Chimia 54 (2000) 663–668

ISSN 0009–4293

Extended Substrate Acceptance of *Herpes Simplex Virus Type 1* Thymidine Kinase: a New Chance for Gene and Antiviral Therapy

Leonardo Scapozza*, Patrick Ballmer, Regula Johner, Remo Perozzo, Beatrice Pilger, Pavel Pospisil, Andrea Prota, Pierre Schelling, Loredana Spadola, Christine Wurth, and Gerd Folkers

Abstract: *Herpes simplex virus type 1* thymidine kinase (HSV1-TK) has become increasingly important as a target in medicinal chemistry because of its links to therapy of viral infection, gene therapy of cancer and allogeneic transplantation. These applications are based on the differences in binding properties between the human and the viral enzyme. Several problems have been encountered in the clinic, *e.g.* the increase of resistance for antiviral drugs and the immunosuppressive effects of the dosages needed for tumor regression. Thus intensive efforts have been directed towards understanding substrate diversity to overcome the clinical limitations. In this context, kinetic and thermodynamic studies revealed that substrates bind in compulsory order and that the binding event is enthalpy driven. The structural evaluation of aciclovir resistant HSV strains shows that loss of electrostatic interactions, change in steric accessibility and modification of the 3D conformation of HSV1-TK are responsible for the encountered resistance. Further crystallography studies revealed the role of water in substrate binding, the advantage of a fixed ribose ring and that substrate acceptance of HSV1-TK is extended to all five nucleobases. The reviewed results give new rationale for the design of novel prodrugs and engineered HSV1-TK for antiviral and gene therapy.

Keywords: Antiviral therapy · Gene therapy · Pharmaceutical chemistry · Protein Engineering · Structures · Thymidine Kinase

1. Introduction

Herpes simplex virus type 1 (HSV1) is a well-characterized widespread infectious agent in human populations [1][2]. HSV1 infections are associated with oral-facial and skin lesions. Especially in immunocompromised hosts, HSV1 infections can cause severe pathologies namely blindness and central nervous system damage [3]. During the past decade, potent agents against herpes virus infections have been found. The mode of action of these drugs is based on the different substrate preference between viral and human thymidine kinase.

Thymidine kinase (TK, EC 2.7.1.21) is the key enzyme in the pyrimidine salvage pathway catalyzing the phosphorylation of thymidine (dT) to thymidine monophosphate (dTMP) in the presence of Mg^{2+} and ATP [4]. HSV1-TK accepts a broad range of substrates in contrast to cellular TK [5–7]. The antiviral compounds are selectively activated through phosphorylation by HSV1-TK to finally act in their triphosphorylated form as DNA polymerase inhibitor as well as DNA chain terminators [5][6][8]. The broad clinical use of aciclovir has led to the emergence of drug-resistance, mainly linked to HSV1-TK mutations [9].

The rising resistance poses increasing problems for immunocompromised patients [10][11]. Several new prodrugs have been developed and documented in large clinical trials [10][12][13]. All of them are aciclovir analogs with improved oral bioavailability, however, showing cross-resistance because they are chemically similar and bind similarly to HSV1-TK [14–16]. Further searches for resistance repellent drugs led to HSV1-TK-circumventing monophosphate analogs [17] such as adefovir and cidofovir, which are active against a broad spectrum of DNA viruses, including *herpes simplex virus* (types 1 and 2). A novel series of drugs is composed by conformationally rigid compounds that differ as little as possible from the natural substrate [18–21]. Several of them were synthesized, tested and showed antiviral activity against HSV1, HSV2, human cytomegalovirus and Epstein-Barr virus [18][19][21].

The enzyme-prodrug gene therapy using HSV1-TK as suicide gene [22-24] mimics the classical antiviral therapy. In

*Correspondence: Dr. L. Scapozza
Department of Applied BioSciences
Swiss Federal Institute of Technology (ETH)
Winterthurerstr. 190
CH-8057 Zurich
Tel.: +41 1 635 60 36
Fax: +41 1 635 68 84
E-Mail: scapozza@pharma.anbi.ethz.ch

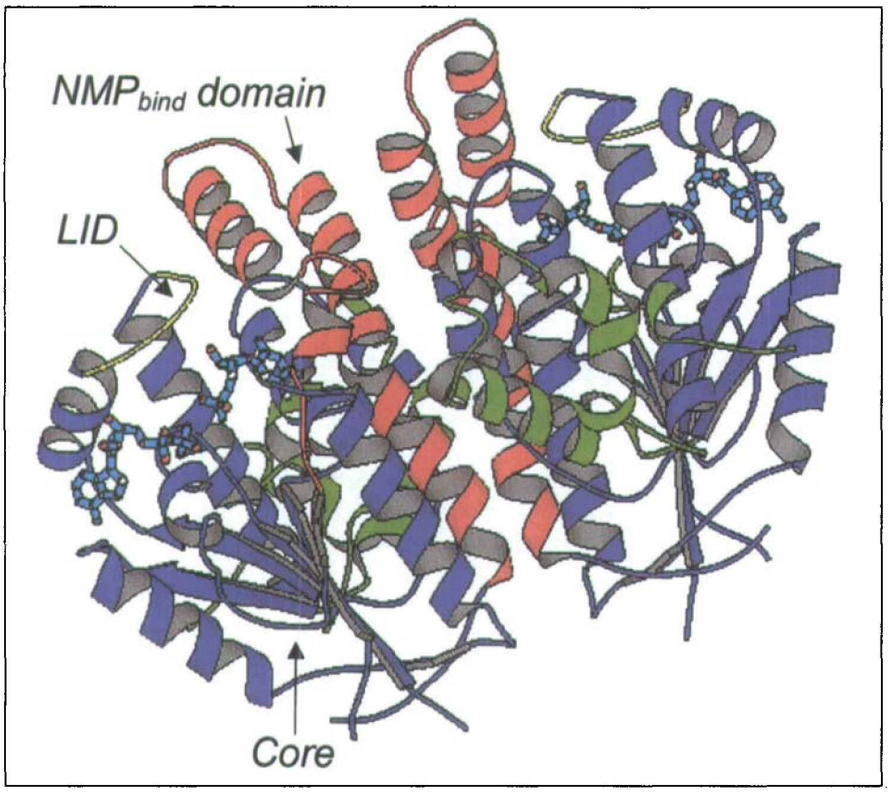

Fig. 1. Ribbon diagram of the symmetric HSV1-TK dimer with bound ADP and dTMP. (PDP entry 1VTK, [14]) The domains defined as for other NMP-kinases are indicated. The five stranded parallel β-sheet is depicted with arrows and the surrounding helices are displayed. Substrate and co-substrate are depicted as stick models. The picture was generated with the program MOLSCRIPT [52].

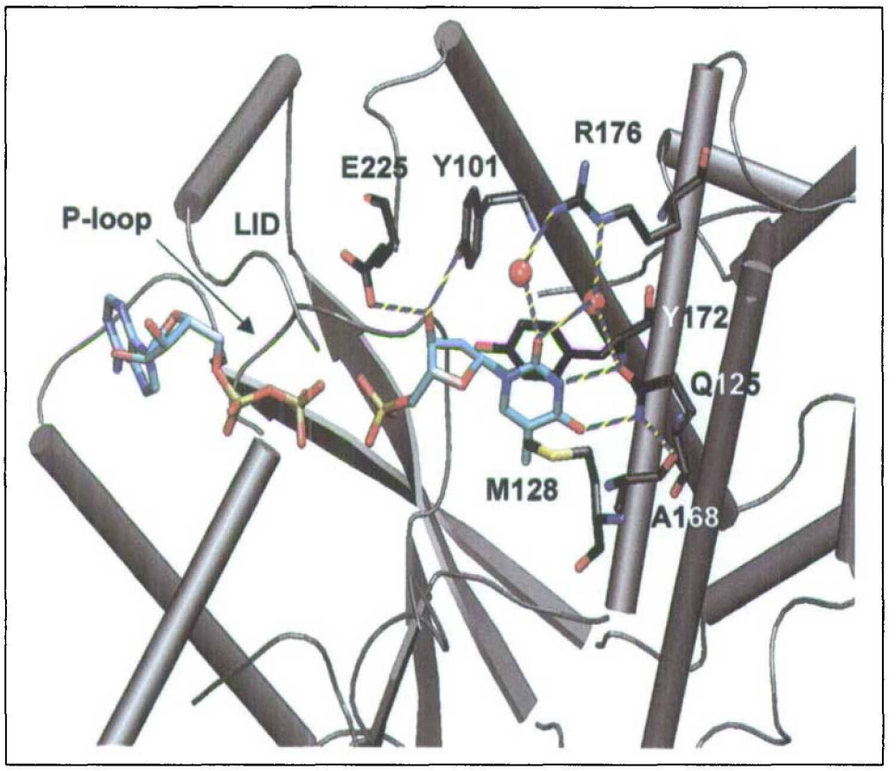

Fig. 2. The binding motif of the substrate and cosubstrate in wild type HSV1-TK (PDB entry 1 VTK, [14]. dTMP as representative of the substrates and ADP are shown as stick models. The hydrogen bond network is displayed with dotted lines. Water moleculesare represented as spheres. The glycine rich loop (P-loop), the LID domain and the residues involved in binding are labelled.

this approach, the HSV1-TK gene is introduced into tumor cells by retroviral or adenoviral vectors. Thus, dividing cells that express HSV1-TK are capable of converting non-toxic nucleoside analogs such as ganciclovir into their nucleoside triphosphates which inhibit cellular polymerases leading to cell death and consequently tumor ablation [25–27]. Clinical trials with ganciclovir were hampered by unfavorable side-effects such as immunosuppression caused by the large dosages required. Clinical results with HSV1-TK used as control system of graft-*versus*-host disease in the context of allo-BMT [24] revealed some intrinsic limitations related to the HSV1-TK suicide gene [28]. The first one is immunogenicity of the HSV1-TK. Some patients treated late after transplant at a time of immunocompetence, developed an immune response against the genetically-modified cells. The second limitation is that ganciclovir (GCV) was required in the management of cytomegalovirus disease and resulted in the unwanted elimination of genetically modified cells. The question we address in this paper is whether an increased knowledge of substrate acceptance is a new chance for overcoming the limitation in gene and antiviral therapy allowing the enzyme [29] as well as the prodrugs to be improved.

2. The Structure

HSV1-TK appears in the crystal structure as a homodimer with 376 amino acids per subunits with a typical α,β fold with a central five-stranded parallel β-sheet surrounded by twelve helices [14–16][30] (Fig. 1). HSV1-TK is a member of the family of NMP-kinases and contains the classical mononucleotide (NMP) binding fold [31]. The central five β-strand domain is referred to as the CORE domain. Other domains are the LID domain and the NMP$_{bind}$ domain [32]. Thymidine binding is characterized by a sophisticated hydrogen bond network, dominated by the Watson-Crick-like interaction between Gln125 and the base, and a peculiar sandwich-like complex formed by Met128 and Tyr172 with the base (Fig. 2). Guanine lies in the same plane between Met128 and Tyr172 as thymine does [15]. ATP binding is governed by the interaction of the phosphate moiety with the glycine rich loop that is highly conserved throughout the whole family of nucleoside kinases, and the LID domain covering it (Fig. 2).

3. Substrates Binding to HSV1-TK

Kinetic analyses were performed under equilibrium conditions to elucidate the different association steps that the enzyme undergoes during binding of substrates and co-substrate [33]. This study strongly indicated an ordered sequence of substrate binding, namely thymidine binding prior to ATP addition. Alternatively, there is no preferred substrate-binding pathway for aciclovir phosphorylation, thus showing a substrate-dependent binding pathway for the mechanism of binding that is possible only if conformational changes during the catalytic cycle are assumed [33].

Thermodynamics of substrate binding of HSV1-TK were studied using isothermal titration calorimetry (ITC) [34] because a comprehensive analysis of the thermodynamics of binding and a meaningful correlation of thermodynamics with structure is the key to the understanding of molecular recognition. These results showed once more that binding follows a sequential pathway in which dT binds first and ATP second [35]. The free enzyme does not bind ATP, whose binding site only becomes accessible in the HSV1-TK:dT complex. At pH 7.5 and 25 °C, the binding constants are 1.9×10^5 M^{-1} for dT binding to the apo enzyme and 3.9×10^6 M^{-1} for ATP binding to the binary HSV1-TK:dT complex. Binding of both substrates is enthalpy-driven and opposed by a large negative entropy change [35]. The heat capacity change (ΔCp) obtained from ΔH in the range 10–25 °C, is –360 cal K^{-1} mol^{-1} for dT binding and –140 cal K^{-1} mol^{-1} for ATP binding. These large ΔCp-values are incompatible with a rigid body binding model in which the dT and ATP binding sites pre-exist in the free enzyme [35]. The values of ΔCp and $T\Delta S$ strongly indicate large scale conformational adaptation of the active site in sequential substrate binding. The conformational changes seem to be more pronounced in dT binding than in the subsequent ATP binding. Considering the crystal structure of the ternary HSV1-TK:dT:ATP complex, a large movement in the dT binding domain and a smaller but substantial movement in the LID domain are proposed to take place when the enzyme changes from the substrate-free, presumably more open and less ordered conformation, to the closed and compact conformation of the ternary enzyme-substrate complex [33][35]. These thermodynamic data nicely confirm the kinetic findings and the assumption of movement.

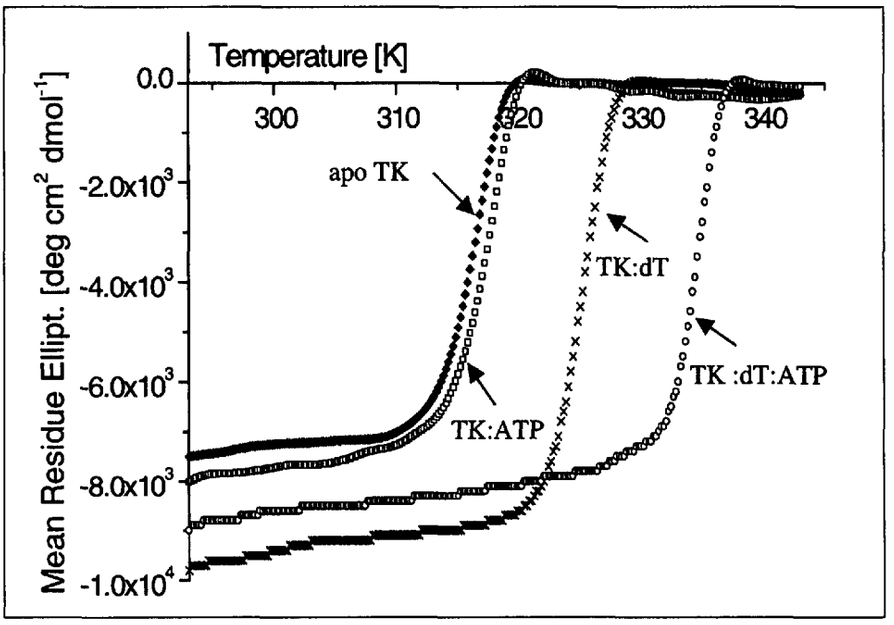

Fig. 3. Thermal denaturation curves of ligand-free and ligated HSV1-TK (0.4 mg/ml) in TBSE. The CD signal was recorded at 223 nm in a temperature range from 20 to 70 °C. Influence of the natural HSV1-TK substrates ATP and thymidine on the melting temperature. The melting curves of HSV1-TK without substrates (□), HSV1-TK with 1mM ATP (♦), HSV1-TK with 1mM dT (X), HSV1-TK with 1mM ATP and 1mM dT (O) are displayed. This figure has been reproduced with the permission of the authors [36].

Further biophysical studies have been undertaken to gain more insight into the kinematics of the enzyme. The effect of substrate binding on the conformation and structural stability of HSV1-TK has been measured by thermal denaturation experiments, far UV, CD and fluorescence. The results indicate that the conformation of the ligand free HSV1-TK is less ordered and less stable compared to that of the ligated enzyme (Fig. 3) [36]. In the same study it was shown that binding affinity and binding mode of the ligand correlate strikingly with thermal stability of the complex [36]. Furthermore, the structure of the unligated enzyme, but complexed with sulfate has been solved at 1.9 Å resolution [37]. This structure contains four water molecules in the thymidine pocket and reveals a small induced fit on substrate binding. It shows an increased mobility of the residues involved in substrate binding (Fig. 2) which gives an insight for intrinsic 3D-movement [37]. HSV1-TK has been also crystallized with a new ligand HMTT ((*R*,*R*)-6-(6-hydroxymethyl-5-methyl-2,4-dioxo-hexahydro-pyrimidin-5-yl-methyl)-5-methyl-1H-pyrimidin-2,4-dione) and refined to 2.2 Å [36]. The HSV1-TK:HMTT complex displays a unique conformationally altered active site resulting in a lowered thermal stability of this complex [36] and for the first time, showed structural evidence of the conformational movement indicated by the kinetic, thermodynamic and folding studies [33][35][36].

4. The Rationale of Substrate Diversity

The Role of Met128 and Tyr172 in Nucleobase Fixation

From a multiple alignment study of type I thymidine kinases (long TK) of different species *e.g.* herpes simplex virus type 1;2 (HSV 1;2), marmoset herpes virus (MHV), equine herpes virus type 4 (EHV), varicella-zoster virus (VZV) and Epstein-Barr virus (EBV), it was striking that Gln125 was conserved over all species while the triad H58/M128/Y172 was conserved only by strains with broad substrate diversity (HSV1, HSV2 and MHV). In contrast, strains with narrow substrate specificity (EHV, VZV and EBV) had X58/F128/Y172 as consensus triad, where X is an hydrophobic amino acid but never histidine [38]. To gain a better understanding of the nature of the interactions of thymine with Tyr172 and Met128, *ab initio* calculations in the frame work of density functional theory were performed. These calculations showed strong polarization on thymine, no polarization on the sulfur atom of Met128 and the absence of π-π interactions between Tyr172 and thymine. This

indicated that Tyr172-thymine interactions are dominated by electrostatics and the role of Met128 is purely steric and hydrophobic [39].

Based on this information, the mechanisms of substrate binding of HSV1-TK were studied by means of site-directed mutagenesis combined with thermodynamic measurements performed using ITC [38]. The results show the link between the exceptionally broad substrate diversity of HSV1-TK and the presence of structural features such as the residue triad H58/M128/Y172. The mutation of Met 128 into a Phe as well as the double mutant M128F Y172F result in mutants that have lost their activity [38]. However, by exchanging His58 to form the triple mutant H58L/M128F/Y172F the enzyme regains activity. Strikingly, this triple mutant becomes resistant towards aciclovir. The titration experiments clearly revealed a change in the entropy contribution depending on the mutation. The inactive mutant showed a significantly increased entropy of the system whereas the enthalpy contribution was diminished [38]. However, by complete transposition of the triad X58/F128/F172, the entropy contribution adapts to the wild type [38]. These results suggest that conformational changes are essential for binding and that the accommodation of the base within the plane formed by M128 and Tyr172 is indeed a prerequisite for the correct formation of hydrogen-bonds. These results have been confirmed by the crystal structure of the Q125N mutant ligated with dT solved at 2.4 Å resolution [37]. This structure clearly shows that the nucleobase binds exactly as in the wild type enzyme while a rearrangement of the hydrogen bond network occurs (Fig. 4) [37]. All results point to sequential binding events with formation of the sandwich-like complex occurring prior to hydrogen bonds formation.

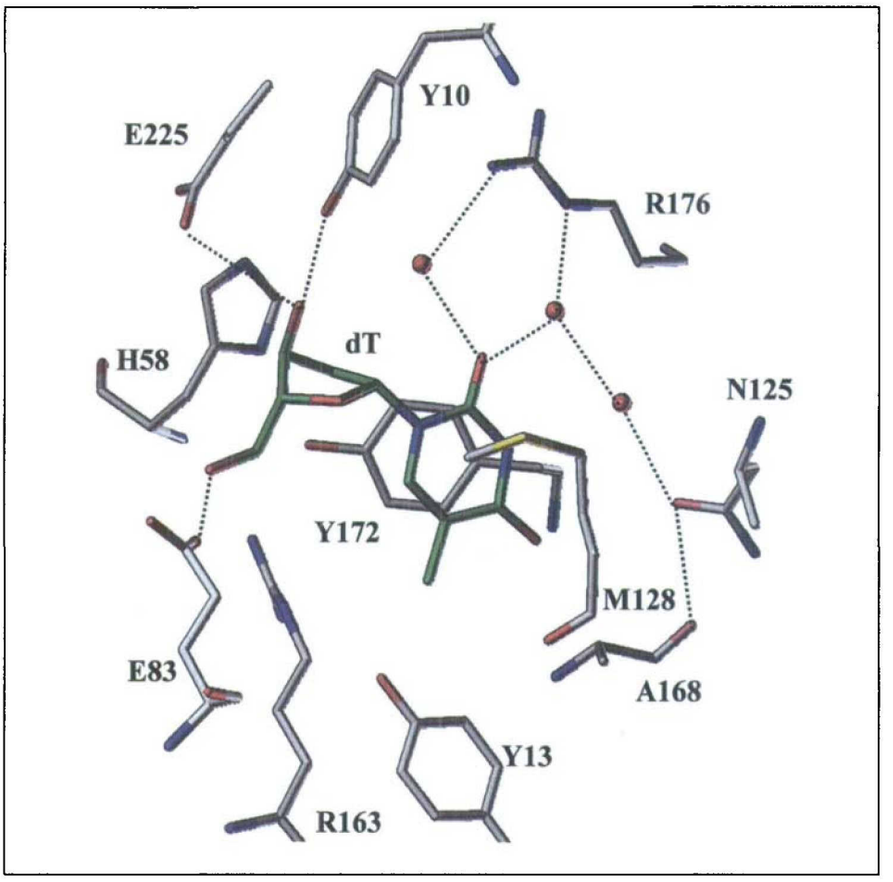

Fig. 4. Binding mode of dT in the Q125N mutant of HSV1-TK (PDB entry 1e2j, [37]). dT and the residue involved in binding are labelled. Water molecules are shown as spheres. The hydrogen bonds are represented as dotted lines.

The Electrostatics

Several drug resistant strains of *herpes simplex virus type 1* (HSV1) isolated *in vivo* or from tissue culture, have exhibited a mutated thymidine kinase (TK) [40-43]. Moreover, various site-directed mutagenesis experiments conducted with HSV1-TK allowed the assignment of specific amino acid residues to specific functional properties [44]. At this point a range of hypotheses were generated related to substrate binding of TK at the molecular level.

A site-directed mutagenesis study on Glu125 revealed that while Q125L is only able to phosphorylate thymidine, the mutant Q125N accepts both thymidine and aciclovir as substrates. The mutant Q125E shows no phosphorylation activity [45]. Moreover the mutation R176Q previously identified as relevant in drug resistance shows a 10 fold decrease in binding affinity towards dT and aciclovir. On the one hand, the structural analysis at molecular level revealed that the loss in affinity measured for Q125L, Q125N and R176Q is consistent with the number and type of lost hydrogen bonds [45]. On the other hand, it shows that the mutation Q125E causes not only the loss of two hydrogen bonds but it also introduces repulsive forces. The combination of mutagenesis experiments, molecular modelling and crystallographic study of these mutations [37][45] clearly showed that the free binding energy contribution of hydrogen bonds, once they are formed, range between the classical 2 and 6 KJ/mol as stated in the text books [46].

Steric Accessibility

The mutation A168T confers resistance towards prodrugs such as bromvinyl-deoxyuridine that have a bulky substituent at C(5) of the nucleobase. The structural analysis at the molecular level reveals that the mutation alters steric accessibility, influencing the binding orientation of the prodrug with bulky substituent and thus, substrate acceptance [45].

5. Substrate Diversity at the Sugar-Moiety Level

The Conformation

In solution, the sugar ring of nucleosides and nucleotides equilibrates between two extreme forms, a 2'-*exo*/3'-*endo* (North) conformation and a 2'-*endo*/3'-*exo* (South) conformation [47]. Upon binding into the active site, one particular conformation is fixed causing an unfavourable entropy contribution. The bound conformation can be mimicked in solution by introduction of a conformational restricted sugar-moiety reducing the unfavourable entropy contribution. For this purpose, a variety of nucleobases with a bicyclo[3.1.0]hexane carbocyclic analog were synthesized [18] and their antiviral activity was tested against several types of DNA viruses, such as HSV1 and

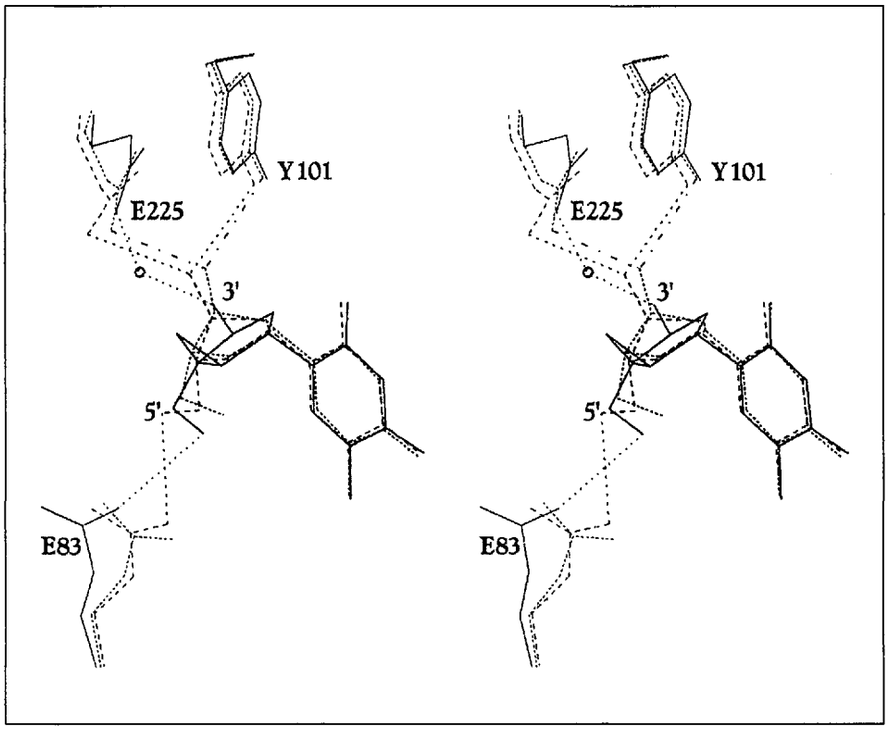

Fig. 5. Superposition of HSV1-TK:n-MCT and the HSV1-TK:dT structures. The structure of HSV1-TK:n-MCT is shown as continuous lines, HSV1-TK:dT in the same space group $C222_1$ [15] is shown in narrow dotted lines, the structure of HSV1-TK:dT:ADP in spacegroup $I4_1$ [14] is depicted in wide dotted lines. Hydrogen bonds are depicted as dashed lines, the water molecule as a black circle. For sake of clarity, the dT hydrogen bonds are only depicted for the 3'OH and 5'OH groups. The hydrogen pattern for the thymine moiety is the same for all complexes and corresponds to this reported in Fig. 2. This figure has been reproduced with the permission of the authors [48].

HSV2, human CMV and Epstein-Barr virus. Among them, 2'-*exo*-methanocarba-thymidine (n-MCT) showed a high and reproducible antiviral activity against HSV1 and HSV2 as measured by plaque reduction assays. Its potency surpassed that of aciclovir [18]. The crystal structure of HSV1-TK in complex with n-MCT solved at 1.7 Å resolution clearly showed that n-MCT shares the same binding mode as dT and there is a loss of direct hydrogen bonds towards Glu225 and Tyr101 (Fig. 5) [48]. Furthermore it shows that the bicyclo-system assumes a flat 2'-*exo* envelope conformation that clearly differs from the 2'-*endo* conformation observed for dT (Fig. 5). These results clearly point out that HSV1-TK shows no selectivity for the conformation of the sugar moiety.

The Role in Catalysis

Since the first structure of HSV1-TK in complex with dT and ATP was solved in 1995 [30], several structures of HSV1-TK have shed light on the binding mode of variety of ligands. A striking finding was the inhibitor 9-(4-hydroxybutyl)-N(2)-phenylguanine (HBPG) sharing the same binding mode as structurally related substrates such as aciclovir [16]. Thus, an intriguing question remains as to what is the structural basis for the different properties.

Ab initio quantum chemistry calculations within the density functional theory (DFT) framework have been revealed as a powerful tool to elucidate nucleobase binding to HSV1-TK [39]. The combined biochemical and quantum chemical study on the binding of different sugar like moiety of the nucleoside analogs within the binding site of HSV1-TK points out the crucial role of the ligand dipole within the active site and its interaction with amino acids Glu225 [49]. This amino acid is not directly involved in the kinase catalytic machinery because it is not directly interacting with the 5'-OH group. Site-directed mutagenesis study performed at position 225 confirms the theoretical calculation and the importance of Glu225 in catalysis [38].

6. Substrate Acceptance Extended to All Five Bases

It has been known for a long time that HSV1-TK was able to phosphorylate thymine, uridine, cytidine and guanine analogues. Recent crystallographic work revealed that the substrate acceptance is also extended to adenine analogues. In fact, a structure solved at 1.9 Å resolution showed for the first time a bound adenine analogue (9-(2-hydroxypropyl)adenine, HPA) after numerous complexes with thymine and guanine analogues were reported [37]. The crystal structure of HSV1-TK:HPA revealed the presence of multiple binding modes of HPA which correspond to its poor binding affinity (Ki = 5.3 mM) compared with guanine analogues (Ki = 0.17 μM for aciclovir) [33]. A comparison of the binding modes of HPA and dT shows that the stacking interaction of the bases are similar and that the two water molecules between the bases and Arg176 are essentially the same [37]. The fact that HPA is phosphorylated despite its poor and diffuse binding behavior demonstrates again the broad substrate acceptance of HSV1-TK. Furthermore, HPA can be seen as new lead compound for prodrugs to be used in the gene therapy approach, if we consider that a closely related monophosphorylated analogue PMEA is a potent inhibitor of the cellular DNA polymerase [50].

7. Conclusion

The presented work shows that by using an integrated approach involving molecular biology, biophysics and biocomputing it was possible to understand the structural feature of HSV1-TK, and at molecular level binding and its thermodynamics. Furthermore, first results show how the enzyme regulates catalytic activity. Based on the presented knowledge enzymes have been already improved for gene therapy [48][51] and new substrates of HSV1-TK have been found [36]. These results represent a complete set of information that will allow a rational approach to clinical limitations in the field of antiviral and gene therapy.

Received: October 13, 2000

[1] B. Roizman, *Proc. Natl. Acad. Sci. U.S.A.* **1996**, *93*, 11307.
[2] B. Roizman, A. Sears, 'Herpes simplex viruses and their replication', 3rd Ed. 'Fields virology', Eds. B.N. Fields, D.M. Knipe, P.M. Howley, Lippincott-Raven Publishers, Philadelphia, **1996.**
[3] K. Umene, H. Sakaoka, *Arch. Virol.* **1999**, *144*, 637.
[4] R. Okazaki, A. Kornberg, *J. Biol. Chem.* **1964**, *239*, 275.
[5] G.B. Elion, P.A. Furman, J.A. Fyfe, P. de Miranda, L. Beauchamp, H.J. Schaeffer, *Proc. Natl. Acad. Sci. U.S.A.* **1977**, *74*, 5716.
[6] J.A. Fyfe, P.M. Keller, P.A. Furman, R.L. Miller, G.B. Elion, *J. Biol. Chem.* **1978**, *253*, 8721.
[7] J.E. Reardon, T. Spector, *J. Biol. Chem.* **1989**, *264*, 7405.
[8] Y.C. Cheng, S.P. Grill, G.E. Dutschman, K. Nakayama, K.F. Bastow, *J. Biol. Chem.* **1983**, *258*, 12460.
[9] D.M. Coen, *Antiviral Res.* **1991**, *15*, 287.
[10] P. Wutzler, *Intervirology.* **1997**, *40*, 343.
[11] M. Ida, S. Kageyama, H. Sato, T. Kamiyama, J. Yamamura, M. Kurokawa, M. Morohashi, K. Shiraki, *Antiviral Res.* **1999**, *40*, 155.
[12] R.A. Vere Hodge, D. Sutton, M.R. Boyd, M.R. Harnden, R.L. Jarvest, *Antimicrob. Agents Chemother.* **1989**, *33*, 1765.
[13] K.H. Fife, R. A. Barbarash, T. Rudolph, B. Degregorio, R. Roth, *Sex. Transm. Dis.* **1997**, *24*, 481.
[14] K. Wild, T. Bohner, G. Folkers, G.E. Schulz, *Protein Sci.* **1997**, *6*, 2097.
[15] J.N. Champness, M.S. Bennett, F. Wien, R. Visse, W.C. Summers, P. Herdewijn, E. de Clerq, T. Ostrowski, R.L. Jarvest, M.R. Sanderson, *Proteins: Struc. Func. Gen.* **1998**, *32*, 350.
[16] M.S. Bennett, F. Wien, J.N. Champness, T. Batuwangala, T. Rutherford, W.C. Summers, H. Sun, G. Wright, M.R. Sanderson, *FEBS Lett.* **1999**, *443*, 121.
[17] E. De Clercq, A. Holy, I. Rosenberg, T. Sakuma, J. Balzarini, P.C. Maudgal, *Nature* **1986**, *323*, 464.
[18] V.E. Marquez, M.A. Siddiqui, A. Ezzitouni, P. Russ, J. Wang, R.W. Wagner, M.D. Matteucci, *J. Med. Chem.* **1996**, *39*, 3739.
[19] N. Ono, S. Iwayama, K. Suzuki, T. Sekiyama, H. Nakazawa, T. Tsuji, M. Okunishi, T. Daikoku, Y. Nishiyama, *Antimicrob Agents Chemother.* **1998**, *42*, 2095.
[20] L. Scapozza, G. Folkers, *International Antiviral News.* **1998**, *6*, 210.
[21] T. Sekiyama, S. Hatsuya, Y. Tanaka, M. Uchiyama, N. Ono, S. Iwayama, M. Oikawa, K. Suzuki, M. Okunishi, T. Tsuji, *J. Med. Chem.* **1998**, *41*, 1284.
[22] K.W. Culver, Z. Ram, S. Wallbridge, H. Ishii, E.H. Oldfield, R.M. Blaese, *Science* **1992**, *256*, 1550.
[23] F.L. Moolten, *Cancer Gene Ther.* **1994**, *1*, 279.
[24] C. Bonini, G. Ferrari, S. Verzeletti, P. Servida, E. Zappone, L. Ruggieri, M. Ponzoni, S. Rossini, F. Mavilio, C. Traversari, C. Bordignon, *Science* **1997**, *276*, 1719.
[25] D. Klatzmann, *Hum. Gene Ther.* **1996**, *7*, 255.
[26] H. Nagy, Y. Panis, M. Fabre, H. Perrin, D. Klatzmann, D. Houssin, *Surgery* **1998**, *123*, 19.
[27] P. Tiberghien, *Curr. Opin. Hematol.* **1998**, *5*, 478.
[28] S. Verzeletti, C. Bonini, S. Marktel, N. Nobili, F. Ciceri, C. Traversari, C. Bordignon, *Hum. Gene Ther.* **1998**, 9, 2243.
[29] L.P. Encell, D.M. Landis, L.A. Loeb, *Nat. Biotechnol.* **1999**, *17*, 143.
[30] K. Wild, T. Bohner, A. Aubry, G. Folkers, G.E. Schulz, *FEBS Lett.* **1995**, *368*, 289.
[31] G.E. Schulz, *Curr. Opin. Struc. Biol.* **1992**, *2*, 61.
[32] C. Vonrhein, G.J. Schlauderer, G.E. Schulz, *Structure* **1995**, *3*, 483.
[33] S. Kussmann-Gerber, C. Wurth, L. Scapozza, B.D. Pilger, V. Pliska, G. Folkers, *Nucleosides Nucleotides* **1999**, *18*, 311.
[34] T. Wiseman, S. Williston, J.F. Brandts, L.N. Lin, *Anal. Biochem.* **1989**, *179*, 131.
[35] R. Perozzo, I. Jelesarov, H.R. Bosshard, G. Folkers, L. Scapozza, *J. Biol. Chem.* **2000**, *275*, 16139.
[36] C. Wurth, U. Kessler, J. Vogt, G. Schulz, G. Folkers, L. Scapozza, *Protein Sci.* **2000**, in press.
[37] J. Vogt, R. Perozzo, A. Pautch, A. Prota, P. Schelling, B. Pilger, G. Folkers, L. Scapozza, G. Schulz, *Proteins: Struc. Func. Gen.* **2000**, in press.
[38] B. Pilger, R. Perozzo, F. Alber, C. Wurth, G. Folkers, L. Scapozza, *J. Biol. Chem.* **1999**, *274*, 31967.
[39] F. Alber, O. Kuonen, L. Scapozza, G. Folkers, P. Carloni, *Proteins: Struc. Func. Gen.* **1998**, *31*, 453.
[40] B.A. Larder, D. Derse, Y.C. Cheng, G. Darby, *J. Biol. Chem.* **1983**, *258*, 2027.
[41] B.A. Larder, Y.C. Cheng, G. Darby, *J. Gen. Virol.* **1983**, *64 Pt 3*, 523.
[42] J.A. Fyfe, S.A. McKee, P.M. Keller, *Mol. Pharmacol.* **1983**, *24*, 316.
[43] G. Darby, H.J. Field, S.A. Salisbury, *Nature* **1981**, *289*, 81.
[44] Q.Y. Liu, W.C. Summers, *Virology* **1988**, *163*, 638.
[45] S. Kussmann-Gerber, O. Kuonen, G. Folkers, B.D. Pilger, L. Scapozza, *Eur. J. Biochem.* **1998**, *255*, 472.
[46] H.J. Böhm, G. Klebe, H. Kubinyi, *Wirkstoffdesign*, Akademie Verlag GmbH, Berlin, **1996**.
[47] W. Saenger, 'Principles of nucleic acid structure', Springer advanced texts in chemistry, Springer-Verlag, New York, **1984**.
[48] A. Prota, J. Vogt, R. Perozzo, B. Pilger, C. Wurth, V. Marquez, P. Russ, G. Schulz, G. Folkers, L. Scapozza, *Biochemistry* **2000**, *39*, 9597.
[49] M. Sulpizi, P. Schelling, G. Folkers, P. Carloni, L. Scapozza, *Proc. Natl. Acad. Sci. U.S.A.* **2000**, submitted.
[50] S. Hatse, E. De Clercq, J. Balzarini, *FEBS Lett.* **1999**, *445*, 92.
[51] F.C. Christians, L. Scapozza, A. Crameri, G. Folkers, W.P. Stemmer, *Nat. Biotechnol.* **1999**, *17*, 259.
[52] P.J. Kraulis, *J. Appl. Cryst.* **1991**, *24*, 946.