for an additional 30 min. To stop the reactions, an equal volume of 95% formamide/20 mM EDTA was added and the products resolved on 16% sequencing gels (28).

Photoshop images of the dried and autoradiographed gels were quantified using the ImageJ, version 1.345 (NIH) program. UDG activity was calculated on the basis of the percent cleavage of the substrate obtained in most cases from the linear portion of the cleaved product versus the enzyme concentration plot.

**Immunoprecipitations**—Untransformed cells (3 ml) were grown to 0.6 of final volume for 60 min at 4 °C with constant rotation. The immunoprecipitates was retained in the immunoprecipitate.

**Protein Purification**—Host was mixed with glutathione-Sepharose 4 B (Amersham Biosciences) previously equilibrated with phosphate-buffered saline and 1% Triton X-100 and incubated with GST or GST-Ung proteins for 60 min at room temperature. The GST-Sepharose-bound proteins were washed five times with 20 volumes of 50 mM Tris-HCl, pH 8.0, 2 mM EDTA, and protease inhibitors in the presence of lysozyme, DNase, and RNase as described in the above purification protocols. The lysates were cleared by centrifugation followed by the addition of 3 µl of purified rabbit polyclonal anti-Ndk antibody (gift of I. Cascu, University of Bordeaux, Bordeaux, France) and incubation for 60 min at 4 °C with constant rotation. The immunoprecipitates (200 µl) were mixed with 100 µl of washed and pre-equilibrated swollen protein G-agarose beads (25 mg) and incubated for an additional 60 min at 4 °C. Following centrifugation, the supernatant was removed and the rest of the beads washed twice with a total of 30 volumes of 10 mM phosphate-buffered saline and 1% Triton X-100 and incubated with a total of 30 volumes of binding buffer and then eluted with 200 mM NaOH for an additional 30 min. To stop the reactions, an equal volume of 95% formamide/20 mM EDTA was added and the products resolved on 16% sequencing gels (28).

**Far Western Analysis**—Proteins fractionated on 15% SDS-PAGE gels were electroblotted onto nitrocellulose membrane in the same manner as for Western blotting. Membranes were washed in water, and the bound proteins were further denatured for 30 min at room temperature in the presence of 6 M guanidine hydrochloride (Fluka), 25 mM Hepes-KOH, and 5 mM MgCl₂ plus 1 mM DTT and then renatured by successive 30-min incubations with 3, 1.5, 0.75, 0.325, 0.15, and 0.05 M guanidine hydrochloride in the same buffer. The membranes were washed again and then blocked in blocking buffer (20 mM Hepes-KOH, pH 7.4, 100 mM KCl, 2.5 mM MgCl₂, 0.1 mM EDTA, 1 mM DTT, 0.1% Tween 20, and 2% normal goat serum (Vector laboratories)) and further incubated with Ndk purified from ung⁺ E. coli that was freshly autophosphorylated at the His-117 residue as described for the human protein (34). Briefly, 20 µl of Ndk (37 mg/ml) in 0.1 HM buffer was mixed with 2 µCi of [γ-³²P]ATP on ice for 1 h. Unincorporated ATP was removed by spin filtration (Centricon 10, Amicon), and the labeled protein was added to the blots in blocking buffer at a final concentration of 150 µg/ml and ~10⁸ Cerenkov counts/min/ml. The membranes were incubated with the labeled protein with gentle shaking at room temperature for 60 min, the labeling solution then removed and the filters washed three times with blocking buffer and then dried and exposed to x-ray film.

**RESULTS**

The Majority of UDG Activity Associated with Ndk from Wild-type E. coli Is Not Intrinsic—It has been previously suggested that recombinant Ndk overexpressed and purified from wild-type E. coli cells possesses a UDG activity that is Ung-like, *i.e.* sensitive to the Ung-specific inhibitor Ugi (28). These findings were questioned on the basis that Ndk purified from Ung-deficient bacteria did not possess UDG activity (32, 33). Our results here obtained from experiments using Ndk purified from various Ung-deficient mutant E. coli (ung⁻, ung⁻ mug⁻, and ung⁻) hosts and from the isogenic wild type confirm that the majority of UDG activity associated with Ndk is not an intrinsic property of the protein (Fig. 1).
Our data also indicate that a residual amount of intrinsic activity does exist in Ndk that is not due to Mug and is Ugi-sensitive (Fig. 1, lanes 6–9; see Fig. 7A). Its specific activity is $3 \sim 4$ orders of magnitude lower than that of purified Ung ($\sim 5$ nmol of substrate oligonucleotide cleaved/mg/h by Ndk versus $1 \times 10^4$ nanomoles cleaved/mg/h by Ung). The origin of this residual uracil-cleaving activity of Ndk is not yet fully known, although on the basis of several biochemical observations and its dependence on the NDP kinase catalytic activity, one can reasonably assume that it is Ndk-derived.4

Ndk and Ung Co-purify through Sequential Three-column Chromatography—To elucidate the nature of the robust UDG activity of recombinant Ndk purified from wild-type E. coli (Fig. 1, lanes 4 and 5) (28), we investigated the possibility that this activity may have arisen as a consequence of a physical interaction between Ung and Ndk. First, we confirmed previous observations (28) that Ung and Ndk co-purify through sequential ion exchange, hydroxyapatite, and size exclusion column chromatography while also showing the Ndk and UDG activity profiles from all three of the columns (Fig. 2). Of note here is the UDG activity profile of the hydroxyapatite column, showing two distinct, rather than overlapping, peaks (Fig. 2B). One peak, representing $\sim 30\%$ of the total UDG activity, co-elutes with recombinant Ndk in the flow-through of this column, whereas the second larger peak, comprising $\sim 70\%$ of the activity, binds to the hydroxyapatite column from which it elutes with 40–80 mM potassium phosphate. Under ordinary circumstances, Ndk and Ung should have been completely resolved by hydroxyapatite chromatography, as Ndk does not bind to hydroxyapatite at neutral pH (28, 32), whereas Ung does (Ref. 32 and these results). Moreover, when an extract of cells containing only endogenous Ndk was subjected to this same chromatographic procedure, all of the UDG activity was retained by the hydroxyapatite column from which it was eluted with the same potassium phosphate concentrations as in the presence of excess amounts of recombinant Ndk (data not shown). Also, in Fig. 2B, $\sim 5\%$ of the Ndk activity remained associated with the major UDG peak on the hydroxyapatite column. Further, despite their divergent molecular masses ($\sim 26$ kDa of Ung versus $\sim 64$ kDa of Ndk), Ung and Ndk were also co-eluted from the final gel filtration column (Fig. 2C). Overall we have noted that, although the final ratios of Ung and Ndk activities may vary between purification experiments, the phenomenon of copurification of recombinant Ndk and cellular Ung is reproducible and therefore unlikely to be the product of accidental association (i.e. contamination of Ndk by Ung). Rather, it seems that, under conditions of gentle low pressure chromatography,

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4 S. C. Goswami and E. H. Postel, unpublished data.
significant amounts of endogenous Ung can associate with Ndk quite stably.

Ndk and Ung Co-purify by Ni\textsuperscript{2+} Chelate Affinity Chromatography—E. coli Ndk expressed as a recombinant histidine-tagged protein and purified by Ni\textsuperscript{2+} chelate affinity chromatography (Fig. 3A) also carried with it a Ugi-sensitive UDG activity (Fig. 3B), indicating that Ung also co-purifies with histidine-tagged Ndk protein. Because affinity co-purification generally signifies a stable association between two interacting proteins, these findings substantiate the conclusions drawn above that Ndk and Ung can stably associate in a cellular context.

Endogenous Ndk and Ung Co-immunoprecipitate from Growing E. coli Cells—To further investigate whether Ndk is in an endogenous complex with Ung under physiological conditions, lysates were prepared from untransformed, growing isogenic wt and ung\textsuperscript{a} E. coli cells. Ndk from both extracts was then immunoprecipitated with antibody raised against E. coli Ndk, and the immunoprecipitated proteins were bound to protein G-agarose. Following extensive washings, aliquots of the beads carrying the bound complexes were analyzed both for NDP kinase activity by the addition of the kinase assay mixture and for UDG activity by the addition of DNA substrate and reaction buffer. The results of the UDG assay (Fig. 4) demonstrate clearly that a substantial amount of Ung activity was retained by the immunoprecipitates from the wild-type extracts (Fig. 4, lanes 3–6), whereas virtually none was detected in the mutant cell extracts (lanes 7–10), indicating that uracil excision in these immunoprecipitated samples was due to the Ung enzyme alone. The trace amount of residual cleavage in lane 10 by the highest concentration of immunoprecipitated ung\textsuperscript{a} extract is most likely because of the residual activity of endogenous Ndk. In this experiment, ~25% of the initial Ndk activity was retained in the final immunoprecipitates, and ~20% of the total Ung activity was also brought down by the Ndk antibody. However, it is not possible to conclude from these data that the molar ratio of the proteins in the complex is 1:1, because Ndk activity is measured in a coupled spectrophotometric enzyme assay using four different substrates and uracil excision activity is coupled to strand cleavage by another enzyme and is only approximated by image analysis densitometry. Nonetheless, this experiment conclusively demonstrated that Ndk antibodies specifically and quantitatively co-precipitated Ung with Ndk, supporting the conclusion that these two proteins interact with each other in the cell. The reciprocal experiment could not be performed because of the universal unavailability of Ung-specific antibody.

Ndk Binds to Glutathione-Sepharose-bound GST-Ung but Not to Glutathione-Sepharose-bound GST—To demonstrate that a direct protein/protein interaction takes place between Ndk and Ung, we performed an in vitro binding assay using purified glutathione-Sepharose-bound GST-Ung and histidine-tagged Ndk proteins (Fig. 5). The results of this experiment show that Ndk can bind specifically, as verified by immunodetection on a Western blot, to glutathione-Sepharose-bound GST-Ung but not to glutathione-Sepharose-bound GST (Fig. 5B). Under similar conditions, the amount of native (untagged) Ndk bound to GST-Ung was significantly higher; however, for reasons that we do not understand, the untagged full-length 16-kDa Ndk polypeptide was cleaved during the interaction process, resulting in the release of an ~13-kDa fragment, which was confirmed by Western blotting to be Ndk-derived (data not shown).

Purified Native Ndk Binds to Ung in a Far Western Protein Blotting Assay—To further probe for direct protein/protein interaction, we used a far Western protein blotting technique
developed for this purpose, which makes use of the unique auto-
phosphorylating activity of NDP kinases. In this assay, purified
GST-Ung resolved on SDS-PAGE gel was transferred to a nitro-
cellulose membrane, which, following extensive renaturation of
the bound proteins, was incubated with $^{32}$P-labeled (at histidine
phosphorylating activity of NDP kinases. In this assay, purified
His-Ndk ($10^3$ nM Ung).

Ung catalytic activity, we carried out enzymatic assays with
Ung with and without native Ndk purified from ung$^*$ E. coli.
As illustrated in Fig. 7A, when 2 nM Ung, which cleaved 17%
of the substrate (lane 1), was supplemented with increasing
concentrations of Ndk over a 10$^3$-fold range (Fig. 7A, lanes 5–11), an overall 4–5-fold stimulation of Ung activity was
observed (lanes 14–19). Other experiments showed that,
when lesser amounts of Ung were present, the fold stimulation
with Ndk was even higher. The supplemented amounts
of Ndk alone provided only the residual UDG activity and
only at the highest of concentrations (Fig. 7A, lanes 10 and
11). A semilog plot of the percent of substrate cleaved versus
the Ndk:Ung molar ratio in this experiment (Fig. 7B) sug-
ests a slightly sigmoidal response, indicative of co-operat-
ivity between the two enzymes.

To examine some of the important kinetic parameters of
the augmentation reaction, we have obtained velocity versus sub-
strate concentration curves for Ung alone and for Ung + Ndk.
One of these plots is shown in Fig. 7C. From an inspection of
the curves, a $K_m$ value for Ung is estimated as $7.7 \times 10^{-8}$ M, which

FIGURE 4. Endogenous Ndk and Ung co-immunoprecipitate from untransfected cell extracts. Immunoprecipitated (IP) cell extracts were
extensively washed and diluted aliquots of the beads tested directly for UDG
activity. Samples in lanes 3–6 contain serial dilutions (0.2–0.8 µg/reaction) of the
immunoprecipitated complex. Lane 2 shows Ung control (1 pmol). Ndk activity
was similar in both the wild-type (wt) and mutant cell extracts and their
immunoprecipitates.

Ung and Ndk Interact Functionally—The above data
showing direct molecular interactions between Ung and Ndk
suggested that, as a consequence of this interaction, the two
enzymes might also affect each other’s catalytic activity. Thus,
to investigate whether Ndk is capable of modulating

FIGURE 5. Ndk binds to GST-coupled Ung. Binding assay using 2 µg of each
purified recombinant GST, GST-Ung, and His-Ndk proteins. A, Coomassie
Blue-stained SDS-PAGE of the input (lanes 1) and eluted (lanes 2) protein
samples. B, Western analysis of a duplicate of each input and bound sample. The
GST-Ung protein in A contains additional proteins carried over during purifi-
cation that do not interact with Ndk (see also Fig. 6).

FIGURE 6. Ndk interacts with Ung by far Western analysis. Samples were
resolved on duplicate SDS-PAGE gels with one stained with Coomassie Blue
(A) and the other transferred to nitrocellulose and probed with purified $^{32}$P-
labeled Ndk (B) as described under “Experimental Procedures.” Lane 1, marker
proteins (M); lanes 2 and 3 each represent 1 µg, and lanes 4 and 5, 3 µg of each
GST and GST-Ung protein analyzed.
is lowered by ~5-fold by the addition of Ndk, to an estimated $K_m$ value for the augmented enzyme of $1.7 \times 10^{-8}$ M. At the same time, the maximum velocity of Ung approaches the $V_{max}$ of Ung + Ndk at the higher substrate concentrations. We can thus reasonably conclude from these data that Ndk lowers the $K_m$ value of Ung, thereby increasing the affinity of Ung for this oligonucleotide substrate, which points to a specific and regulatory, rather than a perfunctory, effect.

Augmentation is also evident with the catalytically deficient mutant Ndk H117F and K11Q (Fig. 7D, lanes 4 and 5), indicating that the active site of the phosphotransferase is not required for this increase of Ung catalytic activity. Stimulation of Ung activity by bovine serum albumin or with other nonspecific proteins was minimal, below ~100 μg/ml concentrations (Fig. 7D, lane 6; and Fig. 7E). Interestingly, in a reverse experiment, the addition of Ung had no effect on Ndk catalytic activity (data not shown).

DISCUSSION

We have confirmed in this paper two previously made but conflicting observations, 1) that recombinant Ndk purified from wild-type *E. coli* cells contains significant amounts of UDG activity (28) and 2) this UDG activity is not intrinsic to Ndk (32, 33), with the exception that we also find a trace amount on the order of 0.001%, activity that is ascribable to...
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Ndk. Attempts to resolve the inconsistency between the two published observations led us to the discovery of a molecular and functional interaction between Ung and Ndk.

We established by conventional multicolour and by nickel-nitrilotriacetic acid affinity chromatography a propensity of both Ung and Ndk to co-purify, suggesting strongly that this association is not due to accidental contamination of Ndk with Ung but rather to a tight and possibly specific binding in vivo. We have therefore used several different approaches to demonstrate such a protein/protein interaction. One approach was co-immunoprecipitation of endogenous Ung and Ndk from E. coli cells that had not been transformed to overexpress recombinant Ndk. The results of these co-immunoprecipitation experiments provided unequivocal evidence that Ung and Ndk are present in E. coli in a physiologically relevant complex.

We have also searched for evidence of a direct in vitro interaction between purified Ung and Ndk, both by GST pulldown and by far Western protein blotting analyses. In the pulldown assays, Ndk bound directly to GST-Ung but not to the GST control protein alone, as validated by Western blotting. Using a far Western technique, we observed strong and quantitative binding of 32P-autophosphorylated Ndk to Ung coupled to GST but not to GST protein alone. These experiments confirmed unequivocally that a direct and substantial molecular interaction takes place between these two proteins, as was observed both by Ung binding to Ndk (co-purification and co-immunoprecipitation) and by Ndk binding to Ung (GST pulldown and far Western analyses).

To further demonstrate the physiological relevance of this Ndk-Ung interaction, we tested the effect of Ndk supplementation on uracil excision by the purified Ung enzyme. The results of these experiments show a significant and dose-dependent augmentation of Ung catalytic activity by Ndk, suggesting the possibility that Ung and Ndk can act synergistically to remove uracil from DNA. Interestingly, the converse of this effect is not true, as Ung was unable to stimulate Ndk activity as measured in a coupled NDP kinase assay.

We do not yet understand the mechanism of these molecular and functional interactions. The lowering effect by Ndk on the $K_m$ value of Ung implies a specific and regulatory effect as the basis of the interaction, although an additional facilitating and stabilizing effect cannot be ruled out. Because two catalytically inactive point mutants of Ndk were able to augment Ung activity as well as did the wild-type protein, we infer that the Ndk catalytic function is not involved in the protein/protein interaction that results in augmentation and that the domain of Ndk that regulates this process most likely involves regions other than the active site of the molecule. One such region may be the so-called “Kpn loops” located on the north and south poles of the NDP kinase multimer (2), to which modulating functions have already been assigned both in the classical case of the killer-of-prune (kpn) synthetic lethal interaction between PRUNE, a phosphodiesterase (36), and AWD, the Drosophila NDK (8, 37), and also in a case of NM23-H2 human NDK (20). From a physiological perspective, this interaction is not surprising, as the presence of both enzymes has been previously implicated in association with a DNA replication complex (Ndk with a T4 bacteriophage replication complex (22) and Ung in the proximity of other components of the DNA repair machinery of the E. coli chromosome (38)). From the genetic viewpoint, however, it is a puzzle, because, although mutant E. coli cells lacking either Ndk or Ung have mutator phenotypes, the mutations they accumulate differ (27, 39).

Our results and conclusions are at odds with those of Bennett et al. (32) and Kumar et al. (33) in other respects. Most important, these papers argued strongly against protein/protein interaction between Ung and Ndk as a possible explanation for the observed association of Ung activity with Ndk (28) on the basis of only one negative result in which Ndk did not bind to a Ugi-Ung complex. Of note also is the fact that, although Kumar et al. (33) did observe the co-purification of Ung and Ndk by nickel-nitrilotriacetic acid affinity chromatography, a result that in the literature is usually interpreted to mean a functional interaction between the co-purifying proteins, they provided no explanation for this finding. And, although Bennett et al. (32) have also reported some degree of co-purification of Ung with Ndk, they have separated the two proteins by high pressure chromatography on a hydroxyapatite matrix, a process that could have disrupted an existing biological complex.

The amount of the residual UDG activity that immunoprecipitated with endogenous Ndk from Ung-deficient cells (Fig. 4) and that seen in recombinant Ndks prepared from these mutant cells (Figs. 1 and 7A) is 3–4 orders of magnitude lower than the augmented Ung activity. And, although the residual activity appears to be intrinsic to Ndk, further experiments are needed to sort out their overall significance and their effect, for instance, on the augmentation reaction. Nevertheless, it would not be surprising if Ndk were able to perform an auxiliary uracil excision function, as Ung- and Mug-deficient E. coli cells can grow normally, and one might expect therefore that, in these cells, other repair enzymes would step in to catalyze uracil excision. Ndk would be a good candidate to perform such a repair reaction, as it is highly abundant and efficient and is equipped with multiple active sites that are nonspecific with respect to binding substrate nucleotides (2, 3).

In summary, the results presented in this paper support a model in which Ndk and E. coli Ung interact physically and functionally to facilitate uracil repair in the cell. It is notable that, although Ndk already has several established interacting partners in DNA metabolism (23, 25), this is the first indication of Ndk acting as a regulator in a specific DNA repair pathway. More important, to our knowledge, this constitutes the first report of an interaction involving the E. coli Ung enzyme with another E. coli protein. Preliminary data already indicate that, at least at the level of purified proteins, a similar interaction takes place between human NDK and human UDG proteins.5

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