

Uracil excision repair pathway activities are not intrinsic to nucleoside diphosphate kinases from *Mycobacterium tuberculosis* and *Escherichia coli*.

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Abstract

E. coli nucleoside diphosphate kinase (*Eco*NDK) is an important cellular enzyme required to maintain balanced nucleotide pools in the cells. Recently, it was reported that *Eco*NDK is also a multifunctional base excision repair enzyme, possessing a uracil-DNA glycosylase (UDG), an AP-endonuclease and a deoxyribose-phosphodiesterase activities. We investigated for the presence of similar activities in *M. tuberculosis* NDK (*Mtu*NDK), which shares 45.2 % identity, and 52.6 % similarity with *Eco*NDK. In contrast to the robust uracil excision activity reported for *Eco*NDK, *Mtu*NDK preparation exhibited very poor excision of uracil from DNA. However, this activity was undetectable when *Mtu*NDK was purified from an *ung*⁻ strain of *E. coli*, or when the assays were performed in the presence of extremely low amounts of a highly specific proteinaceous inhibitor, Ugi which forms an extremely tight complex with the host Ung (UDG), showing that *Mtu*NDK preparation was contaminated with UDG. Reinvestigation of uracil processing activities in *Eco*NDK purified by conventional, and by Ni-NTA affinity techniques, showed that even *Eco*NDK lacked UDG activity. All preparations of NDK were shown to be active by their autophosphorylation. Ugi neither displayed a physical interaction with *Eco*NDK nor did it affect autophosphorylation of NDKs. Further, neither of the NDK preparations processed the AP-DNA generated by UDG treatment of the uracil containing DNA duplexes. However, partially purified preparations of NDK did process such DNA substrates.

Introduction

DNA glycosylases excise damaged or unconventional bases from DNA and initiate base excision repair (BER) pathway (Friedberg, 1978). In BER pathway, cleavage of the N-glycosidic bond by a DNA glycosylase is followed by an AP-endonuclease, a deoxyribose-phosphodiesterase (dRpase), a DNA polymerase and a DNA ligase functions (Dianov et. al., 1994). The DNA glycosylases are highly specific for the unusual base(s) they excise from DNA, and based on the associated activities, they have been grouped into monofunctional or bifunctional enzymes (Lindahl, Krokan, Duncan, reviews Friedberg, 1995). The monofunctional enzymes possess only the glycosylase activity whereas the bifunctional enzymes, in addition, possess AP lyase and/or dRpase activities. The enzymes that remove uracil from DNA are collectively referred to as uracil DNA glycosylases (UDGs). UDGs have common evolutionary origin with a distinct α/β structural fold, and constitute a super family of monofunctional DNA-glycosylases (arvind and koonin, 2000; Pearl 2000, sakumi and sekiguchi, 1990). Based on the primary structure analysis, UDGs have been classified into five families (Pearl, 2000). The family 1 comprises of UDGs with a high homology to *E. coli* Ung (referred to hereafter as *EcoUDG*), which are specifically inhibited by a proteinaceous inhibitor, Ugi (uracil DNA glycosylase inhibitor) encoded by *Bacillus subtilis* phages PBS -1 and -2 (Mosbaugh; crystal structures). The binding of Ugi to *EcoUDG* is essentially irreversible under physiological conditions, and stable to even 8 M urea at room temperature. Such a tight interaction of Ugi with UDG is necessary for complete inhibition of UDG activity of the complex. Mutations in *EcoUDG* or Ugi that weaken the stability of the complexes, result in incomplete inhibition of *EcoUDG* (Mosbaugh, Narottam). Further, despite a common evolutionary origin and a conserved three dimensional fold among the members of UDG super family, only the members of the *EcoUng* family are known to be inhibited by Ugi (Barret, family 4; Jiricny).

Recently, nucleoside diphosphate kinase from *E. coli* (*Eco*NDK) was reported to be a multifunctional protein. In addition to its NDK activity, it was shown to possess uracil processing, AP- site processing and dRPase functions, suggesting it to be the first member of a novel family of UDGs (Postel, 2003) which utilized both single- and double- stranded uracil-DNA as substrates. The primary function of *Eco*NDK is to maintain cellular levels of nucleoside triphosphates for the synthesis of DNA and RNA (Hama H, 1991). It catalyzes transfer of γ -phosphate from nucleoside triphosphates to 5' position of ribo- and deoxyribo-nucleoside phosphates (Almaula N 1995). It also undergoes autophosphorylation at H117, S119, and S121 (Inouye et al, J Bacteriol, 1995). A deletion of *ndk* gene in *E. coli* results in an increased rate of spontaneous mutations and elevation of cellular levels of CTP, dCTP and dGTP (Bernard MA 2000, Lu Q 1995 JMB).

The genome sequence of *Mycobacterium tuberculosis* (Cole, ST et al. 1998) revealed that it is missing orthologs of many of the DNA repair proteins (Mizrahi V 1998), known to play important functional roles in *E. coli*. Recently, we showed that UDG repair pathway is crucial for survival of mycobacteria in the host macrophages especially under the conditions of increased reactive nitrogen intermediate production (Venkatesh et al). *M. tuberculosis* NDK (*Mtu*NDK) shares 45.2 % identity, and 52.6 % similarity with *Eco*NDK (). The potential implication of NDK in uracil excision repair in mycobacterium biology encouraged us to carry out a comparative biochemical characterization of *Mtu*NDK and *Eco*NDK (for their possible role in uracil processing). Contrary to the findings of Postel *et. al.*, but consistent with a more recent independent study on *Eco*NDK (Bennett *etal*), by using NDKs from a diverse group of bacteria, we show that neither *Mtu*NDK nor *Eco*NDK possess detectable uracil excision activities. In addition, we demonstrate that these NDKs do not process the AP-site containing DNA duplexes generated by UDG, thereby ruling out a direct role of the NDKs in uracil excision repair pathway.

Materials and Methods

Plasmids and biochemicals: The *Eco*NDK expressing plasmids *pndkec*[pJC20] and *pndkec*[pJC20HisC] were kindly provided by Dr. M. Konrad (Max Planck Institute for Biophysical Chemistry, Goettingen, Germany). Plasmid, pACT7 was generated by Dr. S. Karnik in the laboratory of Dr. H. G. Khorana, M.I.T., Cambridge, USA. *E. coli* strain BL21 (DE3) was from Novagen, BW310 (*ung*⁻, Duncan 1985) was obtained from *E. coli* Genetic Stock Center (New Haven, USA). *Eco*UDG and Ugi were earlier purified in the laboratory (sudipt, priya). Exonuclease III (Roche, Germany); T4 polynucleotide kinase (NEB); [γ -³²P]ATP (6,000 Ci/mmol, Perkin Elmer); Ni-NTA agarose (QIAGEN); hydroxyapatite (BioRad); Sephadex, Superdex-75, Superdex-200, DEAE-Sepharose, Source 15Q (Amersham Biosciences); were purchased from the respective companies.

DNA substrate: Oligonucleotides with two modified phosphorothioate bonds (indicated by '~') towards each end, 12U30 (5'- A~T~ATACCGCGGUCGGCCGATCAAGCTTA~T~T - 3') and its complimentary strand, 12U30c (3'- T~A~TATGGCGCCGGCCGGCTAGTTC-GAAT~A~A -5') were obtained from Microsynth (Switzerland). Oligomer, 12U30 was end labeled using [γ -³²P]ATP and T4 polynucleotide kinase (Chaconas, 1980), purified by passing through Sephadex G50 mini spin column, and annealed with 3 fold molar excess of its complementary strand by heating at 90 °C for 5 min followed by slow cooling to 25 °C (Dianov G, 1992). Formation of the duplex was verified by electrophoresis on a 15 % polyacrylamide gel (Kumar NV, 1994).

Cloning and purification of *M. tuberculosis* NDK (*Mtu*NDK). Using *M. tuberculosis* genomic DNA as template, Rv2445c open reading frame was amplified by PCR with DNA oligomers, 5'- GGGGGATCCGTGACCGAACGGACTCTGGTACTGATCAAG -3' and 5'- CCGGAATTCGGCGCCGGGAAACCAGAGCG 3' containing *Bam*HI and *Eco*RI sites, respectively. The PCR product was cloned into pBluescript-KS between *Bam*HI and *Eco*RI

sites, mobilized into pQE-30 (QIAGEN) as *Bam*HI and *Hind*III fragment and verified by DNA sequencing. The *Mtu*NDK expression construct, *pQE30Mtu-ndk*, was introduced into *E. coli* M15/pREP4 (*ung*⁺) or *E. coli* BW310 (*ung*⁻) by transformation. Transformants were grown in 2YT medium (1 L) (Sambrook, molecular cloning) and harvested after induction with IPTG (1 mM) for 3-4 h. The cells were lysed by sonication, and NDK was purified using Ni-NTA agarose (Y Singh, 2003).

Purification of *E. coli* NDK-His₆ (*Eco*NDK-His₆): The construct *pndkec*[pJC20HisC] was introduced into an *E. coli* BL21 (DE3, *ung*⁺), or into the BW310 (*ung*⁻) harboring pACT7, a low copy compatible plasmid for expression of T7 RNA polymerase. Isolated colonies were grown to a density of ~0.6 OD₆₀₀ in 2 YT medium (250 ml) and induced with IPTG (0.5 mM) for 4-5 h. The culture was lysed by sonication in 10 ml buffer (50 mM potassium phosphate, pH 7.4) and the cell debris was removed by centrifugation (20, 000 X g, 30 min). The supernatant was bound to 200 µl Ni-NTA-agarose affinity matrix for 30 min with slow mixing, packed in a 2 ml mini column and washed with 10 ml buffer (50 mM potassium phosphate, pH 7.4). The column was washed again with 10 ml of the same buffer containing 20 mM imidazole, the proteins were eluted with 0.5 M imidazole in the same buffer and dialyzed against 50 mM potassium phosphate (pH 7.4).

Purification of *E. coli* NDK (*Eco*NDK): The plasmid *pndkec* [pJC20] was introduced into *E. coli* BL21(DE3), and the transformants were grown in 2YT medium (1.8 L) to ~ 0.6 (OD₆₀₀), induced with IPTG (0.5 mM) for 3-4 h before harvesting. The cells were lysed in ~30 ml of 10 mM potassium phosphate pH 7.2, 1 mM Na₂EDTA, 2 mM β- mercaptoethanol using french press (20K cell at 1000-1100 psi) and subjected to centrifugation at 14,000 X g for 90 min. Proteins from the supernatant were subjected to precipitation by ammonium sulfate to 60% saturation, recovered by centrifugation and dissolved in ~10 ml buffer (10 mM potassium phosphate pH 7.2, 1 mM Na₂EDTA, 2 mM β-mercaptoethanol and 10% glycerol)

and dialysed against the same buffer. Further purification of NDK by anion exchange column chromatography (DEAE-sepharose/Source 15Q) and hydroxyapatite column chromatography was carried out essentially as described by Postel and Ab.,(2003) to obtain preparation 'A'. Preparation 'A' was dialyzed against 20 mM Tris-HCl (pH 7.5) and 2 mM β -mercaptoethanol and subjected to hydroxyapatite column chromatography according to Bennet el. Al. The NDK eluted in the flow through to provide preparation 'B' of *Eco*NDK. Both preparations were used for UDG assays.

Uracil DNA glycosylase activity assays: UDG assays were performed on single stranded 5' end labeled 12U30 oligomer (5,000 cpm mixed with 0.5 pmol of unlabeled oligomer) in a 15 μ l reaction containing 10 mM HEPES, pH 7.8, and varying amounts of proteins at 37 °C for 30 min, and stopped by addition of 5 μ l of 0.2M NaOH and heating at 90 °C for 30 min (Acharya, 2002, JMB). The reactions were mixed with 20 μ l 80% formamide dye, separated on 15% polyacrylamide gel containing 8 M urea (Sambrook) and visualized by electronic autoradiography on BioImage analyzer (FLA 2000, Fuji, Japan). For Ugi inhibition assay, 10 μ g of the NDK preparation was incubated with Ugi at room temperature for 15 min followed by on ice for 15 min (Bennett, 1992; Roy S, 1998). In some assays, NDK preparations from *ung⁻* strains were mixed with *Eco*UDG.

Autophosphorylation of NDK: NDK (1 μ g) was incubated with or without 200 ng of Ugi at room temperature for 15 min followed by on ice for 15 min and subjected to autophosphorylation in 15 μ l reactions containing 20 mM HEPES, pH 7.8; 1 mM Na₂EDTA; 100 mM NaCl and 0.5 μ Ci [γ -³²P]ATP (Amlaula, 1995) for 10 min on ice. Reactions were terminated by the addition of 5 μ l of 5 X SDS sample buffer and heating at 90 °C for 5 minutes, and analyzed on SDS-PAGE (15%), stained with Coomassie brilliant blue, destained

extensively using 10 % acetic acid and 10% methanol, and subjected to electronic autoradiography on BioImage analyzer (FLA 2000, Fuji, Japan).

AP-endonuclease assays: The oligomer, 12U30 was 5' end labeled, annealed to the complementary strand as described above, and treated with UDG in 100-200 μ l reactions consisting of 0.4 nM *Eco*UDG, 15 mM HEPES (pH 7.8) and 7.5 mM $MgCl_2$ to generate the AP-DNA. Aliquots (10 μ l) from this reaction containing 0.5 pmol (5,000 cpm) of *Eco*UDG treated duplex were mixed with 5 μ l of NDK preparations (0.2 to 10 μ g protein) or exonuclease III (0.1 unit) and incubated at 37 $^{\circ}C$ for 20-30 min (as indicated in the figure legends) and stopped with 15 μ l of stop solution (90% formamide, 25 mM Na_2EDTA , 20 mM sodium borohydride). A marker for cleavage at AP-site was generated by heating the UDG treated oligomer with 50 mM NaOH at 90 $^{\circ}C$ for 30 min. The products were separated by electrophoresis on a 15% polyacrylamide gel containing 8 M urea and visualized by BioImage analyzer (Fuji).

Results

Purification of NDK proteins: NDK proteins (*Mtu*NDK-His, *Eco*NDK-His and *Eco*NDK) were purified from *ung*⁺ (Fig. 1 A, lanes 1, 3 and 5) as well as *ung*⁻ (Fig. 1 A, lanes 2, 4 and 6) strains of *E. coli*. As shown in Fig. 1 B, all preparations were readily autophosphorylated suggesting that they were functionally active and that they retained native conformation during purification. Importantly, treatment of none of the NDK preparations with Ugi resulted in any apparent change in their autophosphorylation activity (Fig. 1 B, compare lanes 1, 3, 5, 7, 9 and 11 with lanes 2, 4, 6, 8, 10 and 12, respectively).

Uracil processing activity of NDK from *M. tuberculosis*: We used a 5' ³²P- labeled uracil containing synthetic DNA oligomer (12U30) as substrate (S) to detect the uracil excision activity. Cleavage of the glycosidic bond between uracil and the deoxyribose sugar by the UDG generates an apyrimidinic (AP) site in the DNA, which is sensitive to alkaline conditions and results in two fragments, one of which (³²P-labeled) is detected as faster migrating product band (P) upon gel electrophoresis (Fig. 2 A, lanes 1 and 2). Similar assays using *Mtu*NDK-His₆ from *ung*⁺ *E. coli* revealed that 2 μg of the preparation produced detectable UDG activity (lanes 3), and 5 to 10 μg protein resulted in significant uracil processing activity (lanes 4 and 5). Interestingly, as low as 2 ng Ugi (~0.2 pmol) was sufficient to completely abrogate this activity (lanes 6) from 10 μg of *Mtu*NDK-His₆ preparation (>500 pmol of NDK monomer). Hence, it appeared unlikely that physical interaction of Ugi with NDK (with a molar stoichiometry of Ugi:NDK at ~1:2500) accounted for the complete loss of uracil excision activity of *Mtu*NDK preparation. On the other hand, both biochemical and three-dimensional structural characterization of complexes of Ugi with the family I UDGs from various organisms have shown that they interact in a molar stoichiometry of 1:1 (**crystal structures**). Thus, it was important to analyze whether the uracil processing activity of *Mtu*NDK preparation was a result of the presence of contaminating

amounts of *Eco*UDG, known to be a highly efficient enzyme (Lindahl, 1977). Therefore, we purified *Mtu*NDK-His₆ from an *ung*⁻ strain of *E. coli*. Despite the fact that in an autophosphorylation assay, this protein was as active as the one purified from the *ung*⁺ strain (Fig. 1 B, lanes 3 and 4), and despite the fact that the assays were performed under the conditions used by Postel and ... (2003), it showed no detectable uracil processing activity (Fig. 2 A, lanes 8 to 10). Further, when we supplemented this *Mtu*NDK-His₆ with low amounts of *Eco*UDG (2 and 20 ng), it showed complete cleavage of the substrate (lanes 11 and 12) ruling out that lack of uracil processing activity of this preparation was due to the presence of some inhibitors that co-purified with *Mtu*NDK-His₆ from the *ung*⁻ strain. These observations clearly suggest that the UDG activity is not intrinsic to *Mtu*NDK-His₆.

Uracil processing activity of NDK from *E. coli*: Observation that the uracil processing activity of *Mtu*NDK was a consequence of its purification from the *ung*⁺ host, and because the primary sequence of *Mtu*NDK is very similar to that of *Eco*NDK it necessitated re-investigation of the uracil processing activity in *Eco*NDK. Hence, we purified *Eco*NDK-His₆ from *ung*⁺ and *ung*⁻ strains of *E. coli* (Fig. 1). As was the case with the *Mtu*NDK-His₆ preparation, the *Eco*NDK-His₆ preparation from *ung*⁺ *E. coli* showed uracil processing activity (Figs. 2 B, lanes 3 to 5). However, no such activity was observed in the preparation obtained from the *ung*⁻ strain (Fig. 2 B, lanes 8-10). It was also observed that uracil processing activity of up to 10 μg of *Eco*NDK-His₆ prepared from *ung*⁺ strain could be completely inhibited by 2 ng of Ugi (Fig. 2 B, lane 6). And, supplementation of *Eco*NDK-His₆ preparation from *ung*⁻ *E. coli* with 2 or 20 ng of *Eco*UDG resulted in complete processing of the substrate (Fig. 2 B, lanes 11 and 12).

To gain further insights, we repeated our experiments on *Eco*NDK (native primary sequence), which was purified by conventional methods essentially as in an earlier study (). Not unexpectedly, the preparation showed UDG activity (Fig. 2 C, lane 3) which was

completely eliminated by very low levels of Ugi (lanes 4 and 5) in the reaction. Importantly, when the *Eco*NDK preparation was purified further (Bannet), no UDG activity was detectable in *Eco*NDK preparation (lanes 8-10) despite the fact that it retained efficient autophosphorylation activity (Fig. 1 B, lanes 11 and 12). Taken together, these results clearly show that neither *Eco*NDK nor *Mtu*NDK possess intrinsic UDG activities.

Elution profiles of *Eco*NDK and Ugi on Superdex 200: To analyze if *Eco*NDK interacted with Ugi, we chromatographed *Eco*NDK, Ugi, and the mixture of the two proteins on Superdex-200 gel filtration column. As shown in Fig. 3, on this column, NDK elution peaked in fraction 28 and Ugi elution peaked in fraction 34 (*panels a and b*, respectively). When the two proteins were incubated together and chromatographed on Superdex-200 under the same conditions, the NDK and Ugi elution still peaked in fractions 28 and 34, respectively (*panel c*). Furthermore, analysis of the peak fractions on SDS-PAGE (Fig. 3 B) clearly shows that the two proteins eluted independently as distinct peaks. This analysis provided no indication of co-elution of the two proteins suggesting that the two proteins do not interact with one another to any detectable level.

Assays for AP-endonuclease activity using *Mtu*- and *Eco*- NDKs. While the experiments of the kind shown in Fig. 3, did not reveal any interaction between *Eco*NDK and *Eco*UDG (*data not shown*), the observation of detectable UDG activities in the Ni-NTA affinity column purified NDK preparations from *ung*⁺ strain of *E. coli*, could be argued to imply a weak interaction between the two proteins. Hence, to check whether NDKs played any functionally relevant role in the DNA repair pathway at a step downstream of the UDG, we decided to check our NDK preparations for the presence of the reported AP-endonuclease activity. The substrate for AP-endonuclease assays was generated by pretreating the uracil containing duplex DNA (12U30 duplex) with *Eco*UDG. It may be noted that because of the unstable nature of the AP sites, a background level of buffer mediated cleavage, producing

two product bands (Varshney and van de Sande, 1991), is seen in the UDG treated sample (compare lanes 1 and 2). Therefore, the protein specific effects should be observed over and above this background. As a control, when UDG processed oligomer was treated with NaOH, all of the substrate, as expected, was cleaved to produce a single product band (lane 3), suggesting that UDG treatment was complete and generated the desired AP-DNA substrate. Treatment of the UDG processed substrate with exonuclease III (lane 4) served as yet another control, which showed a faster migrating band when compared with NaOH treated sample (lane 3). More importantly, treatment of the AP-DNA with 1 to 10 μg of *Mtu*NDK-His₆, resulted in no apparent increase (over and above the background) in the intensity of any of the bands corresponding to cleavage of AP-site cleavage (compare lanes 5-7 with 2 and 3). Moreover, if the AP-endonuclease activity was present in the NDK preparation, band intensity in lane 7 (10 μg protein) should have been more than that in lane 6 (5 μg protein), which in turn should have been more than the intensity of the bands in lane 5 (1 μg protein). Contrary to these expectations, the intensity across the lanes (lane 5 to 7) was uniform. Similar to *Mtu*NDK-His₆, *Eco*NDK-His₆ also showed no detectable AP-endonuclease activity above the background (lanes 11-13). As a control, no product was detected when the substrate was not pretreated with *Eco*UDG (lanes 8-10, and 14-16). Similar results were obtained when assays were performed in the absence of Mg^{++} (*data not shown*).

To further substantiate that NDKs do not possess AP-endonuclease activity, we assayed the protein pools from various purification steps of one of our preparations of *Eco*NDK (native primary sequence). Analysis of equal amounts of total proteins from various pools on SDS-PAGE, as expected, showed enrichment of *Eco*NDK at each step of purification (Fig. 5 A, lanes 2-6, fractions I to V). The AP-endonuclease assays performed with 0.2 μg of total proteins from various fractions are shown in Fig. 5 A. The assays using Fraction I-III showed efficient AP-DNA processing activities (compare product bands in lane

7 to 9, with the control products obtained by treatment of AP-DNA with NaOH, lane 4, and exonuclease III, lane 6). However, at the purification step resulting in Fraction IV, the AP-DNA processing activity was nearly eliminated (lane 10); and by Fraction V of *Eco*NDK preparation, this activity was undetectable even when 5 μ g preparation was used in the assay (lanes 11-13). As a control, when checked for autophosphorylation activity, Fraction V showed efficient autphosphorylation of *Eco*NDK (Fig. 5 C). Thus, the successive steps of purification of *Eco*NDK resulted in gradual decrease in the AP-DNA processing activity. Had this activity been intrinsic to NDK, we would expect an increase in its specific activity with each step of purification.

Discussion.

Nucleoside diphosphate kinase is an important enzyme in nucleotide metabolism in the cells. Recent studies showed that it has additional activities of uracil DNA glycosylase, AP-endonuclease and dRpase, implicating its role in DNA repair pathways (Postel). To study such DNA repair functions, we purified *Eco*NDK and *Mtu*NDK, with and without hexahistidine tag from *ung*⁺ and *ung*⁻ background of *E. coli* (Fig. 1 A). The autophosphorylation assay with each one of the preparations indicated that the proteins retained their native conformation (Fig 1 B). The *Eco*NDK as well as *Mtu*NDK preparations from *ung*⁺ strains showed detectable UDG activity. However, this activity was fully inhibited by extremely low levels of Ugi protein (2 ng Ugi in 10 µg of NDK preparation). Analysis of *Eco*NDK and Ugi on Superdex-200 column (Fig. 3) showed that the two proteins do not interact with one another. Thus, the Ugi effect (especially when it is added to NDK in more than 2000 fold molar shortage) could not be via its physical interaction with NDK, as argued (Postel). A hypothetical possibility that presence of Ugi somehow altered the conformation of NDK, can be ruled out by the fact that the autophosphorylation activity of NDK was not inhibited even in the presence of 200 ng of Ugi in 1 µg NDK preparation (Fig. 1 B, *panel ii*). Also, the observation that elution profile of NDK on Superdex-200 remained unchanged by the presence of Ugi supports that it does not alter the overall folding of *Eco*NDK (Fig. 3 B).

On the other hand, as Ugi interaction with family I UDGs is highly specific, and it forms a complex with them, which is essentially irreversible under the physiological conditions (Mosbaugh, Narottam, JMB), a straightforward interpretation of these observations is that the NDK preparations from *ung*⁺ strains retained contaminating amounts of Ung. Addition of extremely low levels of Ugi results in complete sequestration of the contaminating Ung into a highly stable complex, which no longer binds DNA (), and therefore, results in total inhibition of UDG activity. More importantly, the NDK preparations

from *ung*⁻ background fail to show any detectable UDG activity, which provides conclusive evidence that UDG activity in the NDK preparations from *ung*⁺ strain was a consequence of contaminating amounts of UDG. As the elution properties of NDK on DEAE-Sepharose and hydroxyapatite, under the conditions used by Postel and III() are common with those of *E. coli* UDG (Ung) on such matrices (Lindahl, 1977), it is quite logical to argue that NDK preparation (Postel//, 2003) might have remained contaminated with UDG. And, because UDG has a very high turnover of ~800 per minute (Lindahl, 1977), it is not surprising to detect its activity even when it is present in extremely low amounts. From a physiological perspective, majority of the mutations in NDK deficient *E. coli* have been shown to be AT to GC transition mutations (Miller JH, 2002, Lu Q 1995 JMB). On the contrary, the loss of UDG activity results in GC to AT mutations (Miller, Bhagwat). Taken together, these observations clearly show that the NDK from *E. coli* as well as *M. tuberculosis* do not possess an intrinsic UDG activity. A recent independent study by Bennett et. al. (2004) on *Eco*NDK also supports the conclusion that UDG activity is not intrinsic to NDK.

The other DNA repair activities reported to be present in *Eco*NDK led us to examine the possibility of AP-endonuclease activity in NDK proteins. However, the data in Fig. 5, are quite convincing to suggest that even such an activity is not intrinsic to NDK. On the other hand, the data () that led to a proposal that *Eco*NDK formed a covalent complex with AP-site containing DNA and catalyzed the lyase reaction apparently lack identification of the shifted band (detected by autoradiography) to correspond to NDK.

Finally, while a recent independent report (Bennett et. al. 2004) also showed that *Eco*NDK lacked UDG activity, it did not address the aspect of the reported AP-endonuclease activity of *Eco*NDK or the interaction of Ugi and *Eco*NDK. In our studies, we used NDK from two diverse groups of bacteria (*E. coli* and *M. tuberculosis*) to establish that UDG activity is intrinsic to neither of these proteins. Furthermore, we show that the *Eco*-, and *Mtu*-

NDKs lack an intrinsic AP endonuclease activity, thereby ruling out a direct role of NDK in base excision repair pathway. Additionally, our studies highlight the essentiality of the use of *ung⁻* strains for testing of novel UDG activities.

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Figure legends

Figure 1: (A) Analysis of purified NDKs (~10 µg each) on a 15 % polyacrylamide-SDS gel. Lanes: 1 & 2, *Mtu*NDK-His₆; 3 & 4, *Eco*NDK-His₆; 5 & 6, *Eco*NDK (native primary sequence) from *ung*⁺ strain of *E. coli*, preparations A (lane 5) and preparation B (lane 6). **(B)** Autophosphorylation assay. Panel (i) shows Coomassie brilliant blue stained gel with ~1 µg of each NDK proteins on a polyacrylamide-SDS gel. Panel (ii) shows electronic autoradiograph obtained by BioImage analyser. Lanes: 1-4, *Mtu*NDK-His₆; 5-8, *Eco*NDK-His₆; 9 & 10, *Eco*NDK (preparation A); and 11 & 12, *Eco*NDK (preparation B). Genetic background of *E. coli* (*ung*⁺ and *ung*⁻) from which the proteins were purified are as indicated above the lanes. The symbols – and + indicate absence or presence of the treatment of NDK preparations (1 µg) with Ugi (0.2 µg) prior to autophosphorylation.

Fig. 1A

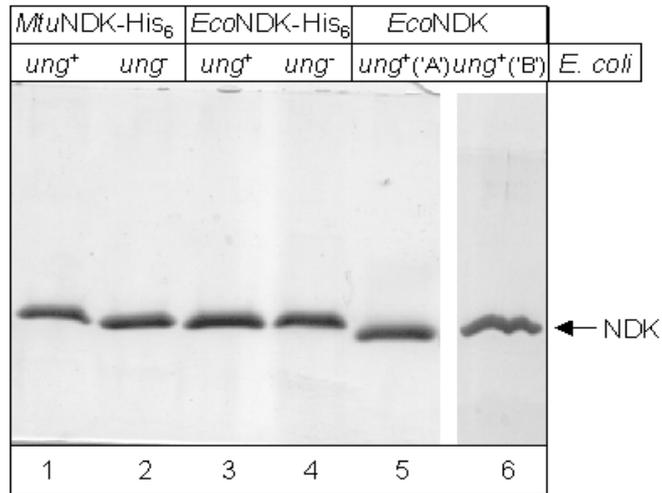


Fig. 1B

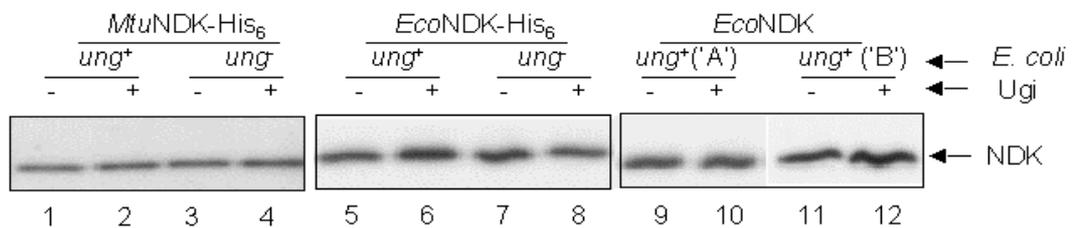


Figure 2: Analysis of NDKs for uracil excision activity. **(A)** Lanes: 1, substrate control; 2, product control; 3-5 assays with 2, 5 and 10 μg of *Mtu*NDK-His₆ (from *ung*⁺ strain), respectively; 6-7, assays with 10 μg of *Mtu*NDK-His₆ (from *ung*⁺ strain) pretreated with 2 and 20 ng of Ugi, respectively; 8-10 assays with 2, 5 and 10 μg of *Mtu*NDK-His₆ (from *ung*⁻

strain), respectively; 11-12, assays with 10 μg of *Mtu*NDK-His (*ung*⁻ strain) supplemented with 2 and 20 ng of *Eco*UDG, respectively. **(B)** Lanes: 1, substrate control; 2, product control; 3-5 assays with 2, 5 and 10 μg of *Eco*NDK-His₆ (from *ung*⁺ strain), respectively; 6-7, assays with 10 μg of *Eco*NDK-His₆ (from *ung*⁺ strain) pretreated with 2 and 20 ng of Ugi, respectively; 8-10 assays with 2, 5 and 10 μg of *Eco*NDK-His₆ (from *ung*⁻ strain), respectively; 11-12, assays with 10 μg of *Eco*NDK-His (*ung*⁻ strain) supplemented with 2 and 20 ng of *Eco*UDG, respectively. **(C)** Lanes: 1 and 6, substrate controls; 2 and 7, product controls; 3, assays with 10 μg of *Eco*NDK (preparation 'A'), respectively; 4 and 5, assays with 10 μg of *Eco*NDK (preparation 'A') pretreated with 2 and 20 ng of Ugi, respectively; 8-10, assays with 1, 5 and 10 μg of *Eco*NDK (preparation 'B'), respectively; 11-12, assays with 10 μg of *Eco*NDK (preparation 'B') supplemented with 2 and 20 ng of *Eco*UDG, respectively.

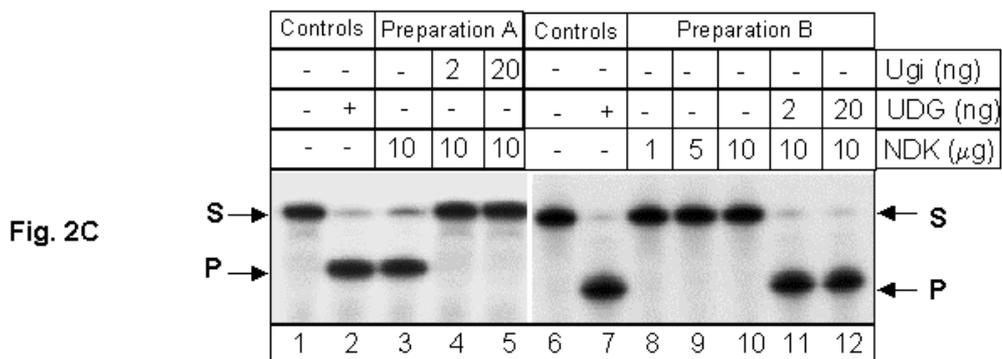
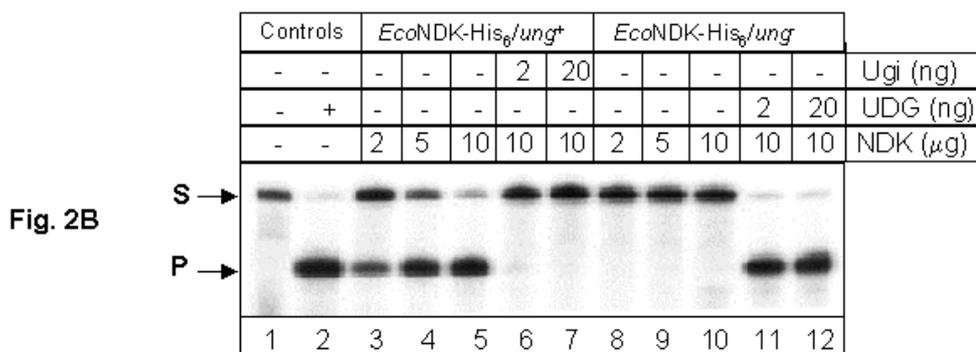
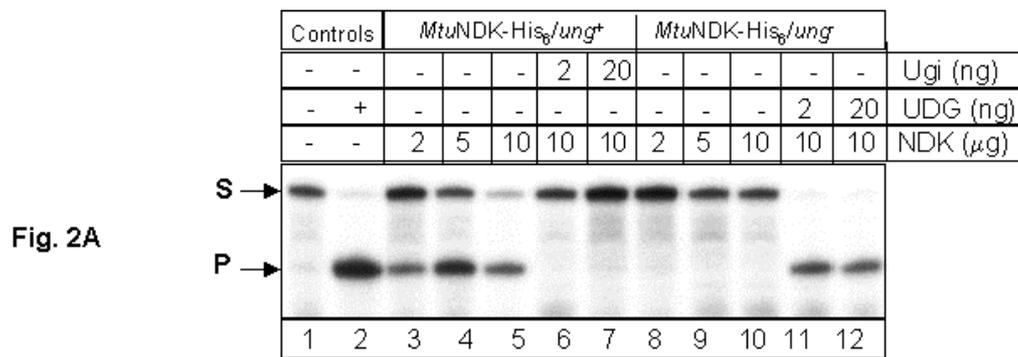


Figure 3: (A) Analytical gel filtration using Superdex-200 (HR 10/30, Amersham Biosciences). Elution profiles of approximately 100 μg of *Eco*NDK (*panel a*), 100 μg Ugi (*panel b*), and a mixture of *Eco*NDK and Ugi, 100 μg each (*panel c*) using 10 mM potassium

phosphate, pH 7.2, 1 mM Na₂EDTA, 2 mM β-mercaptoethanol and 150 mM KCl as elution buffer. To analyze interaction between the two proteins, *Eco*NDK and Ugi were mixed and incubated at room temperature for 30 min followed by on ice for 30 min. All the samples were applied on to the column using a 100 μl sample loop. Fractions (0.5 ml) were collected, and 100 μl aliquots from each were quantified in an ELISA plate set-up using Bradford's reagent (BioRad). The intensity of the resulting color was read at 620 nm, and plotted on the Y-axis against the fraction numbers on the X-axis. **(B)** SDS-PAGE analysis of aliquots (20 μl) from the peak fractions corresponding to gel filtration chromatography shown in *panel c* (fraction numbers are indicated above the lanes).

Fig. 3A (i)

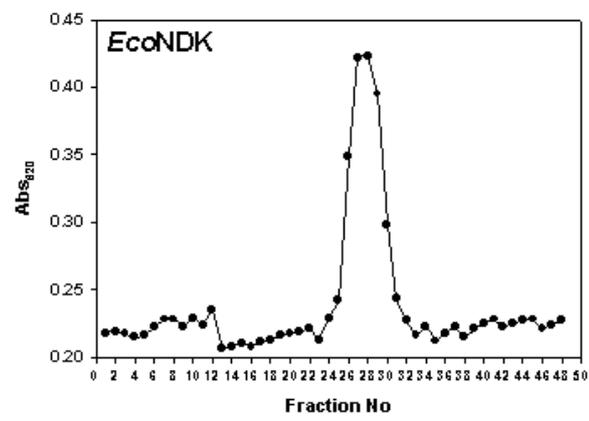


Fig. 3A (ii)

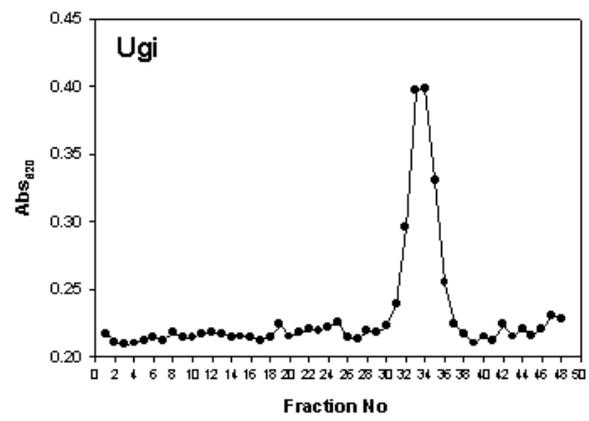
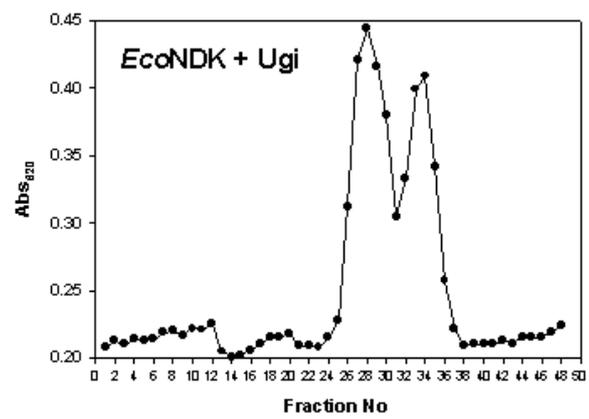


Fig. 3A (iii)



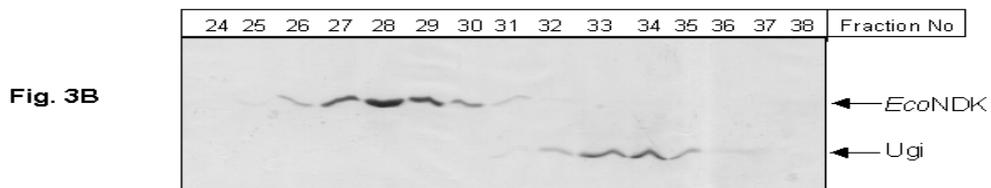


Figure 4: Analysis of AP-endonuclease activity. Assays were carried out as described in Materials and Methods, for 30 min at 37 °C. Lanes: 1, substrate control without *EcoUDG*; 2, substrate control treated with *EcoUDG*; 3, Alkali mediated cleavage of UDG treated sample (positive control); 4, Exonuclease III treatment of UDG treated substrate; 5-7, assays with 1, 5 and 10 µg of *MtuNDK-His₆*; 8-10, incubation of uracil containing DNA oligomer (untreated with UDG) with 1, 5 and 10 µg of *MtuNDK-His₆*; 11-13, assays with 1, 5 and 10 µg of *EcoNDK*; 14-16, incubation of uracil containing DNA oligomer (untreated with UDG) with 1, 5 and 10 µg of with *EcoNDK*.

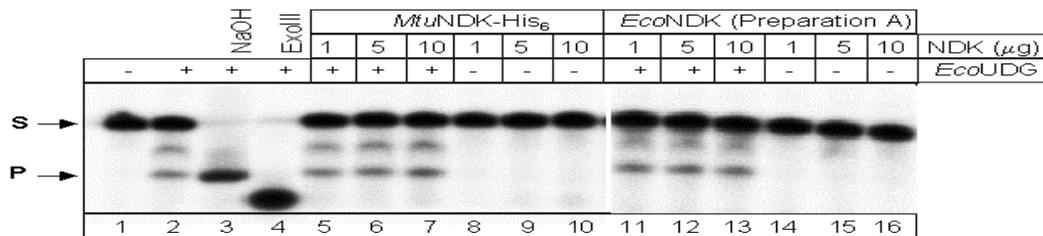


Fig. 4

Figure 5: (A) Analysis of purification of *Eco*NDK. Total proteins (~10 μg) from successive steps of purification were separated by SDS-PAGE (15%) and visualized by Coomassie brilliant blue staining. Lanes: 1, standard markers (M-0671, Sigma Aldrich) corresponding to ~150, ~100, ~75, ~50, ~35, ~25 and ~15 kDa molecular sizes; 2, S100 extract (Fraction I); 3, dialyzed extract following ammonium sulfate fractionation (Fraction II); 4, enriched fractions from Source 15Q column (Fraction III); 5, enriched fractions from hydroxyapatite column (Fraction IV); 6, fraction IV was further purified on DEAE-sepharose and applied to Superdex-75 column. Enriched fractions from Superdex-75 constituted Fraction V. **(B)** Analysis of AP-endonuclease activity in NDK preparations. Assays were carried out as described in Materials and Methods for 20 min at 37 °C. Lanes: 1, 5' end labeled 12U30, uracil containing DNA oligomer; 2, *Eco*UDG and NaOH treated 12U30 as product marker; 3,

12U30 untreated with *Eco*UDG; 4, 12U30 duplex treated with *Eco*UDG and NaOH; 5, 12U30 treated with *Eco*UDG alone; 6, 12U30 treated with *Eco*UDG and exonuclease III; 7-11, *Eco*UDG treated 12U30 was supplemented with ~200 ng proteins from various steps of purification of NDK, respectively; lanes 12 & 13 reactions supplemented with 1 and 5 µg of Fraction V, respectively. **(C)** Autophosphorylation activity of Fraction V. Fraction V (1 µg) was autophosphorylated, electrophoresed of SDS-PAGE, stained with Coomassie brilliant blue (lane 1, CBB) and subjected to electronic autoradiography on BioImage analyzer (lane 2, auto).

Fig. 5A

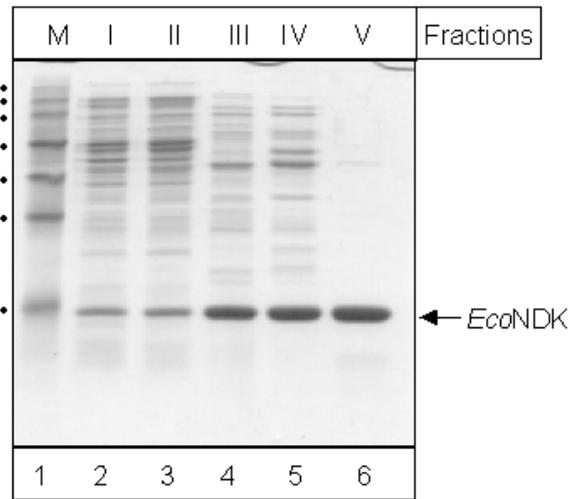


Fig. 5B

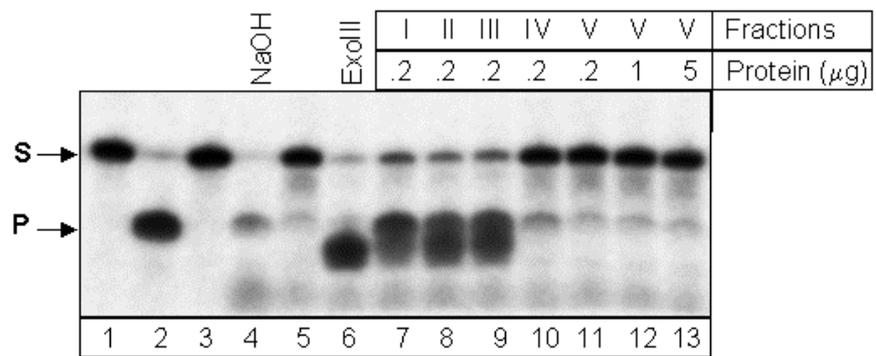


Fig. 5C

