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Mutational consequences of dNTP pool imbalances in *E. coli*

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ABSTRACT

The accuracy of DNA synthesis depends on the accuracy of the polymerase as well as the quality and concentration(s) of the available 5′-deoxynucleoside-triphosphate DNA precursors (dNTPs). The relationships between dNTPs and error rates have been studied *in vitro*, but only limited insights exist into these correlations during *in vivo* replication. We have investigated this issue in the bacterium *Escherichia coli* by analyzing the mutational properties of *dcd* and *ndk* strains. These strains, defective in dCTP deaminase and nucleoside diphosphate kinase, respectively, are characterized by both disturbances of dNTP pools and a mutator phenotype. *ndk* strains have been studied before, but were included in this study, as controversies exist regarding the source of its mutator phenotype. We show that *dcd* strains suffer from increased intracellular levels of dCTP (4-fold) and reduced levels of dGTP (2-fold), while displaying, as measured using a set of *lacZ* reversion markers in a mismatch-repair defective (*mutL*) background, a strong mutator effect for G·C→T·A and A·T→T·A transversions (27- and 42-fold enhancement, respectively). In contrast, *ndk* strains possess a lowered dATP level (4-fold) and modestly enhanced dCTP level (2-fold), while its mutator effect is specific for just the A·T→T·A transversions. The two strains also display differential mutability for rifampicin-resistant mutants. Overall, our analysis reveals for both strains a satisfactory correlation between dNTP pool alterations and the replication error rates, and also suggests that a minimal explanation for the *ndk* mutator does not require assumptions beyond the predicted effect of the dNTP pools.

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1. Introduction

Cells employ multiple mechanisms to ensure an appropriate low error rate during the replication of their genome [1–3]. The responsible DNA polymerases (replicases) avoid errors through a combination of error prevention at the insertion step (base selection) and error correction *via* the exonucleolytic removal of erroneous insertions (proofreading or editing). Following polymerization, postreplicative mismatch repair (MMR) is capable of further reducing the error rate by detecting and correcting mispairs that escaped the proofreading step. In addition, cells employ numerous DNA surveillance and repair pathways aimed at maintaining the DNA in a damage-free state, so as to minimize the entry of damaged, and likely mutagenic, DNA into the replication fork [4].

One important additional factor that is relevant to DNA replication fidelity and has been receiving increasing interest is the status of the DNA precursors, the 5′-deoxynucleoside-triphosphates (dNTPs), both qualitatively and quantitatively. Quality-wise, the cellular dNTPs are 'sanitized' to rid the precursor pool of modified or damaged dNTPs that upon incorporation into the DNA may be toxic and/or mutagenic [5,6]. One example of such sanitizing activity is the MutT protein that removes 8-oxodGTP from the pools [5,7].

In terms of quantities, each of the dNTPs is kept at relatively low concentration in the micromolar range (at only a few % of the corresponding rNTPs). The reason why the dNTPs are kept low is an interesting question that may well relate to the need to keep the error rate low. In addition to overall low levels, the relative ratios of the four dNTPs (dATP, dTTP, dCTP, dGTP) are kept under control, although not at equimolar levels. In general the dGTP level is the lowest in both prokaryotes and eukaryotes [8,9] with the exception of mitochondria [10,11].

The regulation of the cellular dNTP pool occurs at a number of levels. One critical step involves allosteric regulation of the enzyme ribonucleotide reductase (RNR), which performs the important reduction of the ribonucleotides to the corresponding deoxyribonucleotides in an allosterically controlled fashion that (i) limits overall dNTP production and (ii) balances the reduction of the four substrates such that the resulting dNTPs are present in the cell at the desired proportions [3]. In both *Escherichia coli* and human cells this reduction occurs at the level of the nucleoside diphosphates (NDP→dNDP). Once formed, the dNDPs are converted to the dNTP level by the enzyme nucleoside diphosphate kinase (NDK).

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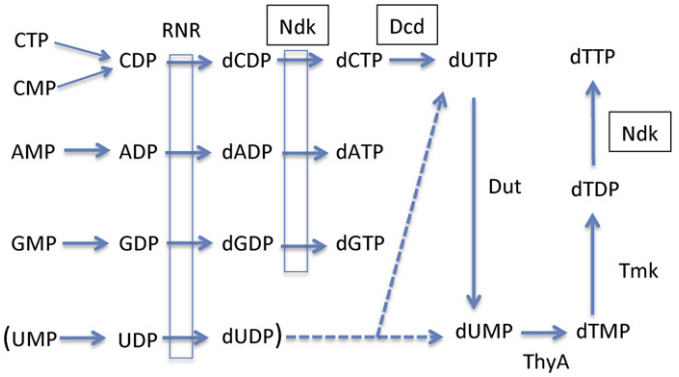

Fig. 1. Pathways for dNTP biosynthesis in *E. coli*. RNR = ribonucleotide reductase, Ndk = nucleoside diphosphate kinase, Dcd = dCTP deaminase, Dut = dUTPase, ThyA = thymidylate synthase, Tmk = dTMP kinase. The pathway in parentheses along with the stippled paths indicates the minor pathway (~20%) for dUMP (and dTTP) synthesis. The major pathway for dTTP synthesis is *via* dCTP and dCTP deaminase.

RNR is a unique enzyme containing two allosteric regulatory sites. The first one regulates overall activity through an (ATP/dATP-controlled) on/off switch (this site is termed the activity site). The second allosteric site determines which substrate can be reduced at any given time (called the specificity site) [3]. Recent studies have described strains of *E. coli* [12] and *S. cerevisiae* [13] carrying amino acid substitutions at sites of RNR allosteric regulation and, consequently, displaying a mutator phenotype (increased mutation rates). Likewise, overproduction of RNR itself is mutagenic [8,14], further supporting the important role of RNR in controlling the cellular mutation rate.

While RNR is a critical component in the dNTP metabolism, it is embedded within a larger network of nucleotide metabolizing pathways, the components of which may also affect the dNTP pools. We have outlined part of this network in Fig. 1. Of keen interest to our present study into the role of dNTPs and mutagenesis are two genes: *dcd*, encoding dCTP deaminase (Dcd) [15], and *ndk*, encoding nucleoside diphosphate kinase (Ndk) [16,17]. As shown in Fig. 1, dCTP deaminase is responsible for converting dCTP to dUTP, representing the first step in the *de novo* synthesis of dTTP [15]. dUTP is rapidly converted to dUMP by dUTPase (*dut* gene product); dUMP is converted to dTMP by thymidylate synthase, and dTMP is converted by two consecutive kinase reactions to the final product dTTP. Altered dNTP pools in *E. coli dcd* mutants lacking dCTP deaminase activity were reported early on [18,19]. As expected, these strains accumulate dCTP and, when grown on synthetic medium, suffer from reduced dTTP levels [18,19]. However, no studies on the mutation rates of *dcd* mutants have been reported, and therefore this strain was chosen presently to analyze relationships between dNTP pools and mutagenesis.

The second genetic defect that we chose for an in-depth analysis of mutagenesis and dNTP pools is *ndk*. Ndk is a multipurpose enzyme capable of converting each of the (d)NDPs to the corresponding triphosphates [20]. Interestingly, in *E. coli* the enzyme is dispensable as its function can be substituted, at least in part, by other kinases, particularly adenylate kinase [21,22]. Deletion of *ndk* caused both dNTP pool changes and a mutator phenotype, which was ascribed to the dNTP pool changes [22,23]. However, this particular explanation for the *ndk* mutator effect has been controversial [24,25]. One other study showed that complementation of the *E. coli ndk* defect with the human NDK homolog hNm23-H2 abolished the mutator phenotype but did not restore the dNTPs to their normal levels [24]. Hence, the dNTP pool alteration was not considered the main cause of the mutator phenotype. Instead, an alternative explanation was forwarded in which the main cause of the mutator effect is related to an increased production of dUTP and its incorporation into DNA instead of dTTP [24,25]. As noted (Fig. 1), dUTP resulting from the deamination of dCTP, is hydrolyzed by dUTPase (*dut* gene product) to yield dUMP, which is then converted to dTMP and eventually dTTP. As dUTP is a close analog of dTTP, differing only in the lack of the 5-methyl group, it is readily incorporated by DNA polymerase, and rapid destruction of dUTP is therefore necessary to prevent frequent incorporation. In additional support for the dUTP hypothesis, a synergistic interaction was reported between *ndk* and the *dut-1* defect, resulting in further enhancement of mutagenesis [24]. On the other hand, the intrinsic mutagenicity of uracil in DNA is low, as is the case for the abasic sites that result from its removal by uracil DNA glycosylase. Therefore, the precise mode of mutagenesis in *ndk* strains remains open to interpretation and is addressed by this study.

In this paper, we investigate in some detail the mutational properties of the *dcd* and *ndk* strains, focusing on the precise types of mutations that are enhanced (mutational specificity). In parallel, we determine the dNTP pool changes in the strains, and subsequently attempt to make meaningful comparisons between the pool changes and the observed mutational specificity. The mutation measurements are made in mismatch-repair-deficient strains (*mutL*) in order to more accurately correlate the dNTP data with replication error rates. Overall, the data indicate that meaningful correlations between dNTP pool changes and the induced replication errors can be derived.

2. Materials and methods

2.1. Strains and media

The *E. coli* strains used are derivatives of strain KA796 (Δ*pro-lac*, *ara*, *thi*) [26]. Strain constructions by P1 transduction were done using P1*virA*. Two mismatch-repair-deficient (*mutL*) derivatives of KA796 were used: NR9559 (*mutL211*::Tn*5*) [27] and NR12443 (*mutL218*::Tn*10*). The *mutL* markers in the two strains derive, respectively, from strains ES1301 and ES1481 [28]. The *dcd-12*::Tn*10dkan* allele [15] was introduced into KA796 and NR9559 (*mutL*) from BW1040 [15] (provided by Dr. Bernard Weiss, University of Michigan) yielding, respectively, NR12572 (*dcd*) and NR12573 (*mutL dcd*). The Δ*ndk*::*cam* allele was introduced into KA796 and NR12443 (*mutL*) from QL7623 [22] yielding, respectively, NR11814 (*ndk*) and NR11816 (*mutL ndk*). Into each of these strains we introduced, by conjugation, the series of F'*prolacZ* episomes originally present in strains CC101 through CC106 [29].

Liquid LB and minimal media (MM) were prepared using standard recipes [30]. Solid medium contained 1.5% agar (Difco). MM medium contained 1× Vogel-Bonner salts [31], 0.4% glucose (MM-Glu) or 0.4% lactose (MM-Lac) as a carbon source, and 5 μg/ml of thiamine. For experiments with *dcd* strains the solid media (LB, MM-Glu, MM-Lac) contained additionally 50 μg/ml of thymidine to improve colony growth on the plates (larger colony sizes). Liquid media, used for generation of mutant frequencies and extraction of cellular dNTP pools (see below), did not contain any added thymidine. Antibiotics, when required during strain constructions, were added at 25 μg/ml (kanamycin), 12.5 μg/ml (tetracycline), or 20 μg/ml (chloramphenicol); LB-Rif plates used for the scoring of rifampicin-resistant mutants contained 100 μg/ml rifampicin (Sigma–Aldrich).

2.2. Mutant frequency determinations

Mismatch-repair-deficient (*mutL*) strains were used for these series of experiments. For each strain, a total of 12–20 independent LB cultures (1 ml each) were initiated from single colonies (one colony per tube). The cultures were grown to saturation on a rotator