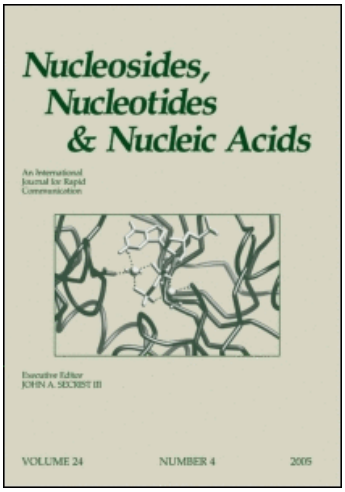

Nucleosides, Nucleotides and Nucleic Acids
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597286

Interaction of the Recombinant Herpes Simplex Virus Type 1 Thymidine Kinase with Thymidine and Aciclovir: A Kinetic Study

Susanna Kussmann-gerber[a]; Christine Wurth[a]; Leonardo Scapozza[a]; Beatrice D. Pilger[a]; Vladimir Pliška[b]; Gerd Folkers[a]
[a] Department of Pharmacy, Swiss Federal Institute of Technology (ETH), Zürich, Switzerland [b] Department of Animal Science, Swiss Federal Institute of Technology (ETH), Zurich, Switzerland

To cite this Article Kussmann-gerber, Susanna , Wurth, Christine , Scapozza, Leonardo , Pilger, Beatrice D. , Pliška, Vladimir and Folkers, Gerd(1999) 'Interaction of the Recombinant Herpes Simplex Virus Type 1 Thymidine Kinase with Thymidine and Aciclovir: A Kinetic Study', Nucleosides, Nucleotides and Nucleic Acids, 18: 3, 311 — 330
To link to this Article: DOI: 10.1080/15257779908043078
URL: http://dx.doi.org/10.1080/15257779908043078

NUCLEOSIDES & NUCLEOTIDES, 18(3), 311-330 (1999)

INTERACTION OF THE RECOMBINANT HERPES SIMPLEX VIRUS TYPE 1 THYMIDINE KINASE WITH THYMIDINE AND ACICLOVIR: A KINETIC STUDY

Susanna Kussmann-Gerber[$‡], Christine Wurth[$‡], Leonardo Scapozza[$*], Beatrice D. Pilger[$], Vladimir Pliška[#], and Gerd Folkers[$]

[$] Department of Pharmacy, Swiss Federal Institute of Technology (ETH), Winterthurerstr. 190, CH-8057 Zürich, Switzerland

[#] Department of Animal Science, Swiss Federal Institute of Technology (ETH), ETH Zentrum, CH-8092 Zürich, Switzerland

ABSTRACT

Herpes Simplex Virus type 1 thymidine kinase (HSV 1 TK) is a key target for antiviral therapy and it phosphorylates a broad spectrum of nucleosides and nucleotides. We report the results from kinetic and inhibition experiments with HSV 1 TK, and show that there is a preferred, but not exclusive, binding order of substrates, i.e. dT binds prior to ATP. Furthermore, the results provide new informations on the mechanism of binding suggesting that HSV1 TK undergoes conformational changes during the catalytic cycle.

INTRODUCTION

Thymidine kinase (TK) (EC 2.7.1.21) is a key enzyme in the pyrimidine salvage pathway catalyzing the γ-phosphate transfer from ATP to thymidine (dT) and thus yielding thymidine monophosphate (dTMP). In contrast to the highly specific human cellular TK (TK1), the Herpes Simplex Virus type 1 thymidine kinase (HSV 1 TK) phosphorylates a broad spectrum of nucleosides and nucleotides including thymidine (dT),

---

[‡] Both authors contributed equally to the presented work.

[*] Author to whom correspondence should be addressed: Phone: ++41-1-635 6071, fax: ++41-1-635 6884, e-mail: scapozza@pharma.ethz.ch

thymidine monophosphate (dTMP), deoxycytidine (dC), and several purine-derived antiviral drugs such as aciclovir (ACV) and ganciclovir (GCV) [1-3]. The differences in substrate specificity for the human TK1 and the HSV 1 TK can be exploited and therefore used to establish a molecular basis for selective antiviral therapy. Furthermore, HSV 1 TK has been used as a suicide enzyme in gene therapy of cancer [4-7] and AIDS [8].

Thymidine kinase isoforms are members of the family of nucleotide binding proteins (NBP). Recently, structures of the HSV 1 TK complexing different substrates were solved [9-11]. The NBP-family includes the HSV 1 TK, the adenylate kinase (ADK), and the elongation factor Tu (EF-Tu), the crystal structure all of which have been solved. The HSV 1 TK exhibits several structural features shared by the NBPs, including (i) a core of a parallel five-stranded β-sheet, (ii) several surrounding α-helices, (iii) a glycine-rich loop (phosphate-binding loop), and (iv) an arginine-rich helix [11]. The active center of HSV 1 TK is formed by an ATP and a dT binding site. The ATP binding site is more solvent exposed than the thymidine binding site [9, 11]. This is in agreement with the approximately one hundred fold higher K_m value for ATP (values between 12μmol/L and 118μmol/L are reported [1, 3] compared to the K_m value of 0.2μmol/L for thymidine [1, 12].

Although the crystal structure of the recombinant HSV 1 TK complexed with thymidine and ATP has been solved [11], some structure-function relationships remain elusive. The structure shows a closed conformation with respect to the binding sites. This would appear to indicate an ordered binding of the substrates, with thymidine addition occurring prior to the binding of ATP. In contrast, a Random Bi Bi binding mechanism with an ordered substrate release (ADP is released first) has been postulated for the native HSV 1 TK [1]. These conclusions, however, were based on a rather inaccurate graphical analysis (double reciprocal plots) of the kinetic data by which the data points are unequally weighted.

Nonetheless, it has been demonstrated that nucleoside monophosphate (NMP) kinases such as ADK undergo extensive conformational changes during their catalytic cycles. In a detailed study it was revealed that the ADK apo-enzyme has a larger solvent-accessible surface area as a consequence of its more open conformation, compared to the conformation when complexed with the transition state inhibitor Ap_5A [13]. In contrast to the closed conformation, the open form is able to accept random docking of the

substrates. This is in agreement with results of kinetic experiments, including those with the transition state inhibitor Ap_5A. These studies provided support for the Random Bi Bi mechanism for muscle ADK [14, 15].

TK displays similar three-dimensional features with ADK. Therefore, it can be assumed that TK also shares some conformational flexibility with ADK. In this communication, we report the results from kinetic and inhibition experiments with HSV 1 TK, and show that HSV 1 TK reveals a preferred, but not exclusive, binding order of substrates, i.e. dT binds prior to ATP addition.

METHODS

Materials: [methyl 1',2'-^{3}H] thymidine (100-130 Ci/mmol) was purchased from Amersham and [side chain-2-^{3}H] aciclovir (15-35 Ci/mmol) from NEN DuPont. Aciclovir was obtained from Wellcome, London. The plasmid pGEX2T was purchased from Pharmacia. The plasmid pBR322-TK containing the gene for HSV 1 strain F TK was a gift from Dr. S. McKnight.

Expression and purification of HSV 1 TK: The bacterial expression vector pGEX2T-TK was constructed as described earlier [16]. The purification was achieved by a one-step procedure as previously described [17]. Expression and purification was followed by SDS-PAGE.

Thymidine kinetics: Initial velocities of products formation were used for estimation of kinetic and equilibrium constants of HSV 1 TK - ligand interactions. Reaction mixtures containing 50 mM Tris pH 7.2, 5 mM $MgCl_2$, 3 mg/mL BSA, 0.10 ng (= 0.25 pmol) TK and various concentrations of [^{3}H]-thymidine (0.1-1.2 μM) and ATP (10-80 μM) in a final volume of 30 μL were incubated at 37°C. Aliquots of 5 μL were removed every 3 minutes (five time intervals in total for each reaction) and inactivated by transferring them immediately to DEAE-cellulose paper squares (9 mm x 9 mm) placed on the bottom of the scintillation vials. The DEAE-paper squares were dried at room temperature, washed, cellulase digested and the retained radioactivity was counted as described earlier [18].

Aciclovir kinetics: Reaction mixtures for initial velocity measurements containing 50 mM Tris pH 7.2, 5 mM $MgCl_2$, 3 mg/mL BSA, 0.10 μg (= 0.25 nmol) TK and various concentrations (50-800 μM) of [^{3}H]-aciclovir (1:250 mixture of labeled and unlabeled

aciclovir) and ATP (10-80 μM) in a final volume of 30 μL were incubated at 37°C. Aliquots of 5 μL were removed and treated as described for the thymidine kinetics.

Aciclovir inhibition study: Initial velocities of [^{3}H]-thymidine phosphorylation were measured in the absence and presence of aciclovir at three concentrations (0, 50, 150 and 400 μM or 0, 300, 700 and 1000 μM, respectively). ATP was constant at a concentration of 30 μM (below K_m of the enzyme). For all 16 concentration combinations of four [^{3}H]-thymidine (0.1, 0.2, 0.5 and 1 μM) and the four aciclovir (ACV) concentrations, initial rate measurements were performed as described for the thymidine kinetic.
All kinetic experiments were repeated at least twice with different TK batches.

TK assay by thin-layer chromatography (TLC): For thymidine TLCs, reaction mixtures containing 50 mM Tris pH 7.2, 5 mM $MgCl_2$, 0.5 mg/mL BSA, 5 mM ATP, 60 μM [^{14}C]-thymidine and 44 ng/mL (= 1.1 pmol/mL) HSV 1 TK were incubated at 37°C. The aciclovir assay mixtures contained 50 mM Tris pH 7.2, 5 mM $MgCl_2$, 0.5 mg/mL BSA, 1 mM ATP, 1 mM aciclovir, 1 μM [^{3}H]-aciclovir and 10 μg/mL (= 25 nmol/mL) TK. After 60 minutes, an additional 2mM ATP was added to the aciclovir reaction mixture. Aliquots of 5 μL were collected at regular time intervals, boiled for 3 minutes and centrifuged. The supernatant was transferred to PEI-cellulose plates, which had been pre-spotted with unlabeled thymidine, dTMP and dTDP. The plates were successively developed in 0.2 M LiCl for 2 minutes, 1 M LiCl for 6 minutes and 1.6 M LiCl for 22 minutes. The TLC plates were directly subjected to autoradiography. The signals were intensified by treating the TLC plates with a 2 M sodium salicylate solution [19] prior to exposure to X-ray film.

Data analysis: The data from the time-course experiments were converted to units of product formation per minute (initial velocities). These data sets were then fitted by a non-linear regression routine to the appropriate initial rate equation. The SYSTAT software Version 5.02 for Windows (Systat, Inc., Evanston, IL, U.S.A.) was utilized.

RESULTS

The thymidine kinase (TK) of the wild-type strain F of Herpes Simplex Virus type 1 (HSV 1) was expressed in *E. coli* as a thrombin-cleavable glutathione S-transferase fusion

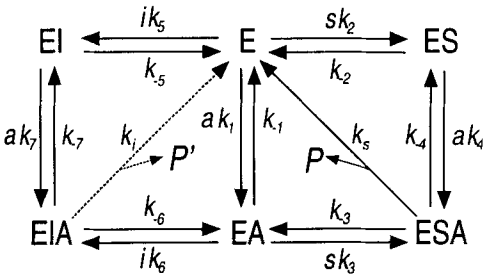

FIGURE 1

Scheme of the concerted action model: Rate constants k for the corresponding reaction steps are indicated at the arrows denoting direction of these steps. Indices 1 to 7 stand for association steps (the corresponding rate is $k_i \times$ ligand concentration; see below), -1 to -7 for dissociation steps; k_s and k_i are rate constants of thymidine and aciclovir phosphorylation (P and P' are corresponding phosporylated products). Symbols s, a and i stand for thymidine, ATP and aciclovir concentrations, respectively. Broken arrow on the left-hand side symbolizes a slow reaction (inhibition step).

protein, and purified to homogeneity in a one-step procedure described previously [17]. Expression and purification was controlled by SDS-PAGE (data not shown).

The activity of the recombinant TK was evaluated by quantifying the conversion of radioactively labeled substrates to their phosphorylated products. Substrate and product were separated by means of two procedures: adsorption onto DEAE-cellulose paper discs, a technique first described by Furlong [20], and by thin-layer chromatography (TLC) on PEI-cellulose plates according to Fitt *et al.* [21]. In contrast to the first of these methods, the TLC separation enabled differentiation between mono-, di-, and triphosphorylated products but it did not allow simple quantitative determination of the tritiated material. Nevertheless, the formation of dTDP during the initial velocity measurements was excluded (data not shown).

Thymidine kinetics (partial model):

Kinetic scheme on fig. 1 shows individual steps of the thymidine/ATP and aciclovir/ATP interaction with the thymidine kinase. The steady state in this complex system is described by several equilibria and Michaelis constants. In the absence of the inhibitor I (i = 0), two "pure" equilibrium constants describe the dissociation of the

enzyme-ATP (K_1) and enzyme-thymidine (K_2) complexes,

$$K_1 = k_{-1}/k_1 \tag{1a}$$

$$K_2 = k_{-2}/k_2 . \tag{1b}$$

Furthermore, the Michaelis constants which define the reaction paths EA → ESA → E and ES → ESA → E are

$$K_{3m} = (k_s + k_{-3})/k_3 \tag{2a}$$

$$K_{4m} = (k_s + k_{-4})/k_4 ; \tag{2b}$$

where k_s is the rate constant of the decomposition of the ternary complex ESA. The algebraic expression for the initial reaction rate (in pmol/min/μg protein) becomes very complex under conditions of a Michaelis-Menten type mechanism,

$$v_0 = k_s[ESA] , \tag{3}$$

(brackets denote concentrations of intermediate complexes) when taking into account all reaction steps of this multiple cycle system [22]. However, approximations can be achieved when considering the two pathways of the first cycle separately. For the pathway E → ES → ESA → E is

$$[ESA] = \frac{e_0}{\frac{K_{4m}}{a}\left(\frac{K_2}{s}+1\right)+\frac{k_s}{k_2}\frac{1}{s}+1} \tag{4a}$$

(e_0 is the total enzyme concentration). The other pathway, E → EA → ESA → E, yields

$$[ESA] = \frac{e_0}{\frac{K_{3m}}{s}\left(\frac{K_1}{a}+1\right)+\frac{k_s}{k_1}\frac{1}{a}+1} . \tag{4b}$$

Equation 4a describes the substrate binding mechanism in which thymidine binds prior to ATP, equation 4b the one for ATP binding prior to thymidine. Symbols e_0, s, a, i stand for the total enzyme concentration and concentrations of thymidine, ATP and aciclovir, respectively. Comparison of the denominators leads to the definition of several constraints which reduce the degrees of freedom with respect to the equilibrium and Michaelis constants, and define interrelationships between them. First, it proves that

$$K_1 K_{3m} = K_2 K_{4m} \tag{5}$$

which is a generally valid condition for cyclic systems of this type, declaring that the sum of Gibbs free energies in the two directions (E → EA → ESA and E → ES → ESA) must be equal. Second, obviously,

TABLE 1

Parameter values associated with thymidine kinetics, fitted to equation 7

The two independent initial velocity data sets were analyzed by nonlinear regression using the computer program SYSTAT. The corresponding equation 7 is given in the results section. The true k_s values have been calculated starting from eq. 3 using V_{max} as v_0 with the steady-state assumption [ESA]=[E_0] and are shown in s^{-1}, the dissociation and Michaelis constants in μmol/L. A.S.E. represent asymptotic standard errors computed from means of the Hessian matrix after the termination of the iterations.

	set 1	A.S.E.	set 2	A.S.E.	geometric mean
k_s	0.356	0.022	0.340	0.019	0.348
K_1	45.14		77.85		59.3
K_2	0.123	0.053	0.158	0.060	0.139
K_{3m}	0.056	0.023	0.033	0.018	0.043
K_{4m}	20.55	3.55	16.26	2.85	18.3
r^2	0.976		0.973		
n	20		20		

$$K_{3m} = k_s/k_2 \qquad (6a)$$

and

$$K_{4m} = k_s/k_1 \,. \qquad (6b)$$

This facilitates the numeric estimates of the individual reaction descriptors: for computational reasons, eq.4a can be used in the form

$$v_0 = \frac{V_{max}}{\frac{K_{4m}}{a}\left(\frac{K_2}{s}+1\right)+\frac{K_{3m}}{s}+1} \,, \qquad (7)$$

where V_{max} stands for maximal initial reaction rate; value of K_1 follows from eq. 5. And finally, the comparison yields an interesting, although practically less important, constraint for rate constants in the two directions:

$$\frac{k_4-k_1}{k_1}\times\frac{1}{k_{-4}} = \frac{k_3-k_2}{k_2}\times\frac{1}{k_{-3}} \,. \qquad (8)$$

In order to evaluate the Michaelis-Menten constants for the thymidine phosphorylation reaction, two independent data sets were individually fitted to equation 7. The results are summarized in table 1. The dissociation constants for thymidine were determined as

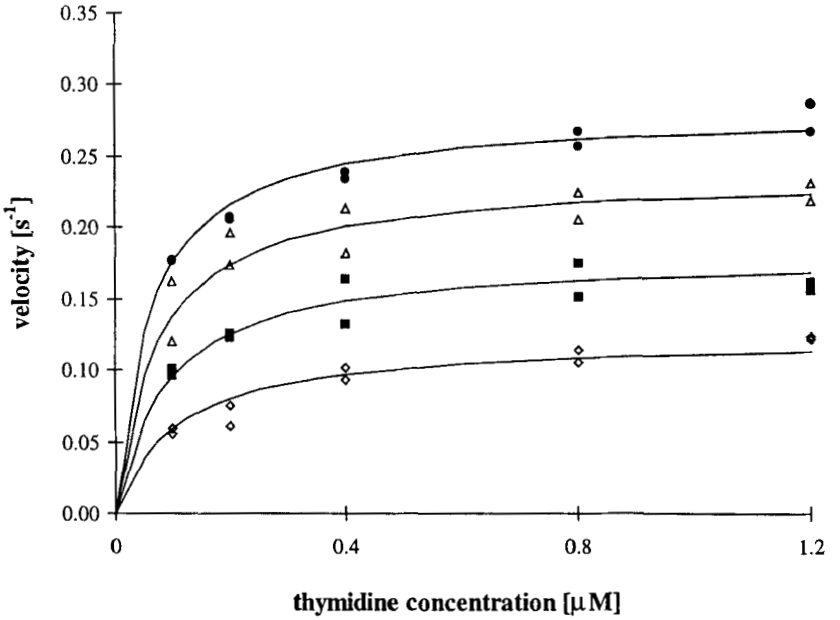

FIGURE 2
Thymidine kinetics: correlation between experimental and computed values using the direct plot.
Actual measured data points are represented by symbols (◊: 10µM ATP; ■:20µM ATP; Δ: 40µM ATP; ●: 80µM ATP). The plot of the eq.7 using the determined parameters from table 1 is given as a line.

0.139 µmol/L for the binary thymidine-enzyme complex, and 0.043 µmol/L for the ternary thymidine:ATP-enzyme complex. For the co-substrate ATP, a value of 59.3 µmol/L was obtained for the binary ATP-enzyme complex and 18.3 µmol/L was determined for the ternary ATP:thymidine-enzyme complex. k_s revealed a value of 0.348 s^{-1}. The obtained parameter values were verified graphically by comparing the plot of the mathematical function with the experimental data points (fig. 2). The correlation between estimated and computed values ($r^2 > 0.97$) indicated that the mathematical model describes sufficiently well the kinetics of the system. The increase in the binding affinity of thymidine, in the presence of ATP, suggests a rearrangement of the active site. This occurs when both substrate and co-substrate are bound. Moreover, the increase in the binding affinity of thymidine, in the presence of ATP, and thus, the suggestion of rearrangement has been

TABLE 2

Parameter values associated with thymidine kinetics, fitted to the model where thymidine binds prior to ATP

The two independent initial velocity data sets were analyzed by nonlinear regression using the computer program SYSTAT. The corresponding equation 10a is given in the results section. The true $k_s$values are shown in s^{-1}, the dissociation constants K_2 and K_{4m} in µmol/L.

	set 1	A.S.E.	set 2	A.S.E.	geometric mean
k_s	0.316	0.014	0.315	0.012	0.315
K_2	0.30	0.064	0.28	0.059	0.290
K_{4m}	14.18	2.31	12.57	1.91	13.3
r^2	0.964		0.967		
n	20		20		

confirmed by isothermal titration calorimetry (ITC) measurements. The equilibrium association constant (K_B) of dT in absence and in presence of ATP, measured by ITC, was $1.9x10^5$ M^{-1} and $190x10^5$ M^{-1} respectively [23].

As for any enzymatic reaction, it holds

$$k_1 >> k_s << k_2. \tag{9}$$

In case that k_s is fully negligible compared to the on-rate constants, eq. 4 becomes

$$v_0 = \frac{V_{max}}{\frac{K_{4m}}{a}\left(\frac{K_2}{s}+1\right)+1} \tag{10a}$$

and

$$v_0 = \frac{V_{max}}{\frac{K_{3m}}{a}\left(\frac{K_1}{s}+1\right)+1}\ , \tag{10b}$$

representing two alternative penultimate mechanisms in which one of the intermediate complexes, EA (eq. 10a) and ES (eq. 10b), is *not* formed.

The results of the fit of two thymidine data sets to equation 10a (substrate binding prior to ATP) are given in table 2. As expected, the obtained values for the dissociation

TABLE 3

Parameter values associated with aciclovir phosphorylation kinetics, fitted to equation 11

The two independent initial velocity data sets were analyzed by nonlinear regression using the computer program SYSTAT. The true $k_s$values are shown in s^{-1}, the dissociation and Michaelis constants in µmol/L. The corresponding equation 11 is given in the results section.

	set 1	A.S.E.	set 2	A.S.E.	geometric mean
k_s	0.085	0.015	0.120	0.025	0.101
K_1	45.9		34.8		40.0
K_5	204.7	173.0	128.7	115.6	162.3
K_{6m}	180.6	86.7	242.7	114.2	209.4
K_{7m}	40.5	19.9	65.6	29.1	51.5
r^2	0.932		0.939		
n	20		20		

constants K_2 and K_4 (0.29 µmol/L and 13.3 µmol/L, respectively) are consistent with the values obtained with equation 4a. Both data sets revealed a close correlation to the experimental data. The mathematical fit of the thymidine data sets to equation 10b (ATP binding prior to thymidine) failed to converge. In addition, only negative values for K_1 were obtained and correlation coefficients of approximately 0.2 were determined (data not shown).

Aciclovir kinetics (partial model):

In analogy to eq. 7 the aciclovir phosphorylation kinetics can be described by

$$v_0' = \frac{V'_{max}}{\frac{K_{7m}}{a}\left(\frac{K_5}{i}+1\right)+\frac{K_{6m}}{i}+1} \quad . \qquad (11)$$

Michaelis constants K_{7m} and K_{6m} are analogical to K_{4m} and K_{3m} (eqs 2), respectively. Two independent data sets were analysed and the resulting parameters are given in table 3.

Dissociation constants of 162.3 µmol/L and 209.4 µmol/L were determined for the aciclovir-enzyme complex and the ternary aciclovir:ATP-enzyme complex, respectively. A value of 40.0 µmol/L was determined for the binary ATP-enzyme, and 51.5 µmol/L for the ternary ATP:aciclovir-enzyme complex. The k_s value, 0.101 s^{-1}, is about three-times lower compared to the k_s of the thymidine phosphorylation (cf. tab. 2). Nevertheless, the HSV 1 TK phosphorylates aciclovir with a still considerable turnover rate and thus, eq. 11 permits to estimate the Michaelis constants K_{7m}, K_{6m}, and K_5.

Aciclovir inhibition study (concerted action model):

The initial rate of thymidine phosphorylation in the presence of all three components, thymidine, ATP and aciclovir, is given by

$$v = \frac{V_{max}}{\frac{K_{4m}}{a}\left(\frac{K_2}{s}+1\right)+\frac{K_{3m}}{s}\left(\frac{i}{K_{6m}}\left(\frac{K_{7m}}{a}+1\right)+1\right)+1} \qquad (12)$$

As mentioned above, aciclovir is still considerably phosphorylated by the HSV 1 TK. Thus, the two Michaelis constants K_{7m} and K_{6m} gain a character of equilibrium dissociation constants K_7 and K_6. Together with K_5, they can simply be assigned as inhibition constants.

Parameters obtained from the independent thymidine and ACV kinetics were used as the initial values for the computeral analysis. Three independent initial velocity data sets from aciclovir inhibition experiments were fitted to equation 12; results are summarised in table 4. The constant for the dissociation of thymidine from the binary thymidine-enzyme complex (K_2) revealed an identical value as for the independent thymidine kinetic experiment, namely 0.139 µmol/L. The constant for the dissociation of thymidine from the ternary thymidine:ATP-enzyme complex was calculated as 0.075 µmol/L and the parameters for ATP revealed values of 30.0 µmol/L for the binary and 16.2 µmol/L for the ternary complex. The constants for the dissociation of ACV from the ternary ACV:ATP-enzyme complex (K_6) was found to be 150.7 µmol/L for the dependent model (tab. 4) and 209.4 µmol/L independent model (tab. 3). K_5 showed the poorest correlation between the dependent and independent model with values of 56.2 and 162.3 µmol/L,

TABLE 4

Parameter values associated with the aciclovir inhibition study, fitted to equation 12
The three independent initial velocity data sets were analyzed by nonlinear regression using the computer program SYSTAT. The true k_s values are shown in s^{-1}, the dissociation and Michaelis constants in µmol/L. The corresponding equation 12 is given in the results section. The values of K_1 have been obtained by solving equation 5. K_5 has been calculated using the interrelationship ($K_1 K_{6m} = K_5 K_{7m}$) that is analogue to eq. 5.

	set 1	set 2	set 3	geometric mean
k_s	0.341	0.319	0.319	0.346
K_1	29.2	33.8	27.3	30.0
K_2	0.115	0.190	0.123	0.139
K_{3m}	0.083	0.071	0.072	0.075
K_{4m}	21.1	12.6	16.0	16.2
K_5	42.3	76.1	55.2	56.2
K_{6m}	92.8	178.5	206.9	150.7
K_{7m}	64.0	79.3	102.5	80.4
r^2	0.963	0.938	0.983	
n	16	16	16	

respectively. Moreover, the matrix for the asymptotic correlation of the parameters (calculated by the program SYSTAT) showed that K_{6m} and K_{7m} can be only poorly predicted.

Initial velocities obtained experimentally were correlated with those computed by means of estimated steady-state parameters (fig. 3). As expected, the regression coefficient (slope) of this correlation was close to 1 (i.e. 1.0015). The correlation was highly significant ($r^2 = 0.969$). Thus, the determined parameters accurately reflect the recorded kinetic data.

Qualitative thin-layer chromatography:

The TLC assay revealed that HSV 1 TK phosphorylates both thymidine and aciclovir firstly to the monophosphate and then to the diphosphate form (fig. 4). For the

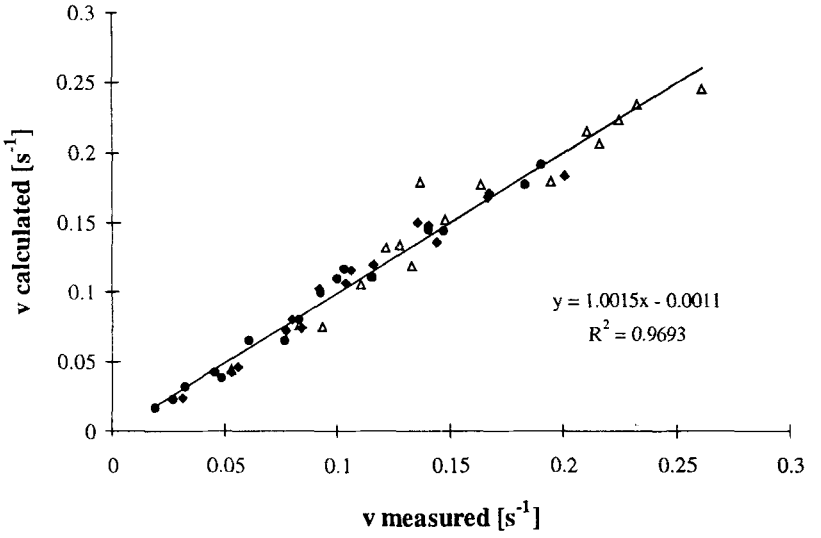

FIGURE 3
ACV inhibition study: correlation between initial velocities measured experimentally and those computed by means of estimated steady-state parameters. The data points of the three independent data sets are represented by symbols (♦,●, and Δ). The trend line is calculated using linear regression and the resultant equation is indicated.

substrate analogue aciclovir, a much higher enzyme concentration was required in order to convert the majority of the aciclovir to the monophosphate form. In contrast to the formation of thymidine diphosphate, aciclovir diphosphate could only be detected when the major portion of the ACV had been converted to the monophosphate form (fig. 4B).

DISCUSSION AND CONCLUSIONS

In the earlier kinetic studies performed with enzyme-saturating ATP concentrations (1 - 5mM), the apparent K_m for thymidine was determined as 0.2 μmol/L [1, 12, 24, 25]. This value is in the same range as the calculated K_2 value in the present study. When using such enzyme-saturating ATP concentrations, one would expect to determine the K_{3m} parameter according to figure 1, since the major portion of the enzyme would be present as an enzyme-ATP complex. These findings suggest that thymidine addition prior to ATP binding is the preferred order of substrate docking. Consistent with this is that earlier

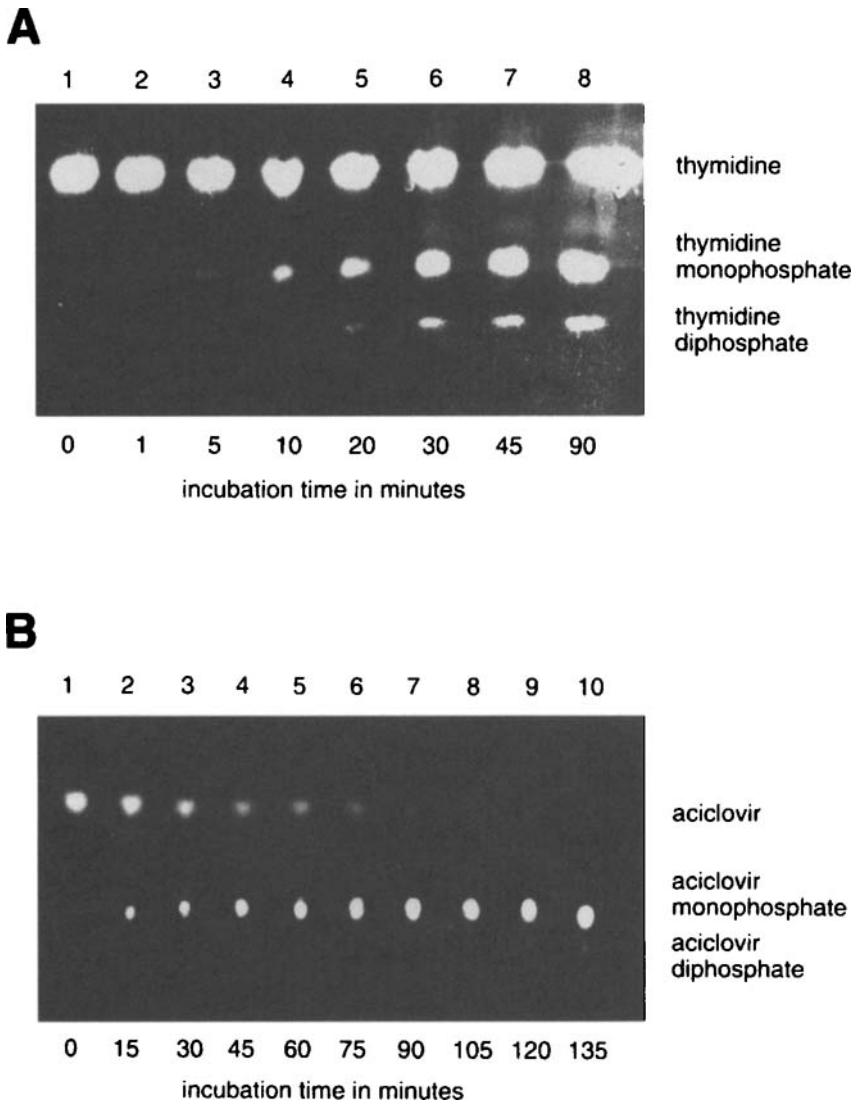

FIGURE 4

Semiquantitative TK assay by thin layer chromatography on PEI-cellulose plates for the substrate thymidine (A) and aciclovir (B).

(A) Reaction mixture containing 50 mM Tris 7.2, 5 mM ATP, 5 mM $MgCl_2$, 60 µM [^{14}C]-thymidine and 44 ng/mL recombinant HSV 1 TK was incubated at 37°C. At regular time intervals, samples of 5 µL were removed, boiled for 3 minutes and transferred to PEI-cellulose plates. The TLC plates were developed successively in 0.2 M LiCl for 2 minutes, 1 M LiCl for 6 minutes and 1.6 M LiCl for 22 minutes. They were evaluated by autoradiography and the X-ray film was used as a negative for the glossy print. The thymidine conversion in TMP and TDP over the time can be clearly depicted. (B) 1 mM aciclovir, 1 µM [^{3}H]-aciclovir and 10 µg/mL recombinant HSV 1 TK were used in the reaction mixture. After 60 minutes another 2 mM ATP was added. Before exposure to the X-ray film, the TLC plate was treated with a 2 M sodium salicylate solution in order to enhance the signals. The time course for the phosphorylation of ACV to ACVMP and ACVDP is shown.

recorded apparent K_m for ATP vary from 13 µmol/L to 28 µmol/L [1, 24, 26] which are in the same range as K_{4m} in the present study. The results of the mathematical fit of the two thymidine data sets to equation 10a and 10b support the hypothesis of a preferred, but not exclusively used sequential order of substrate binding, with thymidine binding prior to ATP addition. However, we must address the question as to whether our model reflects the physiological situation, since the ATP concentration in the cell may be up to 2 orders of magnitude higher than the concentrations used in the kinetic assays. Therefore, initial velocity data experimentally obtained with 5mM ATP[12] were compared to the corresponding calculated values obtained by using eq. 7 and the parameters from tab. 1. The correlation (r^2) was found to be 0.986 indicating that the ATP concentration has no influence on the overall mechanism. Furthermore, ITC titration of HSV1 TK with dT in presence of ATP in a range varying from 30 µM to 2 mM showed that the binding affinity of dT to TK does not depend from the used ATP concentration [23]. Thus, the used model reflects indeed the physiological conditions.

The determined values for the partial model of the aciclovir kinetics are consistent with earlier published data. Purified native HSV 1 TK showed a K_i for aciclovir of 100 - 200 µmol/L [24, 25, 27]. Reported values in the literature were recorded at enzyme-saturating ATP concentrations, thus representing apparent K_i constants. As expected, the values of the ATP dissociation constants computed for the binary ATP-enzyme complex of both the dT and ACV partial model (K_I = 40.0 and 59.3 µmol/L) are numerically similar. In contrast to the thymidine kinetics, no preference of a pathway could be postulated for aciclovir and ATP binding. Aciclovir shows the same affinity to the free enzyme as to the enzyme-ATP complex. This is also valid for ATP.

According to the aciclovir inhibition study, all the thymidine kinetic-related parameters from the concerted action model, and those obtained with the partial thymidine kinetic model are in close agreement. The ACV related values revealed a larger deviation when the independent ACV kinetics were compared to the dependent ACV inhibition experiments. The prediction of K_{6m} and K_{7m} from the other parameters was poor because only the thymidine phosphorylation was recorded. Improved conformity of K_{6m} and K_{7m} with the partial (independent) aciclovir model could be achieved by measuring the phosphorylation reaction of the ternary ACV:ATP-enzyme complex. Moreover, the aciclovir inhibition analysis further supports the hypothesis of a preferred pathway in the

case of thymidine phosphorylation, since it revealed an overall satisfying congruity with the two independent thymidine and aciclovir kinetics.

Using qualitative thin-layer chromatography we could demonstrate that thymidine conversion to TDP is a process that occurs via the intermediate TMP. The K_D values for thymidine are 0.139 µmol/L and 0.043 µmol/L (this study), whereas the apparent K_m value for TMP is 28 µmol/L[1]. The thymidine concentration used in the TLC assay was 60 µmol/L. Remarkably, TMP is phosphorylated even in the presence of high thymidine concentrations. This can only be explained if ADP is released first, as postulated by Chen *et al.* [1].

In our model we assume that no dTDP is formed during the initial velocity measurements, since no dTDP could be detected when analyzing the reaction mixtures using the TLC assay (data not shown). Compared to the initial velocity measurements a more than 10 times higher enzyme concentration needs to be applied to the reaction mixtures in order to demonstrate that dTDP is actually formed. Nonetheless, under these harsh conditions dTDP could be only detected after 25 minutes whereas the last sample for the initial velocity measurements was taken after 15 minutes.

Structure - function considerations:

The ACV conversion is approximately 3 times slower than thymidine phosphorylation. A plausible explanation for this finding can be attributed to the missing 3'-OH group in the ACV molecule. The amino acids E225 and Y101 interact via a hydrogen bond with the 3'-OH of the ribose moiety of thymidine [9-11]. The absence of the 3'-OH group in the ACV molecule increases the freedom of motion of its acyclic carbohydrate moiety (which corresponds to the ribose moiety in dT) and also the entropy of the system. Thus, the loss of entropy upon binding of an acyclic substrate compared to the more rigid cyclic substrate is smaller because of the absent hydrogen bonds with E225 and Y101. These missing directional interactions account for a lost of two order of magnitude in K_m compared with dT. As a consequence, the HSV 1 TK generally exhibits a smaller binding energy for the acyclic substrates and a decreased rate of phosphorylation compared to the natural substrate thymidine. When GCV (an analogue of ACV) is bound,

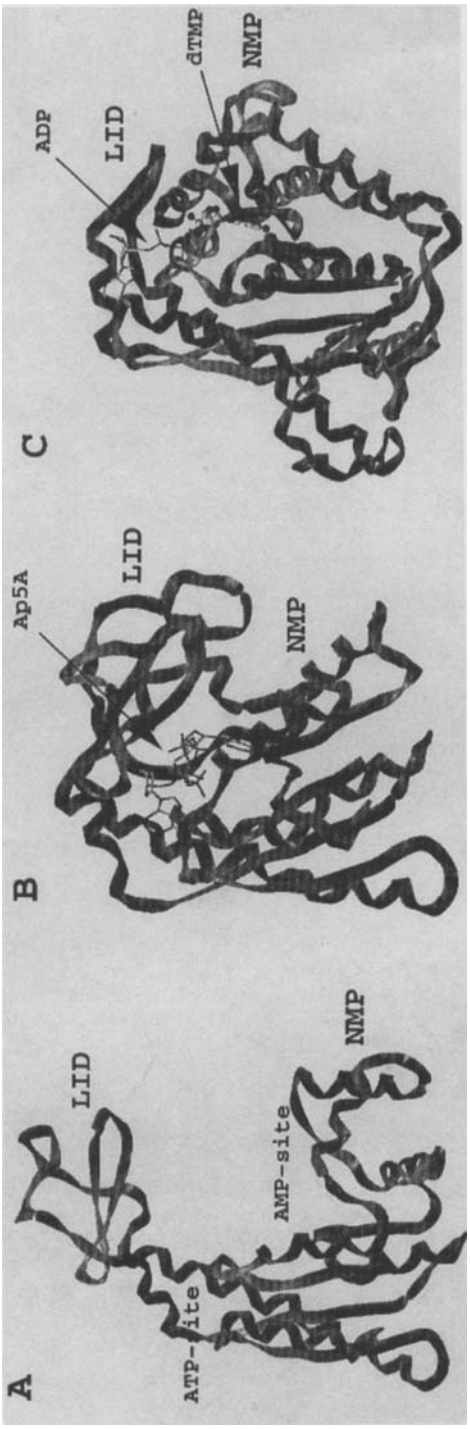

FIGURE 5

Ribbon representation of the Cα backbone of adenylate kinase from *E. coli* without bound substrate (apo-AKeco; PDB entry 4AKE) (A), with the transition sate inhibitor Ap_5A (PDB entry 1AKE) (B) and HSV 1 thymidine kinase with bound substrates (dTMP and ADP) (PDB entry 1VTK)[11] (C).

(A) The NMP binding and the LID domains (region from amino acid 120 to 160 for ADK and from 219 to 226 for TK) as well as the ATP- and AMP-binding sites of apo-AKeco are indicated. The open conformation of the active site can be clearly depicted [13].

(B) Cα backbone of adenylate kinase from *E. coli* complexed with the transition state inhibitor Ap_5A (PDB entry 1AKE). The close conformation is very similar to the conformation of HSV 1 TK complexed with its substrates.

(C) This HSV 1 TK structure is representative for all TK structures solved until now. ADP and dTMP bound to TK are represented as stick and ball-and-stick models, respectively. TK with bound substrates presents a closed conformation of the binding site which is similar to that reported for the ADK structure of *E. coli* complexed with the bi-substrate analog Ap5A. In contrast to the closed conformation of ADK, the open form was crystallized without bound substrate [13].

the amide group of Q125 rotates 180° [10] in order to form two hydrogen bonds with the nucleobase. This rotation results in a loss of a hydrogen bond between Q125 and the backbone of A168. Thus, the so induced increase in mobility of residue Q125 adds to the loss of the two hydrogen bonds resulting in the low binding affinity of ACV.

Does the kinetic finding correlate with the available structural information? All the TK structures complexed with substrate (dT, GCV, IdU) and co-substrate (ATP) or sulfate ion [9-11] show a closed binding site conformation (fig. 5C). In a lock and key system this would suggest an ordered sequence of binding for the two components with thymidine binding prior to ATP addition, and an ordered sequence of product release with ADP being released first. It has been demonstrated, however, that nucleoside monophosphate (NMP) kinases such as adenylate kinase (ADK) undergo extensive conformational changes during their catalytic cycle. This includes a large movement of the LID domain [13]. It was shown that the ADK apo-enzyme has a larger solvent-accessible surface area as a consequence of its more open conformation, compared to the conformation when complexed with the transition-state inhibitor Ap_5A (fig. 5A and B). In contrast to the closed conformation of the ADK, the open form is able to accept random docking of the substrates in the corresponding binding site.

The presented kinetic analyses strongly indicate an ordered sequence of substrate binding, namely thymidine binding prior to ATP addition. Alternatively, there is no preferred substrate-binding pathway for aciclovir phosphorylation. The kinetic experiments are congruent with the available structural information. Furthermore, from the accessible structures we know that HSV1 TK has a common reaction centre for dT and ACV and that ATP binds in a substrate independent binding orientation to the enzyme. Considering this structural knowledge, the kinetic experiments showing a substrate-dependent binding pathway add a new information on the mechanism of binding suggesting that HSV1 TK is not a typical lock and key enzyme and undergoes conformational changes during the catalytic cycle.

ABBREVIATIONS

ACV, aciclovir; ACVMP, aciclovir monophosphate; ADK, adenylate kinase; AP_5A, p1,p5-(diadenosine-5')-pentaphosphate; A.S.E., asymptotic standard error; HSV 1,

Herpes Simplex Virus type 1; NBP, nucleotide binding proteins; NMP, nucleotide monophosphate; PEI, polyethyleneimine; dT, thymidine; TK, thymidine kinase; TMP, thymidine monophosphate.

REFERENCES

1. Chen, M. S.; Walker, J.; Prusoff; W. H. *J. Biol. Chem.* **1979** ***254 (21):*** 10747-10753
2. Smee, D. F.; Boehme, R.; Chernow, M.; Binko, B. P.; Mattews, T.R. *Biochem. Pharmacol.* **1985** ***34(7):*** 1049-1056
3. Waldman, A. S.; Häusslein, E.; Milman, G. *J. Biol. Chem.* **1983** ***258 (19):*** 11571-11575
4. Huber B.E.; Richards, C.A.; Krenitsky T.A. *Proc. Natl. Acad. Sci. USA* **1991** ***88***: 8039-8043
5. Takamiya, Y.; Short, P.; Moolten, F.L.; Fleet, C.; Mineta, T.; Breakefield, X.O.; Martuza R.L. *J. Neurosurg.* **1993** ***79 (1)***: 104-110
6. Boviatsis, E.J.; Park, J.S.; Sena-Esteves, M.; Kramm, C.M.; Chase, M.; Efird, J.T.; Wei, M.X.; Breakefield, X.O; Chiocca, E.A. *Cancer Res.;* **1994** ***54 (22)***: 5745-5751
7. Tong, X.W.; Agoulnik, I.K.; Contant, C.F.; Hasenburg, A.; Runnebaum, L.B.; Stickeler, E.; Kaplan, A.L.; Woo, S.L.; Kieback, D.G. *Anticancer Res.* **1997** ***17 (2A):*** 811-813
8. Caruso, M.; Salomon, B.; Zhang, S.; Brisson, E.; Clavel, F.; Lowy, I.; Klatzmann, D. *Virology* **1995** ***206 (1)***: 495-503
9. Wild, K.; Bohner, T.; Aubry, A.; Folkers, G.; Schulz, G.E. *FEBS Lett.* **1995** ***368 (2)***: 289-292
10. Brown, D. G.; Visse, R.; Sandhu, G.; Davis, A.; Rizkallah, P.J.; Meliz, C.; Summers, W.C.; Sanderson, M.R. *Nature Struct. Biol.,* **1995** ***2 (10):*** 876-881
11. Wild, K.; Bohner, T.; Folkers, G.; Schulz, G.E. *Protein Science* **1997** ***6 (10)***: 2097-2106
12. Michael, M.; Fetzer, J.; Folkers, G. *Europ. J. Biochem.* **1994** ***226 (1):*** 219 - 226
13. Müller, C.W.; Schlauderer, G.J.; Reinstein, J.; Schulz, G.E. *Structure* **1996** ***4***: 147-156
14. Purich, D.L.; Fromm, H.J. *Biochim. Biophys. Acta* **1972** ***276 (2):*** 563-567

15. Lienhard, G.E.; Secemski, I.I. *J. Biol. Chem.* **1973** ***248 (3):*** 1121-1123

16. Fetzer, J.; Folkers, G. *Pharm. Pharmacol. Lett.* **1992** ***2***: 112-114

17. Fetzer, J.; Michael, M.; Bohner, T.; Hofbauer, R.; Folkers G. *Prot. Expr. Purif.* **1994** ***5***: 432-441

18. Gerber, S.; Folkers, G. *Biochem. & Biophys. Res. Com.* **1996** ***225:*** 263-267

19. Lucher, L.A.; Lego, T. *Analyt. Biochem.* **1989** ***178***, 327-330

20. Furlong, N. B. *Analyt. Biochem.* **1963** ***5***: 515-522

21. Fitt, P. S.; Peterkin, P. I.; Grey, V. L. *J. Chromatogr.* **1976** ***124:*** 137-140

22. Volkenstein M.V.; Goldstein, B.N. *Biochim. Biophys. Acta* **1966** ***115:*** 478-485

23. Perozzo, R.; Folkers, G.; Scapozza, L. paper in preparation

24. Larder, B.A.; Cheng, Y.; Darby G. *J. gen. Virol.* **1983** ***64***: 523-532

25. Larder, B.A.; Derse, D.; Cheng, Y.; Darby G. *J. Biol. Chem.* **1983** ***258 (3):*** 2027-2033

26. Kussmann - Gerber, S.; Kuonen, O.; Folkers, G.; Pilger, B. D.; Scapozza, L. Eur. J. Biochem., **1998** ***255***: 472-481

27. Fyfe, J.A.; McKee, S.S.; Keller P.M. *Molecular Pharmacology* **1983** ***24***: 316-323

Received 9/16/98
Accepted 12/28/98