Antiviral nucleoside analog therapies rely on their incorporation by viral DNA polymerases/reverse transcriptase leading to chain termination. The analogs (3’-deoxy-3’-azidothymidine (AZT), 2’,3’-dideoxy-2’,3’-dideoxymethylene (d4T), and other dideoxynucleosides) are sequentially converted into triphosphate by cellular kinases of the nucleoside salvage pathway and are often poor substrates of these enzymes. Nucleoside diphosphate (NDP) kinase phosphorlylates the diphosphate derivatives of the analogs with an efficiency some $10^4$ lower than for its natural substrates. Kinetic and structural studies of *Dictyostelium* and human NDP kinases show that the sugar 3’-OH, absent from all antiviral analogs, is required for catalysis. To improve the catalytic efficiency of NDP kinase on the analogs, we engineered several mutants with a protein OH group replacing the sugar 3’-OH. The substitution of Asn-115 in Ser and Leu-55 in His results in an NDP kinase mutant with an enhanced ability to phosphorylate antiviral derivatives. Transfection of the mutant enzyme in *Escherichia coli* results in an increased sensitivity to AZT. An x-ray structure at 2.15-A resolution of the *Dictyostelium* enzyme bearing the serine substitution in complex with the $R_o$-beta-triphosphate derivative of AZT shows that the enhanced activity reflects an improved geometry of binding and a favorable interaction of the 3’-azido group with the engineered serine.

Nucleotide analogs such as dideoxynucleosides, AZT, and d4T are widely used in clinics for their antiviral effects, in particular in the treatment of AIDS. Because the sugar moiety of these nucleoside reverse transcriptase inhibitors (NRTI) lacks a 3’-OH group, their incorporation by viral DNA polymerase or reverse transcriptase leads to DNA chain termination. To be substrates of DNA synthesis, an analog must first be converted to the 5’-triphosphate form, which is done intracellularly by kinases of the nucleoside salvage pathway. Whereas the first two phosphorylation steps are catalyzed by enzymes specific for the nucleobase, the γ-phosphate is added by nucleoside diphosphate (NDP) kinase, which exhibits little specificity toward the nucleobase and the sugar moiety (1). The γ-phosphate transfer from N$_1$TP to N$_2$DP catalyzed by NDP kinase involves a phosphohistidine intermediate,

$$E + N_1TP \rightleftharpoons E \sim P + N_2DP \quad \text{REACTION 1}$$

$$E \sim P + N_2DP \rightleftharpoons E + N_1TP \quad \text{REACTION 2}$$

All eukaryotic NDP kinases are homohexamers with a 17-kDa subunit (2). In humans, where eight isoforms have been reported, the major isoforms NDPK-A and NDPK-B, respectively encoded by the genes *nm23-H1* and *nm23-H2*, display 88% sequence identity and have very similar kinetic parameters (3). They closely resemble the NDP kinase from the lower eukaryote *Dictyostelium discoideum* (Dd-NDPK), which for most purposes is as a reliable model of other eukaryotic NDP kinases (4), easier to purify and crystallize than human NDP kinases. NDP kinases have a very high turnover rate on natural nucleotides, but their catalytic efficiency drops by a factor of $10^4$ on the analogs AZT diphosphate or ddNDP (5). This is attributed to the substrate-assisted catalysis mechanism of NDP kinase, where the 3’-OH plays a major role. Using fluorescence stopped-flow experiments to study the two half-reactions (Reactions 1 and 2), we have previously shown that affinity is reduced 10-fold and phosphotransfer 500- to 1000-fold slower in the absence of the 3’-OH (6). The poor activation of NRTI by NDP kinase, resulting in low amounts of the triphosphate form of NRTI within infected cells, is of clinical importance. It is a major cause of incomplete suppression of viral DNA synthesis, allowing the selection of resistance mutations (7).

To overcome this limitation, we designed new NRTIs with increased reactivity toward NDP kinase: the $\alpha$-bromo derivations of AZT-TP, Rp-$\alpha$-bromo-AZT triphosphate; NDP kinase, ATP, nucleoside diphosphate phosphotransferase (EC 2.7.4.6); PK, phosphoenolpyruvate kinase (EC 2.7.4.0).
tives of AZT and d4T (8). Alternatively, we may consider modifying the kinase based on its known structure and reaction mechanism. In the present study, we attempt to substitute a hydroxyl group of the enzyme for the missing 3'-OH of the analogs. The 3'-OH of natural nucleotides is involved in a key hydrogen bonds network with the β-phosphate and two side chains of the active site. We hypothesized that the presence of a hydroxyl provided by the protein could compensate for the absence of the 3'-OH and enhance either the substrate binding or the rate of phosphotransfer for antiviral analogs activation.

**EXPERIMENTAL PROCEDURES**

**Chemicals**—Natural nucleotides (NDP and NTP) and dideoxynucleoside triphosphates were from Roche Molecular Biochemicals. The synthesis of the diphospho- and triphospho-derivatives of AZT, d4T, and acyclovir was as described previously (5). Pyruvate kinase was purchased from Fluka, and lactate dehydrogenase was from Sigma Chemical Co. Rp-α-boron-AZT-triphosphate (RB-AZT-TP) synthesis has been described in Ref. 8.

Expression and Purification of Wild-type and Mutated NDP Kinases—Human NDPK-A mutants were obtained by PCR method using the overlap extension strategy. The oligonucleotides 5′-ATACAAGTT-GGCAGGAGCATTATACGTGGCAGT-3′ and 5′-GGACACTGCGTGCACTGGCAATCATTCC-3′ and their complementaries were used to introduce N115S and L55H mutations, respectively, in NDPK-A. Mutations in Dd-NDPK were introduced using site-directed mutagenesis (9) with the oligonucleotides 5′-ATGGTTGGTAGAACCATCAGCCGT-3′, 5′-ATGGTTGGTAGAACCATCAGCCGT-3′, and 5′-ATGGTTGGTAGAACCATCAGCCGT-3′ for the N119S, N119T, and N119Y mutations, respectively. Changes from the wild type sequence are underlined in boldface. Sequences were checked by automatic sequencing. The IE-N119S mutant was obtained by mutation of the previously described F64W/H122G double mutant (called IE) devoid of catalytic properties (10).

The wild type and mutant human NDPK-A were expressed and purified according to Ref. 10. Wild type and mutant Dd-NDPK were obtained as described previously (6) except for the N119Y mutant, which was partially purified by Q-Sepharose FF chromatography. Each protein was characterized by SDS-PAGE electrophoresis. Enzyme concentrations were expressed as 17-kDa subunits, based either on the Bradford assay (11) or on the absorbance coefficient: ΔA_{280} = 1.249 for a 1 mg/ml solution of human NDPK-A, or ΔA_{280} = 0.55 for Dd-NDPK.

**Steady-state Kinetic Experiments**—The activity of NDP kinase was measured at 20 °C with ATP and dTDP as substrates using coupled enzymes (pyruvate kinase and lactate dehydrogenase) (12). One unit is measured at 20 °C with 3 mg/ml of human NDPK-A, or 1 mg/ml in the case of Dd-NDPK.

**Crystal Structure of the Complex of R.p-boron-AZT Triphosphate with IE-N119S**—The H122G-N119S-F64W (IE-N119S) variant of Dicysteolium NDP kinase was cocrySTALLized with R.p-boron-AZT triphosphate (RB-AZT-TP). Crystals appeared within 1 week in hanging drops containing 8 mg/ml protein, 100 mM MES, pH 6.5, 20 mM MgCl_{2}, 10 mM zine sulfate, and 12% polyethylene glycol 550 monomethylether in the same buffer. They belong to the hexagonal space group P6_{3}, with unit cell a = b = 71.2 Å, c = 106.7 Å. The asymmetric unit contains a dimer.

X-ray diffraction data from a single crystal were collected at 1.54 Å resolution (10) by an Enraf-Nonius CAD-4 diffractometer. The data were collected in a single exposure measuring 2θ = 0–90°. All data were merged with the program DENZO and SCALEPACK (13) and further processed using the CCP4 program (14). Although overall statistics are good for the 2.15 Å resolution limit (Table I), the presence of ice rings on some of the images affected the data quality in the range 2.8–2.5 Å. Molecular replacement was done with AMoRe and the 1.8 Å model of wild type Dd-NDPK (15). Electron density maps were examined using Turbo-FRODO (16). The first (2F_{o} – F_{c}) electron density map showed easily interpretable density at the three mutation sites and for the bound nucleotide, which was initially built as AZT triphosphate. The presence of Mg^{2+}, and that of boron with five electrons replacing the R_{b} oxygen (eight electrons) in the α-phosphate, became apparent after few cycles of refinement. Water molecules were gradually added during further conjugate gradient refinement with CNS (17). Residues 2–5 are missing in the final model for each monomer. The model has good stereochemistry; the relatively high value of R_{cryst} (21.2%) and R_{free} (29.5%) at 2.15 Å resolution, is largely due to data in the range 2.8–2.5 Å.

**AZT Toxicity Screening in Escherichia coli**—The sensitivity to AZT of E. coli transformed with NDP kinase expression vectors was evaluated. Bacteria BL21(DE3) (Stratagene) were transformed by heat shock with pJC20 vectors expressing either the wild type NDPK-A (pJC20-RA), the mutant enzyme N115S (pJC20-N115S), the double mutant enzyme L55H-N115S (pJC20-L55H-N115S) or without insertion (pJC20). Bac-
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Fig. 1. Active site of Dictyostelium NDP kinase. Part of the subunit in the complex of the wild type enzyme with thymidine diphosphate (21) (PDB entry 1NDC). The nucleotide interacts with a Mg$^{2+}$ ion and several amino acid side chains drawn in ball-and stick representation. His-122 becomes phosphorylated during catalysis, quenching the fluorescence of Trp-137 nearby.

Table II

Changes in the kinetic parameters of N119S mutant NDP kinase

| N119S NDP kinase, $k_2/K_s$ | N119S-IE, $K_A$ |
|-----------------------------|---------------|
| $\mu^{-1} \cdot s^{-1}$     | $\mu^{-1}$    |
| ATP                         | 2.5 $\times$ 10$^3$ (0.05) | 4.2 $\times$ 10$^3$ (0.08) |
| ddATP                       | 2700 (2)       | 3.8 $\times$ 10$^2$ (1.8)  |
| GTP                         | 7 $\times$ 10$^3$ (0.01)  | 9.4 $\times$ 10$^3$ (0.14) |
| ddGTP                       | 3500 (1.5)     | 2 $\times$ 10$^3$ (1.7)    |
| Acy-TP                      | 1650 (4.7)     | 5 $\times$ 10$^2$ (9)      |
| TTP                         | 4.3 $\times$ 10$^3$ (0.07) | 1.9 $\times$ 10$^3$ (0.2)  |
| AZT-TP                      | 1100 (4)       | 4.5 $\times$ 10$^3$ (13)   |

Fig. 2. Pre-steady-state kinetics of phosphotransfer between the phosphorylated NDPK and NDP analogs. $A$, kinetics of reaction of phosphorylated Dictyostelium wild type and N119S NDP kinases by 100 $\mu$m acyclovir diphosphate (Acy-DP). The phosphorylated enzyme (one P per subunit) was prepared according to Ref. 22 in buffer T (50 mM Tris-HCl, pH 7.5, 5 mM MgCl$_2$, and 75 mM KCl). The increase in fluorescence upon mixing the phospho-enzyme (1 $\mu$m, final concentration) with Acy-DP in buffer T at 20 °C was monitored with stopped-flow analysis. The solid lines represent the best fit of each curve to a monoeponential. $B$, concentration dependence of the rate constant on Acy-DP concentration. The pseudo-first order rate constant for the reaction ($k_{o,b}$) was plotted against Acy-DP. Fits indicate that data can be analyzed as a second order reaction with apparent constants of 670 $\mu^{-1} \cdot s^{-1}$ for the wild type NDP kinase (●) and 4500 $\mu^{-1} \cdot s^{-1}$ for the N119S mutant (■).

Strategy for Improving the Enzyme Specificity Toward Nucleoside Analogues—We decided to introduce an OH group at a location in the NDP kinase active site where it could substitute for the missing 3'-OH of the antiviral nucleotide analogs. The choice of the residue to be mutated arose from structural and catalytic considerations. Previous work indicated that the 3'-OH of the substrate receives two hydrogen bonds from the conserved side chains of Lys-16 and Asn-119 (Dictyostelium NDP kinase numbering) and donates one to the oxygen bridging the β- and γ-phosphates (18) (Fig. 1). The latter hydrogen bond activates the phosphate oxygen for transfer and is crucial for catalytic efficiency. Its absence in the nucleotide analogs drastically affects the rate of phosphate transfer (5, 6). Removing the Lys-16 side chain, which also interacts with the γ-phosphate, is less drastic, but the loss of activity is still large, a factor of 100 in the K16A mutant (19). In contrast, the deletion of the amide group in the N119A mutant causes little loss of catalytic efficiency (19, 20). Thus, we introduced OH-bearing side chains (Ser, Thr, or Tyr) at position 119. The N119Y mutant protein proved to be unstable and poorly active (0.05% of wild type activity) and was not further studied. The N119T and N119S mutants were expressed and purified to homogeneity.

RESULTS

Mutations in the Active Site of the Catalytic Subunit—Part of the subunit in the complex of the wild type enzyme with thymidine diphosphate (21) (PDB entry 1NDC). The nucleotide interacts with a Mg$^{2+}$ ion and several amino acid side chains drawn in ball-and stick representation. His-122 becomes phosphorylated during catalysis, quenching the fluorescence of Trp-137 nearby.

teria were grown at 37 °C in M9 liquid medium supplemented with casamino acids in exponential phase, then 10 μM isopropyl-thio-β-galactopyranoside was added. After 1 h, AZT (10$^{-7}$ to 10$^{-4}$ mg/ml) was added to cells. After 4-h incubation, the cell viability was measured by plating 1 ml of bacteria onto LB agar. The cells were counted after overnight incubation at 37 °C.

RESULTS

Strategy for Improving the Enzyme Specificity Toward Nucleoside Analogues—We decided to introduce an OH group at a location in the NDP kinase active site where it could substitute for the missing 3'-OH of the antiviral nucleotide analogs. The choice of the residue to be mutated arose from structural and catalytic considerations. Previous work indicated that the 3'-OH of the substrate receives two hydrogen bonds from the conserved side chains of Lys-16 and Asn-119 (Dictyostelium NDP kinase numbering) and donates one to the oxygen bridging the β- and γ-phosphates (18) (Fig. 1). The latter hydrogen bond activates the phosphate oxygen for transfer and is crucial for catalytic efficiency. Its absence in the nucleotide analogs drastically affects the rate of phosphate transfer (5, 6). Removing the Lys-16 side chain, which also interacts with the γ-phosphate, is less drastic, but the loss of activity is still large, a factor of 100 in the K16A mutant (19). In contrast, the deletion of the amide group in the N119A mutant causes little loss of catalytic efficiency (19, 20). Thus, we introduced OH-bearing side chains (Ser, Thr, or Tyr) at position 119. The N119Y mutant protein proved to be unstable and poorly active (0.05% of wild type activity) and was not further studied. The N119T and N119S mutants were expressed and purified to homogene-
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The catalytic efficiencies were measured at the pre-steady state by fluorescence stopped-flow experiments. Italicized numbers in parentheses indicate the fold change in catalytic efficiency when compared to wild type NDPK-A. The specificity change \( R \) reflects the ability of a mutant enzyme to prefer the analog rather than the natural nucleotide according the expression, \( R = \frac{([\text{CE}_{\text{drug}}]/[\text{CE}_{\text{nucleotide}}]_{\text{mutant}})}{([\text{CE}_{\text{drug}}]/[\text{CE}_{\text{nucleotide}}]_{\text{wt enzyme}})} \), where \( R \) is the ratio of the specificity factors of the mutant compared to the original enzyme. The specificity factor of an enzyme is defined as the ratio of the catalytic efficiencies \( (CE = k_{cat}/K) \) for a nucleotide analog and for the natural nucleotide.

| NDPK-A, \( k_{cat}/K_N \) \( \times 10^6 \) s\(^{-1}\) |  | L55H | N115S | L55H-N115S |
|-----------------|-----------------|--------|--------|-------------|
| TTP              | 1.2 \times 10^6 | 2 \times 10^6 (1.6) | 1 \times 10^5 (0.08) | 4.5 \times 10^5 (0.33) |
| ddTTP            | 20              | 200 (10) | 170 (6) | 2800 (140)  |
| AZTTP            | 75              | 930 (12) | 900 (12) | 6770 (90)   |
| ddTTP            | 700             | 1290 (1.8) | 6290 (9) | 55400 (80)  |
| dGTP             | 3.6 \times 10^6 | 6.8 \times 10^6 (2) | 2.4 \times 10^5 (0.07) | 5 \times 10^5 (0.14) |
| AcyTP            | 25              | 160 (6)  | 250 (10) | 2600 (100)  |
| ddGTP            | 190             | ND*     | 750 (4) | 4200 (22)   |

* ND, not determined.

The enzyme phosphorylation and dephosphorylation (half-reactions; Reactions 1 and 2) were measured by stopped-flow following the intrinsic protein fluorescence. Table II shows that, in the N119S mutant, the rate of phosphorylation decreases by a factor of 10 to 20 when the phosphate donor is a natural substrate (ATP, GTP, and TTP). On the other hand, it increases by a factor of 1.5 to 5 when the donor is an analog lacking a 3’-OH (dATP, ddGTP, acyclovir, or AZT triphosphates). No such gain was observed in the N119T mutant (data not shown). Acyclovir triphosphate is the best analog substrate of N119S NDP kinase. Acyclovir is a guanosine analog used against herpes simplex virus. The enhanced reactivity of acyclovir derivatives with the N119S mutant is also observed at the dephosphorylation step (Fig. 2). At any given concentration of acyclovir diphosphate, the rate of phosphate transfer is increased by a factor of 7 in the N119S mutant as compared with wild type NDP kinase.

The effect of the mutation on the binding affinity was measured on the IE variant (H122G-F64W) of Dictyostelium NDPK, which lacks the catalytic histidine and has a tryptophan at the site where the nucleobase moiety of the substrate binds. In this mutant, the substrate cannot transfer a γ-phosphate, but its binding can be followed by monitoring tryptophan fluorescence (10). The N119S mutation was inserted in IE, and the serine contribution to binding was evaluated (Table II). In the mutant, affinity dropped by a factor of 4 to 12 for the natural substrates, whereas it improved for the antiviral analogs: by a factor of 2 for ddTTP, 9 for acyclovir triphosphate, and 13 for AZT triphosphate. This suggests that most of the change in catalytic efficiency \( (k_{cat}/K_N) \) results from changes in affinity. Unlike the wild type, the mutant protein does not discriminate against the dideoxy derivatives, and it actually binds the AZT derivative better than the natural substrate dTTP.

**Improving Human NDP Kinase—Dictyostelium NDP kinase has a higher specific activity (2000 units/mg) than the human type A and type B enzymes (1200–1400 units/mg). The active sites of the three proteins are identical except for one residue, Leu-55 in the human enzymes replacing His-59 in Dictyostelium. His-59 interacts with the α-phosphate (21) (Fig. 1), and this interaction is lost in the human enzyme. Aiming to enhance the activity of human NDP kinase and improve its ability to phosphorylate nucleotide analogs, we replaced Leu-55 by His and Asn-115 (equivalent to Asn-119 in Dictyostelium enzyme) by Ser. The N115S, L55H, and L55H-N115S variants of human NDPK-A were expressed in E. coli and purified to homogeneity. Their activity on natural substrates ATP and dTDP was studied in the steady state. L55H, N115S, and L55H-N115S had specific activities of 1900, 140, and 240 units/mg, respectively, under standard test conditions. Thus, the L55H substitution results in a 1.6-fold activation, and suffices to reproduce the high activity of the Dictyostelium wild type enzyme. The N115S substitution causes a 10-fold drop in activity as it does in the N119S Dictyostelium mutant. The N115S substitution was also made in human NDPK-B, with similar results.**

Phosphorylation of the catalytic histidine of NDPK-A quenches the intrinsic protein fluorescence (22). The amplitude was somewhat lower (5%) in the two variants carrying the L55H mutation, but still sufficient to monitor protein phosphorylation in the stopped-flow as previously described (6). With the natural substrates dTTP and dGTP, the same effects were observed under pre-steady state and steady-state conditions: the L55H mutation increased the catalytic efficiency of protein phosphorylation by a factor of 1.6 to 2; the N115S mutation decreased it by a factor of 12 to 15. In contrast, with the dideoxy, AZT, d4T, and acyclovir derivatives, each mutation separately proved to be beneficial. L55H improved catalytic efficiency by a factor of 2 to 12, N115S by 4 to 10 (Table III). Moreover, the effects of the two mutations proved to be additive. Thus, the double mutant has a much improved catalytic efficiency relative to wild type NDPK-A, by a factor of 22 for ddGTP, 140 for ddTTP, and about 100 for the derivatives of AZT, d4T, and acyclovir. These effects are illustrated in Fig. 3 in the case of d4T triphosphate.

The improvement in enzyme performance in the mutant proteins is even more important when comparing the specificity factors, defined as the catalytic efficiency on a given analog divided by that of the corresponding natural substrate. In Table III, \( R \) is the ratio of the specificity factors of the double mutant to that in the original enzyme. The L55H mutation in human NDP kinase has modest effects on \( R \) ranging from 6 to 10 and the N115S mutation has a much larger effect, near 100 (not shown). This results in values of \( R \) of up to 460 for the double mutant. It is remarkable that the double mutant is almost as efficient as d4T-TP as with TTP, illustrating the specificity switch of the mutated enzyme.

**Structural Consequences of the N119S Mutation on Analog Binding—**We determined the 2.15-Å x-ray structure of the H122G-N119S-F64W (IE-N119S) variant of Dd-NDPK to further analyze the effect of the N119S mutation. The absence of the active site histidine enables crystallization with a triphosphate derivative, here with \( R^+\alpha\)-borano-AZT-triphos-
phosphate (RB-AZT-TP). As can be seen in Fig. 4A, the nucleotide analog binds at the same site and in the same orientation as the natural substrate thymidine diphosphate. In this complex, the thymine base stacks on the aromatic ring of Phe-64. In IE-N119S, the same interaction takes place, the base stacking on Trp-64, which replaces the phenylalanine. The triple mutation has essentially no effect on the protein conformation, the root mean square deviation of the Cα positions being root mean square = 0.57 Å compared with the wild type enzyme complexed with dTDP (21). This validates the use of the F64W mutant in kinetic and equilibrium studies of NDP binding and of the IE variant for NTP binding.

The geometry of binding of RB-AZT-TP to IE-N119S is detailed in Fig. 4A. The triphosphate moiety of RB-AZT-TP and the bound Mg2+ ion superimpose on those of other complexes with nucleoside triphosphates where Mg2+ ligates all three phosphate groups (8). The BH3 group in Rα position of the α-phosphate, apparent in the electron density, which is lower
at the $R_p$ than at the $S_p$ position, makes no interaction with the protein. In the $S_p$ position, the BH$_3$ modification would interfere with metal binding and yield an inactive analog (8).

In Fig. 4B, RB-AZT-TP bound to IE-N119S is compared with AZT diphosphate bound to N119A Dd-NDPK (20). Significant differences are observed, which relate to the presence of a Ser in position 119. The base and phosphate moieties of the analogs superimpose to within 1 Å, but the modified sugar ring shifts by 1.5 Å and it rotates in its plane, displacing the azido group in the 3' position by up to 4 Å. In IE-N119S, the azide group receives a hydrogen bond from the hydroxy group of Ser-119 (Fig. 4B). This bond replaces the bond made by the 3'-OH of a natural substrate with the amide group of Asn-119 in the wild type enzyme. The movement of the azido group also enables the side chain of Lys-16 to retain the extended conformation observed in complexes with NTP substrates and interacts with the $\gamma$-phosphate. In the N119A-AZT diphosphate complex, the lysine side chain moves away to make room for the azido group and does not interact with the ligand.

**Effect of L55H and N115S Mutations on E. coli Sensitivity to AZT**—The effect of the mutant NDP kinases in a cellular background was tentatively evaluated. We asked whether the change in specificity introduced by the mutations in NDP kinase could result in increased toxicity of an analog due to its incorporation during cellular DNA synthesis (23). We tested the sensitivity to nucleoside analogs of E. coli expressing human NDPK-A. AZT was chosen for this assay instead of d4T, because d4T phosphorylation by thymidine kinase is slow and is possibly limiting (24), whereas the step catalyzed by NDP kinase is limiting with AZT.

Wild type and mutant NDPK-A were overexpressed in E. coli, and the sensitivity of exponentially growing cells to AZT was assayed after induction of NDP kinase expression by isopropyl-1-thio-$\beta$-D-galactopyranoside. The viability was estimated by plating the cells and counting. The levels of expression were checked by Western blot and found to be similar for the wild type and mutant enzymes (not shown). As shown in Fig. 5, bacteria transfected by the plasmid without insertion (pJC20) are insensitive to AZT up to a concentration of 2.5 mg/ml. Sensitivity increases in bacteria overexpressing wild type NDPK-A and further increases with the L55H-N115S double mutant. In the 5–10 mg/ml range of AZT concentration, the enhanced sensitivity of the mutant roughly correlates with the specificity factors cited in Table III.

**DISCUSSION**

The substitution of a serine at the Asn-119 (Dictyostelium) or Asn-115 (human) position improves the capacity of NDP kinase to use as substrates nucleotide analogs lacking a 3'-OH. The gain in catalytic efficiency is less than an order of magnitude in Dd-NDPK, but it reaches two orders of magnitude when the N115S and L55H mutations are combined in NDPK-A. It can be noted that directed mutagenesis aimed at a small number of sites leads here to an efficient improvement of the kinetic parameters of the target. The improvement factor is actually larger than was obtained by directed evolution methods when, for instance, the thymidine kinase of herpes virus was modified for instance the thymidine kinase of herpes virus was modified to incorporate proximity effects: the activation free energy decreases as the substrate and the enzyme catalytic groups are held in the right orientation and position for the reaction (27). The correlation between affinity and catalytic efficiency is excellent for NDP kinase.$^2$ Other major enhancements of the affinity of an enzyme for a transition state analog by a single hydroxyl group have been reported (for example see Ref. 28).

Our results suggest that the N115S and L55H mutations

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$^2$ S. Gallois-Montbrun, B. Schneider, Y. Chen, V. Giacomini-Fernandes, L. Mulard, S. Morera, J. Janin, D. Deville-Bonne, and M. Veron, unpublished results.
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might be of particular interest for improving the cellular activation of AZT or d4T. Gene transfer of such a potentiated mutant could improve the cytotoxicity of the analog on the transfected cells. An application could be cell therapy strategies in reative medicine (29). The clinical potential of cell therapies is widely accepted for neurodegenerative and metabolic disorders such as diabetes mellitus, Parkinson’s disease, and others. The use of embryonic or bone marrow stem cells as a therapy presents a problem of proof of safety, linked to proliferation and differentiation (30). The improved NDP kinase gene could render cells used in reative medicine sensitive to AZT doses that are otherwise harmless for non-transfected cells. The mutant NDP kinase could then play the role of a suicide enzyme in case of uncontrolled proliferation (31, 32). The overexpression of the mitochondrial deoxyguanosine kinase in human pancreatic adenocarcinoma cell lines was reported to enhance sensitivity of the cells to CdA, araG, and dFdG (33). Promising results have already been obtained by transfection with herpes simplex thymidine kinase that in-

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