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A Single Amino Acid Substitution Abolishes Feedback Inhibition of Vaccinia Virus Thymidine Kinase*

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Exploitation of differences in the substrate specificity of the type I and type II thymidine kinases (EC 2.7.1.21, TK) expressed by the *Herpesviridae* and *Poxviridae* (and human cells), respectively, has lead to the development of effective antiherpetic drugs such as acyclovir and gancyclovir. Analysis of type I TK protein sequences reveals a consensus sequence which corresponds to domain IV of type II TK proteins such as that encoded by vaccinia virus (VV). The type I descriptor (X_{pho} − + + X_{pho}) differs at the second position from the type II consensus sequence (X_{pho} X_{phi} + + X_{pho}) by having an aspartic acid residue (D) substituted for a glutamine (Q). To test the hypothesis that this substitution may be responsible for the observed differences in substrate specificity of these enzymes and as a approach to identify the nucleoside binding site of the type II VV TK, site-directed mutagenesis was employed to alter glutamine 114 (Q114) within domain IV of VV TK to a histidine (Q114H) or an aspartic acid (Q114D). All of the mutant enzymes retained full enzymatic activity as compared to wild-type VV TK when thymidine or bromodeoxyuridine were used as substrates. However, unlike the wild-type herpes simplex (type 1) TK enzyme, neither wild-type nor domain IV VV TK mutants were able to phosphorylate acyclovir or cytidine substrates. Surprisingly, the domain IV VVTK mutants displayed a dramatic loss of feedback inhibition by dTTP. Mutations of the Q114 position also lead to a difference in ATP binding as demonstrated by an altered elution pattern of Q114H and Q114D from an ATP-agarose affinity column with dTTP. Taken together, these results suggest that domain IV of VV TK is not involved directly in substrate discrimination but instead participates in feedback inhibition by dTTP.

Thymidine kinase is a key enzyme in the salvage pathway of nucleotide metabolism catalyzing the transfer of a phosphate moiety from ATP to thymidine to produce dTMP. The thymidine kinase enzyme (TK)[1] from vaccinia virus (VV) is a 80-kDa homotetrameric complex which is feedback-inhibited by the distal product of this pathway, dTTP. In general, all thymidine kinases can be grouped into two classes or types on the basis of size, quaternary structure, feedback sensitivity, and substrate specificity (Black and Hruby, 1990a). The type I TKs comprise members of the *Herpesviridae* family (Wagner *et al.*, 1981; Gompels and Minson, 1986; Darby *et al.*, 1986; Kit *et al.*, 1983; Otsuka and Kit, 1984; Littler *et al.*, 1986; Davison and Scott, 1986; Nunberg *et al.*, 1989). These enzymes are approximately 40 kDa in size, are functional as dimers, and are not subject to feedback inhibition by dTTP. Because they have a low substrate specificity, being able to phosphorylate cytidine in addition to thymidine, they are more properly considered deoxypyrimidine kinases (Robertson and Whalley, 1988). HSV-1 TK is the prototype type I thymidine kinase. The type II thymidine kinases comprise a large group of enzymes from human (Flemington *et al.*, 1987), mouse (Lin *et al.*, 1987), chicken (Kwoh *et al.*, 1984)) *Escherichia coli* (Black and Hruby, 1991), bacteriophage T4 (Valerie *et al.*, 1986), and members of the *Poxviridae* family (Esposito and Knight, 1984; Weir and Moss, 1983; Upton and McFadden, 1986; Boyle *et al.*, 1987; Gershon and Black, 1989). Type II TK polypeptides are approximately 20 kDa in size and assemble into enzymatically active tetrameric complexes. In addition, type II TKs are sensitive to feedback inhibition by dTDP and dTTP and exhibit a high substrate specificity with thymidine or closely related thymidine analogs such as bromodeoxyuridine being the only acceptable substrates for phosphorylation by this enzyme. Vaccinia virus TK is the prototype type II thymidine kinase.

The efficacy of antiherpetic drugs is largely due to the difference in substrate specificity between the Herpes Simplex virus thymidine kinase (type I) and the host cell counterpart (type II). The development of such antiviral drugs utilizes the ability of HSV-1 TK to phosphorylate guanosine analogs such as acyclovir or gancyclovir which the cellular enzyme is incapable of phosphorylating. One question that arises is what is the molecular basis of the difference in substrate specificity between the type I and type II thymidine kinases, *i.e.* which residues or structures are involved in substrate binding and/or phosphorylation.

When several type II thymidine kinases were aligned seven regions of complete identity were identified and denoted as domains I through VII (Black and Hruby, 1990b). An alignment of predicted protein secondary structures of HSV-1 and monkeypox (a very close relative of VV) thymidine kinases and adenylate kinase by Folkers and Trumpp (1987) lead them to suggest one region as having a possible role in nucleoside binding. This region corresponds to domain IV in vaccinia virus thymidine kinase. Folkers and Trumpp (1987) postulated that the key aspartic acid (D162) found in this region in HSV-1 TK, but which is replaced by a glutamine (Q114 in VVTK) in domain IV of type II thymidine kinases is the basis of substrate specificity and hence antiviral drug

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[1] The abbreviations used are: Vaccinia virus, VV; thymidine kinase; TK, herpes simplex virus type I, HSV; kb, kilobase(s); ORF, open reading frame.

specificity between type I and type II enzymes. In the present study we were unable to provide evidence that domain IV is involved in nucleoside binding and/or substrate discrimination. Rather, mutations in domain IV of the VVTK demonstrated a greatly reduced sensitivity to the type II allosteric feedback inhibitor, dTTP, and altered binding of ATP.

EXPERIMENTAL PROCEDURES

Materials—[*methyl*-^{3}H]Thymidine (specific activity, 83 Ci/mmol) for standard TK assays and L-[^{35}S]methionine (specific activity, 1248 Ci/mmol) for determination of protein synthesis levels were purchased from Amersham. The other radioisotopes ([*side chain*-2-^{3}H] acyclovir (specific activity, 25 Ci/mmol), 5-[6-^{3}H]bromodeoxyuridine (specific activity, 23.1 Ci/mmol), [5-^{3}H]deoxycytidine (specific activity, 24.2 Ci/mmol) and [*methyl*-^{3}H]thymidine (specific activity, 20 Ci/mmol)) were purchased from Du Pont-New England Nuclear. T7 RNA polymerase, T4 DNA ligase, Klenow fragment of DNA polymerase, and ATP were obtained from Boehringer Mannheim. Other chemicals and the ATP-agarose (attached at C-8 with a six-carbon spacer) were purchased from Sigma. Oligonucleotides used for mutagenesis were synthesized with an Applied Biosystems 380A synthesizer at the Center for Gene Research and Biotechnology at Oregon State University (Corvallis, OR).

Vectors for In Vitro Transcription—The VVTK vector used as a template for introducing mutations and as the wild-type TK control for *in vitro* transcription was pT7:TKII and is described in Wilson *et al.* (1989). The herpes simplex virus thymidine kinase open reading frame (3.4-kb *Bam*HI insert in pBR322, pHSV106) was provided by Dr. Gary Merrill (McKnight, 1980). Using the *Eco*RI sites within the vector polylinker region, the HSVTK open reading frame was moved into *Eco*RI-linearized pUC118 to allow for production of ssDNA template for mutagenesis procedures. An *Nco*I site was introduced by site-directed mutagenesis at the initiating methionine codon. In a two-step subcloning procedure an *Nco*I-*Bam*HI fragment containing the 3′ end of the open reading frame (ORF) was first subcloned into the pT7:TKII vector which had the VVTK open reading frame excised. In the second step, an *Nco*I fragment containing the majority of the 5′ portion of the gene was subcloned into the vector containing the *Nco*I-*Bam*HI 3′ end of the HSVTK. This vector then contains the entire authentic HSVTK ORF in place of the VVTK ORF in pT7:TKII and was designated pT7:HSVTK.

Site-directed Mutagenesis—The HSVTK-*Nco*I, Q114H, and Q114D mutants were created by site-directed mutagenesis using a modified version of the protocol of Kunkel (1985) as described in Black and Hruby (1990b). The single-stranded DNA template was prepared as described previously from the expression plasmid (Wilson *et al.*, 1989). The oligonucleotides were prepared for mutagenesis as described in Black and Hruby (1990b) and in the case of Q114H and Q114D, were designed to introduce a silent mutation which results in the creation of an *Ava*II site in addition to creating the desired alterations at Q114. All transformants were screened initially by restriction analysis for the presence of a novel *Ava*II site within the VVTK ORF. The position and nature of the mutations introduced were then confirmed by dideoxy sequencing (Sanger *et al.*, 1977).

Enzyme Activity Assays—Production of wild-type and mutant enzymes by *in vitro* transcription and translation reactions was according to Wilson *et al.* (1989). Analysis of protein synthesis and enzyme activity levels using [*methyl*-^{3}H]thymidine (specific activity, 83 Ci/mmol) was according to Black and Hruby (1990b). A full description of the thymidine kinase filter binding assay was reported in Hruby and Ball (1981). For the thymidine kinase assays using acyclovir, bromodeoxyuridine, cytidine, and thymidine, tritiated nucleosides with specific activities of ~25 Ci/mmol were used and the assays incubated for 4 h at 30 °C. For the dTTP inhibition assays, approximately equivalent amounts of cell free translation products of each RNA were assayed in a standard TK assays ([^{3}H]thymidine; specific activity, 83 Ci/mmol) in the presence of a range of dTTP concentrations (0, 5, 10, 25, 50, and 100 μM) for 1 h at 30 °C.

ATP-Agarose Chromatography—Adenosine 5′-triphosphate-agarose (500 mg) was swollen overnight at room temperature in binding buffer (10 mM morpholinepropane sulfonic acid, 1 mM EDTA, 10 mM NaF, 1 mM dithiothreitol, 0.1% Triton X-100, 1 mM benzamidine, and 50 μM thymidine at pH 7.4 (Ballou *et al.*, 1988)). The swollen beads were packed into a disposable polypropylene column and rinsed thoroughly with binding buffer. Radiolabeled translation products (15 μl) were diluted into 1 ml of binding buffer and loaded onto the column three times. The column was then washed with two 2.5-ml aliquots of binding buffer. A 0–50 μM gradient of dTTP was passed over the column and ten 1-ml fractions collected. A pulse of 100 mM ATP (1 ml) was then applied to the column followed by five 1-ml washes with binding buffer. The amount of radiolabeled protein in 100 μl of each fraction was determined by hot trichloroacetic acid precipitation and scintillation counting.

RESULTS AND DISCUSSION

Alignment of Type I and Type II Thymidine Kinases within the Type II Domain IV—In general, type I and type II thymidine kinases share little primary amino acid identity. However, alignment of the herpes simplex virus (HSV-1) thymidine kinase (a type I TK), monkeypox thymidine kinase (a type II TK), and adenylate kinase on the basis of secondary structure, reveals several regions that aligned by the position of predicted α helices and β sheets amongst these enzymes (Folkers and Trumpp, 1987). In addition, there were regions which also display shared homology at the primary amino acid level. One of these regions, corresponding to domain IV of VVTK, is found only in the thymidine kinases but not in adenylate kinase. Based on this observation together with the report by Darby *et al.* (1986) that a mutation near this region in HSVTK lead to a change in K_m for thymidine and acyclovir, Folkers and Trumpp (1987) suggested that this region is involved in nucleoside binding. Furthermore, noting that HSVTK has an aspartic acid residue (D162) at the same location in their alignment where the monkeypox thymidine kinase has a glutamine (Q114 in VVTK) lead to the hypothesis that the aspartic acid residue was involved in substrate specificity. Because type I TKs have a low substrate specificity being able to bind and phosphorylate cytidine and guanosine analogs in addition to thymidine, while the type II thymidine kinases have a strict substrate specificity, they postulated that this difference in substrate specificity is due to the presence of an aspartic acid in the type I thymidine kinases *versus* a glutamine in the type II thymidine kinases.

Since a direct comparison of only a single type I and a single type II enzyme has been done, we sought to determine whether the observed similarities between type I and domain IV of the type II thymidine kinases was a general phenomenon. Therefore, alignment of this region was expanded to include a much larger sampling of thymidine kinase enzymes. Twelve thymidine kinases from each type were aligned and a consensus sequence for each generated to aid in comparing these domains. The group of type I thymidine kinases consisted of herpes simplex virus types 1 and 2, marmoset herpesvirus, varicella zoster virus, feline herpes virus, pseudorabies virus, equine herpes virus type I, bovine herpes virus type 1, turkey herpesvirus, Marek's disease virus, herpesvirus saimiri, and Epstein-Barr virus. Complete nucleotide sequences of these type I thymidine kinases and their corresponding references can be found in Balasubramaniam *et al.* (1990). A similar comparison of type II thymidine kinases (vaccinia virus, variola virus, monkeypox virus, swinepox virus, capripox virus, shope fibroma virus, fowlpox virus, entomopox virus, human, mouse, chicken, and African Swine Fever virus) was performed by Gruidl *et al.* (1991). Fig. 1 shows the two consensus sequences with each prototype type TK sequence and the number of corresponding amino acids (either the specific amino acid or the classification of amino acid) from the alignment are in parentheses. For the type I TKs the consensus sequence consists of a hydrophobic residue (X_{pho}) followed by a negatively charged residue (−), then two positively charged residues (+) and ending with another hydrophoic residue (X_{pho} − + + X_{pho}). The type II TK consensus sequence consists of the same features except that the second

TYPE I TK (HSV)	$F_{(6)}$ $Xpho_{(10)}$	$D_{(12)}$ $-_{(12)}$	$R_{(12)}$ $+_{(12)}$	$H_{(12)}$ $+_{(12)}$	$P_{(10)}$ $Xpho_{(12)}$
TYPE II TK (VV)	$Xpho_{(11)}$ $F_{(11)}$	$Xphi_{(9)}$ $Q_{(8)}$	$+_{(11)}$ $R_{(11)}$	$+_{(10)}$ $K_{(9)}$	$Xpho_{(11)}$ $P_{(6)}$

FIG. 1. **Consensus sequences within domain IV of type I and type II thymidine kinases.** In the *top* set of *boxes* are shown the most commonly occurring residues among the type I thymidine kinases and the consensus sequence derived from a comparison of 12 type I thymidine kinases; herpes simplex virus types 1 and 2, marmoset herpesvirus, varicella zoster virus, feline herpes virus, pseudorabies virus, equine herpes virus type I, bovine herpes virus type 1, turkey herpesvirus, Marek's disease virus, herpesvirus saimiri, and Epstein-Barr virus (Balasubramaniam *et al.*, 1990). A consensus sequence of 12 type II thymidine kinases (vaccinia virus, variola virus, monkeypox virus, swinepox virus, capripox virus, shope fibroma virus, fowlpox virus, entomopox virus, human, mouse, chicken, and African Swine Fever virus (Gruidl *et al.*, 1991) was also compiled and is shown in the *lower section* of this figure. The *numbers* in *parentheses* indicate the number of occurrences of either the particular residue shown or the general case of residue (hydrophobic, X_{pho}; hydrophilic, X_{phi}; negatively charged, −; positively charged, +). Standard single letter amino acid nomenclature is used.

VVTK	F Q R K P
Mutants	
Q114H	F H R K P
Q114D	F D R K P
HSVTK	F ● R H P

FIG. 2. **Mutations created within domain IV of VVTK.** The *top line* shows the carboxyl terminus of the VVTK domain IV and the 2 adjacent residues. The *second* and *third lines* show the alterations made at position 114 with the new residues (Q114H and Q114D) *boxed* and *highlighted* with the wild-type VVTK. The *bottom line* depicts the HSVTK sequence corresponding to domain IV with the key aspartic acid residue *encircled* and *shaded*.

residue within the sequence is hydrophilic (X_{phi}) corresponding to the aspartic acid/glutamine difference described previously (X_{pho} X_{phi} + + X_{pho}). While only nine of 12 were hydrophilic at the second position, two other sequences (swinepox and African Swine Fever virus) were flanked on one side by a hydrophilic residue. Entomopox virus thymidine kinase contains two hydrophilic residues. Perhaps simply the presence of a hydrophilic residue within this domain is sufficient. In any case the type I and type TKs appear to have a strict conservation of specific classes or residues within domain IV with one major difference at the second position suggesting that the difference between the type I and type II thymidine kinases at this position is important.

Domain IV Mutants and TK Activities—To address the question of domain IVs importance in substrate specificity, the VVTK glutamine 114 (Q114) was altered by site-directed mutagenesis to either a histidine (Q114H) or an aspartic acid (Q114D) residue (Fig. 2). The hydrophilic glutamine 114 thereby was altered to a negatively charged aspartic acid to reflect the HSVTK sequence (D162) in this region (Q114D) or altered to a positively charged residue (Q114H). Analysis of enzyme activity of VVTK, Q114H, Q114D, and HSVTK using [^{3}H]thymidine as the substrate demonstrated that all enzymes were fully active. Analysis of radiolabeled cell free translation products by glycerol gradient sedimentation or sodium dodecyl sulfate-polyacrylamide gel electrophoresis showed no difference between VV wild-type and mutant thymidine kinases (data not shown). Interestingly, in a recent report Zimmermann *et al.* (1991) described that the exchange of HSVTK D162 to asparagine resulted in a total loss of activity. Because both Q114H and Q114D reflect mutations from a hydrophilic residue to a charged residue it is unclear why the reverse alteration (charged to hydrophilic residue) might display such a dramatic difference in activity. While Zimmermann *et al.* (1991) reported what was in a dissertation, it is unknown whether the mutant enzyme was capable of forming a dimeric complex. We have noted previously that often mutations in VVTK lead to a loss of structural integrity (Black and Hruby, 1990b). In our case however, the presence of full activity in the mutants along with demonstration of tetrameric complex formation by glycerol gradient sedimentation allows examination of the substrate specificity question.

Determination of Substrate Specificities—To address whether the difference in substrate specificity between HSVTK and VVTK is due to a difference at a key residue, aspartic acid (D162) in HSVTK *versus* a glutamine (Q114) in VVTK, VVTK, Q114H, Q114D, and HSVTK enzymes produced *in vitro* were assayed for their ability to phosphorylate acyclovir, bromodeoxyuridine, cytidine, or thymidine (Fig. 3). While both thymidine and bromodeoxyuridine were phosphorylated to the same extent by all enzymes tested, cytidine and acyclovir were only phosphorylated by HSVTK. Therefore it appears that Q114 is not directly involved in nucleoside binding or substrate specificity.

With respect to the HSVTK mutant reported by Zimmermann *et al.* (1991), Robertson and Whalley (1988) had previously predicted that the DHR motif may be involved in MgATP binding primarily on the basis that the region looks like a Mg^{2+} binding site with a key aspartic acid residue preceded by several hydrophobic residues. However, in the case of VVTK, analysis of mutants at the key aspartic acid residue 82 (D82) strongly suggests that D82 is involved in binding magnesium as a MgATP complex (Black and Hruby (1992) in press). Given that, the question then arises as to what the function of this highly conserved motif may be.

It is likely that the domain IV motif has been evolutionarily maintained in both type I and type II thymidine kinases because of its participation in catalysis and/or regulation of enzyme activity. In addition, the maintenance of the difference within domain IV (either aspartic acid or glutamine) appears to be of some importance. The role proposed by Folkers and Trumpp (1987) for the involvement of domain IV residues in substrate specificity is not supported by experimental data provided here. Alternatively, one other biochemical difference between type I and type II thymidine kinase activities may involve domain IV participation. HSVTK and VVTK differ in sensitivity to feedback inhibition by dTTP. VVTK enzyme activity is inhibited noncompetitively at about 50% at 10 μM dTTP or dTDP and is completely inhibited at 50 μM concentrations (Hruby, 1985). Because HSVTK is also a thymidylate kinase, being able to phosphorylate dTMP to dTDP, it is not surprising that HSVTK lacks sensitivity to at least dTDP. This then raises the question of whether domain IV is involved in feedback inhibition.

Loss of dTTP Sensitivity by Q114H and Q114D—Wild-type HSVTK, VVTK, and the two VVTK domain IV mutants, Q114H and Q114D, were assayed for TK activity in the presence of various concentration of dTTP (0, 5, 10, 25, 50, and 100 μM). Fig. 4 shows the results of such an experiment. HSVTK is not inhibited by dTTP as expected. VVTK is

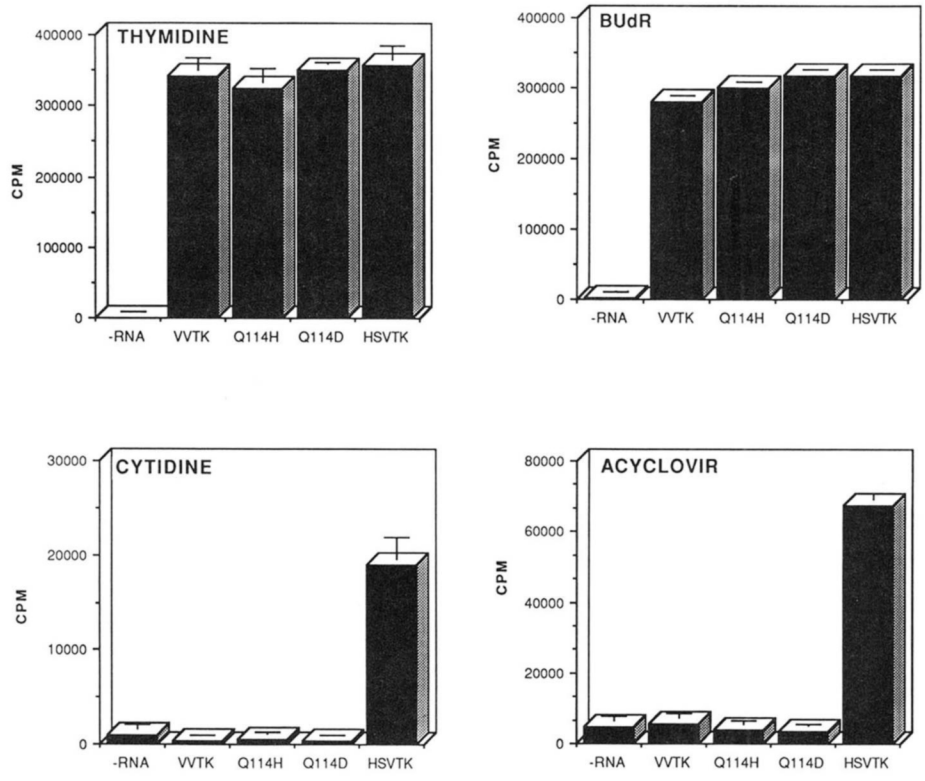

FIG. 3. **Specificity of VVTK, Q114H, Q114D and HSVTK for nucleoside substrates.** HSVTK, mutant, and wild-type VVTK translated *in vitro* were assayed for their ability to phosphorylate thymidine, bromodeoxyuridine (*BUdR*), cytidine, or acyclovir (see "Materials and Methods"). As a negative control, equivalent amounts of a cell free translation carried out in the absence of any added RNA was used (−RNA). Assays were carried out at 30 °C for 4 h. The *bars* represent the standard deviation from triplicate assays.

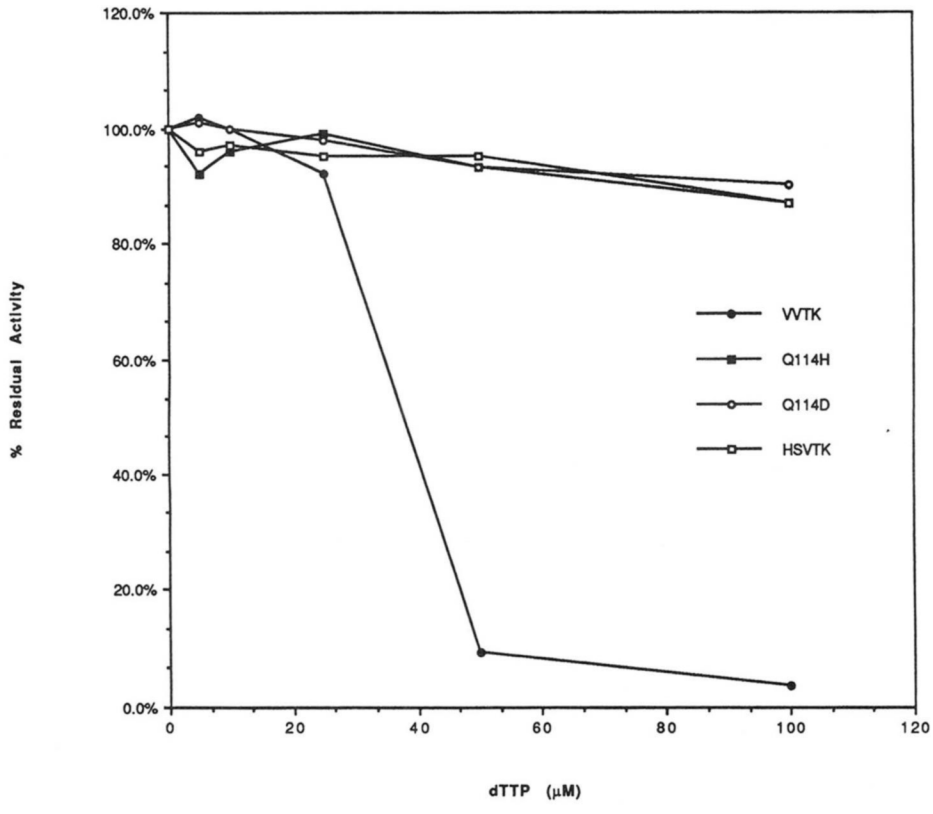

FIG. 4. **dTTP inhibition of wild-type and mutant thymidine kinases.** For the dTTP inhibition assays, approximately equivalent amounts of cell free translation products of each RNA (VVTK, Q114H, Q114D, and HSVTK) were assayed in a standard TK assays ([^{3}H]thymidine, specific activity 83 Ci/mmol) in the presence of a range of dTTP concentrations (0, 5, 10, 25, 50, and 100 μM) for 1 h at 30 °C.

almost completely inhibited in assays performed in the presence of 50 μM dTTP. Surprisingly, both Q114H and Q114D displayed no inhibition by dTTP even at levels as high as 100 μM. In fact, the domain IV mutants appear much like the HSVTK in response to dTTP additions. Clearly these mutants have an altered sensitivity to feedback inhibition and suggests that a glutamine residue at position 114 is responsible at least in part for the feedback inhibition phenotype.

Interaction of HSVTK, VVTK, Q114H, and Q114D with ATP and dTTP—In light of these results, the question arises as to whether this loss of inhibition in Q114H and Q114D is due to a lack of ability to bind dTTP. Black and Hruby (1990b) had previously shown that wild-type VVTK bound to an ATP-agarose column could be eluted with dTTP. The

elution pattern of the individual TK enzymes from an ATP-agarose column should give insight into the mechanism of feedback inhibition from the standpoint of the interaction between enzyme, dTTP, and ATP. dTTP appears to act on VVTK by causing a conformational change within the active site (ATP binding site) and thereby results in release of the nucleotide (Black and Hruby, 1990b). If the feedback-insensitive mutant enzymes remain bound to ATP in the presence of dTTP, it would suggest that dTTP no longer binds to the enzyme. Alternatively, dTTP could bind the mutant enzymes but no longer fully elicit the correct conformational change. Fig. 5*A* shows the elution profile of radiolabeled VVTK and HSVTK from an ATP-agarose column first over a 0–50 μM gradient of dTTP followed by a pulse of 100 mM ATP (>10-fold excess ATP is required to elute VVTK) and washes with buffer (see "Materials and Methods" for details). Fig. 5*B* shows the elution profiles of Q114H and Q114D under the same conditions. As expected VVTK is eluted primarily with dTTP while HSVTK is only eluted in the presence of ATP. For Q114H and Q114D it appears that more dTTP is required to elute the mutants from the column since the peak of radioactivity is in the third to fourth fraction while the majority of VVTK radioactivity elutes in the second fraction. A large amount of the mutant enzymes remains bound to the column and is eluted with ATP. The mutant elution patterns fit neither the VVTK nor the HSVTK elution profile but lie somewhere between the two. Along with the direct dTTP inhibition assays shown in Fig. 4, these results suggest that dTTP is still capable of binding to the mutant enzymes, albeit in a dysfunctional fashion, and yet is unable to inhibit enzyme activity. Furthermore, the delay in elution indicates that the affinity for dTTP by the mutants is increased and less TK is released from the column than VVTK. Therefore, it appears that dTTP is still capable of binding to Q114H and Q114D but with a higher affinity than wild-type VVTK such that normal interaction(s) with ATP is altered.

Possible Thymidine Binding Sites—In an earlier alignment of type II thymidine kinases, seven regions of complete amino acid identity were identified (Black and Hruby, 1990b). Of these seven domains, experimental results suggest that at least two domains interact to bind MgATP (domains I and III) (Black and Hruby, 1990b; Black and Hruby, 1992), and, as presented in this paper, domain IV appears to be involved in feedback inhibition. Of the four remaining domains (II, V, VI, and VII) very little can be surmised as to their role(s) in enzyme catalysis and/or regulation.

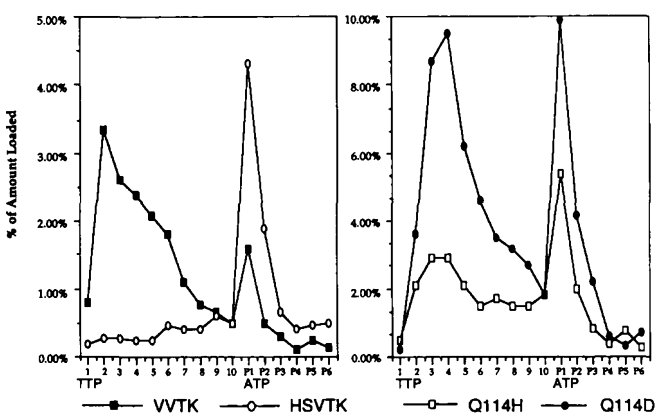

FIG. 5. **Elution profile of L-[^{35}S]methionine-labeled wild-type and mutant TKs from an ATP-agarose affinity column with dTTP.** Cell free translation products of VVTK (■), HSVTK (○) (*left panel*), Q114H (□) or Q114D (●) (*right panel*) were loaded onto an ATP-agarose affinity column and eluted with a 0–50 μM gradient of dTTP (fractions 1–10). A pulse of 100 mM ATP (1 ml, P1) was then applied to the column followed by five 1-ml washes with binding buffer (P2–P6). 100-μl aliquots of the collected fractions were analyzed and adjusted to the percentage of counts per min loaded onto the column.

The corresponding sequence found in domain II (RRVRR, residues 23–27 in VVTK) is not present in HSVTK and also is likely to be sterically too close to the putative ATP binding site, domain I, to function in nucleoside binding. Domain V (VVKLTAVCM, residues 131–139 in VVTK) is also not found in HSVTK nor the type II *E. coli* or bacteriophage T4 thymidine kinase amino acid sequences. However, a bromodeoxyuridine-selected TK minus vaccinia virus mutant was isolated, and sequence analysis of the TK open reading frame revealed a 3-base pair deletion which altered residues K133/L134 to a single isoleucine.[2] Extensive analysis of this mutant has not been performed so it is as yet unknown what role, if any, domain V plays in thymidine kinase activity.

The conserved KRL motif of domain VI is found in type II and in a few type I thymidine kinases. However, most type I thymidine kinases diverge from the KRL motif quite substantially from this highly conserved sequence in type II thymidine kinases. It is unclear what importance this region has.

The strongest contender after domain IV for participation in thymidine binding is domain VII (SVCR, residues 168–171 in VVTK). This motif is highly conserved in type II thymidine kinases. In type I thymidine kinases only the cysteine residue is conserved. Darby *et al.* (1986) reported a cysteine 336 to tyrosine 336 mutation in HSV-1 TK which had a marker effect on ATP and nucleoside binding. This result lead them to suggest involvement of this region within the catalytic site. Mutagenesis studies with VVTK at C170 demonstrated that, of the five mutations introduced, only one was capable of maintaining structural integrity.[3] This result was similar to what was observed with mutations within the putative ATP binding site suggestive of a functional role either in structure or catalysis (Black and Hruby, 1990b). Furthermore, the C170S mutant demonstrated a 2.5-fold higher level of enzyme activity than wild-type VVTK.[3] Experiments are in progress to determine what this "super TK" activity is due to.

Despite demonstration of the involvement of domain IV residues in feedback inhibition, domain IV may also participate in nucleoside binding since the majority of domain IV was not examined for its role in thymidine binding. It has previously been shown that dTTP is a noncompetitive inhibitor with thymidine (Hruby, 1985). By definition then, dTTP binds to the free enzyme or the enzyme-substrate complex at a site other than the active site, often to deform the enzyme such that normal substrate binding or product release is impaired (Lehninger, 1975). Therefore, because of the proximity of residues at the amino terminus of domain IV to Q114 and the noncompetitive nature of dTTP inhibition, it seems unlikely that domain IV residues are also involved in substrate binding.

While many of the conserved domains or motifs discussed above may be essential in catalysis and regulation, the thymidine binding site may be comprised of several, nonlinear residues which, upon folding or subunit association, come together to form the binding site. Purification of the VVTK for x-ray crystallography should greatly aid determination of the residues involved in nucleoside binding.

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