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Communication

Modifying Human Thymidylate Kinase to Potentiate Azidothymidine Activation*

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Based on the knowledge of the crystal structures of yeast and *Escherichia coli* thymidylate kinases (TmpKs) and the observation that TmpK from *E. coli* can phosphorylate azidothymidine monophosphate (AZT-MP) much more efficiently than either the yeast or the highly homologous human enzyme, we have engineered yeast and human TmpKs to obtain enzymes that have dramatically improved AZT-MP phosphorylation properties. These modified enzymes have properties that make them attractive candidates for gene therapeutic approaches to potentiating the action of AZT as an inhibitor of human immunodeficiency virus (HIV) replication. In particular, insertion of the lid domain of the bacterial TmpK into the human enzyme results in a pronounced change of the acceptance of AZT-MP such that it is now phosphorylated even faster than TMP.

One of the main reasons for the less than optimal properties of nucleoside prodrugs in the treatment of HIV[1] infection is their poor phosphorylation to the active triphosphate form by cellular enzymes. In the case of 3′-azido-3′-deoxythymidine (AZT), the bottleneck in its activation appears to be addition of the second phosphoryl group to AZT-monophosphate (AZT-MP) by thymidylate kinase (TmpK) (1, 2). Earlier work suggested that a specific and unique feature of the highly homologous yeast and human TmpKs (Fig. 1) is responsible for the low efficiency of these enzymes in phosphorylating AZT-MP. This is the fact that an arginine at position X_3 of the P-loop (the $GX_1X_2X_3X_4GKS(T)$ motif involved in fixing the α- and β-phosphates of ATP (3)) appears to play an important catalytic role but is mispositioned because of steric hindrance between the azido group of AZT-MP and the preceding carboxylic acid side chain of Asp-14 (position X_2) (4, 5). A catalytic role was assigned to Arg-15 of yeast TmpK based on the observations that in the structure with the bisubstrate inhibitor P^1-(5′-adenosyl)-P^5-(5′-thymidyl)pentaphosphate (TP_5A) this residue interacts with the γ-phosphate of the ATP moiety and that its mutation to glycine results in a 200-fold decrease of phosphorylation activity with the natural substrate, dTMP (Table I). Interestingly, TmpK from *E. coli* has no arginine in its P-loop motif but rather several arginine residues in its lid region, a flexible stretch of amino acids that in adenylate kinase becomes ordered upon ATP binding (6). One or more of these arginines in *E. coli* TmpK were predicted to assume the catalytic role assigned to the P-loop arginine of the yeast enzyme. Therefore, we anticipated that the bacterial TmpK would be less affected by the presence of the azido group in AZT-MP, and this prediction proved to be correct (7). The crystal structure of the *E. coli* enzyme suggested that the role of Arg-15 in the yeast enzyme is taken over by Arg-153 from the lid region (8). Using this knowledge we have engineered yeast and human TmpKs to obtain enzymes that have dramatically improved AZT-MP phosphorylation properties.

EXPERIMENTAL PROCEDURES

Cloning, Generation of Mutants, and Protein Purification—Yeast thymidylate kinase was overproduced and purified as described (4). To facilitate purification, the pJC20 vector was modified (pJC20HisC) such that a Gly-Ser-$(His)_6$ tail is fused to the C terminus of the authentic yeast protein. This extension has no influence on the activity of the wild type protein. All DNA inserts were cloned as *Nde*I/*Bam*HI-restricted fragments. Mutants were generated by established polymerase chain reaction methods, notably by using the gene fusion by overlap extension strategy. All constructs were verified by automatic DNA sequencing (Applied Biosystems 373 Sequencer) and data analysis to confirm the presence of the desired mutation and clone integrity.

Wild type and mutant forms of human thymidylate kinase were produced as GST-fusion proteins in *E. coli* strain BL21(DE3) using a modified expression plasmid pGEX-2T (Amersham Pharmacia Biotech), which allowed insertion of *Nde*I/*Bam*HI-restricted fragments into its multiple cloning site. The coding region of the human gene to be transferred into this vector was generated by polymerase chain reaction from a cDNA clone that was a generous gift of Dr. R. A. Sclafani. Expression of the recombinant protein was induced with 0.5 mM isopropyl-1-thio-β-D-galactopyranoside at 25 °C overnight. Bacteria were lysed by sonication, and cleared cell lysates were passed over a glutathione-Sepharose column. After extensive washing with phosphate-buffered saline, the protein was cleaved from the beads with thrombin (using a molar ratio of about 1:500) for 1–3 h. The cleavage reaction was stopped by the addition of benzamidine-Sepharose (Amersham Pharmacia Biotech). The protein thus obtained carries a Gly-Ser-His extension at the N terminus; it was stored at −20 °C in the presence of 20% glycerol. Nucleotide sequencing of the entire coding region of human wild type thymidylate kinase (cDNA clone) revealed several deviations from the published data (9, 10) (GenBank™ accession numbers X54729 and L16991) at amino acid positions 31–37, 58, 183/184, and 190/191. The sequence that we determined for our clone is identical to that published by Huang *et al.* (10), with the exception of amino acid 58, which in our case is Gln instead of Lys in agreement with the publication of Su and Sclafani (9).

Kinetic Measurements—The catalytic activity of TmpK was measured at 25 °C using a modified coupled colorimetric assay essentially as described (11) with the following assay buffer: 100 mM Tris-HCl, pH 7.5, 100 mM KCl, 10 mM $MgCl_2$, 0.5 mM phosphoenol pyruvate, 0.25 mM NADH, 5 units of lactate dehydrogenase, 4 units of pyruvate kinase, 2 mM ATP, and either 1 mM dTMP or AZT-MP.

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[1] The abbreviations used are: HIV, human immunodeficiency virus; AZT, azidothymidine; AZT-MP, AZT-monophosphate; TmpK, thymidylate kinase; TP_5A, P^1-(5′-adenosyl)-P^5-(5′-thymidyl)pentaphosphate.

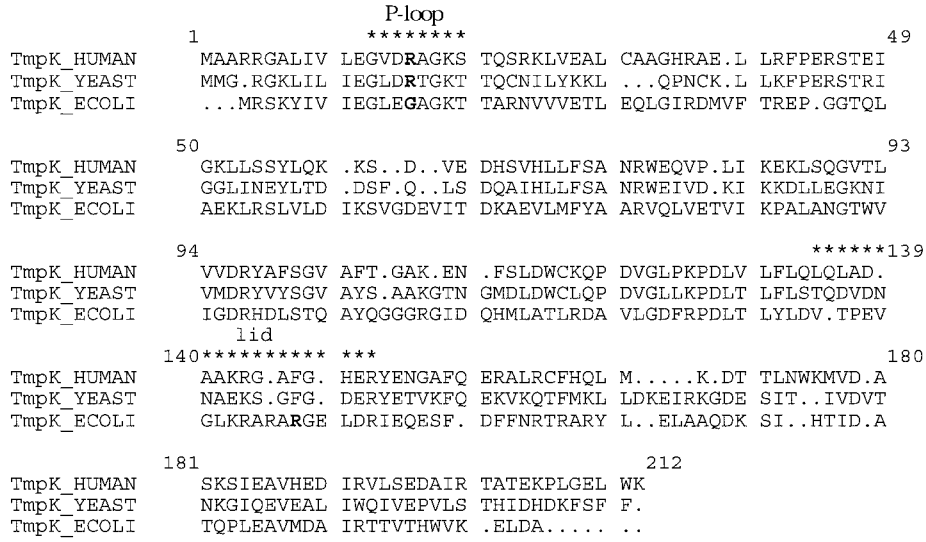

FIG. 1. **Structure-based sequence alignment of human, yeast, and *E. coli* TmpK.** The P-loop and lid sequences are marked with *stars*, and the *numbering* is according to the human enzyme. Both yeast and human TmpK have an arginine in the P-loop (shown in *bold*), which in the yeast TmpK-TP_5A complex structure (7) was observed to interact with the phosphate that would correspond to the γ-phosphate of ATP. *E. coli* TmpK lacks this arginine, having a glycine instead, but compensates with an arginine that originates from the lid region (*bold*). Note the longer lid region of the *E. coli* enzyme with respect to the yeast enzyme (1 amino acid shorter) and the human TmpK (2 amino acids shorter)

TABLE I

Steady state kinetics of yeast thymidylate kinase

Steady state kinetics were measured with the physiological substrate dTMP and the partially activated prodrug AZT-MP. WT, wild type.

	k_{obs} for dTMP	k_{obs} for AZT-MP	Ratio k_{obs} for dTMP/k_{obs} for AZT-MP
	s^{-1}	s^{-1}	
WT	35	0.175	200
D14X (Ala, Ile, Asn, Ser, Cys, His, Glu)	<0.01	<0.01	
R15G	0.175	<0.01	
R15M	0.035	<0.01	
R15K	2.8	<0.01	
R15G + Q142R	1	<0.01	
R15G + K143R	1	<0.01	
R15G + S144R	0.175	<0.01	
R15G + G145R	<0.01	<0.01	
R15G + F146R	<0.01	<0.01	
R15G + F146[A+R]	<0.01	<0.01	
WT + *E. coli* lid[a]	1.4	<0.01	
R15G + *E. coli* lid[a]	8.75	0.7	12.5

[a] Residues ^{131}FLSTQDVDNNAEKSGFGDE^{149} of yeast TmpK were replaced by ^{138}YLDVTPEVGLKRARARGELD^{157} from *E. coli* TmpK.

RESULTS AND DISCUSSION

The rationale of our initial design of a yeast TmpK variant with enhanced AZT-MP activity was to prevent the azido group-induced P-loop displacement by mutating Asp-14 to a smaller amino acid. However, all Asp-14 mutant proteins were catalytically inactive (see Table I). Therefore, we decided to pursue a new approach that entailed the modification of the yeast enzyme to mimic the *E. coli* TmpK, thus, hopefully, alleviating the detrimental effect of the P-loop mispositioning by providing catalytic residues from the lid region. The chosen strategy was to avoid the steric clash between the introduced arginines in the lid region with the P-loop arginine by first mutating Arg-15 to glycine. The R15G mutation results in a 200-fold reduced k_{obs} for dTMP but attempts to recover activity by systematically mutating lid residues (residues 142–146) to arginines did not increase the dTMP activity appreciably (AZT-MP activity was not detectable). On close structural comparison between the lid regions of the yeast and *E. coli* TmpKs (8), we noticed that the lid region of *E. coli* TmpK is one residue longer, and that the most promising position for the introduced arginine (such that it could potentially interact with the γ-phosphate group of ATP) corresponds to the position of Phe-146. However, the attempt to incorporate this information, by the simultaneous mutation of Phe-146 to arginine and the insertion of an alanine residue prior to it, also failed to recover activity with either dTMP or AZT-MP.

The exact positioning required by the catalytic residues, which was probably not achieved in our initial efforts of simple modifications of the yeast lid region, prompted the replacement of the entire yeast lid region (residues 131–149) by that of the *E. coli* lid (residues 138–157) (Fig. 2). The variant that retains the P-loop arginine exhibits only 4% of wild type activity with dTMP, with no improvement in AZT-MP phosphorylation. However, the variant that in addition to the replaced lid has the P-loop Arg-15 exchanged by a glycine recovers 25% of wild type activity and, most encouragingly, results in a 400% increase of the AZT-MP phosphorylation rate. This result suggests that the presence of arginines in both P-loop and lid leads to steric interference but that the loss of activity caused by removal of Arg-15 can be partially restored by an arginine in the lid region. In summary, we have created a mutant of yeast TmpK that phosphorylates AZT-MP four times more efficiently than wild type, and at the same time the ratio between the k_{obs} for dTMP and AZT-MP is improved 16-fold.

For the long term aim of gene therapeutic potentiation of AZT effectiveness, there would be significant advantages from the use of a modified human enzyme. We therefore extended the mutational studies to the human enzyme (Table II). Here, too, mutation of the P-loop carboxylic acid (Asp-15) completely inactivates the enzyme. Contrary to all expectations arising from the studies on the yeast enzyme, replacement of Arg-16 (equivalent to Arg-15 in the yeast enzyme) does not lead to a loss in catalytic activity. At present, we have no experimentally based explanation for this observation. However, it is possible

that it is related to the fact that k_{obs} for the human enzyme is much lower than for the yeast enzyme (0.7 *versus* 35 s^{-1}), which is already very slow when compared with other nucleoside monophosphate kinases (*e.g.* 570 s^{-1} for human adenylate kinase (12). Because the catalytic machinery in both enzymes appears to be identical, as shown by sequence comparison and three-dimensional structural determination,[2] we speculate that it is possible that the chemical step (*i.e.* phosphoryl transfer) is not rate-limiting, but rather another step such as product release; so that slowing a relatively rapid chemical step, by removing the P-loop arginine, might not have any influence on the overall rate.

More in keeping with expectations arising from the yeast TmpK data was the fact that introduction of the *E. coli* lid region without removing Arg-16 leads to a decrease in catalytic activity. Most dramatically, the combination of the *E. coli* lid with the replacement of Arg-16 by glycine not only restores full wild type catalytic activity, but it results in a protein that is more efficient with AZT-MP than with dTMP (for the *E. coli* large lid variant, see Table II). The increase in activity with AZT-MP is approximately 200-fold over wild type. This mutant thus has properties that are highly attractive for improving the potency of AZT, because the efficiency of AZT-MP phosphorylation is improved dramatically with only a minor increase in dTMP phosphorylation activity. The latter is an important aspect, because AZTTP must compete with dTTP for HIV reverse transcriptase-catalyzed addition to the end of a growing viral DNA chain.

Interestingly, an increase of AZT-MP phosphorylation with a concomitant reduced activity for dTMP is achieved by the F105Y mutant (Table II). The rationale for constructing this mutant was that in the yeast enzyme, the corresponding residue (at position 102) is a tyrosine, and its hydroxyl group interacts with the carboxylate side chain of Asp-14, which (like Asp-15 in the human enzyme) appears to be an essential residue for the catalytic mechanism. In the hope for synergy, we combined our lid modification with the F105Y point mutation (*i.e.* R16G + *E. coli* large LID + F105Y), and in fact, this variant shows the best catalytic ratio of AZT-MP to dTMP.

The mutants of TmpK described so far, showing altered specificity for AZT-MP and dTMP, were produced according to rational considerations based on comparative structure-function studies of TmpK from three different sources. Despite certain gaps in our understanding of the mechanistic features of TmpK (*e.g.* the role of Arg-16 in the human enzyme), mutants have been obtained that have almost ideal properties for enzymes that potentiate the action of AZT, and these are presently being tested in appropriate cell culture experiments. Further unraveling of the details of the mechanism of human TmpK may lead to the design and construction of mutants that have an even more pronounced reversal of specificity, *i.e.* that can phosphorylate AZT-MP but not dTMP. Earlier attempts to improve the inhibitory effect of AZT on HIV replication (13), by transfecting human cells with the herpes simplex virus thymidine kinase (which is several orders of magnitude slower in AZT-MP phosphorylation than our best human TmpK variant), have been partially successful, demonstrating the feasibility of this approach.

Our goal of designing mutants of human TmpK with improved AZT-MP-phosphorylating properties has been criticized on several grounds (14). One of these is that knowledge of the structure of the homologous but not identical yeast enzyme would not be enough to design mutants of the human enzyme with the desired properties. Although this is a well founded cautionary note in the absence of evidence to the contrary, the

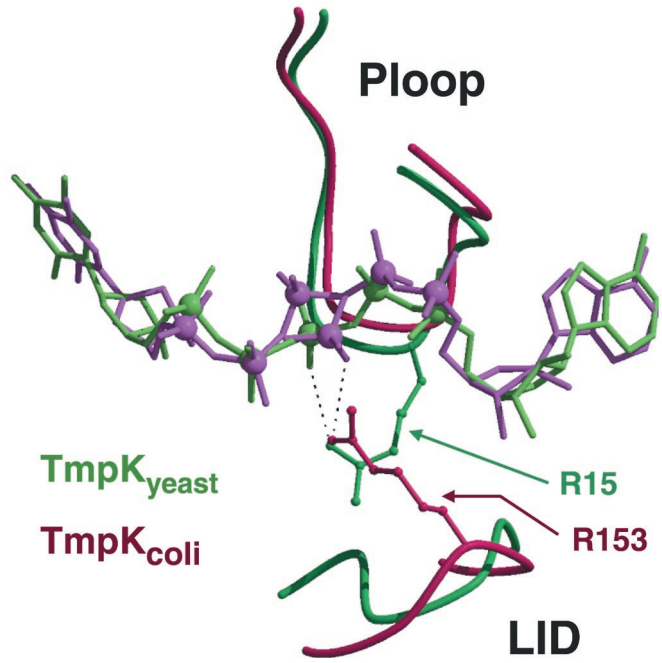

FIG. 2. **Arginines that may interact with the γ-phosphate of ATP originate from the P-loop in the case of the human and yeast enzymes but from the lid region in the case of the *E. coli* TmpK.** The figure shows an overlay of the yeast TmpK (7) (*green*) and *E. coli* TmpK (8) (*red*) structures, both determined in the presence of the bisubstrate inhibitor TP_5A. The spatial position of the guanidinium group of Arg-15 of the yeast TmpK and that of Arg-153 of *E. coli* TmpK are nearly identical, enabling similar interactions with the third phosphate (two conformations were observed for the middle phosphate in the *E. coli*-TP_5A complex structure).

[2] A. Lavie, R. Brundiers, T. Veit, J. Reinstein, I. Schlichting, N. Ostermann, R. S. Goody, and M. Konrad, manuscript in preparation.

TABLE II
Steady state kinetics of human TmpK mutants

	k_{obs} for dTMP	k_{obs} for AZT-MP	Ratio k_{obs} for dTMP/ k_{obs} for AZT-MP	Ratio k_{obs} mutant/ WT for AZT-MP
	s^{-1}	s^{-1}		
WT	0.73	0.012	61	1
D15X (Ala, Asn, Glu)	<0.001	<0.001		
R16G	1.0	<0.01		
WT + *E. coli* small lid[a]	0.15	0.003	50	0.25
WT + *E. coli* large lid[b]	0.17	0.002	85	0.17
R16G + *E. coli* small lid[a]	0.7	0.22	3.2	18
R16G + *E. coli* large lid[b]	1.5	2.14	0.7	178
F105Y	0.17	0.25	0.7	21
R16G + *E. coli* large LID[b] + F105Y	0.33	0.60	0.55	50

[a] Residues ^{145}AFGH^{148} of human TmpK were replaced by ^{151}RARGEL^{156} from *E. coli* TmpK.
[b] Residues ^{136}QLADAAKRGAFGH^{148} of human TmpK were replaced by ^{142}TPEVGLKRARARGEL^{156} from *E. coli* TmpK.

present work shows that incorporation of knowledge gained from *E. coli* TmpK, which can phosphorylate AZT-MP readily, has indeed allowed our aim to be achieved at the level of enzyme activity, as predicted by Lavie *et al.* (7, 8). It is of interest to note that a similar undertaking with the thymidine/thymidylate kinase from herpes simplex, in which DNA family shuffling was used to generate enzymes capable of phosphorylating AZT more efficiently than the wild type protein, were considerably less successful than the work presented here in achieving the desired improvement (15).

Another potential area of application of nucleoside analogs is that of cancer chemotherapy (reviewed in Ref. 16). The approach adopted here is to use an analog that is not phosphorylated by cellular kinases and to combine this with transfection with herpes simplex thymidine kinase, which is less specific in its requirements concerning substrate structure. Promising results have been obtained in cell culture experiments with the combination of transformation with herpes simplex thymidine kinase and the open chain guanosine analog ganciclovir. With the help of the viral kinase, the analog is phosphorylated to its highly cytotoxic triphosphate. However, ganciclovir is actually a very poor substrate for herpes simplex thymidine kinase, and its use may limit the effectiveness of this approach. The results reported here suggest that a better strategy might be to generate and use mutants of the appropriate human kinases for the activation of such potentially cytotoxic prodrugs. This would have the advantage, presumably, of lower immunogenicity and, judging by the results of mutation of human Tmpk, potentially higher activity.

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