Compulsory Order of Substrate Binding to Herpes Simplex Virus Type 1 Thymidine Kinase

A CALORIMETRIC STUDY*

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Isothermal titration calorimetry has been used to investigate the thermodynamic parameters of the binding of thymidine (dT) and ATP to herpes simplex virus type 1 thymidine kinase (HSV1 TK). Binding follows a sequential pathway in which dT binds first and ATP second. The free enzyme does not bind ATP, whose binding site becomes only accessible in the HSV1 TK:dT complex. At pH 7.5 and 25 °C, the binding constants are \(1 \times 10^3\) \(\text{M}^{-1}\) for dT and \(3.9 \times 10^4\) \(\text{M}^{-1}\) 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. The heat capacity change (\(\Delta C_p\)) obtained from \(\Delta H\) in the range of 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 \(\Delta C_p\) values are incompatible with a rigid body binding model in which the dT and ATP binding sites pre-exist in the free enzyme. Values of \(\Delta C_p\) and \(\Delta S\) 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.

Molecular recognition phenomena are at the heart of biological reactions. Key to the understanding of molecular recognition is a comprehensive analysis of the thermodynamics of binding and a meaningful correlation of thermodynamics with structure. A close insight into the thermodynamics of a binding process provides guide marks for structure-based molecular design strategies. The forces that govern a binding reaction are the free energy change (\(\Delta G\)), the enthalpy change (\(\Delta H\)), the entropy change (\(\Delta S\)), and the heat capacity change (\(\Delta C_p\)). \(\Delta C_p\) is an approximate measure of the surface area buried in an association reaction and can be used to predict conformational rearrangements in associating protein molecules. An example of high medicinal interest where such information is essential is thymidine kinase from herpes simplex virus type 1 (HSV1 TK). The structure of this enzyme is known at high resolution in complex with a series of ligands, including various substrates (natural and non-natural) and inhibitors (1–5).

Thymidine kinases (EC 2.7.1.21) catalyze the phosphorylation of thymidine (dT) to dTMP in the presence of magnesium ions by transferring the \(\gamma\)-phosphate group of ATP to the 5'-OH group of dT. Herpesviruses encode their own thymidine kinases, which differ considerably from the enzyme of the human cellular host (human cellular thymidine kinase). While the human enzyme is highly specific, HSV1 TK is a multifunctional enzyme of broad substrate specificity. It shows deoxycytidylate kinase and thymidylate kinase activity (6) and phosphorylates a broad spectrum of pyrimidine as well as purine analogs (7–12). Moreover, HSV1 TK displays low stereochromic specificity. The enzyme accepts modified ribose moieties, acyclic side chains, and the \(\alpha\)-stereoisomer of the deoxyribose of dT (13). The preferred phosphate donor is ATP, yet HSV1 TK also shows high affinity for cytidine triphosphate, uridine triphosphate, and guanosine triphosphate and their deoxy analogs.

Therapeutic applications involving HSV1 TK make use of the broad substrate diversity of the viral enzyme in the background of strict substrate selectivity of the host cell enzyme. Therefore, a detailed thermodynamic analysis of substrate binding to the viral kinase is a prerequisite for the successful design of new clinically useful compounds.

HSV1 TK is a homodimer with 376 residues per subunit (Fig. 1). The constituent subunits display the general \(\alpha/\beta\) folding pattern. A central five-stranded parallel \(\beta\)-sheet is flanked on either side by helices. HSV1 TK is a member of the family of NMP kinases and contains the classical mononucleotide (NMP) binding fold (14). In this enzyme family, three-dimensional structures are known for adenylate kinase (15, 16), guanylate kinase (17), uridylylate kinase (18), bacteriophage T4 deoxynucleotide kinase (19), and thymidylate kinase (20). The central five-\(\beta\)-strand domain is referred to as the CORE domain. Other domains are the LID domain and the NMP\(_{\text{bind}}\) domain (21). Main chain superposition of HSV1 TK and adenylate kinase reveals substantial similarity in the CORE domain. Major differences exist in the NMP\(_{\text{bind}}\) domain, where HSV1

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The abbreviations used are: HSV1, herpes simplex virus type 1; TK, thymidine kinase; dT, thymidine; GST, glutathione S-transferase; HPLC, high performance liquid chromatography; ITC, isothermal titration calorimetry; PIPES, 1,4-piperazineethanesulfonic acid; MOPS, 4-morpholinepropanesulfonic acid; TES, 2-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]aminoethanesulfonic acid; NMP, nucleoside monophosphate.
TK has extensive insertions. The LID domain of HSV1 TK consists of only eight residues, reminiscent of other small variants of NMP kinases. Further differences from adenylate kinase arise from the following unique structural features of HSV1 TK: a 45-residue-long amino-terminal segment, which is not resolved in the crystal structure and is not necessary for catalytic activity (22) but plays a role in migration within the cell (23); a partially mobile segment of 72 residues between residues 250 and 322; and an additional 29 C-terminal residues (2). Interestingly, many of these differences appear to be located close to the dimer interface.

Adenylate kinases undergo large conformational changes upon substrate binding as shown by crystallography (24). The substrate-free enzyme has a more open conformation, and substrate binding leads to a closed conformation. The NMP\textsubscript{bind} domain and the LID domain rearrange upon binding of AMP and ATP, whereas the conformation of the CORE domain remains unchanged (21, 24). The three-dimensional structures of HSV1 TK known to date have been solved for ternary complexes with natural and non-natural substrates, inhibitors, cofactors, or sulfate ions mimicking the $\beta$-phosphate of ATP (1–5) and correspond to the closed conformation of NMP kinases. The structure of the free apoenzyme is not known. Since structural similarities in the CORE domain and substrate binding pockets are substantial in the NMP kinase family, one may assume that substrate-free HSV1 TK also exists in an open conformation and that conformational changes take place when HSV1 TK is converted to the closed conformation during substrate binding.

Here we present a comprehensive thermodynamic analysis of nucleoside (dT) and cofactor (ATP) binding to HSV1 TK. Substrate binding, which was followed by ITC, is shown to be strictly sequential, with dT binding first and ATP second. Combining the thermodynamic parameters with the three-dimensional structure of the ternary HSV1 TK\textsubscript{dT}\textsubscript{AMP} complex demonstrates that the sequential binding pathway is accompanied by significant structural rearrangements of the enzyme.

**EXPERIMENTAL PROCEDURES**

**Materials**— All chemicals were of analytical grade and were used without further purification. Thymidine, ATP, glutathione, buffer reagents, and glutathione-agarose (SH-coupled via 12 C-spacer) were from Sigma; dithiothreitol and EDTA were from Fluka.

**Expression and Purification**— The bacterial expression vector pGEX2T-TK was constructed as described previously (25, 26). HSV1 TK was expressed as glutathione S-transferase fusion protein (GST-TK) in *Escherichia coli* strain BL21 and purified by glutathione affinity chromatography (25). After isolation of the fusion protein from the crude extract by glutathione-affinity chromatography, the protein was directly (on column) exchanged into the experimental buffer by thoroughly rinsing the column with an excess of buffer. The protein was eluted by the addition of 5 mM glutathione and used for titration experiments. The total protein concentration was determined using a dye-binding assay (27). The concentration of active enzyme present in the protein samples used for ITC was determined by ATP affinity chromatography in the presence of dT (25). Inactive enzyme does not bind to this column. The amount of inactive enzyme was 20–30% of the total protein concentration and was corrected for in the calculation of the binding constants ($K_b$) and enthalpy of binding ($\Delta H$) from ITC raw data.

**Isothermal Titration Calorimetry**— ITC experiments were carried out using an OMEGA titration microcalorimeter (Microcal Inc., Northampton, MA) equipped with a nanovolt preamplifier to reduce electrical noise (28). The reference cell was filled with water containing 0.01% sodium azide, and the calorimeter was calibrated using standard electrical signals recommended by the manufacturer. All solutions were degassed for 10 min with gentle stirring under vacuum. Solutions of the fusion protein were filled in the sample cell (1.34-mL volume) and titrated with dT or ATP. Substrate solutions were prepared in the buffer from the final step of protein purification. The substrate concentration in the injection syringe was usually 25 times higher than the concentration of protein binding sites. A typical experiment consisted of a first control injection of 1 $\mu$L followed by 19 injections, each of 4 $\mu$L and 15 s duration, with a 4-min interval in between.

ITC measurements were routinely performed in 50 mM Tris, pH 7.5, 4 mM EDTA (to suppress enzymatic activity), 5 mM glutathione, 1 mM dithiothreitol. Heat contributions due to coupled protonation events upon binding were evaluated by calorimetric experiments in various buffers at different ionization enthalpies under otherwise identical conditions. The buffers and their ionization enthalpies (in kcal mol\textsuperscript{-1}) at 25 °C were as follows: PIPES (2.7), MOPS (4.9), TES (7.7), and Tris (11.34) (29). The pH of the buffer was adjusted at the experimental temperature. Buffer concentrations were 50 mM, and the ionic strength was similar for all buffers. In control experiments, the ligand was injected into buffer. The observed heat effects were concentration-independent and were identical to the heat signals detected after complete saturation of the protein. Therefore, the nonspecific background was usually estimated by averaging the small heats at the end of the titration. Raw data were collected, corrected for ligand heats of dilution, and integrated using the Microcal Origin software supplied with the instrument. Since protein concentration was expressed on a subunit basis, a single-site binding model was fit to the data by a nonlinear regression analysis to yield binding constants ($K_b$), enthalpies of binding ($\Delta H$), and stoichiometry of interaction.

**HPLC Assay**—High-performance liquid chromatography was used for concentration determination of thymidine and ATP in the final ligand solutions and to monitor potential phosphorylation products during calorimetric experiments using a modified protocol of a previously published method (30). Nucleotides were determined by reverse-phase ion-paired chromatography using a C\textsubscript{18} column (LiChrospher 100 RP-18, 5 $\mu$m, 250 × 4 mm; Merck) in 0.2 $\mu$L NaH\textsubscript{2}PO\textsubscript{4}, 25 mM tetrabutylammonium, 3% (v/v) methanol at 1.0 mL/min and detection at 254 nm. Ligand concentrations were calculated by means of calibration curves from standard solutions showing linearity in the range of 0.04 to at least 2 mM. The detection limit for dT, dTMP, and ATP was <20 nM (31).

**Calculation of Solvent-accessible Surface Area**—The program NACCESS (32), an implementation of the Lee and Richards solvent accessibility algorithm (33), was used with a probe radius of 1.4 $\AA$ and a slice width of 0.25 $\AA$. $\Delta$ASA<sub>ap</sub> and $\Delta$ASA<sub>pol</sub> were calculated using the coordinates of the ternary complex TK\textsubscript{dT}\textsubscript{AMP} (1) and removing either dT or ATP or both.

**RESULTS**

**Experimental Setup**—The GST fusion protein of HSV1 TK was used in this study to facilitate protein purification and to improve stability during storage. To rule out artifacts caused by the presence of GST in the construct, control experiments

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**FIG. 1. Ribbon diagram of the symmetric HSV1 TK dimer with bound ADP and dTMP (Protein Data Bank entry 1VTK; Ref. 2).**

The domains are defined as for other NMP kinases (21). The CORE domain is depicted in blue, the NMP\textsubscript{bind} domain in red, and the LID domain in yellow. The additional residues (250–322) are shown in green. Substrate and cofactor are depicted as cyan ball and stick models. The image was generated with the program MOLESCRIPT (53).
were performed with the HSV1 TK obtained after on-column cleavage of the affinity tag. Thermodynamic parameters determined by ITC were identical within error for cleaved HSV1 TK and for the GST fusion protein (data not shown). The influence of glutathione in concentrations up to 5 mM on HSV1 TK kinetics was investigated as previously described (25, 34). Glutathione in concentrations up to 5 mM does not change the kinetics was investigated as previously described (25, 34). Glutathione in concentrations up to 5 mM does not change the kinetics was investigated as previously described (25, 34). Glutathione in concentrations up to 5 mM does not change the kinetics was investigated as previously described (25, 34). Glutathione in concentrations up to 5 mM does not change the kinetics was investigated as previously described (25, 34).

**Fig. 2.** Formation of the ternary enzyme-substrate complex TK-dT-ATP. The two ordered sequential pathways are i plus ii and iii plus iv, respectively. In a random binding mechanism, all four reactions take place.

In the cell, HSV1 TK is exposed to both substrates at the cell membrane. For the purpose of clarity, only one representative result of triplicate measurements is shown. Values on the y axis (kcal/mol of injectant) are raw data and are not yet corrected for protonation effect (Equation 1).

The reaction was again exothermic and yielded values of $K_B = 3.9 \times 10^6 \text{ M}^{-1}$ and $\Delta H_{\text{bind}} = -13.8 \text{ kcal mol}^{-1}$ (Table I; corrected for protonation effect; see below).

In the cell, HSV1 TK is exposed to both substrates at the cell membrane. For the purpose of clarity, only one representative result of triplicate measurements is shown. Values on the y axis (kcal/mol of injectant) are raw data and are not yet corrected for protonation effect (Equation 1).
Thermodynamics of HSV1 Thymidine Kinase

TABLE I
Thermodynamic parameters for the binding of dT and ATP to HSV1 TK at pH 7.5

| Temperature (°C) | TK + dT (reaction i) | TK-dT + ATP (reaction ii) | TK + dT/ATP* |
|------------------|----------------------|---------------------------|--------------|
|                  | ΔH kcal mol⁻¹ | ΔG kcal mol⁻¹ | TΔS kcal mol⁻¹ | ΔH kcal mol⁻¹ | ΔG kcal mol⁻¹ | TΔS kcal mol⁻¹ | ΔH kcal mol⁻¹ | ΔG kcal mol⁻¹ | TΔS kcal mol⁻¹ |
| 10               | -13.6            | -7.1                 | -6.5         | -11.7         | -8.9         | -2.8         | -25.4         | -18.9         | -6.5          |
| 15               | -15.3            | -7.0                 | -6.8         | -12.1         | -8.9         | -3.2         | -27.9         | -18.9         | -9.0          |
| 20               | -17.4            | -7.1                 | -10.2        | -13.0         | -9.1         | -3.9         | -30.5         | -17.2         | -13.3         |
| 25               | -19.1            | -7.2                 | -11.9        | -13.8         | -9.0         | -4.8         | -33.1         | -16.8         | -16.3         |

ΔCp (kcal K⁻¹ mol⁻¹)
-0.36
-0.14
-0.51

K_B is the binding constant determined by ITC. Uncertainty of ΔG is within ±0.35 kcal mol⁻¹ of the mean. Errors of ΔH are about ±5% and mainly reflect the error in ligand concentration. Maximal possible errors of TΔS are 1.5 kcal mol⁻¹. Errors of ΔCp were estimated by reduction of the data set by one data point at a time and were, on average, >0.92 kcal K⁻¹ mol⁻¹, i.e. within 5–15% of the reported mean.

The ITC experiment provides the binding constant K_B for a single-site reaction, and ΔG values of reactions i and ii were calculated from ΔG = -RT × ln K_B. ΔS was obtained from ΔG = ΔH - TΔS. Reactions i and ii were driven by favorable negative changes in binding enthalpy and strongly opposed by unfavorable entropic contributions. Although the reaction with the 1:1 mixture of dT and ATP was more complex, it could still be treated as a single-site reaction if one considered dT plus ATP as one ligand. In this case, K_B obtained from ITC equaled (K_i × K_{ii})¹/² where K_i and K_{ii} were the binding constants for reactions i and ii, respectively. Hence, the apparent binding constant for the coupled reactions i and ii was K_B², and ΔG equaled -RT × ln K_B².

Change of Protonation State—Substrate binding may cause the enzyme to take up or release protons, for example through changes of side chains accompanying the binding reaction. This will contribute to the overall heat change, ΔH_{ion}, measured in the ITC experiment. If ionizable groups undergo pK_a changes on complex formation, protons will be exchanged with the buffer. The heat of protonation/deprotonation depends on the ionization enthalpy of the buffer, ΔH_{ion}, according to the equation:

ΔH_{obs} = ΔH_{bind} + n_{H^+} × ΔH_{ion}  
(Eq. 1)

where n_{H^+} designates the number of protons that are released (n_{H^+} > 0) or taken up (n_{H^+} < 0) by the buffer (35). To study such protonation effects, titration experiments were repeated in various buffers of different ΔH_{ion}. The intrinsic enthalpy of binding, ΔH_{bind}, was obtained from the intercept (ΔH_{ion} = 0) of a plot according to Equation 1. The results are shown in Fig. 4. Protonation/deprotonation was negligible in the case of dT binding to the free enzyme (Fig. 4, line B). Hence, ΔH_{obs} = ΔH_{bind} for reaction i. An uptake of 0.31 protons was observed with ATP binding to the TK-dT complex in reaction ii (Fig. 4, line A). Titration with the 1:1 mixture of dT and ATP leads to the uptake of 0.35 protons (Fig. 4, line C). It follows that proton uptake occurred with ATP binding but not with dT binding. Changes of ΔCp from ITC experiments were corrected accordingly (Table I).

Phosphate buffer behaved anomalously and influenced the thermodynamic parameters significantly. Therefore, data collected in phosphate buffer were not included in the analysis because of obvious differences in the interaction mechanism.

Temperature Dependence of Thermodynamic Parameters—ITC measurements were performed at 10, 15, 20, and 25 °C.

The results are presented in Fig. 5 and are summarized in Table I. ΔH and TΔS depended strongly on temperature, while ΔG was almost insensitive to temperature due to enthalpy-entropy compensation. Values of ΔCp were calculated from the slopes of the regression lines of ΔH_{bind} versus temperature (Fig. 5). Binding of dT to the free enzyme (reaction i) was characterized by ΔCp = -360 cal K⁻¹ mol⁻¹. ΔCp = -140 cal K⁻¹ mol⁻¹ was measured for ATP binding to the TK-dT complex (reaction ii), and ΔCp = -510 cal K⁻¹ mol⁻¹ for the titration of the enzyme with a 1:1 mixture of dT and ATP (Table I). The latter value was very close to the sum of the ΔCp values of reactions i and ii, in agreement with a thermodynamic cycle described by the three reactions.

Correlation between ΔCp and Surface Area Buried on Substrate Binding—In protein folding, the changes in enthalpy, entropy, and heat capacity can be accounted for in terms of changes in solvent-accessible polar and apolar surface area...
Ordered Binding of Thymidine and ATP to HSV1 TK—The calorimetric titrations presented here provide a comprehensive description of the energetics of substrate binding to HSV1 TK. Substrate binding is strictly ordered. ATP could bind only after the TK-dT complex had been formed. No ATP binding to the apoenzyme was observed. One might argue that ATP binding was entropy-driven and that \( \Delta H \) for ATP binding was so small that it escaped detection by ITC. This possibility could be ruled out because the entire set of thermodynamic parameters satisfied the cycle shown in Fig. 6. Fig. 6 summarizes the energetics of binding of dT and ATP at 25 °C and pH 7.5. Summation of the parameters for reactions i and ii equaled almost exactly the parameters determined independently for the single reaction of the apoenzyme with dT and ATP together. The free energy change for dT binding was smaller than for ATP binding, \(-7.2 \text{ versus } -9.0 \text{ kcal mol}^{-1}\), but the enthalpy, entropy, and heat capacity changes were significantly larger for the initial dT binding. Comparing absolute values, one notes that \( \Delta H \) was 1.4 times larger, and \( \Delta T S \) and \( \Delta C P \) were 2.5 times larger for dT binding than for the subsequent ATP binding. This means that dT binding, which induced the formation of a tight ATP binding site, was driven by a large enthalpy change but at a high cost in entropy.

Substrate-induced Conformational Changes Deduced from Large \( \Delta C P \) Values—In the relatively narrow temperature range studied, \( \Delta H \) became more favorable and \( \Delta T S \) more unfavorable with increasing temperature. As a result, \( \Delta G \) remained remarkably insensitive to temperature through entropy/enthalpy compensation. This is a ubiquitous phenomenon seen in many association reactions and is thought to be directly related to the role of solvent water molecules in the association process (47). According to the laws of thermodynamics, the temperature dependence of \( \Delta H \) and \( \Delta S \) results from substantial changes in heat capacity. In almost all association processes with proteins, \( \Delta C P \) has a negative sign if the free components are the reference state (45). In the present case, \( \Delta C P \) of the overall reaction was \(-510 \text{ cal K}^{-1} \text{ mol}^{-1}\). Binding of dT contributed 70%, and ATP binding contributed 30% to this large negative heat capacity change. Recently, it has been shown that the enthalpy slope observed in ITC experiments may include contributions arising from temperature-induced changes in the heat capacities of all participants of the association reaction over the considered temperature range (48). The quantitative information that is needed to correct the experimental \( \Delta C P \) for such effects could be obtained by DSC, given that the individual components undergo a reversible thermal unfolding. Unfortunately, the thermal unfolding of HSV1 TK was fully irreversible, with an apparent midpoint at 43 °C. However, the far and near CD spectra remained unchanged, and the enzyme retained full enzymatic activity over the relatively narrow temperature interval of this study. We believe, therefore, that the measured \( \Delta H \) and \( \Delta C P \) values genuinely reflect the formation of the specific ligand-protein complex, including the structural rearrangements of the enzyme to accommodate the ligands and do not contain significant contributions from preexisting temperature-dependent conformational equilibria.

Since, in general, \( \Delta C P \) correlates well with the amount of surface area buried at the complex interface (49–52), we have tried to calculate \( \Delta C P \) from the surface area of HSV1 TK buried by dT and ATP, respectively, and by both substrates together. The calculation (Equation 2) is based on a large body of structural and thermodynamic data for protein folding, protein-protein association, and protein-ligand binding.

Since the three-dimensional structure of substrate-free HSV1 TK is not known, we could not relate the calculated

\[
\Delta C P = \Delta C P_{\text{ap}} + \Delta S_{\text{ap}} - \Delta C P_{\text{pol}} + \Delta S_{\text{pol}} \tag{Eq. 2}
\]
The mobile segment between residues 250 and 322 contribute to the NMPbind domain. While these considerations remain speculative before the structure of substrate-free HSV1 TK is known, recent crystallographic studies of adenylyl kinases, to which the viral thymidine kinase is homologous, have indeed demonstrated major changes induced by substrate binding in the NMPbind domains and the LID domains as well as small changes in the CORE domains (21, 24).

**CONCLUSION**

This is the first report providing a comprehensive thermodynamic description of substrate and cofactor binding to HSV1 TK, a representative of the large family of nucleotide and nucleoside kinases. The results obtained by titration microcalorimetry reveal an extreme case of positive heterotropic interaction. Formation of a binary complex of thymidine with HSV1 TK is a stringent prerequisite for ATP binding. Since the ATP binding site is in fact generated by thymidine binding, one would expect the enzyme to undergo considerable conformational rearrangements. This has been supported by the analysis of the observed heat capacity changes, which were large and negative and indicated burial of molecular surface to an extent much larger than expected if the substrate binding sites preexisted on the apoenzyme and no rearrangement would occur (rigid body binding model). The findings support the view that substrate binding to HSV1 TK leads to a conformational closing of the substrate binding sites to bring thymidine and ATP into an orientation appropriate for catalysis. The details of the predicted rearrangements must await a firm structural foundation. Work is in progress in this laboratory to solve the crystal structure of the substrate-free apo form of HSV1 TK.

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