Binding of Nucleotides to Nucleoside Diphosphate Kinase: A Calorimetric Study‡

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Received October 18, 2000; Revised Manuscript Received January 22, 2001

ABSTRACT: The source of affinity for substrates of human nucleoside diphosphate (NDP) kinases is particularly important in that its knowledge could be used to design more effective antiviral nucleoside drugs (e.g., AZT). We carried out a microcalorimetric study of the binding of enzymes from two organisms to various nucleotides. Isothermal titration calorimetry has been used to characterize the binding in terms of $\Delta G°$, $\Delta H°$ and $\Delta S°$. Thermodynamic parameters of the interaction of ADP with the hexameric NDP kinase from *Dictyostelium discoideum* and with the tetrameric enzyme from *Myxococcus xanthus*, at 20 °C, were similar and, in both cases, binding was enthalpy-driven. The interactions of ADP, 2′deoxyADP, GDP, and IDP with the eukaryotic enzyme differed in enthalpic and entropic terms, whereas the $\Delta G°$ values obtained were similar due to enthalpy−entropy compensation. The binding of the enzyme to nonphysiological nucleotides, such as AMP−PNP, 3′deoxyADP, and 3′-deoxy-3′-amino-ADP, appears to differ in several respects. Crystallography of the protein bound to 3′-deoxy-3′-amino-ADP showed that the drug was in a distorted position, and was unable to interact correctly with active site side chains. The interaction of pyrimidine nucleoside diphosphates with the hexameric enzyme is characterized by a lower affinity than that with purine nucleotides. Titration showed the stoichiometry of the interaction to be abnormal, with 9−12 binding sites/hexamer. The presence of supplementary binding sites might have physiological implications.

Nucleoside diphosphate (NDP)[1] kinases catalyze the reversible phosphorylation of nucleoside diphosphates by nucleoside triphosphates, via a ping-pong mechanism involving a phosphohistidine intermediate (*1*−*5*).

$$N_1TP + E\text{-}His \leftrightarrow N_1DP + E\text{-}His\text{-}P \quad (1a)$$

$$E\text{-}His\text{-}P + N_2DP \leftrightarrow E\text{-}His + N_2TP \quad (1b)$$

$$N_1TP + N_2TP \leftrightarrow N_1DP + N_2TP \quad (1)$$

These ubiquitous enzymes play a key role in the metabolism of nucleotides, their principal function being the synthesis of (deoxy)nucleotide triphosphates from ATP and (deoxy)nucleotide diphosphates. Thus, for example, they supply GTP to G-proteins in signal transduction and substrates to nucleic acid synthesis (*6*, *7*).

Several crystal structures have been obtained for free NDP kinase (*8*−*11*), the phosphorylated intermediate (*12*), the enzyme complexed with ADP (*9*, *10*), with GDP (*13*), with TDP (*14*), with 3′-azido-3′-dTDP and 3′-F-2′,3′-dideoxy UDP (*15*), and the enzyme complexed with ADP and AlF_3, a putative transition state analogue (*16*). These structures are reviewed in Janin et al. (*17*). The contacts between the nucleotides and the protein are remarkably conserved in enzymes from different sources, from bacteria to humans, as is overall protein subunit structure. Eukaryotic enzymes are hexameric, whereas some bacterial enzymes are tetrameric (*9*). No major conformational differences were observed when the structures of the free, phosphorylated and nucleotide-bound enzymes were superimposed, except for a movement of the αA−α2 helices required for nucleotide binding. The base moiety lies in a hydrophobic crevice and makes no specific polar interactions with the protein. The substrate 2′-OH is not required for binding or catalysis, being replaced by a water molecule. This fits well with the broad specificity of NDP kinases, necessary for in vivo function. The 3′-OH is within hydrogen-bonding distance of the oxygen bridging the phosphates β and γ of the substrate NTP. Rapid-mixing studies (*18*) and steady-state experiments (*5*, *19*) have shown that the absence of the ribose 3′-OH

‡ Atomic coordinates have been deposited in the Protein Data Bank (filename 1hiy).

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[1] Abbreviations: NDP, nucleoside diphosphate; D.d., *Dictyostelium discoideum*; M.x., *Myxococcus xanthus*; AZT-DP, 3′-azido-3′-deoxythymidine diphosphate; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-(2-ethane-sulfonic acid); 3′-NH_2-ADP, 3′-amino-3′-deoxyADP; AMP-PNP, 5′-adenylylimido-diphosphate; DSC, differential scanning calorimetry; ITC, isothermal titration calorimetry.

10.1021/bi002432s CCC: $20.00
Published on Web 03/23/2001

dramatically decreases the catalytic efficiency of the enzyme. This is important in anti-AIDS therapy, because all the nucleoside analogues used lack this hydroxyl group (*20*). In particular 3′-deoxythymidine, 2′,3′-dehydro-3′-deoxythymidine (D4T) and AZT are used as anti-AIDS drugs. After being activated by conversion to triphosphates by cellular kinases, they become substrates of the viral reverse transcriptase and block elongation and DNA synthesis when they become incorporated into the growing chain. NDP kinases have been implicated in this cellular activation of nucleoside analogues.

Steady-state kinetic studies (*1*) have suggested that the various NDP kinases examined have low specificity for the heterocyclic moiety of their substrates. In contrast, stopped-flow kinetic studies have demonstrated large differences with various nucleotides (*18*). In addition, differences in the rates of enzyme phosphorylation and dephosphorylation with nucleotides containing different bases appear when using 2′,3′-dideoxynucleotides, poor substrates for NDP kinase (*18*). This may be because, with natural nucleotides, which are good substrates, the rate-limiting step is probably diffusion, whereas with poor substrates, the chemical step becomes rate limiting (*5*). A further complication is that reactions 1a and 1b may both be partially rate limiting. Extensive kinetic studies have been performed on NDP kinases, but fewer studies have dealt with the affinity of these enzymes for the various natural substrates and their analogues (*18*).

The knowledge of substrate specificity of the enzymes involved in the phosphorylation steps is therefore of primary importance. To design drugs capable of maximum interaction with the enzyme, we need to have a thorough understanding of the nature of the forces and of the molecular features that determine binding.

To obtain this information we have carried out a calorimetric titration study of two NDP kinases (the hexameric enzyme from *Dictyostelium discoideum* and the tetrameric enzyme from *Myxococcus xanthus*) with ADP and of the hexameric enzyme with various nucleoside diphosphates. These are the best characterized NDP kinases in terms of X-ray structure, in both the free and complexed states. In addition, the difference in quaternary structure of these two enzymes is an interesting albeit unexplained feature.

Complexes with nucleoside triphosphates cannot be studied by calorimetry because reaction 1a occurs. Nucleoside diphosphates are dead-end inhibitors when bound to the free enzyme, but their relative affinity should be similar to that for the phosphorylated enzyme and that of triphosphates for the free enzyme. Owing to technical difficulties, most of the experiments had to be performed in the absence of added magnesium ions, so the results obtained do not exactly reflect physiological conditions.

Finally, to determine the reasons for the observed lack of affinity for 3′-NH_2-ADP, we solved the crystal structure of this nucleotide with D.d. NDP kinase.

MATERIALS AND METHODS

Most nucleoside diphosphates were purchased from Sigma. AZT-DP and 3′-amino-3′-deoxyADP were obtained as previously described (*5*, *21*). 3′-deoxyADP was prepared by chemical phosphorylation of the nucleoside. The monophosphate was purified by ion-exchange chromatography on QAE Sephadex eluted with an ammonium bicarbonate gradient. The monophosphate was dried by evaporation and enzymatically converted to triphosphate, using creatine phosphate and creatine kinase as the regenerating system. It was not possible to stop the reaction at the diphosphate stage because the 3′-deoxyADP is a good substrate for creatine kinase. The triphosphate was converted to the diphosphate derivative by the hexokinase reaction.

The presence of contaminating material was checked by FPLC on a Mono Q column and found to be less than 1% for all substrates.

Recombinant D.d. and M.x. NDP kinases (*5*) were expressed in *Escherichia coli* and purified by negative adsorption on Q-Sepharose columns. It was necessary to desalt the crude extract on a Sephadex G25 column to obtain pure enzyme preparations in a single step. Proteins were stored as precipitates in saturated ammonium sulfate solutions. The concentration of the enzymes was determined spectrophotometrically using the extinction coefficients (determined by amino acid analysis) $E^{0.1\%} = 0.38$ and 0.73, respectively, for the M.x. (64 kDa) and the D.d. (100 kDa) enzymes, at 280 nm. Nucleotide concentration was measured spectrophotometrically using published extinction coefficients.

The pK_a of the 3′-amino group was estimated using a chromatographic technique (*5*) and was found to be 7.5. Binding of Mg^{2+} by the 3′-NH_2-ADP was measured by competition with 8-hydroxyquinoline in Hepes-KOH buffer at pH 8.0.

Enzymatic activity was routinely assayed immediately before calorimetric titration using a coupled assay at 25 °C and pH 7.5 (*22*).

DSC experiments were performed with a MicroCal MC-2 differential scanning microcalorimeter (MicroCal Inc., Nothampton, MA) as described by Giartosio et al. (*23*). Proteins were extensively dialyzed against 50 mM HEPES buffer, pH 8, and gently degassed before scanning. Protein concentration was 2 mg/mL and scan speed was 60 °C/h. No reversibility of thermal unfolding was observed, and no thermodynamic parameters (ΔCp or ΔH) could be calculated, due to aggregation of the denatured proteins.

We carried out isothermal calorimetric titration with nucleotides, using the Omega isothermal titration calorimeter (MicroCal, Northampton, MA). Proteins were extensively dialyzed against the buffer of choice (50 mM HEPES, or 20 mM phosphate, at pH 8, in the presence or absence of 0.1 M NaCl) and the dialysis buffer was used to dissolve the nucleotides. The pH of nucleotide solutions was carefully checked. Protein and nucleotide diphosphate solutions were degassed before use. The protein solution was placed in the sample cell, and the reference cell was filled with 0.1% sodium azide. Titrations were performed at various temperatures in the 12−35 °C range. The temperature of the coolant surrounding the titration and reference cells was kept at least 5 °C below the temperature of the cells.

Aliquots of 3−10 μL of the ligand were injected into the cell by means of a stirrer-syringe, at 4 min intervals. The concentration of protein used was 0.05−0.07 mM as hexamer or tetramer, and ligand concentrations were in the 20−60 mM range. In typical experiments at room temperature, the *C* value (product of the binding constant and binding site

concentration) (*24*) was between 3 and 5. The apparent heat change after each injection was determined by integration and corrected for the heat of dilution of the nucleotide. In some cases, the injection volume was changed from 3 to 10 μL after the first 10 additions of ligand, to allow a direct measurement of both the heat of binding and the heat of dilution in a single experiment. If the final addition did not result in saturation, an independent experiment was performed to determine the correct heat of dilution. The heat of dilution obtained by adding buffer to the protein solution was found to be negligible.

The titration curves were analyzed using the Origin software provided by MicroCal. Theoretical curves represent the best fitting to the experimental data, in terms of ΔH (binding enthalpy per mole of ligand), K (apparent equilibrium binding constant), and n (number of binding sites per hexamer or tetramer). ΔCp, the heat capacity at constant pressure, was determined from the slope of the linear fit of ΔH values plotted as a function of temperature.

Buffers of different heats of ionization were used to determine whether the binding was accompanied by the release or uptake of protons. Since most experiments were performed at pH 8, HEPES and phosphate were chosen as appropriate buffers, after verifying that activity measurements and thermograms were not affected by their presence in the solvent. If the enthalpy values differed between buffers, the observed heat of reaction was corrected by subtracting the heat of ionization of the buffer according to the expression $\Delta H_{corr} = \Delta H_{obs} - np\Delta H_{ion}$, where np is the number of exchanged protons and ΔH_{ion} is the deprotonation enthalpy [5.0 and 1.2 kcal mol^{-1}, respectively, for HEPES and for phosphate at 25 °C (*25*)].

Crystals of D.d. enzyme complexes with 3′-NH_2-ADP were obtained by cocrystallization in a hanging drop containing 17 mM 3′-NH_2-ADP, 5 mg/mL protein, 20 mM $MgCl_2$, 50 mM Tris-HCl, pH 8, and 15−16% PEG550. PEG6000 also yielded crystals, but an electron density map showed no bound ligand. X-ray diffraction data to 2.6 Å resolution were collected on the W32 station of the LURE-DCI synchrotron radiation center (Orsay, France). The data were processed with DENZO (*26*). The crystals were of space group $P3_121$ with parameters $a = b = 71.6$ Å, $c = 153.8$ Å, with three subunits (half of the hexamer) in the asymmetric unit. They were isomorphous to the crystals of the complex with ADP−AlF_3 (*16*), which was the starting point for refinement. An omit map showed 3′NH_2-ADP bound at each of the three active sites present in the asymmetric unit, with weaker density and possible incomplete occupancy at site B. All data (99.7% complete at 2.6 Å) were used for refinement with X-plor (*27*), except 7% left out for monitoring R_{free}. The final model had $R = 22.5\%$ and $R_{free} = 30.5\%$ with good stereochemistry (root-mean square deviations from library values: 0.012 Å for bond lengths, 1.84° for bond angles).

RESULTS AND DISCUSSION

Binding of ADP to NDP Kinases. DSC thermograms of the enzymes from D.d. and M.x. in the presence and absence of ADP (Figure 1) show that nucleotide binding shifts the denaturation peak by about 5°, stabilizing both the D.d. and the M.x. enzyme. As thermal denaturation is not reversible under these conditions, the thermograms do not yield useful values of ΔH. However, the DSC thermogram of free NDP kinase shows that, in the titration temperature range (12−35 °C), the heat capacity increases steadily. This phenomenon, not evident in the thermogram of the M.x. enzyme (except at higher temperatures) or in protein−ligand complexes, has important implications for determination of the ΔCp of binding (*28*).

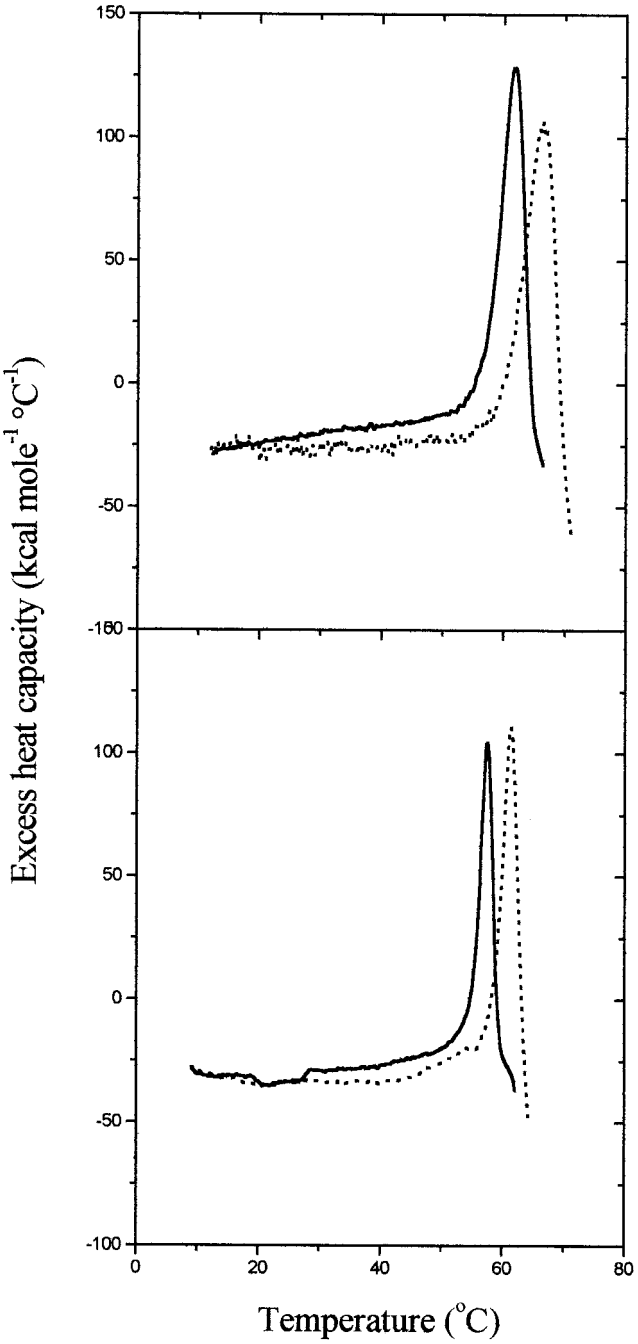

FIGURE 1: Effect of ADP on the temperature dependence of the molar heat capacity of D.d. and M.x. NDPKs. (Upper panel) D.d. NDPK with 0 (solid line) and 1 mM (dotted line) ADP. (Lower panel) M.x. NDPK with 0 (solid line) and 5 mM (dotted line) ADP.

The result of a typical calorimetric titration experiment is shown in Figure 2. In the upper panel, the heat pulses observed upon addition of ADP to NDP kinase are plotted as a function of injection order. The area of the pulses decreases progressively, until it reaches a constant value. The

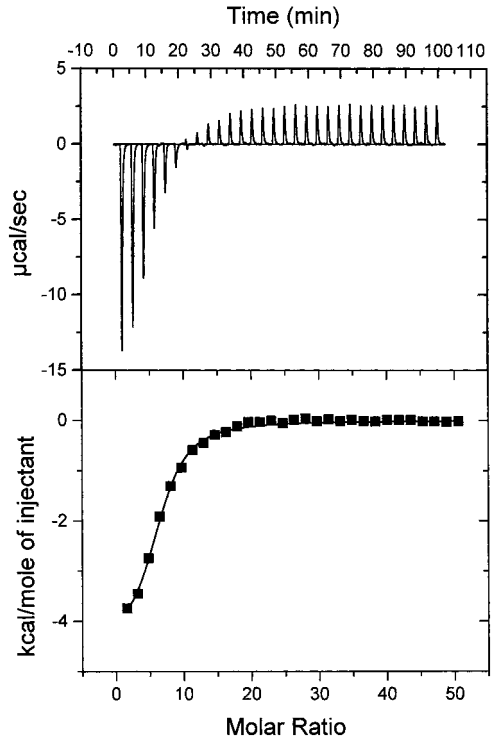

FIGURE 2: Calorimetric titration of 6.1 mg/mL (61 μM) D.d. NDPK with 27.3 mM ADP in 50 mM HEPES buffer, 100 mM NaCl, pH 8, at 17.8 °C. (Upper panel) Heat evolved upon injection of ADP into NDPK, plotted as a function of injection order. (Lower panel) Integrated heats of reaction plotted against the molar ratio of total ligand concentration to total hexameric protein concentration. The solid line shows the best fit to the data, according to a model that assumes a single set of identical sites, for $n = 6.00$ ($\pm$ 0.11) sites/mol, $K = 17.6$ ($\pm$ 1.5) $\times$ 10^3 M^{-1}, $\Delta H = -4.55 \pm 0.11$ kcal mol^{-1}ADP.

final value is the heat of dilution of the ligand into the cell and can be subtracted to obtain the corrected heat effect upon each addition. The lower panel shows the integrated heats of reaction plotted against the molar ratio of total ligand concentration to total oligomeric protein concentration, and the simulated curve obtained by best fitting the data according to a model assuming a single set of identical sites. The values of K, n, and ΔH were obtained from the best fit curve, whereas ΔG and ΔS were calculated from the basic equation $\Delta G = -RT \ln K = \Delta H - T\Delta S$. Measurements were performed at various temperatures.

The stoichiometry was always, as expected, 6 for ADP and the hexameric enzyme (5.9 $\pm$ 0.4 at 20 °C) and 4 for the tetrameric enzyme (3.7 $\pm$ 0.3 at 20 °C), with the low accuracy of some results probably due to errors in the active protein concentration.

For the D.d. enzyme the thermodynamic values at 20 °C obtained from the linear fits in Figure 3 where the results are plotted as a function of temperature, are $\Delta G = -5.4 \pm 0.1$ kcal mol^{-1}, $\Delta H = -4.3 \pm 0.6$ kcal mol^{-1}, $T\Delta S = 1.1 \pm 0.7$ kcal mol^{-1}.

The observed ΔCp is very low (-10 to -20 cal mol^{-1} K^{-1}). This is a consequence of the dependence on temperature of the heat capacity of the free protein in the temperature range of the titration experiments (Figure 1). Correction of the ΔH values as described by Privalov et al. (*28*), although affected by large errors, yields a larger

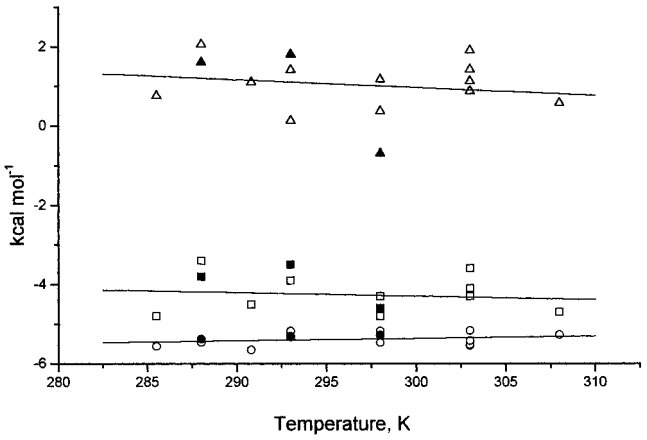

FIGURE 3: Temperature dependence of the interaction of D.d. NDPK with ADP. Experimental points indicate ΔG (○), ΔH (□), and $T\Delta S$ (△). (Open symbols) HEPES buffer; (closed symbols) phosphate buffer. Solid lines are linear least-squares regressions.

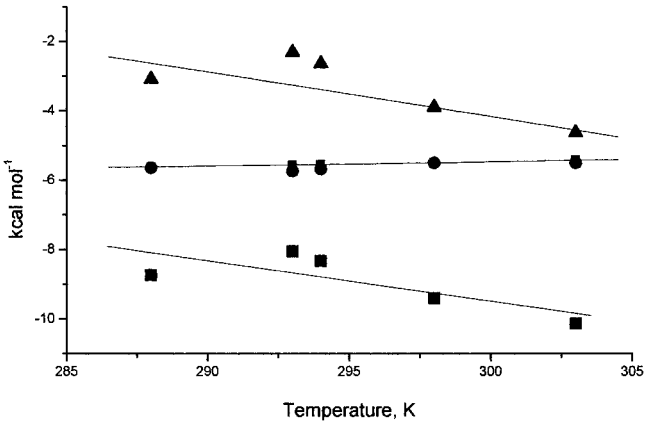

FIGURE 4: Temperature dependence of the interaction of M.x. NDPK with ADP in HEPES buffer. Experimental points indicate ΔG (○), ΔH (□), and $T\Delta S$ (△). ΔH values have been corrected for buffer protonation (see Materials and Methods). Solid line for ΔH is a linear least-squares fit. Solid lines for ΔG and $T\Delta S$ are theoretical curves based on non linear fits of the data obtained from equations $\Delta G(T) = \Delta H(298) + \Delta Cp(T - 298) - T\Delta S(T)$ and $\Delta S(T) = \Delta S(298) + \Delta Cp \times \ln(T/298)$, respectively, which assume that ΔCp is constant in the examined temperature range.

negative ΔCp value in the range -100 to -200 cal mol^{-1} K^{-1}.

The use of two buffers of different heats of ionization had no noticeable effect on ΔH values, indicating that few, if any, protonation events occur during binding.

ΔG for ADP binding to the tetrameric prokaryotic enzyme is similar to that of the eukaryotic enzyme (Figure 4). ΔH values in two buffers of different heats of ionization indicate an uptake of about 0.3 protons from the buffer per bound ADP at 25 °C. While data on only two different buffers prevent accurate determination of the proton uptake, a tentative correction was performed. The data in Figure 4, plotted as a function of temperature, have been corrected for buffer deprotonation. The thermodynamic parameters of binding for the tetrameric prokaryotic enzyme at 20 °C, obtained from the linear fits of the data in Figure 4, are $\Delta G = -5.6 \pm 0.1$ kcal mol^{-1}, $\Delta H = -8.7 \pm 0.6$ kcal mol^{-1}, $T\Delta S = -3.0 \pm 0.7$ kcal mol^{-1}, at 20 °C. ΔCp is negative (-116 ± 54 cal mol^{-1} K^{-1}). The higher binding enthalpy of this enzyme might depend on the protonation of some group(s) on the protein and/or on concomitant effects

Table 1: Thermodynamic Data for the Interaction of D.d. NDP Kinase with Various Nucleotides at 20 °C, pH 8, in Hepes Buffer

ligand	*n* (sites mol^{-1})	ΔH (kcal mol^{-1})	K (M^{-1})	ΔG (kcal mol^{-1})	$T\Delta S$ (kcal mol^{-1})
ADP[a]	5.9 ± 0.4	−4.2 ± 0.6	8540	−5.27 ± 0.1	+1.1 ± 0.6
GDP	4.9	−5.5	19 200	−5.74	+0.3
IDP	7.2	−3.0	9850	−5.35	+2.4
Mg-ADP	5.4	−2.5	21 900	−5.8	+3.3
2′deoxyADP	6.0	−2.6	5050	−4.95	+2.4
3′deoxyADP	5.6	−2.4	3300	−4.72	+2.3
3′NH_2ADP	6.9	−0.6	1200	−4.13	+3.5
AMP−PNP	5.6	−0.6	4200	−4.86	+4.3
TDP[a]	9.2 ± 0.3	−3.2 ± 0.2	3100	−4.68 ± 0.1	+1.5 ± 0.3
UDP	9.9	−1.7	1840	−4.38	+2.7
CDP	10.7	−1.0	860	−3.93	+2.9
AZTDP	4.7	−3.3	1100	−4.08	+0.8

[a] Mean value of three independent measurements ± s.e.

involving solvent molecules.

The association of ADP with the two NDP kinases is enthalpy-driven at all temperatures studied, showing that van der Waals interactions and H-bonds play a major role in the binding. The free energy change depends very little on temperature for either enzyme. In the 1 M standard state, the entropy change is small and favorable for the D.d. enzyme and small and unfavorable for the M.x. enzyme.

Interaction of D.d. NDP Kinase with Other Natural Purine Nucleoside Diphosphates. The interactions of 2′deoxyADP, GDP, and IDP with D.d. NDP kinase at 20 °C are characterized by ΔG values not very different from those obtained for ADP (Table 1) The binding constants obtained are between 5×10^3 M^{-1} and 22×10^3 M^{-1}, consistent with values obtained by dye-displacement spectroscopy (*29*) and tryptophan quenching fluorimetric titration (*18*, *30*). Calorimetry confirms that GDP is the best ligand for the D.d. enzyme, followed by IDP, then ADP and finally 2′deoxyADP. This may be due to the additional interaction, in hexameric NDP kinases, involving the exocyclic amino group of the guanine base bound at one active site and the C-terminal glutamate of a neighboring subunit (*13*). This interaction cannot occur with bases other than guanine or in tetrameric NDP kinases.

Association of the other natural nucleoside diphosphates with D.d. NDP kinase at 20 °C is accompanied by favorable enthalpy and entropy changes, as for ADP. Binding is enthalpy-driven, because entropic contributions are always positive but very small.

The enthalpy−entropy plot for the four natural purine substrates tested indicates a common compensation mechanism (Figure 5). This phenomenon, known as enthalpy−entropy compensation, is typical of processes involving binding to biological molecules in aqueous solution (*31*).

Interaction of D.d. NDP Kinase with Modified Adenosine Nucleotides. The modified adenosine nucleotides 3′-deoxyADP, AMP−PNP (an analogue of ATP) and 3′-NH_2-ADP show low affinity for D.d. kinase at 20 °C (Table 1). 3′-NH_2-ADP is the weakest ligand of the three. The 2.6 Å X-ray structure of the complex showed that this molecule bound at the same site as ADP, and in approximately the same conformation (Figure 6). The β-phosphate receives a hydrogen bond from the 3′ amino group of the modified sugar, just as it does from the 3′-OH of a normal substrate. However, the whole nucleotide is shifted by nearly 1 Å away from the active site and toward the protein surface. This is made possible by a similar shift of the helix hairpin (helices αA and α2), which forms one wall of the cleft in which the nucleotide binds. Except for this movement, the protein structure is essentially the same as in the ADP complex (*10*) and in other ligated NDP kinases (*17*). The helix hairpin is mobile and it shifts a further 1−2 Å to open the cleft in the absence of a ligand. The substitution of the 3′-OH is probably the immediate cause of the shift seen in the presence of 3′-NH_2-ADP. In a complex with ADP or another natural substrate, the 3′-OH receives a hydrogen bond from the amino group of Lys16. With 3′-NH_2-ADP, the shift relieves a short contact between two amino groups. Substitution of the 3′-OH by a fluoride in 3′fluoro-3′deoxyTDP also results in a rearrangement of the hydrogen bond pattern around the 3′ position, making a bad substrate of the modified nucleotide (*5*).

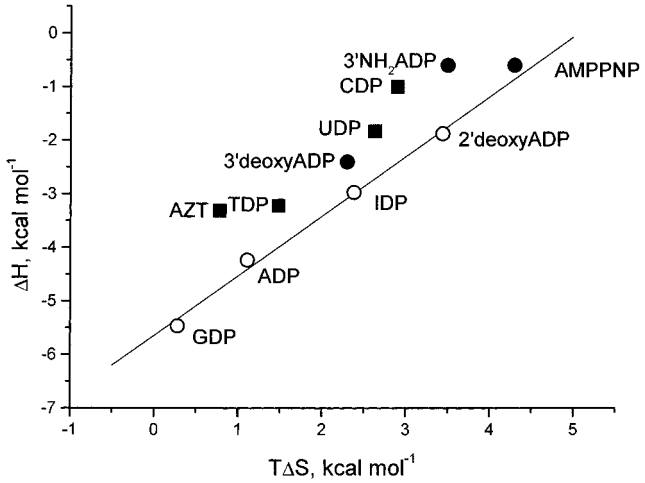

FIGURE 5: Enthalpy-entropy compensation for the interaction of D.d. NDPK with various substrates at 20 °C. The solid line is the linear least-squares regression relative to the natural purine nucleotides (open circles) with a slope of 1.11 and a correlation coefficient of 0.996.

Interaction of D.d. NDP Kinase Pyrimidine Nucleoside Diphosphates. Natural pyrimidine nucleotides have a lower affinity and less negative values of ΔH than do natural purine nucleotides (Table 1). However, several major uncertainties may affect these results, because, in the binding reactions, these substrates behave as if the enzyme had a larger number of sites for pyrimidine nucleotides ($n = 9$ for TDP and 10−11 for UDP and CDP, per hexamer). In phosphate buffer at low ionic strength, only 6 binding sites are titrated with TDP.

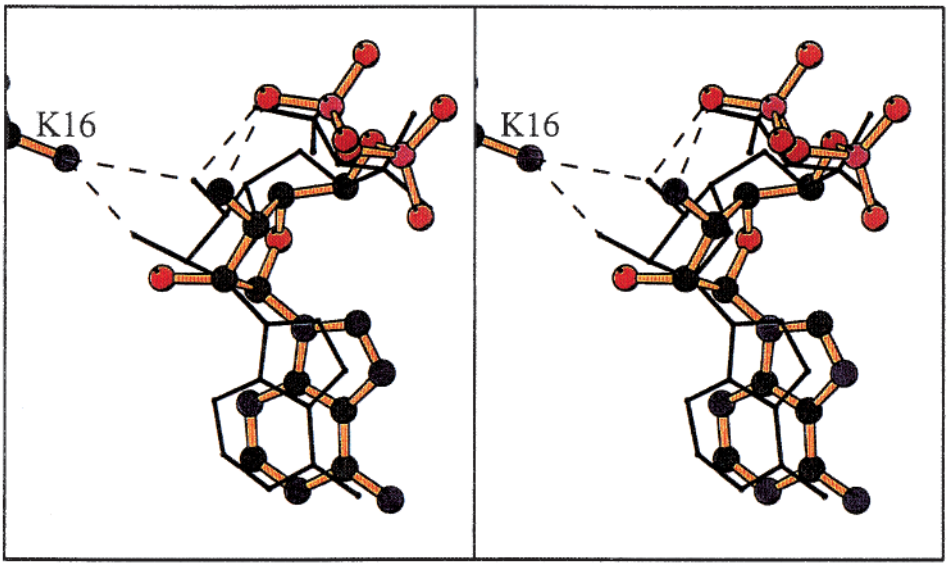

FIGURE 6: Stereoview of 3′-NH_2-ADP bound to D.d. NDP kinase. ADP (thin black lines) is shown for comparison. In both ligands, there is a hydrogen bond (dashed) between the β-phosphate on top and the group in 3′ position. In ADP, both the 3′ and 2′ oxygens receive hydrogen bonds from Lys16 (*10*). The interaction with Lys16 is repulsive in the case of 3′-NH_2-ADP.

This unexpected and puzzling behavior cannot presently be explained, since only one type of sites is always found by titration. It is however worth mentioning, since it could have physiological implications, that human NDP kinase B binds single-stranded DNA oligonucleotides with a pyrimidine-rich sequence (*30*, *32*). The additional sites could be involved in this interaction.

ΔG values of pyrimidine nucleotides are always less negative then those obtained with purine nucleotides (with the exception of 3′-NH_2-ADP), and enthalpic and entropic terms are always favorable. The compensation mechanism shown in Figure 5 does not hold for these substrates.

AZT-DP, which lacks the 3′OH and is kinetically a very poor substrate (*15*, *19*), binds to the hexameric enzyme with normal stoichiometry and an affinity ($K = 1.1 \times 10^3$ M^{-1}) less than that of TDP, largely due to a more positive ΔS for the latter ligand.

CONCLUSIONS

Calorimetry permits a direct measure of thermodynamic constants. By ITC, the heat absorbed or evolved at constant temperature, when the protein is titrated with a ligand, yields the stoichiometry of the reaction, the binding enthalpy and the binding constant.

However, the calorimetric value is the algebraic sum of the heat of interaction and of all the caloric contributions of the system, including dilution heat, conformational changes, solvation changes, ionization of the components and the buffer, aggregation. Only after all or most of these contributions have been taken into account can the data give useful indications.

The results presented here show that, for both the D.d. and the M.x. enzymes, binding constants are consistent with those obtained by other equilibrium methods. No cooperativity is observed for the binding of ADP to the hexameric or to the tetrameric enzyme: all binding sites are identical and behave independently, whatever the quaternary structure of the enzyme. The interaction of pyrimidine nucleoside diphosphates with the hexameric enzyme is characterized by a lower affinity and an abnormal stoichiometry, suggesting additional binding sites which may be those involved in DNA binding. Binding constants for natural purine substrates do not differ by more than 1 order of magnitude, although enthalpic and entropic parameters show large variations, due to the enthalpy−entropy compensation mechanism. Modifications to the ribose moiety do not greatly affect the binding constant, but the lack of the 2′-OH has no effect on the compensation mechanism, whereas this mechanism is markedly modified by any change in the 3′ position (Figure 5). This is consistent with the small difference in position of 3′-NH_2-ADP at the binding site of the D.d. enzyme crystals reported here, and with the known importance of the 3′OH for catalysis.

Antiviral drugs, which usually lack the 3′OH, are poor substrates for NDP kinase and/or for the preceding kinases in the nucleoside phosphorylation pathway.

Using a series of natural substrates differing for small modification in the molecule moiety not involved in the catalytic event, we have measured the thermodynamic constants that characterize the binding. The design of new drugs could be improved by determination of the thermodynamic parameters of binding, since ΔH and ΔS values are more informative than ΔG values for determining the quality of modified nucleotides as substrates.

ACKNOWLEDGMENT

I.L. and J.J. acknowledge support by Agence Nationale pour la Recherche contre le SIDA. Y.X. is grateful to the French-China Programme de Recherches Avancées for financial support and to the staff of the LURE (Orsay, France) synchrotron radiation center for help in data collection.

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BI002432S