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Mutant Varicella-Zoster Virus Thymidine Kinase: Correlation of Clinical Resistance and Enzyme Impairment

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Varicella-zoster virus (VZV) encodes a thymidine kinase (EC 2.7.2.21) which phosphorylates several antiviral nucleoside analogs, including acyclovir (ACV). A mutation in the VZV thymidine kinase coding sequence, resulting in an arginine-to-glutamine substitution at amino acid residue 130 (R130Q), is associated with clinical resistance to ACV. We have expressed the wild-type and the mutant enzymes in bacteria and have studied the kinetic characteristics of the purified enzymes. The arginine-to-glutamine substitution resulted in decreased catalytic activity and altered substrate specificity. The most striking effect was a decrease in the rates of nucleoside phosphorylation to less than 2% of the rates with the wild-type enzyme. This was accompanied by increased apparent K_m values for thymidine and deoxycytidine. ACV was not detectably phosphorylated by the R130Q enzyme but still competed with thymidine for the enzyme. The inability of the R130Q enzyme to catalyze the phosphorylation of ACV correlates with resistance to ACV noted with a clinical isolate of VZV.

Varicella-zoster virus (VZV), a member of the herpesvirus family, encodes a thymidine kinase (TK) similar in sequence homology and molecular weight to the kinases encoded by herpes simplex virus types 1 and 2. These enzymes are necessary for the antiviral activity of acyclovir (ACV). The VZV TK, like the herpes simplex virus nucleoside kinases, catalyzes the phosphorylation of ACV (15) in the initial step of ACV activation to ACV triphosphate (6). ACV-resistant strains of VZV have been isolated in vitro (7, 39). Most laboratory isolates either encode a TK with altered activity or do not express a functional TK. More rarely, resistance to ACV has been attributed to alterations in the virus DNA polymerase (7). ACV-resistant strains of VZV have also been isolated from patients who became unresponsive to ACV therapy (19, 31). Molecular analysis of the TK gene of a plaque-purified variant (7-1-3) from one of these clinical isolates revealed a single G-to-A substitution at nucleotide 389 that resulted in an arginine-to-glutamine amino acid change at amino acid residue 130 (R130Q) (35). Arginine 130 is in the proposed deoxythymidine (dThd)-binding region of TK and is a conserved amino acid in the sequence of TKs from 12 herpesviruses (4, 22). In this report, we describe the expression in *Escherichia coli* of wild-type VZV TK and the R130Q mutant enzyme and the purification of these enzymes. Kinetic analyses revealed that the arginine-to-glutamine substitution altered the substrate specificity for the mutant nucleoside kinase. More significantly, the R130Q alteration produced a mutant TK which had a drastically reduced turnover number. This correlates with resistance found in the clinical isolate (31).

MATERIALS AND METHODS

Genetic manipulations. Plasmid pUC8vztk from J. Ostrove (35) was the source of the wild-type VZV TK gene. This plasmid was digested with *Bam*HI and *Eco*RI (New England Biolabs, Beverly, Mass.); the unique 2.2-kb DNA fragment with the VZV TK gene was isolated and inserted into the multiple cloning site region of M13mp19 (29). An *Nde*I restriction endonuclease site was created at the TK start codon by site-directed mutagenesis (kit RPN.1523; Amersham, Arlington Heights, Ill.). The gene encoding the R130Q TK was also the product of in vitro mutagenesis. The arginine 130 CGA codon of the wild-type TK gene was changed to a glutamine 130 CAG codon. The glutamine codon introduced into the TK gene for bacterial expression was selected on the basis of *E. coli* codon usage (1) and was not the glutamine CAA codon identified upon sequencing the TK gene from the 7-1-3 TK variant (35) purified from an ACV-resistant clinical isolate. The VZV TK was expressed in *E. coli* in a T7 promoter-based system (33). Expression plasmids pRG05 and pRG08 contained wild-type and R130Q VZV TK genes, respectively. The host bacterium for the T7 expression system was *E. coli* BL21(DE3) (33).

Each altered gene was completely sequenced to verify that the expected change was the only alteration in the TK coding sequence. Dideoxy-chain termination sequencing (34) was performed with [α-^{35}S]dATP (1,300 Ci/mmol; New England Nuclear Research Products, Boston, Mass.) and Sequenase version 2.0 (United States Biochemical Corp., Cleveland, Ohio). Samples from the sequencing reactions were electrophoresed through 8% polyacrylamide–8 M urea exponential wedge gels which were maintained at 55°C with a thermostatic plate. Molecular cloning operations were performed according to standard protocols as described by Maniatis et al. (28).

Enzyme purification. The bacteria with wild-type and R130Q VZV TK genes were grown in 2× TY medium (28) containing 0.4% glycerol and 50 μg of kanamycin per ml at 37°C to a final optical density of approximately 12. The cell pellets were suspended in 20 mM Tris (pH 7.5)–1 mM dithiothreitol–10% glycerol (Tris buffer A) at 1 g of cells per ml of buffer, broken by passage through a French press (SLM Instruments, Urbana, Ill.) at 12,000 lb/in^2, and centrifuged at 12,000 × *g* for 10 min. All subsequent steps were conducted on ice or at 4°C except Mono Q column chromatography, which was done at room temperature (22°C). The bacterially expressed VZV TK in the supernatant was partially purified by streptomycin sulfate treatment and ammonium sulfate precipitation as described previously (10). The protein that precipitated with ammonium sulfate (310 mg/ml of supernatant) was dissolved in Tris buffer A and dialyzed

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against the same buffer. A portion of the preparation (50 mg of protein in 1.3 ml of Tris buffer A for the wild type; 40 mg of protein in 1.7 ml of buffer for the R130Q enzyme) was filtered (0.2-μm-pore-size filter) and loaded at a flow rate of 1.27 ml min^{-1} cm^{-2} (1 ml/min) onto an HR10/10 Mono Q FPLC column (Pharmacia, Uppsala, Sweden) equilibrated with Tris buffer A. The Mono Q column was washed with 25 ml of the same buffer, and protein was eluted with a 0 to 1.0 M NaCl gradient in 145 ml of the equilibration buffer at a flow rate of 3.82 ml min^{-1} cm^{-2} (3 ml/min). Fractions (5 ml) were collected into tubes on ice.

The fractions containing the majority of the viral TK activity (eluted in approximately 85 mM NaCl) were dialyzed for 2 h against 20 mM piperazine-*N*,*N*′-bis(2-ethanesulfonic acid) (PIPES; pH 6.5)–1 mM dithiothreitol–10% glycerol and loaded at a rate of 400 μl min^{-1} cm^{-2} onto a 400-μl dThd-agarose column (26) which had been equilibrated with the same buffer at 4°C. The column was washed with 3 column volumes each of equilibration buffer and Tris buffer A containing 1 M NaCl. Enzyme was eluted with 8 column volumes of 600 μM dThd–1 mM ATP–1 M NaCl in Tris buffer A. The column was washed with 4 column volumes of 1.2 mM dThd–2 mM ATP–1 M NaCl in Tris buffer A to ensure all enzyme had been eluted. Portions of each fraction were set aside for protein determinations and analyses by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE). Bovine serum albumin (BSA; essentially fatty acid free; Sigma, St. Louis, Mo.) was added to the remainder of each fraction to 0.5 mg/ml to stabilize the viral TK.

The purified enzyme was stored at −70°C. Prior to use in enzyme assays, the purified VZV TK was dialyzed against Tris buffer A, or the buffer was exchanged with Tris buffer A by exclusion chromatography. No detectable loss of enzyme activity in the desalted preparation was observed after 3 months of storage at −70°C.

Production and analysis of anti-VZV TK antibody. Antibody to wild-type VZV TK was raised in two New Zealand rabbits (Hazleton, Lenexa, Kans.) by injecting 100 μg of purified TK in Freund's complete adjuvant. Two weeks after the initial immunization, the rabbits were injected with enzyme in complete adjuvant, and 2 weeks later they were injected with enzyme in incomplete Freund's adjuvant. The rabbits were bled prior to the onset of the immunization regime (preimmune serum) and 7, 14, and 21 days following injection of enzyme in incomplete Freund's adjuvant.

Pre- and postimmune sera were tested for reactivity to purified VZV TK by the Ouchterlony double-immunodiffusion technique. Postimmune serum samples showing precipitin bands were pooled and purified by ammonium sulfate precipitation. The final ammonium sulfate-precipitated gamma globulin fraction was dissolved in phosphate-buffered saline with 0.1% NaN_3 and dialyzed extensively against the same buffer. Aliquots of the gamma globulin fraction were stored at −70°C.

Enzyme assays. To measure VZV TK activity at different stages of purification, the rates of [2-^{14}C]dThd (56 Ci/mol; Moravek Biochemicals, Brea, Calif.) or [8-^{14}C]6-methoxypurine arabinoside (ara-M, 48 Ci/mol; Wellcome Research Laboratories) (8) phosphorylation were determined by the DEAE-paper procedure (3). Reaction mixtures (40 μl) contained 100 μM labeled nucleoside, 100 mM Tris-HCl (pH 7.5), 5 mM MgATP, 0.5 mM dithiothreitol, 5% (vol/vol) glycerol, and 0.2 to 0.6 mg of BSA per ml. Reactions were started by addition of enzyme and were incubated at 37°C. At timed intervals (a minimum of three points), 10-μl aliquots of the reaction mixture were spotted onto DEAE squares. The squares were washed, and the radioactivity remaining on the paper was counted.

Phosphorylating activities (dThd, TMP, and ara-M phosphorylation) in the chromatography fractions were based on single time point determinations. Because bacterial TK activity is stimulated by dCTP (30), dThd phosphorylation was measured with and without 70 μM dCTP in the reaction mixtures to distinguish between the viral and bacterial TK activities.

The rate of [^{14}C]TMP (56 Ci/mol; Moravek) phosphorylation was determined by polyethyleneimine thin-layer chromatography as previously described (16).

Relative substrate velocities were determined by measuring the rate of transfer of radiolabeled phosphate from [γ-^{32}P]ATP (27 Ci/mmol; New England Nuclear Research Products) to dThd, deoxycytidine (dCyd), ACV, or ara-M at 1 mM MgATP and 1 mM nucleoside substrate (3). Phosphorylation rates were normalized to the rate with dThd (dThd rate = 100).

Apparent K_m (K_m') and K_i (K_i') values were determined as described previously (3) except as noted; K_m' values (at 2 mM MgATP) for dThd, dCyd, and ara-M were determined from the phosphorylation rates of [*methyl*-^{3}H]dThd (53 Ci/mmol; Moravek; 0.02 to 2 μM with wild-type TK; 0.15 to 15 μM with R130Q TK), [2-^{14}C]dCyd (56 Ci/mmol; Moravek; 2 to 10 μM, wild type), or [8-^{14}C]ara-M (33 to 1,050 μM, wild type). K_i' values for dCyd and ACV were determined from the degree of inhibition of [*methyl*-^{3}H]dThd phosphorylation (0.03 to 0.5 μM with wild-type TK; 0.1 to 2.5 μM with R130Q) at six concentrations of [*methyl*-^{3}H]dThd and three concentrations of inhibitor. The velocities were analyzed by weighted least-square fits of the data to hyperbolae and then fit to a competitive inhibition model (11). dCyd and ACV were competitive alternate substrate inhibitors; therefore, $K_m' = K_i'$ (36, 40). Less than 10% substrate or inhibitor was consumed during the reaction. Initial velocities were based on the linear portion of the reactions. The relative maximal velocities were calculated from the relative substrate velocities (v) at 1 mM nucleoside substrate (above) and the measured K_m' or K_i' values [relative $V_{max} = v(s + K_m')/s$].

PAGE. Samples from different stages of purification were analyzed by SDS-PAGE (12% resolving gel) as described by Laemmli (25) and stained with Coomassie blue. Purified wild-type VZV TK was also electrophoresed through nondenaturing 5% polyacrylamide gels prepared by standard Bio-Rad protocols with modifications described by Hackstadt and Mallavia (18). SDS was omitted from all solutions, and dThd and glycerol were added to the gels (10 μM and 10%, respectively) to stabilize the enzyme. dThd (10 μM) was added to the sample treatment buffer, and the concentration of 2-mercaptoethanol was reduced to 10 mM. The Tris concentration in the running buffer was 25 mM. Gels were prerun at 200 V for 15 min at 4°C. ATP was added to the upper buffer to a final concentration of 2.5 mM, 3 μg of purified wild-type VZV TK was loaded per lane, and the samples were electrophoresed for 2 h at 200 V. After electrophoresis, one gel was stained with Coomassie blue and the other was cut into slices of 10 by 2 by 1 mm. The gel slices were incubated in 100 μl of [2-^{14}C]dThd phosphorylation reaction mixture (final concentrations as described for standard conditions) at 37°C for 30 min, and the amount of [2-^{14}C]TMP formation was measured by the DEAE-paper procedure (3).

Protein determinations. Protein concentrations were determined by the Coomassie blue dye method (Pierce reagent;

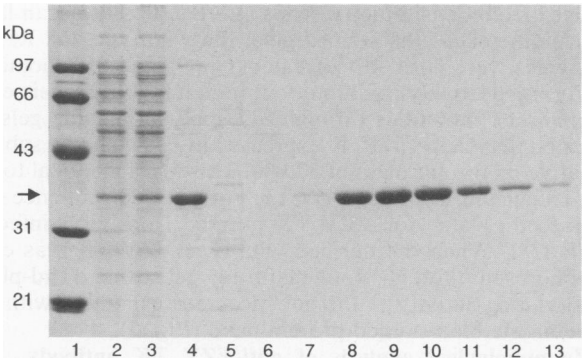

FIG. 1. PAGE analysis of wild-type VZV TK at different stages of purification. Lanes: 1, molecular weight standards; 2, soluble bacterial extract with VZV TK; 3, protein precipitated by 50% ammonium sulfate; 4, proteins remaining after chromatography with a Mono Q column; 5 and 6, proteins in the effluent during loading and washing of the dThd-agarose affinity column; 7 to 13, VZV TK eluted from the dThd-agarose by high NaCl, dThd, and ATP.

Pierce Chemical Co., Rockford, Ill.), with BSA as the protein standard.

RESULTS

Purification of wild-type and R130Q VZV TKs. The bacterially expressed wild-type and R130Q VZV TKs were purified to near homogeneity. Figure 1 shows the SDS-PAGE protein profile of samples from various steps in the purification of wild-type VZV TK. The nucleoside-phosphorylating activities of the wild-type and R130Q enzymes at each step of purification are presented in Table 1. Ara-M, a selective substrate for the wild-type VZV TK (3), was used to assess the activity of the enzyme. In contrast, ara-M was not a substrate for the R130Q TK. Therefore, [^{14}C]dThd phosphorylation rates were determined for the mutant enzyme.

To assure equal recoveries of the viral TK activities, the wild-type and R130Q enzymes were purified under identical conditions. For both enzymes, the specific activity (picomoles of ara-M monophosphate or dTMP formed per minute per microgram of protein) in the redissolved ammonium sulfate pellet was increased twofold. As determined from ara-M phosphorylation, 90% of the original wild-type VZV TK activity was recovered. Recovery of the R130Q enzyme was difficult to assess because a unique substrate for the mutant enzyme was not available; use of dThd as the substrate did not permit a quantitative differentiation between the viral and the bacterial TK activities.

The wild-type VZV TK was purified greater than 20-fold by chromatography on a Mono Q column (Table 1 and Fig. 1, lane 4). To identify and characterize the fractions which contained the VZV TK, each was assayed for dThd (with and without dCTP)-, ara-M-, and dTMP-phosphorylating activities (Fig. 2A). The VZV TK has an associated dTMP kinase activity (16), while *E. coli* has separate TK (dCTP-stimulated [30]) and dTMP kinase activities. Fractions that catalyzed the phosphorylation of ara-M also catalyzed the phosphorylation of dThd and dTMP, three rather different substrates, at a constant ratio of 2:1:1 (ara-M:dThd:TMP). These results indicated the three activities were associated with a single enzyme, the VZV TK. Most of the VZV TK eluted in fractions 15 and 16 (Fig. 2A), although some eluted immediately before and after the major peak of viral TK activity and in fractions 31 to 33 and fraction 35. Use of protease inhibitors and a variety of expression and extraction conditions did not significantly change the elution profile. Similar heterogeneity was also observed with native VZV TK purified from virus-infected cells. When the pooled activities of fractions 15 and 16 were dialyzed and rechromatographed on the Mono Q column, the bacterially expressed viral TK eluted in a single peak and in the same NaCl concentration (data not shown).

Close examination of the data revealed that the bacterial TK and dTMP kinase eluted in fractions 28 to 30 and 30 to 33, respectively; the relative rate of TK activity in the presence of dCTP increased in fractions 28 to 30, indicating the presence of bacterial TK, and the relative rate of dTMP to dThd phosphorylation was elevated slightly in fractions 30 to 33. To verify that these activities were of *E. coli* origin, bacterial extract prepared from the control strain lacking the VZV TK gene was subjected to Mono Q chromatography and assayed for dCTP-stimulated TK and for dTMP-phosphorylating activities (Fig. 2B). *E. coli* TK eluted in fractions 23 to 32, and the bacterial dTMP kinase activity was recovered in fractions 26 to 34, demonstrating that the bacterially expressed VZV TK was well separated from both the *E. coli* TK and dTMP kinase. This result simplified the subsequent

TABLE 1. Purification of VZV TK

Purification stage	Vol (ml)	Protein (mg/ml)	Sp act[a] (pmol min^{-1} μg^{-1})	Purification (fold)	Recovery (%)
Wild-type TK (ara-M phosphorylation)					
Extract	3.4	40	39	1	100[b]
$(NH_4)_2SO_4$	1.3	39	94	2	90
Mono Q	9.5	0.07	2,040	53	26
dThd-agarose	1.2	0.33	2,720	70	20
R130Q TK (dThd phosphorylation)					
Extract	6.2	27	1.6	1	100
$(NH_4)_2SO_4$	1.7	24	2.6	2	38
Mono Q	9.0	0.083	15.4[c]	9[c]	4.2[c]
dThd-agarose	1.6	0.20	20.4	12	2.4

[a] [8-^{14}C]ara-M (100 μM) was the virus-specific TK substrate with preparations containing wild-type enzyme. Extracts of control bacteria without the VZV TK gene did not catalyze the phosphorylation of ara-M (<0.02 pmol min^{-1} μg of protein^{-1}). Since the R130Q enzyme did not catalyze the phosphorylation of ara-M, [*methyl*-^{3}H]dThd was used as the substrate for this enzyme.

[b] Recoveries of wild-type VZV TK as determined from dThd phosphorylation were very similar to recoveries of ara-M phosphorylation (e.g., final recovery of dThd phosphorylating was 17%).

[c] The low recovery of TK activity and the small increase of specific activity with the R130Q enzyme reflected the removal of large amounts of bacterial enzyme relative to the viral enzyme.

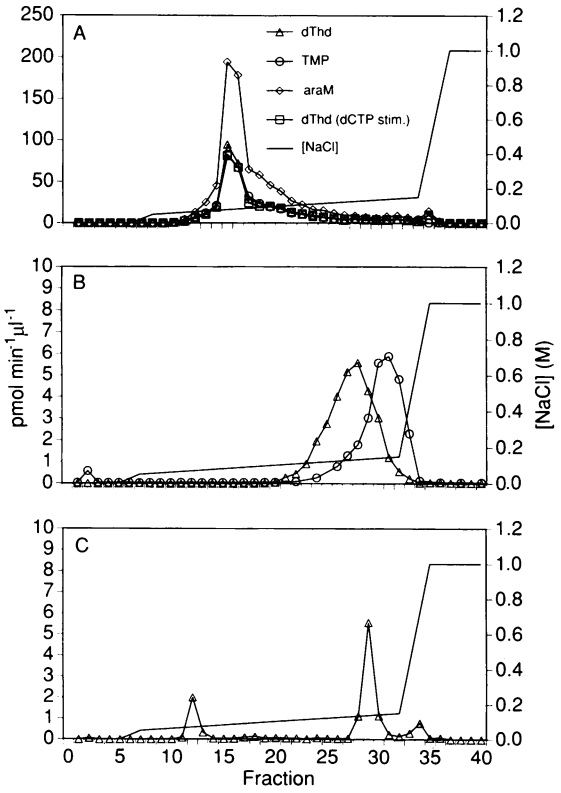

FIG. 2. Elution profile of phosphorylating activities from Mono Q column chromatography. The column was eluted with an NaCl gradient. Fractions (5 ml) were assayed for dThd-, dTMP-, and ara-M-phosphorylating activities as described in Materials and Methods. Sources of the chromatographed ammonium sulfate fractions were bacteria expressing the wild-type VZV TK (A), control bacteria lacking the VZV TK gene (B), and bacteria expressing the R130Q VZV TK (C).

affinity chromatography step, since the only species of TK left to bind to the dThd-agarose was the viral enzyme.

The R130Q TK was also well separated from the bacterial TK by Mono Q chromatography (Fig. 2C). The R130Q activity eluted in about 70 mM NaCl (fractions 12 and 13), which was lower than the salt concentration necessary to elute the wild-type enzyme (85 mM). This difference was consistently observed, but when the R130Q TK was rechromatographed on the Mono Q column, activity eluted in 85 mM NaCl. Therefore, it is unlikely that the chromatographic differences were due to the single amino acid change in the TK, but rather were due to the different complex mixtures added to the column. The specific activity for the R130Q TK was increased fourfold by this chromatographic step (Table 1). Of the total TK activity applied to the Mono Q column, only 11% was recovered as the R130Q TK, while 44% was *E. coli* TK. Thus, recovery of R130Q TK activity was at least 20% (11% as R130Q/[100% TK −44% as *E. coli*]), which was similar to recovery of wild-type TK (29%). The dThd-phosphorylating activity of the R130Q TK after Mono Q purification (Table 1) was only 1.4% of that of the wild-type TK (1,100 pmol of TMP formed min^{-1} µg of $protein^{-1}$). This is consistent with the low specific activity of the purified R130Q TK (see below).

dThd-agarose affinity chromatography yielded nearly homogeneous wild-type VZV TK (Fig. 1, lanes 7 to 13). As judged from densitometry scans (LKB 2202 Ultrascan laser densitometer) of the stained gels, the wild-type and R130Q enzymes were purified to greater than 95 and 85% homogeneity, respectively, by affinity chromatography. Their electrophoretic mobilities through SDS-polyacrylamide gels indicated that each viral TK expressed in *E. coli* had a subunit relative molecular mass of 37,000. This M_r is identical to the M_r calculated from the VZV TK polypeptide sequence (13) and to the value for VZV TK purified from virus-infected cells (38). When the purified wild-type VZV TK was electrophoresed through nondenaturing gels, the dThd-phosphorylating activity in the gel slices corresponded with the Coomassie blue-stained protein band (Fig. 3).

Immunological analysis of anti-VZV TK antibody. The polyclonal antiserum from rabbits immunized with purified wild-type VZV TK was analyzed for reactivity with both wild-type and R130Q kinases. Analysis by the Ouchterlony technique showed no reactivity of the preimmune serum with purified TK, whereas all postimmune sera produced a single, strong precipitin band when tested with the wild-type VZV TK. The gamma globulin fraction of postimmune sera was equally reactive toward wild-type and R130Q enzymes by Western immunoblot analysis and correlated to the Coomassie blue-stained protein band (32). Moreover, this antiserum raised against the bacterially expressed VZV TK precipitated both wild-type and mutant VZV TKs from virus-infected cells (41). In other systems, this polyclonal, anti-VZV TK antiserum effectively detected VZV TK in virus-infected human fetal cell cultures enriched for astrocytes, Schwann cells, or dorsal root ganglia neurons (2) as well as enzyme translated in vitro in a rabbit reticulocyte system (27). Taken collectively, these data indicated that the bacterially expressed VZV TKs are immunologically equivalent to virally expressed VZV TK.

Enzyme kinetics. The most notable effect of the R130Q substitution was the decrease in turnover number (Table 2). The catalytic rate for dThd with the R130Q enzyme was only 1.4% of that with the wild-type enzyme. This, coupled with an increased K_m' value for the R130Q enzyme, decreased the phosphorylation efficiency (relative V_{max}/K_m') with dThd to 0.45% of that for the wild-type TK.

With the other substrates, parallel differences were noted.

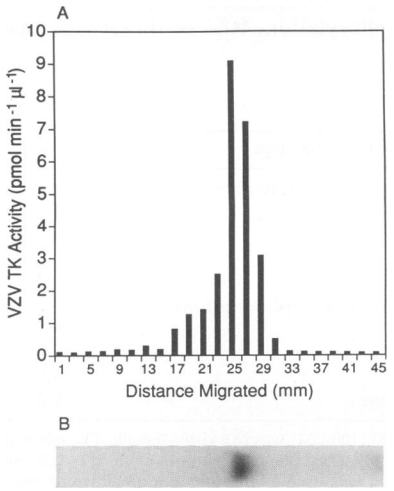

FIG. 3. VZV TK activity in nondenaturing polyacrylamide gels. dThd-phosphorylating activities were analyzed in slices from one gel (A), while the other gel was stained with Coomassie blue (B).

TABLE 2. Kinetic constants for the VZV TKs

TK	Turnover no.[a] (min^{-1})	Substrate specificity		
		K_m' or K_i' (μM)	Relative V_{max}[b]	Efficiency (relative V_{max}/K_m')
Wild type				
dThd	107	0.16	100	625
dCyd		5.3	590	110
ACV		830[c]	37	0.044
Ara-M		200	670	3.35
R130Q				
dThd	1.5	0.5	1.4	2.8
dCyd		720[c]	4.1	0.006
ACV		340[c]	<0.15[d]	—[e]
Ara-M		ND[f]	<0.15[d]	ND

[a] Based on velocities at 100 μM dThd under standard conditions and on the relative molecular weight of the dimer of 75,600.

[b] Calculated from relative substrate velocity (v) and K_m' or K_i' values [$v(s + K_m')/s$]. Velocities at 1 mM phosphate acceptor substrate were normalized to the rate with dThd for each enzyme and then to the relative turnover number of the two enzymes (wild-type relative substrate velocity for dThd = 100).

[c] Determined as K_i' values by measuring the inhibition of [^{3}H]dThd phosphorylation by three concentrations of inhibitor.

[d] Velocity at 1 mM ACV or ara-M relative to the velocity of dThd phosphorylation at 1 mM by wild-type VZV TK.

[e] Efficiency value was not calculated because relative substrate velocity was below detection limit.

[f] ND, not determined.

The relative V_{max} with dCyd as a substrate for the wild-type enzyme was approximately sixfold greater than that obtained with dThd, but the K_m' value was also 33-fold higher, resulting in an overall decrease in efficiency of dCyd phosphorylation relative to dThd phosphorylation. The comparisons between dThd and dCyd also held true with the R130Q TK. The difference in substrate efficiency was even more accentuated for the R130Q enzyme as a result of both higher K_m' and lower relative V_{max} values for dCyd.

ACV, like other purine nucleoside analogs, is a relatively inefficient substrate for the VZV TK (23) because of a low relative V_{max} and a high K_m' value (Table 2). Although ACV was not detectably phosphorylated by the R130Q TK, it was a stronger competitive inhibitor of [^{3}H]dThd phosphorylation with the R130Q enzyme than with the wild-type enzyme. Ara-M, a high-velocity substrate for the wild-type VZV TK, was not detectably phosphorylated by the R130Q enzyme.

DISCUSSION

The TK encoded by gene 36 of VZV plays a critical role in the selective activation of antiviral nucleoside analogs (3, 9, 20, 21, 23, 42). Expression of the VZV TK gene in *E. coli* has enabled us to circumvent the problems that arise from the cell-associated nature of the VZV infection (nonsynchronous and incomplete cell infection) and thereby to produce and purify enzymatically active wild-type and mutant VZV TK in quantities sufficient for kinetic characterization. The direct comparison of purified wild-type and mutant VZV TKs revealed the biochemical consequences of the mutation and thus provides an enzymatic basis for the ACV resistance displayed by the 7-1-3 variant.

It is clear from kinetic studies with the two nucleoside kinases that the activity of the R130Q enzyme has been significantly altered such that this enzyme has a narrower substrate specificity. The most dramatic difference, however, was in the turnover numbers for each enzyme. dThd phosphorylation, under saturating conditions, was reduced 70-fold, and ACV phosphorylation was not detectable with the R130Q enzyme. If ACV were, in fact, phosphorylated by the mutant enzyme at a rate below the detection limit, then the relative V_{max} would be less than 0.20 and the efficiency of the phosphorylation reaction would be less than 0.0006, or less than 1.4% of the efficiency of ACV phosphorylation by the wild-type enzyme.

A strong correlation exists between the susceptibility of wild-type and resistant strains of herpesviruses to antiviral nucleosides and the ability of the virally encoded TK to effectively utilize those compounds as substrates (7, 12, 14, 24, 35, 37). This study extends the correlation for VZV to a clinically relevant example of ACV resistance. VZV isolated from a patient who had become unresponsive to ACV therapy was less sensitive to ACV than was wild-type VZV by a factor of 30 (31). The 7-1-3 TK variant plaque purified from this clinical isolate expressed the R130Q TK (35) and showed an even higher degree of ACV resistance. This variant did not possess the DNA polymerase mutation phenotype (i.e., resistance to adenosine arabinoside and phosphonoformic acid [5]), and the polymerase gene contained only wild-type sequence (17), identical to that published by Davison and Scott (13). Because the only known mutations that confer ACV resistance occur in the viral TK and DNA polymerase genes, these observations, coupled with the lack of detectable ACV phosphorylation by the purified R130Q TK in this study, strongly suggest that the altered activity of the R130Q enzyme contributed substantially to ACV resistance in the clinical isolate.

The R130Q substitution in the mutant enzyme is located in a region of the VZV TK that is proposed to be involved in nucleoside binding (4, 22). Although there is no direct biochemical evidence for nucleoside binding at the proposed site, amino acid substitutions mapping to this region of the herpes simplex virus type 1 TK protein resulted in altered K_m' or K_i' values for dThd and 5-bromovinyl-2′-deoxyuridine without significantly changing the K_m' for ATP (12). Kinetic analysis of the R130Q VZV TK presented in this report demonstrated that the R130Q substitution decreased the turnover number for dThd 70-fold. The kinetic analysis for ATP binding was complex, but the apparent K_m value for ATP (32) was well below the concentration used for these analyses and thus does not affect these considerations. The results of the kinetic analyses are consistent with the proposal that the region of the VZV TK encompassing residues 129 to 146 is important for efficient interaction of nucleoside substrates with this enzyme. Moreover, the fact that the R130Q substitution has a greater effect on the turnover number than on the K_m' for dThd suggests that the arginine at residue 130 is involved in a chemical step of the reaction. One such speculation would be that the positively charged arginine helps position the β-phosphate group of ATP to facilitate transfer of the γ-phosphoryl group to the nucleoside 5′-OH and at the same time stabilizes some of the electron density of the transition state intermediate. That the amino acid substitution affected the chemical step more than the binding step was further exemplified by the effect on ACV as a substrate. In this case, apparent binding was not impaired, but the chemical step (phosphoryl transfer) was no longer detectable. This model is obviously simplified and does not directly account for the additional large K_m' effect with dCyd. Alternatively, it is quite possible that arginine 130 is not located in or near the catalytic site but is necessary for the correct protein conformation to allow residues at the

active site to function for catalysis. Whether arginine 130 functions in either of these possible roles must await tertiary structural information on the wild-type and R130Q VZV TKs.

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REFERENCES

1. **Aota, S., T. Gojobori, F. Ishibashi, T. Maruyama, and T. Ikemura.** 1988. Codon usage tabulated from the GenBank genetic sequence data. Nucleic Acids Res. **16**(Suppl.):315–402.
2. **Assouline, J. G., M. J. Levin, E. O. Major, B. Forghani, S. E. Straus, and J. M. Ostrove.** 1990. Varicella-zoster virus infection of human astrocytes, Schwann cells and neurons. Virology **179**:834–844.
3. **Averett, D. R., G. W. Koszalka, J. A. Fyfe, G. B. Roberts, D. J. M. Purifoy, and T. Krenitsky.** 1991. 6-Methoxypurine arabinoside as a selective and potent inhibitor of varicella-zoster virus. Antimicrob. Agents Chemother. **35**:851–857.
4. **Balasubramaniam, N. K., V. Veerisetty, and G. A. Gentry.** 1990. Herpes viral deoxythymidine kinases contain a site analogous to the phosphoryl-binding arginine-rich region of porcine adenylate kinase; comparison of secondary structure predictions and conservation. J. Gen. Virol. **71**:2979–2987.
5. **Biron, K. K. (Burroughs Wellcome Co.).** 1991. Personal communication.
6. **Biron, K. K., and G. B. Elion.** 1980. In vitro susceptibility of varicella-zoster virus to acyclovir. Antimicrob. Agents Chemother. **18**:443–447.
7. **Biron, K. K., J. A. Fyfe, J. E. Noblin, and G. B. Elion.** 1982. Selection and preliminary characterization of acyclovir-resistant mutants of varicella zoster virus. Am. J. Med. (Acyclovir Symp.) **73**:383–386.
8. **Burnette, T. C., G. W. Koszalka, T. A. Krenitsky, and P. de Miranda.** 1991. Metabolic disposition and pharmacokinetics of the antiviral agent 6-methoxypurine arabinoside in rat and monkey. Antimicrob. Agents Chemother. **35**:1165–1173.
9. **Cheng, Y.-C., G. Dutschman, E. DeClercq, A. S. Jones, S. G. Rahim, G. Verhelst, and R. T. Walker.** 1981. Differential affinities of 5-(2-halogenovinyl)-2′-deoxyuridines for deoxythymidine kinases of various origins. Mol. Pharmacol. **20**:230–233.
10. **Cheng, Y.-C., and M. Ostrander.** 1976. Deoxythymidine kinase induced in HeLa TK^- cells by herpes simplex virus type I and II. II. Purification and characterization. J. Biol. Chem. **251**: 2605–2610.
11. **Cleland, W. W.** 1979. Statistical analysis of enzyme kinetic data. Methods Enzymol. **63**:103–138.
12. **Darby, G., B. A. Larder, and M. M. Inglis.** 1986. Evidence that the 'active centre' of the herpes simplex virus thymidine kinase involves an interaction between three distinct regions of the polypeptide. J. Gen. Virol. **67**:753–758.
13. **Davison, A. J., and J. E. Scott.** 1986. The complete DNA sequence of varicella-zoster virus. J. Gen. Virol. **67**:1759–1816.
14. **Ellis, M. N., P. M. Keller, J. A. Fyfe, J. L. Martin, J. F. Rooney, S. E. Straus, S. Nusinoff Lehrman, and D. W. Barry.** 1987. Clinical isolate of herpes simplex virus type 2 that induces a thymidine kinase with altered substrate specificity. Antimicrob. Agents Chemother. **31**:1117–1125.
15. **Fyfe, J., and K. Biron.** 1980. Phosphorylation of acyclovir by a thymidine kinase induced by varicella-zoster virus, p. 1378–1379. *In* J. D. Nelson and C. Grassi (ed.), Current chemotherapy and infectious disease, vol. 2. American Society for Microbiology, Washington, D.C.
16. **Fyfe, J. A.** 1982. Differential phosphorylation of (*E*)-5-(2-bromovinyl)-2′-deoxyuridine monophosphate by thymidine kinases from herpes simplex viruses types 1 and 2 and varicella zoster virus. Mol. Pharmacol. **21**:432–437.
17. **Gaillard, R., and S. Short.** Unpublished data.
18. **Hackstadt, T., and L. P. Mallavia.** 1978. Deoxypyrimidine nucleoside metabolism in varicella-zoster virus-infected cells. J. Virol. **25**:510–517.
19. **Jacobson, M. A., T. G. Berger, S. Fikrig, P. Becherer, J. W. Moohr, S. C. Stanat, and K. K. Biron.** 1990. Acyclovir-resistant varicella zoster virus infection after chronic oral acyclovir therapy in patients with the acquired immunodeficiency syndrome (AIDS). Ann. Intern. Med. **112**:187–191.
20. **Karlström, A. R., C. F. R. Källander, G. Abele, and A. Larsson.** 1986. Acyclic guanosine analogs as substrates for varicella-zoster virus thymidine kinase. Antimicrob. Agents Chemother. **29**:171–174.
21. **Keller, P. M., J. A. Fyfe, L. Beauchamp, C. M. Lubbers, P. A. Furman, H. J. Schaeffer, and G. B. Elion.** 1981. Enzymatic phosphorylation of acyclic nucleoside analogs and correlations with antiherpetic activities. Biochem. Pharmacol. **30**:3071–3077.
22. **Kit, S.** 1985. Thymidine kinase. Microbiol. Sci. **2**:369–375.
23. **Koszalka, G. W., D. R. Averett, J. A. Fyfe, G. B. Roberts, T. Spector, K. Biron, and T. A. Krenitsky.** 1991. 6-*N*-Substituted derivatives of adenine arabinoside as selective inhibitors of varicella-zoster virus. Antimicrob. Agents Chemother. **35**:1437–1443.
24. **Lacey, S. F., T. Suzutani, K. L. Powell, D. J. M. Purifoy, and R. W. Honess.** 1991. Analysis of mutations in the thymidine kinase genes of drug-resistant varicella-zoster virus populations using the polymerase chain reaction. J. Gen. Virol. **72**:623–630.
25. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**:680–685.
26. **Lee, L.-S., and Y.-C. Cheng.** 1976. Human deoxythymidine kinase. I. Purification and general properties of the cytoplasmic and mitochondrial isozymes derived from blast cells of acute myelocytic leukemia. J. Biol. Chem. **251**:2600–2604.
27. **Mahalingam, R., G. Cabirac, M. Wellish, D. Gilden, and A. Vafai.** 1990. In-vitro synthesis of functional varicella-zoster and herpes simplex viral thymidine kinase. Virus Genes **4**:105–120.
28. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
29. **Messing, J.** 1983. New M13 vectors for cloning. Methods Enzymol. **101**:20–78.
30. **Okazaki, R., and A. Kornberg.** 1964. Deoxythymidine kinase of *Escherichia coli*. I. Purification and some properties of the enzyme. J. Biol. Chem. **239**:269–274.
31. **Pahwa, S., K. Biron, W. Lim, P. Swenson, M. H. Kaplan, N. Sadick, and R. Pahwa.** 1988. Continuous varicella-zoster infection associated with acyclovir resistance in a child with AIDS. JAMA **260**:2879–2882.
32. **Roberts, G., J. A. Fyfe, R. K. Gaillard, and S. A. Short.** Unpublished data.
33. **Rosenberg, A. H., B. N. Lade, D.-S. Chui, S.-W. Lin, J. J. Dunn, and F. W. Studier.** 1987. Vectors for selective expression of cloned DNAs by T7 RNA polymerase. Gene **56**:125–135.
34. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74**:5463–5467.
35. **Sawyer, M. H., G. Inchauspe, K. K. Biron, D. J. Waters, S. E. Straus, and J. M. Ostrove.** 1988. Molecular analysis of the pyrimidine deoxyribonucleoside kinase gene of wild-type and acyclovir-resistant strains of varicella-zoster virus. J. Gen. Virol. **69**:2585–2593.
36. **Segal, I. H.** 1975. Enzyme kinetics: behavior and analysis of rapid equilibrium and steady-state enzyme systems, p. 292, 329, 341, and 810. John Wiley & Sons, New York, N.Y.
37. **Shigeta, S., S. Mori, T. Yokota, K. Konno, and E. DeClercq.** 1986. Characterization of a varicella-zoster virus variant with altered thymidine kinase activity. Antimicrob. Agents Chemother. **29**:1053–1058.
38. **Shiraki, K., T. Ogino, K. Yamanishi, and M. Takahashi.** 1985. Immunochemical characterization of pyrimidine kinase induced

by varicella-zoster virus. J. Gen. Virol. **66**:221–229.
39. **Shiraki, K., T. Ogino, K. Yamanishi, and M. Takahashi.** 1986. Thymidine kinase with altered substrate-specificity of acyclovir-resistant varicella-zoster virus. Biken J. **29**:7–10.
40. **Spector, T., and W. W. Cleland.** 1981. Meanings of K_i for conventional and alternate-substrate inhibitors. Biochem. Pharmacol. **30**:1–7.
41. **Talarico, C. (Burroughs Wellcome Co.).** 1991. Personal communication.
42. **Yokota, T., K. Konno, M. Shuichi, S. Shigeta, M. Kumagai, Y. Watanabe, and H. Machida.** 1989. Mechanism of selective inhibition of varicella-zoster virus replication by 1-β-D-arabinofuranosyl-*E*-5-(2-bromovinyl)uracil. Mol. Pharmacol. **36**: 312–326.