Modifying Human Thymidylate Kinase to Potentiate Azidothymidine Activation*

(Received for publication, August 27, 1999, and in revised form, September 30, 1999)

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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 produgs in the treatment of HIV 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 X4 of the P-loop (the GX4X5X6X7GKS(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 X3) (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 P5-([5’-adenosyl]-P3-([5’-thymidyl])pentaphosphate (TPA) 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 adenylyl 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 (pJC20Hist) such that a Gly-Ser-His (GSH) 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 NdeI/BamHI-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 NdeI/BamHI-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 column. 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improving azt-mp phosphorylation by thymidylate kinase

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 tmpktpa complex structure (7) was observed to interact with the phosphate that would correspond to the y-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).

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 sterical clash between the introduced arginines in the lid region with the p-loop arginine by first removing arg16, 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 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 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 arg16 (equivalent to arg15 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

| 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.

|          | $k_{\text{obs}}$ for dtmp | $k_{\text{obs}}$ for azt-mp | ratio $k_{\text{obs}}$ for dtmp/$k_{\text{obs}}$ for azt-mp |
|----------|---------------------------|-----------------------------|--------------------------------------------------------|
| wt       |                           |                             |                                                        |
| d14x     | 35                        | 0.175                       | 200                                                   |
| r15g     |                           |                             |                                                        |
| r15m     |                           |                             |                                                        |
| r15k     |                           |                             |                                                        |
| r15g + q142r | 1                       |                             |                                                        |
| r15g + k143r | 1                       |                             |                                                        |
| r15g + s144r | 0.175                   |                             |                                                        |
| r15g + g145r |                           |                             |                                                        |
| r15g + f146r |                           |                             |                                                        |
| r15g + f146[r+] |                           |                             |                                                        |
| wt + e. coli lidp | 1.4                     |                             |                                                        |
| r15g + e. coli lidp | 8.75                    | 0.7                         | 12.5                                                  |

* residues 131flstqvdvndnaeksgfgde149 of yeast tmpk were replaced by 138yldvtpveglkrarargeld157 from e. coli tmpk.
that it is related to the fact that $k_{\text{obs}}$ 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, 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 Arg-16, 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

![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$_2$A. 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$_2$A complex structure).](image308x281 to 555x527)

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### Table II

|                  | $k_{\text{obs}}$ for dTMP | $k_{\text{obs}}$ for AZT-MP | Ratio $k_{\text{obs}}$ for AZT-MP | Ratio $k_{\text{obs}}$ mutant/WT for AZT-MP |
|------------------|--------------------------|-----------------------------|---------------------------------|------------------------------------------|
| 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$^d$ | 1.5                  | 2.14                        | 0.7                             | 178                                      |
| F105Y            | 0.17                     | 0.25                        | 0.7                             | 21                                       |
| R16G + E. coli large lid$^d$ + F105Y | 0.33                | 0.60                        | 0.55                            | 50                                       |

$^a$ Residues 145AFGH148 of human TmpK were replaced by 151RARGEL156 from E. coli TmpK.

$^b$ Residues 136QLADAAKRGAFGH146 of human TmpK were replaced by 142PDEVGLKRARARGEL156 from E. coli TmpK.
The 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 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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