Phosphodeoxyribosyltransferases, Designed Enzymes for Deoxyribonucleotides Synthesis

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A large number of nucleoside analogues and 2′-deoxynucleoside triphosphates (dNTP) have been synthesized to interfere with DNA metabolism. However, in vivo the concentration and phosphorylation of these analogues are key limiting factors. In this context, we designed enzymes to switch nucleobases attached to a deoxyribose monophosphate. Active chimeras were made from two distantly related enzymes: a nucleoside deoxyribosyltransferase from lactobacilli and a 5′-monophosphate-2′-deoxyribose nucleoside hydrolase from rat. Then their unprecedented activity was further extended to deoxyribose triphosphate, and in vitro biosyntheses could be successfully performed with several base analogues. These new enzymes provide new tools to synthesize dNTP analogues and to deliver them into cells.

DNA in all known living organisms is synthesized from deoxyribonucleoside triphosphates (dNTPs), the precursor substrates that are condensed by DNA polymerase enzymes, releasing pyrophosphate as co-product. No exception to this biosynthetic scheme was ever encountered in nature, whether nucleoside triphosphates are condensed onto a DNA template acting as co-catalyst, an RNA template, or no template at all. The pyrimidine and purine base moieties attached to the common triphosphodeoxyribosyl moiety of dNTPs are not universally conserved in nature. In addition to the four canonical dNTPs, four noncanonical dNTPs bearing an exotic pyrimidine and one bearing an exotic purine are condensed by bacterial viruses. This shows that DNA can host modified nucleobases that could serve, for example, to extend the genetic code or to induce mutagenesis.

The demand for purified deoxyribonucleotides is high, for example, for DNA synthesis (PCR or DNA microarrays) and reverse transcription in vitro in academic research and medical diagnosis. It extends to numerous nucleoside/nucleotide analogues that are used for DNA mutagenesis or labeling, as well as mechanistic probes to study nucleotide metabolic enzymes. In parallel, these modified precursors are also used as antibiotics, antiviral, and anticancer agents. The diversification of the "biocompatible nucleotides" is also a field in expansion with the development of synthetic biology as illustrated by the development of new functional base pairs or the chemical synthesis and assembly of genomes. Methods for chemical synthesis of nucleotides are evolving, but nucleotides are still difficult to make, isolate, and characterize. Enzyme-mediated syntheses of natural nucleotides and of some analogues have been developed using whole cells or coupled enzymes, but the available repertoire of enzymes is still limited.

The enzymatic synthesis of pure 2′-deoxyribonucleotides and the metabolic engineering for producing such compounds is hindered by the intricacy of biosynthesis and salvage pathways, upstream and downstream of the DNA polymerization step. Each nucleobase A, C, G, and T is indeed processed separately by highly discriminating enzymes that phosphorylate nucleoside monophosphates into diphosphates. In addition, nucleotide reductases are required to convert the ribose moiety of RNA precursors into the deoxyribose moiety of DNA building blocks (rNDP into dNDP or rNTP into dNTP, depending on nucleotide reductase families) through a cumbersome and fragile free radical mechanism. The natural design of this complex metabolic machinery is to prevent the generation of unwanted rNTPs and dNTPs and their incorporation into RNA and DNA. We sought to bypass this intricacy and expand DNA chemistry by elaborating an enzyme that would transfer 5′-phosphorylated deoxyribose between any two nucleobases (N and N′), canonical or synthetic, e.g., dN − (P)x + N ′ ⇌ N + dN′ − (P)x, where x stands for 0, 1, 2, or 3. We proceeded using a structure-based and stepwise approach to build a chimera from two distantly related enzymes: a nucleoside deoxyribosyltransferase (EC 2.4.2.6) from lactobacilli and a 5′-monophosphate-2′-deoxyribose nucleoside hydrolase (EC 3.2.2.1) from rat (Rcl) (Fig. 1). Although

The atomic coordinates and structure factors (code 4HX9) have been deposited in the Protein Data Bank (http://wwpdb.org).

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2 The abbreviations used are: NDT, nucleoside deoxyribosyltransferase; Rcl, 5′-monophosphate-2′-deoxyribonucleoside hydrolase; wtNDT, wild-type NDT; dCTPαS, 2′-Deoxycytidine-5′-O-(1-Thiotriphosphate).
their sequences are globally dissimilar (~18% of sequence identity), these two proteins, whose oligomerization states differ (NDT is an hexamer, and Rcl is a dimer in solution), adopt the same Rossmann fold (Fig. 2A), and they share a common catalytic triad. Both enzymes hydrolyze their substrates via the formation of a deoxyribose (5-phosphate)-enzyme covalent intermediate (10, 11) using a conserved glutamate residue (11). However, these enzymes differ functionally: NDT can act as a transferase on nucleosides (x = 0), whereas Rcl hydrolyzes monophosphate deoxynucleosides (x = 1) (12) (Fig. 1). These structural and functional features are in agreement with Rcl and NDT diverging from a common enzyme ancestor with broader substrate specificity.

Here, we report the structure-based design of Rcl-NDT chimera with mono-, di-, and triphosphate activities. These enzymes can transfer deoxyribose 5-phosphate between canonical bases and also between analogues opening the road to new synthetic pathways of deoxyribonucleotides.

**EXPERIMENTAL PROCEDURES**

**Chemicals**—All of the chemicals were obtained from Sigma-Aldrich except dCTP from Biolog.

**List of Oligonucleotides Used**—The oligonucleotides used were: T7prom, 5'-CGCGAAATTAATACGACTCACTATA-GGGG-3'; T7term, 5'-GGGGTTATGCTAGTTATTGCTCAGGG-3'; F13R, 5'-CTTCGGTGCGGCGTGGCGAGCTGA-CCGCCAAAAC-3'; F13Q, 5'-CTTCGGTGCGGCGTGGCGAGCTGA-CCGCCAAAAC-3'; F13Q-, 5'-TGTTTTGGCGGTCAGTCTGCCAGCCGGCACCGAAG-3'; D92S, 5'-ATCCCTGACGAAAGCGCTGGCATG-3'; N123S, 5'-CTACGGCAAGCCGATCAGCCTCATGAGCTGGGGCG-3'; N123S-, 5'-CGCCCCAGCTCATGAGCTGGATCGGCTTGGCCGTAG-3'; N123T, 5'-CGCCCCAGCTCATGAGGCTGATCGGCTTGGCCGTAG-3'; N123T-, 5'-CGCCCCAGCTCATGAGGCTGATCGGCTTGGCCGTAG-3'; E91Q, 5'-TGTCTACATCCCTGACGAAGAGGACCGGCCTGGGCA-3'; E91Q-, 5'-GGCCGGTCCCTGTTCGTCAGGGATGTAGAC-3'; Y157K, 5'-GCAAGCTTTTACTTTACGGCACCTTCGTAGAAGTCGA-AGC-3'; G92T93, 5'-GTCTACATCCCTGACGAAGAGGACCGGCCTGGGCA-3'; G92T93-, 5'-GTCCCAAGGCCGGTCCCTGTTCGTCAGGGATGTAGAC-3'.

**Construction of the Different Variants**—Variants S92 and R13 S92 were obtained by using plasmid pET24a NDT as DNA template and oligonucleotides D92S and D92S and F13R, respectively, using the QuikChange Multi site-directed mutagenesis kit (Stratagene) according to the manufacturer's protocol. For variants S92 S123, R13 S92 S123, R13 G92 T93, S92 T123, R13 G92 T93 S123, R13 Q91 S92 S123, oligonucleotide N123S-, N123T-, G92 T93-, E91Q-, F13Q-, E91Q-, and T7 prom and oligonucleotide N123S, N123T, G92 T93, E91Q, F13Q, E91Q, and T7 term were used in two separate PCRs using plasmids pET24a NDT S92, pET24a NDT R13 S92, pET24a NDT R13 S92 S123, pET24a NDT R13 G92 T93 S123, pET24a NDT S92 S123, and pET24a NDT Q13 S92 S123 as DNA templates, respectively. The parameters used were...
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1 cycle of 5 min at 95 °C; 25 cycles of 30 s at 95 °C, 30 s at 53 °C, and 30 s at 72 °C; and 1 cycle of 10 min at 72 °C. The annealing temperature was dependent on the pairs of oligonucleotides used. Oligonucleotides T7prom and T7term were used in a second PCR using aliquots of the first one using the same parameters as above with the exception of an annealing temperature of 61 °C.

For variant R13 G92 T93 S123 K157, oligonucleotides T7 prom and V157K were used in a standard PCR using plasmid pET24a NDT R13 G92 T93 S123 as DNA template. The amplified DNA fragments were purified by using the QIAquick PCR purification kit (Qiagen) and then digested with Ndel and BamHI enzymes over 2 h at 37 °C and repurified. Each PCR product was ligated with plasmid pET24a that had been digested with the same restriction enzymes. The ligation mixtures were used to transform strain DH5α. Plasmids with the correct sequence were used to transform strain BLi5.

Overexpression and Purification—Plasmids containing an insert of the correct size were sequenced at the Plateforme Génomique PF1 at the Institut Pasteur. Those with the correct sequences were used to transform strain BLi5.

650 ml of 2× YT medium inoculated with an overnight culture of BLi5 containing any of the pET24a NDT R13 G92 T93 S123 was grown under agitation at 37 °C until A600 = 0.6. Isopropyl-1-thio-β-D-galactopyranoside was added to a final concentration of 1 mM, and the cultures were incubated for 2.5 h. Bacteria were centrifuged, and the pellets were frozen at −20 °C. The cells were resuspended in 20 ml of 50 mM MES, pH 6.0, and broken by one passage through a French press at 14000 p.s.i. Cell debris were pelleted by centrifugation at 12,000 rpm for 20 min at 4 °C.

The total proteins contained in the supernatant were separated by an anion exchange chromatography on a Hi Trap Q HP with a linear gradient from 0 to 300 mM NaCl gradient in 50 mM MES, pH 6.0, at 2 ml min⁻¹ for 100 min. Fractions containing the enzyme were pooled and precipitated at 4 °C by adding solid ammonium sulfate (0.6 mg/ml). The precipitate was pelleted by centrifugation at 12,000 rpm for 20 min at 4 °C. The pellet was resuspended into 50 mM MES, 100 mM NaCl, pH 6.0, and purified on a Hi Load Superdex S200 column at 1 ml min⁻¹. The purified proteins ran as a single band at ~18 kDa, consistent with the predicted molecular weight and was >98% pure as judged by SDS-PAGE with Coomassie Blue staining. Protein concentration was determined spectrophotometrically by UV absorption at 280 nm using an ε^280 = 34,380.

Enzyme Assay—The standard assay for the different NDTs consisted of 5–15 µg of NDT in 50 mM MES buffer, pH 6.0, 3 mM dNTP (dNMP, dNDP, or dNTP), and 1 mM base in a total volume of 50 µl. The assays were incubated for 60 min at 37 °C.

The products of the reactions were analyzed every 7 min by rapid resolution high performance liquid chromatography using a reverse phase column (ZORBAX Eclipse XDB-C18 2.1×50 mm 1.8 µm) with a flow rate of 0.25 ml/min and a linear gradient of 1–12% CH₃CN (2–25% CH₃CN or 1–90% CH₃CN) in 20 mM triethylammonium acetate, pH 7.5, buffer for 7 min. The low resolution mass spectra of the newly synthesized deoxynucleotides were obtained on an Agilent 1200 series LC/MS 6120 quadrupole system using an atmospheric electrospray ionization system. Kinetic parameters kcat and Km were obtained by fitting the initial velocity at various substrate concentrations to a Michaelis-Menten equation using the software KaleidaGraph.

Crystalization and X-ray Data Collection—Crystal growth conditions were screened using cations, anions, and AmSO₄ kits from Qiagen. Crystallization was then optimized in medium concentration of NH₄SO₄ (~1.2 M) in 0.1 M HEPES at pH 7.7, in the presence of glycerol and/or small PEGs (e.g., 2% PEG400). Co-crystallization with ATP or CMP was also attempted. X-ray diffraction data sets were collected from frozen single crystals at the European Synchrotron Radiation Facility (Grenoble, France, Beamline ID29) and processed with the programs MOSFLM (13), SCALA, and TRUNCATE from the CCP4 program suite (14) (see Table 1).

The structure was solved by molecular replacement using the program MolRep (15) and the crystal structure of the wild-type NDT (wtNDT) from Lactobacillus leichmannii (Protein Data Bank code 1F8Y) (16) as a search model. Two crystal forms were obtained both in the I213 trigonal space group, but only the one with the larger cell (a = b = c = 218.5 Å versus 151.3 Å) diffracted well. The structure of PDTT2 was solved at 2.7 Å and refined using the program COOT (17) and the program REFMAC5 (18), using a translation/liberation/screw model (19) (see Table 1). The refined model and structure factors have been deposited in the Research Collaboratory for Structural Biology under the following accession number: Protein Data Bank code 4HX9.

Comparative Modeling and Ligand Docking—Once a crystal structure of a mutated NDT was made available, we used it to model the other variants to analyze the docking of nucleotides. Three-dimensional models were built using @TOME-2 (20). Three-dimensional conformation of the desired deoxynucleosides/deoxynucleotides (adenosine and cytosine series) were extracted from Pubchem (21) in SDF format and rewritten MOLE format to dock them using the software PLANTS (22). The results will be described elsewhere.³

RESULTS

Structure Guided Design of a Monophosphodeoxyribosyltransferase—To have a deoxyribosyltransferase with the broadest substrate specificity, we attempt to reconstruct the hypothetical common ancestor to Rcl and NDT by phylogenetic means using the software PAML 4.3 (PMID:17483113). The resurrected proteins were poorly soluble and inactive (not shown), so an alternative approach was to compare the sequences and structures of NDT and Rcl to create a chimera with the desired activities.

NDT was chosen as a starting template because its transferase activity was more interesting from the synthetic point of view because it exchanges deoxyribose between pyrimidines and purines and, vice versa, between two pyrimidines and to a lesser extent between two purines. Furthermore, it tolerates substitutions on the base. The idea was to keep intact the catalytic core while reshaping the substrate specificity. First, two amino acids, Asp^92 and Asn^123 of NDT, were substituted to neutral and smaller serines. In wtNDT, these aspartate and

³ J. L. Pons and G. Labesse, manuscript in progress.
asparagine interact with the 5′-OH group of 5-methyl-2′-deoxyapurine (Fig. 2B) by forming hydrogen bonds and prevent entrance of a larger and negatively charged phosphate group. At equivalent positions in the active site of Rcl, two series (Ser87 and Ser117, respectively) were found (Fig. 2B). These changes confer to the NDT single mutant D92S (hereafter PDTM1, for phosphodeoxyribosyltransferase monophosphate) and the double mutant D92S,N123S (PDTM2) the ability to transfer deoxyribose 5-phosphate between cytosine and adenine, although with a low efficiency (Table 1). Overlay of the active sites of Rcl and NDT also predicts a steric clash between F13 and an incoming phosphate group (Fig. 2B).

Upon substitution of Phe13 to the positively charged arginine (to give PDTM3 from PDTM1 or PDTM4 from PDTM2), the deoxyribose 5-phosphate transferase activity between C and A is enhanced by a factor of 50. Finally, we also substituted the Phe13 by a polar but neutral glutamine (PDTM5) leading to significant improvements in activity (Table 1).

The phosphodeoxyribosyltransferase activity is not restricted to cytosine as donor and to adenine as acceptor (Table 2). PDTM3 is also able to transfer deoxyribose 5-phosphate between A and C and between C and T but with 2- and 4-fold lower activity than between C and A. All mutants transfer also deoxyribose 5-phosphate between A and G, whereas the reaction is almost undetectable when hypoxanthine is the acceptor base in agreement with previous observations showing that the deoxyribose transfer activity of NDT between a purine and hypoxanthine is low (23). PDTM4 is twice more active than PDTM3, whatever the couple of bases tested. In contrast, PDTM5 transfers deoxyribose 5-phosphate efficiently between C and A and between A and C but is much less active for the transfer from C to T. Both PDTM4 and PDTM5 are much more active with the couple A and G than PDTM3. This improvement is mainly due to a better affinity for dCMP, the $K_m$ for dCMP varying from 22 mm (PDTM3) to 11 or 12 mm (PDTM4 and PDTM5, respectively) (Table 3).

The better affinity of PDTM4 and PDTM5 over PDTM3 confirms the predicted contact involving the 5′-position of the ligand and the residue occupying the position 123 and the requirement for additional space to accommodate the phosphate group. Surprisingly, the mutant PDTM4 and PDTM5, differing by the nature of the residue at position 13 (arginine versus glutamine), show distinct specificity in the type of nucleobases to transfer. The arginine side chain was expected to mainly stabilize the negatively charged phosphate group, whereas the glutamine was predicted to point into the active site and stabilize the entering ligand through hydrogen bonding. Although the arginine is supposed to have little contact

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**TABLE 1**

| Data collection and refinement statistics for PDTT2 |
|-----------------------------------------------------|
| **PDTT2**                                          |
| **Beamline** | ID29 |
| **Data collection** | |
| Space group | I213 |
| Cell dimension, $a=b=c$ (Å) | 218.5 |
| No. of molecules (asymmetric unit) | 8 |
| Wavelength (Å) | 0.9793 |
| Resolution (Å) | 2.68 |
| $R_{merge}$ (%) | 9.0 |
| $I/|I|_{av}$ | 10.9 |
| Completeness (%) | 99.8 |
| Redundance (%) | 5.1 |
| B-Wilson | 64.1 |
| **Refinement** | |
| Resolution (Å) | 2.69 |
| No. of reflections | 48494 |
| $R_{work}/R_{merge}$ (%) | 19.8/24.9 |
| No. of protein atoms | 10091 |
| No. of water molecules | 213 |
| Ligand type | 14 SO4 |
| Ligand type | 8 PEG |
| Protein | 48.1 |
| Ligand (SO4/PEG) | 66.6/75.6 |
| Water | 44.8 |
| Root mean square deviations (%) | 0.10 |
| Bond lengths (Å) | 1.79 |
| Bond angles (°) | 1.17 |

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$^a$ The values in parentheses refer to the outermost resolution shell.

$^b$ $R_{merge} = \frac{\sum_{i} \sum_{j} |I_{i,j} - \langle I_{i,j} \rangle|}{\sum_{i} \sum_{j} I_{i,j}} \times 100$.

$^c$ $R_{work} = \frac{\sum_{i} \sum_{j} |I_{i,j} - \langle I_{i,j} \rangle|}{\sum_{i} \sum_{j} \langle I_{i,j} \rangle} \times 100$.

$^d$ $R_{merge}$ is calculated in the same way on a subset of reflections that are not used in the refinement (5%).

$^e$ Deviation from ideal values.

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**TABLE 2**

| Specific activities of alignment-based PDT variants in the presence of different purines and pyrimidines as donors and acceptors of deoxyribose 5-phosphate |
|--------------------------------------------------------------------------------|
| **Reactions** | Enzymes |
|--------------------------------------------------|--------|
| dCMP + A dAMP + C | wtNDT | 0.1 | 1.8 | 1.7 | 79.7 | 170 | 296 | 46.3 | 78 | 286 |
| dAMP + C dCMP + A | PDTM1 | 34.7 | 77.6 | 206 | 23.7 | 5 | 31 |
| dCMP + T dTAMP + C | PDTM2 | 18.2 | 52 | 1.7 | <0.1 | <0.1 | 1.1 |
| dGMP + A dAMP + G | PDTM3 | 8.5 | 57 | 81 | 49 | 32 | 50 |
| dAMP + Hx dIMP + A | PDTM4 | 2 | <0.1 | <0.1 | <0.1 | <0.1 | <0.1 |
| dCDP + A dADP + C | PDTM5 | <0.1 | 31 | 66 |
| dADP + C dCDP + A | PDTT0 | 12 | 8 |
| dCDP + U dUDP + C | PDTT1 | 6 | 8 |
| dGDP + A dADP + G | PDTT2 | 15 | 33 |
| dADP + Hx dDIP + A | <0.1 | <0.1 |
| dCTP + A dATP + C | <0.1 | 1.7 | 10 | 51 |
| dATP + C dCTP + A | 1 | 1.5 |
| dCTP + T dTTP + C | 1 | 1.4 |
| dGTP + A dADT + G | <0.1 | 25 | 36 |
| dATP + Hx dDTP + A | <0.1 | <0.1 |

$^a$ The donor concentration was 3 mm, and the acceptor concentration was 1 mm.

$^b$ The NDT and PDT sequences are: NDT, Phe13-Glu92-Asp93-Val95-Asn123-Tyr157; PDTM1, Ser117; PDTM2, Ser92; Ser123; PDTM3, Arg13-Ser92; PDTM4, Arg13-Ser92; Ser123; PDTM5, Glu13-Ser92; Ser123; PDTT0, Arg13-Gly92-Thr93 Ser123; PDTT1, Arg13-Gly92-Thr93 Ser123-Lys157; and PDTT2, Arg13-Gln92-Gly92-Thr93 Ser123.
with the incoming nucleobase, the glutamine may contact it. In such a case the hydrophobic nature of the 5-position (methyl in thymine) may disfavor the transfer from C to T. In agreement, 5-halogenated uracils are better incorporated (see below; Table 4).

Accommodation of the Phosphate-binding Site to Di- and Triphosphate—At this stage we reasoned that we should try to build a larger phosphate binding site and take advantage of the macrodipole of the central helix bearing the common catalytic glutamate (Glu98 in NDT) as observed in the Rcl-GMP complex (24). A more drastic reshaping of the active site was attempted by replacing the two amino acids, Asp and Val, with Gly and Thr at positions 92 to 93 (PDTT0) to remove a negatively charged residue and simultaneously make the backbone amide groups more accessible to better accommodate a phosphate group. Subsequently, in the context of the variant PDTT0 (PDTT for phosphodeoxyribosyltransferase triphosphate), we either added another positively charged residue (mutation Y157K; named PDTT1) or removed a negative charge (E91Q; PDTT2) to favor further the entrance of nucleotides.

To track possible structural rearrangements induced by the mutations, several variants (PDTM1, PDTM4, and PDTT2) were tested for crystallization. Crystals of the apo form of PDTM1 and PDTT2 were obtained, and the structures were solved by molecular replacement. The crystals of PDTM1 diffracted at best to 3.5 Å, and the resulting structure appeared identical to the parent one and was not further refined. Conversely, the structure of PDTT2 could be refined to 2.7 Å. This crystal possesses a large unit cell that is composed of one dimer and one biologically relevant hexamer. The dimer belongs to a similar hexamer thanks to the 3-fold crystallographic symmetry.

The overall structure is little affected by the five mutations with a root mean square deviation of 0.9 Å compared with the hexameric and wtNDT (Fig. 3A). The observed variation is in part locally because of the mutations and in part because of the absence of bound ligand. Interestingly, the mutated loop 91–93 either is partially disordered or adopts two distinct conformations. In one conformation (e.g., chains A and B), a sulfate ion is

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**TABLE 3**

Kinetic parameters of PDT variants with an enlarged phosphate binding site in the presence of adenine as acceptor and deoxycytidine 5'-mono-, -di-, and -triphosphate as donors of deoxyribose 5-phosphate

| Enzymes | Reaction | $K_m$ (mM) | $k_{cat}$ (s$^{-1}$) | $k_{cat}/K_m$ ($s^{-1}$ M$^{-1}$) |
|---------|----------|------------|---------------------|-------------------------------|
| PDTM3   | dCMP + A D dAMP + C | 22 | 0.3 | 13.6 |
| PDTM4   | dCMP + A D dAMP + C | 11 | 2.1 | 192 |
| PDTM5   | dCMP + A D dAMP + C | 12 | 1.99 | 166 |
| PDTT0   | dCMP + A dAMP + C | 9.2 | 0.45 | 48.9 |
| PDTT1   | dCMP + A dAMP + C | 4.1 | 0.46 | 112 |
| PDTT2   | dCMP + A dAMP + C | 6 | 0.9 | 153 |
| PDTT1   | dCDP + A D dADP + C | 8.9 | 0.08 | 8.9 |
| PDTT2   | dCDP + A dADP + C | 8.7 | 0.1 | 11.5 |
| PDTT1   | dCTP + A D dATP + C | 5.5 | 0.5 | 103.7 |
| PDTT2   | dCTP + A dATP + C | 2 | 1.3 | 668.3 |

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**TABLE 4**

Specific activities of PTM4, PDTM5, and PDTT2 variants in the presence of different donors and nucleoside analogues as acceptors of deoxyribose 5-phosphate or deoxyribose 5-triphosphate

The donor concentration was 3 mM, and the acceptor concentration was 1 mM except for 2 mM dCTP, 1 mM 5-BrU, 2 mM dAMP, 1 mM 5-FU. Activities are expressed in nanomoles of deoxynucleotides synthesized-per min/pg of protein.
bound at the dimeric interface in between two mutated loops Gln<sup>91</sup>—Thr<sup>93</sup> (data not shown). In the second conformation (e.g., chains E and F), one sulfate ion is observed within both substrate-binding sites. Superposition with the wtNDT in complex with 5-methyl-2′-deoxypseudouridine shows significant local rearrangements. Indeed, the presence of glycine 92 (Asp<sup>92</sup> in wtNDT) allows the glutamine 91 (instead of Glu<sup>91</sup> in Rcl) to reorient its side chain (Fig. 3B). These two residues provide two nitrogen atoms for hydrogen bonding with the incoming sulfate in addition to the backbone nitrogen of glycine 94 and leucine 95. Only one equivalent hydrogen bond is observed between the protein and the phosphate group in the high resolution crystal structure of an inhibited form of Rcl, we recently solved (Protein Data Bank code 4FYI) (47). In fact, in Rcl, serine 87 (corresponding to Ser<sup>92</sup> in PDTM1) partially occupies the place of the sulfate ion observed in the PDTT2 structure (data not shown). Accordingly, the newly formed sulfate-binding pocket in PDTT2 does not match perfectly the phosphate-binding pocket observed in Rcl. Indeed, the overlay of its active site with that of Rcl bound to N<sup>6</sup>-cyclopentyl-AMP (C6C) shows that this sulfate ion localized in close contact with the phosphate group of the bound ligand (Fig. 3C).

To better explore the binding capabilities of each variant, we used a combination of comparative modeling and focused virtual screening using the server @TOME-2 (20) and the software PANTS (22) to dock deoxynucleotides (adenosine and cytosine series) extracted from Pubchem (21). This docking survey suggested that the new variants can accommodate di- and potentially triphosphate nucleotides. The above observations and computations prompted us to test whether PDTT0-PDTT2 variants could accept diphosphate and triphosphate deoxyribonucleotide as substrates.

This hypothesis was confirmed by monitoring the specific activity, in the presence of dCDP or dCTP as donor and adenine as acceptor (Table 2). The kinetic parameters were determined for the best variants with dCMP, dCDP, and dCTP as substrates, at a concentration of A being fixed (Table 3). The <i>K<sub>m</sub></i> for adenine was between 80 and 160 μM when measured using constant and saturating concentrations of dCMP, dCDP, or dCTP, irrespective of the enzyme tested (data not shown).

PDTT0, PDTT1, and PDTT2 are still able to transfer deoxyribose 5-phosphate between bases; however, if the <i>K<sub>m</sub></i> for dCMP is better than those measured for PDTM3, PDTM4, and PDTM5 (9.2 mM for PDTT0, 4.1 mM for PDTT1, and 6 mM for PDTT2), their catalytic efficiencies are not enhanced (Table 3). The enhanced affinity in PDTT1 and PDTT2 over PDTM3, PDTM4, and PDTM5 can be linked to the additional space left to accommodate the phosphate group. However, this may not favor the binding of the phosphorylated deoxyribose in a better orientation for efficient catalysis. The last substitutions may let the phosphate explore a larger space and displace the deoxyribose from an optimal configuration.

In addition to their ability to transfer deoxyribose 5-phosphate, PDTT1 and PDTT2 also exchange deoxyribose 5-di- and -triphosphate between bases. PDTT2 has the highest catalytic efficiency so far for both di- and triphosphorylated nucleotides. The improvement of deoxyribose triphosphate transferase of PDTT2 is mainly due to a better affinity for dCTP (2 mM) and a more efficient catalytic efficiency (668 s<sup>−1</sup> M<sup>−1</sup>). In Vitro Synthesis of Deoxynucleotide Analogues—Because NDT exhibits a broad specificity for the acceptor base, from substitutions on the heterocycle part to non-pyrimidine rings, simplified purines, or expanded guanine (25–28), enzymatic syntheses were attempted using PDTM4, PDTM5, and/or PDTT2, with different deoxynucleotides donors and bases as acceptors (Fig. 4 and Table 4).

All the different variants, tested here, were able to transfer deoxyribose 5-phosphate, deoxyribose 5-triphosphate or deoxy-
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yribose 5-1-thiotriphosphate between cytosine or adenine and analogues of natural bases such as 5-fluorouracil, 5-bromouracil, 2,6-diaminopurine, 6-chloropurine, and 2,6-dichloropurine. The efficiency of the transfer is dependent on the nature of the acceptor base and roughly follows that of NDT with its natural substrates (23).

DISCUSSION

Early studies on N-deoxyribosyltransferases revealed that these enzymes have a broad tolerance toward modifications of the purine or pyrimidine (25, 29, 30). In contrast, they have a strict specificity for 2-deoxyribose as a carbohydrate moiety, even if purine 4’-thionucleosides have been synthesized using L. leichmannii NDT (31). We previously showed that a single mutation was sufficient to enlarge the specificity of NDT to 2,3-dideoxyribose and to 2,3-didehydro-2,3-dideoxyribose (32), which indicated that the plasticity of NDT was even more important than initially thought.

Here, we show that the conservation of overall topology and of the catalytic mechanism of the members of the enzyme family N-deoxyribosyltransferase was essential information to guide the design of the different phosphodeoxyribosyltransferases. As reported by Gerlt and Babbitt, “few success in enzyme (re)design were reported, although many have been attempted” (33). Our work exemplifies how natural evolution can be rewired to design novel biocatalysts, a concept whose applicability has been demonstrated in a few cases so far (34, 35).

These variants are now an alternative to chemical or enzymatic phosphorylation of deoxyribonucleosides. Whereas chemical phosphorylation is widely used, it requires several steps of protection, deprotection, and purification (36). It is only recently that a one-pot synthesis of deoxyribonucleoside 5’-triphosphates without any protection on the nucleosides was reported (37). Enzymatic methods using dNMP have been attempted, but they require dNMP kinases and pyruvate kinases (38, 39). The total synthesis of dNTP from deoxyribonucleosides is even more complex because the first step of deoxyribonucleosides phosphorylation is highly specific. In human, four kinases are required: TK1, TK2, dGK, and dCK (40). Nevertheless, a unique multisubstrate enzyme, Dm-dNK from Drosophila melanogaster, presents a high specific activity and a broad substrate specificity (41, 42). Its ability to phosphorylate nucleoside analogues has made this enzyme an interesting candidate for suicide gene therapy in cancer (43). In this context, the design of efficient phosphodeoxyribosyltransferases opens the road for a promising approach to deliver cytotoxic nucleotides in cancer cells.

In summary, we have added a new branch to the evolutionary tree of the nucleoside 2-deoxyribosyltransferases, which now contains two novel sets of variants: those with slightly altered substrate specificity (i.e., only active on 2’-deoxyribonucleotide 5’-monophosphate such as PDTM4 or PDTM5) and those with a much broader specificity (PDTT1 and PDTT2). The latter have the unique property of transferring deoxyribose 5-mono-, -di-, -triphosphate, and 1-thiotriphosphate between various nucleobases. This property is unlikely to exist in nature because nucleotide metabolic enzymes are highly specialized and recognize usually one particular substrate. To our knowledge only herpesvirus type I thymidine kinase can recognize two different substrates differing by their phosphorylation state: thymidine and thymidylate (44, 45). The two novel enzyme lineages elaborated during this work, monophosphodeoxyribosyltransferase and triphosphodeoxyribosyltransferase, are now ready for improvement of their catalytic activity and diversification of their specificity through directed evolution, once adequately implemented in vivo. The utility of such enzymes is multiple: as tools for chemists to synthesize deoxynucleotides enzymatically; for geneticists to manipulate genomes by controlled mutagenesis; and for synthetic biologists to incorporate unnatural deoxynucleotides in vitro and in vivo, thereby enabling the exploration of genotypic and phenotypic options so far unselected by natural evolution of nucleic acids (46).

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