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Substrate specificity and nucleotides binding properties of NM23H2/nucleoside diphosphate kinase homolog from *Plasmodium falciparum*

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Abstract Nucleoside diphosphate kinases (NDKs) play a key role in maintaining the intracellular energy resources as well as the balance of nucleotide pools. Recently, attention has been directed to NDKs owing to its role in activating various chemotherapeutic agents. The binding affinity of different nucleotides with *P. falciparum* NDK was varied according to the following order ADP~GDP>dGDP>dADP>dTDP>CDP>dCDP>UDP. The binding of purines nucleotides was stronger than pyrimidines. Furthermore, PfNDK showed more preferences to ribonucleotides over deoxyribonucleotides. Pyrimidines showed lower negative free energy compared with that of purines. The interaction of all nucleotides showed favorable enthalpic and entropic terms. However, the enthalpic terms were the main deriving forces for purine nucleotides, while the entropic contributions were the predominant forces for pyrimidines. Interestingly, TDP showed marked affinity and more favorable enthalpic and less entropic contributions. In conclusion, the size of nucleotide was the critical factor in PfNDK ligand affinity.

Keywords Bioenergetics · Nucleoside diphosphate kinase · *Plasmodium falciparum* · Isothermal titration calorimetry

Abbreviations

NDP	Nucleoside diphosphate
PfNDK	*Plasmodium falciparum* nucleoside diphosphate kinase
NDK	Nucleoside diphosphate kinase

Introduction

Recently, attention has been directed to NDKs owing to their various biological functions and their role in activating various chemotherapeutic agents and prodrugs used for antiviral and cancer therapies (Otero 2000; Verslues et al. 2007; Mikkelsen et al. 2008; Hippe et al. 2009; Lee et al. 2009). In order to design new drugs capable of maximum interaction with enzymes, we have to determine the

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substrate specificity and understand the nature and forces governing the substrate binding to the active site of enzymes. ITC became a powerful tool in assigning the molecular aspects of macromolecules-ligands recognition. The wealth of data provided by the technique is crucial for understanding the reaction mechanisms, substrate specificity and ligand affinity of target macromolecules.

Parasitic protozoa are equipped with very efficient metabolic systems allowing them to adopt the habitat in various hosts and different environmental conditions. One of these examples is the Nucleoside diphosphate kinase, which has an important role in cellular energy management. Nucleoside diphosphate kinase b (NDPK, nucleoside diphosphate phosphotransferase, **EC 2.7.4.6**) is involved in energy metabolism by catalyzing the transfer of high-energy γ-phosphate between NTPs and NDPs. Nucleoside diphosphate kinase plays a unique metabolic role as the enzyme performs the last reaction of ribonucleotides and deoxyribonucleotides biosynthesis. The enzyme was found to bind a broad range of ribo and deoxyribonucleotides, thus it can produce all precursors of DNA and RNA synthesis. NDK produces NTP for nucleic acid synthesis, CTP for lipid synthesis, UTP for polysaccharide synthesis and GTP for protein synthesis, signal transduction or microtubules polymerization (Jeudy et al. 2009). Growing evidences have been accumulated that NDP kinases are not only housekeeping enzymes, but also enzymes functioning in the central part of signal transduction in bacteria, fungi, plants, invertebrates and vertebrates (Hasunuma et al. 2003).

Various detection methods were adopted for determining the NDK activity including the spectrophotometric enzyme coupling assays, radiolabled nucleotides, microtitier plates using peroxidase coupled methods (Lascu et al. 1993; Duggleby et al. 1996; Barthel and Walker 1999; Tokarska-Schlattner et al. 2008). The major disadvantages of these methods include (i) it requires at least two coupling enzymes; (ii) the sensitivity is limited to the extinction coefficient of a certain chemical; (iii) many compounds have absorption at the designated wavelength; (iv) in complex mixture like cell extract or partially purified sample, the enzyme coupling systems face a certain degree of inconvenience due to the consumption of one or more of the coupling system components; (v) the most important is that these coupling assays have a limited spectrum for the measurement of all of the used substrates. In enzyme coupling reaction, we are unable to use all substrates of NDK as they can be utilized and phosphorylated by the coupling enzymes; (vi) The methods using the isotope-labeled substrate are not suitable for most accurate scientific work. Thus, in order to determine the substrate specificity for an enzyme like NDK we could go through long and difficult steps involving inaccurate methods, high background activity and possibility of false positive results. Furthermore, we have to combine two or more different methods to cover the broad substrate range of NDK which is not reliable in comparing the substrate specificity.

In this paper, we used ITC as a method for determining the substrate specificity for NDK. This method has several advantages including (i) avoid the use of radiolabelling or hazardous materials; (ii) does not require any coupling enzymes or any other substances except the interacting molecules; (iii) by using ITC only we can test all possible substrates of NDK, thus, increasing the validity of results and avoid the comparison of results from different experimental techniques.

We provide here the substrate specificity, differential ligand affinity and detailed thermodynamic profiles of nucleotides binding to PfNDK. Furthermore, we give an estimate for the possible structural rearrangements occurring during different nucleotides recognition.

Materials and methods

Materials

Talon metal affinity resin was obtained from Clontech (Palo Alto, CA). Nucleoside diphosphates, nucleoside triphosphates and phosphoenolpyruvate were obtained from Sigma. Protein concentrations were determined by using a protein assay reagent (Bio-Rad) with bovine serum albumin as the standard.

Production of recombinant enzyme

PfNDK was prepared as described by (Kandeel et al. 2009). Briefly, the recombinant *E. coli* cells were grown overnight in an LB medium containing 50 μg/ml ampicillin. The culture was diluted to 1:100 with the same fresh medium and cells were grown at 37 °C to mid-log phase (D_{600}=0.6). Induction of expression was carried out by the addition of IPTG to a final concentration of 1 mM and cell growth was continued at 37 °C for 4 h. Cells from 2 liters of culture were harvested by centrifugation at 5,000×g for 15 min and then stored at −20 °C until use. To obtain the cell extract, cells were lysed in an extraction buffer (25 mM Tris-HCl buffer pH 7.2 containing 150 mM NaCl) and disintegrated by sonication for 40 s (3 cycles with 3 min intervals). The lysate was centrifuged at 16,000×g for 15 min at 4 °C and the precipitate is discarded.

The hexahistidine-tagged PfNDK was purified from the soluble cell extract by using talon metal affinity resin. Flow rate was kept constant at 30 ml/min. After binding and washing with the extraction buffer, the protein was eluted using a linear imidazole gradient (from 0 to 150 mM).

Isothermal titration microcalorimetry

ITC measurements were carried out by using VP-ITC (MicroCal Inc., USA). The protein solution, at a concentration of 60 μM, was loaded into a 1.4 ml sample cell and titrated with the ligand (1–2 mM) in the 250 μl injection syringe. Substrate solution was made in the final dialysis buffer of proteins to minimize artifacts due to subtle differences in buffer composition. The protein was diluted to the appropriate concentration with dialysis buffer immediately before titration. ITC experiments were performed at 30 °C with a stirring speed 350 rpm and 300 s duration between each 10 μl injection. Control experiments were performed by injecting the nucleotide into the dialysis buffer to determine the heats of dilution. The apparent heat change after each injection was determined by integration and corrected for heat of dilution of the nucleotide. The data is fitted to a single-binding site model or two set sites model by a non-linear regression analysis to yield the thermodynamic parameters K_a, association constant; ΔH, enthalpy of binding; and n, stoichiometry of binding. The affinity of the nucleotides to protein is given as dissociation constant (K_d=1/K_a). The free energy of binding (ΔG) was calculated from the equations: $\Delta G = RT \ln K_a$ and $\Delta G = \Delta H - T\Delta S$ where ΔS is the entropy changes.

Molecular modeling studies

The structure of PfNDK is modeled according to the known structure of nucleoside diphosphate kinases recognizing both guanine and thymidine bases by using the modeling tools available at ProtMod protein modeling server (http://ffas.burnham.org/protmod-cgi/protModHome.pl). Sequence alignment was carried out with the profile-profile alignment and fold recognition algorithm FFAS. Profile-profile alignments utilize information present in sequences of homologous proteins to amplify the sequence conservation pattern defining the family resulting in detection of remote homologies beyond the reach of other sequence comparison methods. *All atom model* is used in which all template residues are replaced by target residues according to the sequence alignment. The protein structure models were based on SCWRL algorithm. The binding site was defined from coordinates of the ligand in the original structure. Ligand interactions and ligand binding site atoms were created by DS Visualizer (Accelrys Inc. USA).

Other methods

Protein concentrations were determined by using a protein assay reagent (Bio-Rad) with bovine serum albumin as the standard.

Western blotting Purified PfNDK was applied to 17.5% SDS–PAGE, transferred to the PVDF membrane and detected by anti-His tag (10,000-fold dilution). The immunoporbed proteins were detected by HRP conjugated substrate detection kit.

The molecular mass of the recombinant enzyme was determined by using SDS–PAGE (17.5% polyacrylamide). After electrophoresis, the gel was stained with Coomassie Brilliant Blue R-250.

The NDP kinase activity was measured spectrophotometrically using an enzyme coupling assay. The standard assay of 1 ml contained the following: 50 mM Tris-HCl pH 7.2, 0.5 mM phosphoenolpyruvate, 40 mM KCl, 2 mM $MgCl_2$, 0.15 mM NADH, 2U pyruvate kinase, 2U lactate dehydrogenase, and various concentrations of TDP and ATP. Routine assays were considered during purifications and ITC to confirm the activity of obtained enzyme.

Results

PfNDK thermodynamics

Figures 1 and 2 show typical titration of 60 μM PfNDK with the nucleotide ligands. The top panel shows the raw calorimetric data referring to the amount of heat produced following each injection. As the titration proceeds, the heat produced decreases gradually due to progressive occupancy of the active sites by the ligand. The bottom panel shows the integrated amount of heat generated per injection as a function of the molar ratio of ligand to enzyme.

Thermodynamic signatures for the binding of nucleotides with PfNDK are shown in Fig. 3a. The thermodynamic profiles of all tested nucleotides indicate a favorable enthalpic and entropic term and negative free energy change, which is the most favorable thermodynamic profile for tight binding.

Substrates binding affinity

The binding constant varied from 0.63×10^4 M^{-1} to 1.62×10^5 M^{-1}. The strongest binding and high affinity for PfNDK is shown by ADP and GDP (Fig. 3b). The binding of purines nucleotides was stronger than pyrimidines. Furthermore, PfNDK showed more preferences to ribonucleotides over deoxyribonucleotides. Among pyrimidines, dTDP showed the strongest binding affinity of 1.4×10^4 M^{-1} (Table 1).

Binding enthalpies

A negative binding enthalpy was observed all over the test nucleotides (Fig. 3c). Hydrogen bonds and van der Waals interactions are often considered to be the major source for

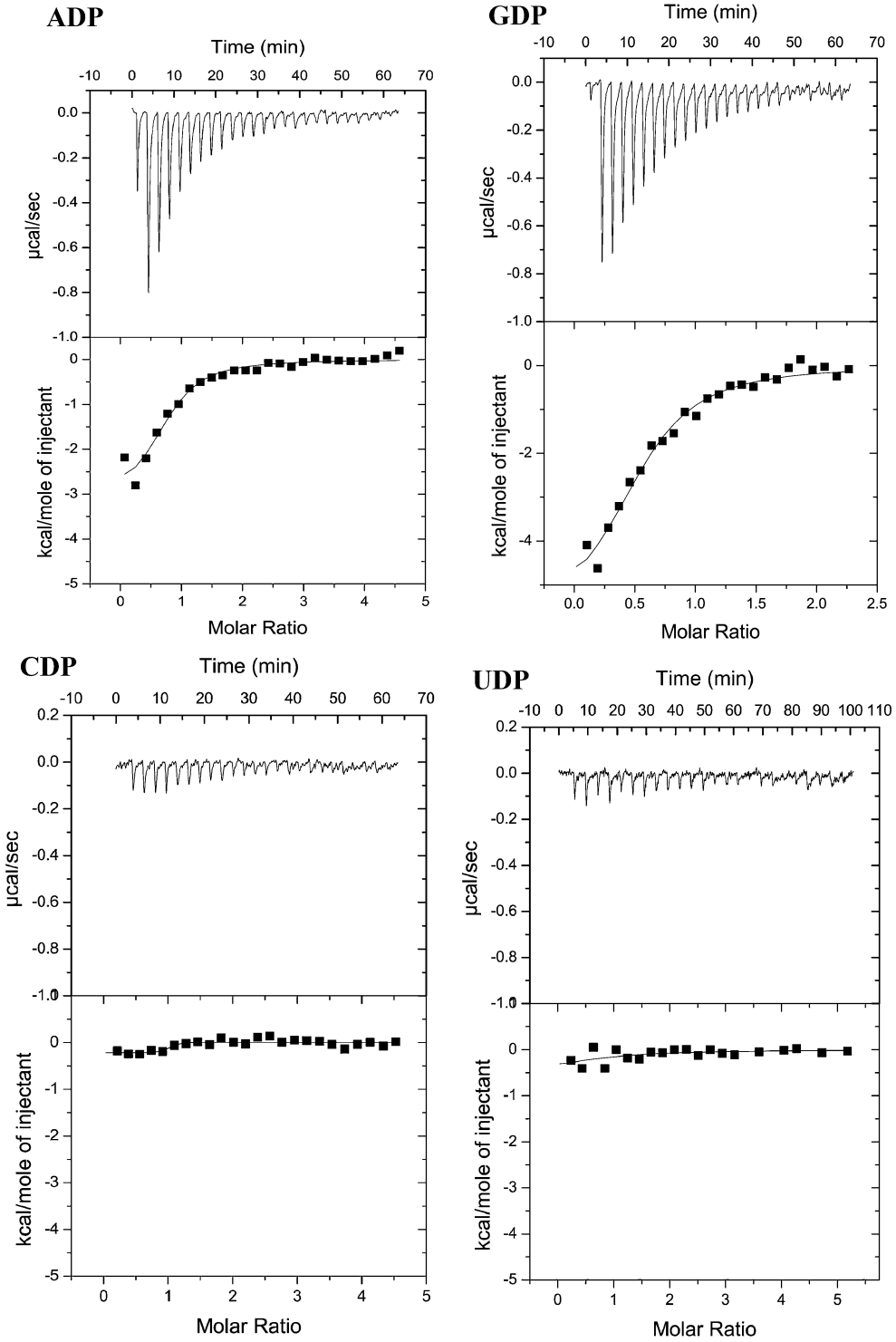

Fig. 1 Thermograms and binding isotherms for the binding of ribonucleotides with PfNDK. The observed binding isotherm is normalized as kilocalories per mole of injectant and plotted against the molar ratio of ligand to macromolecule. The top panels show the raw data heat changes produced by successive injections of nucleotides into PfNDK. The lower panel shows the integrated binding isotherms as a function of the molar ratio of ligand to enzyme. The measured ΔH is corrected for heat changes from control experiments. The heat associated with each injection is calculated by integrating the area under the deflection of the measured signal (amount of heat produced per unit of time, µcal/sec). The heat associated with each injection is calculated by integrating the area under the deflection of the measured signal (amount of heat produced per unit of time, µcal/sec)

negative enthalpies. All purine nucleotides produced a markedly high interaction enathalpies with the active site (> −2 Kcal/mol). GDP showed the highest interaction enthalpy of −5.7 Kcal/mol. All pyrimidines showed weak interaction enthalpy as low as -0.4 kcal/mol. Surprisingly, TDP, which is a pyrimidine compound, showed a markedly high enthalpy change of −3.3 Kcal/mol.

The free energy of binding of different nucleotides

The free energy change of binding showed marked differences among different nucleotides; furthermore, the enthalpic and entropic components of the free energy shows marked distinctions (Fig. 3d and e). The free energy of binding for pyrimidine nucleotides ranged from −5.2 to −5.8 while for

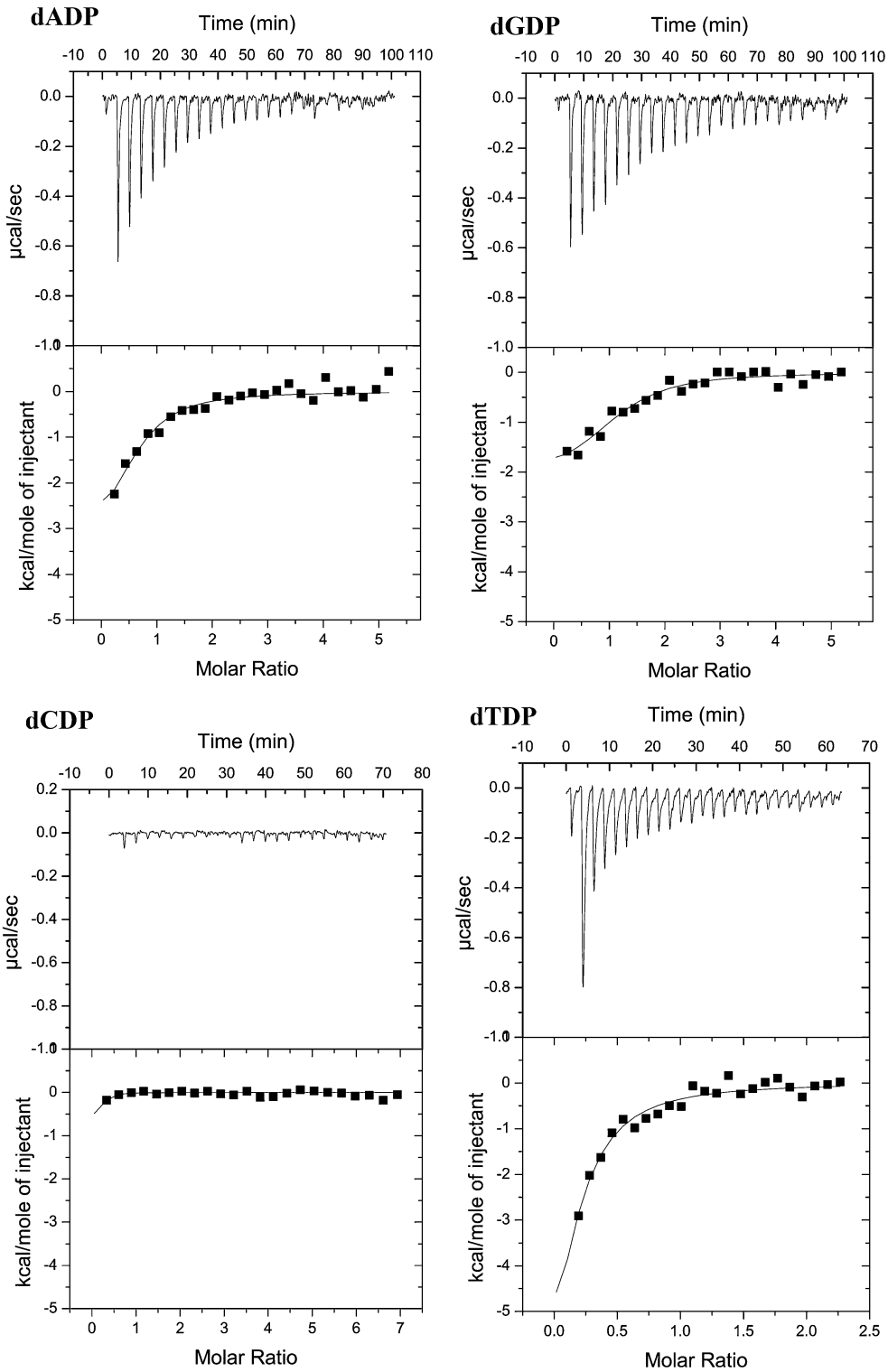

Fig. 2 Thermograms and binding isotherms for the binding of deoxyribonucleotides with PfNDK. The observed binding isotherm is normalized as kilocalories per mole of injectant and plotted against the molar ratio of ligand to macromolecule. The top panels show the raw data heat changes produced by successive injections of nucleotides into PfNDK. The lower panel shows the integrated binding isotherms as a function of the molar ratio of ligand to enzyme

purines it was from −6.6 to −7.3. Thus, pyrimidines showed less negative free energy compared with that of purines.

Enthalpy entropy compensation

For purine nucleotides binding, both binding enthalpy and entropy showed strong correlation.

The plot of ΔH against $T\Delta S$ for the binding of nucleotides with PfNDK deviates from unity, that is ΔG is dependent on the nucleotide. The plot for purine nucleotides compensation is more or less linear while those of pyrimidines greatly deviate from the linear plot indicating the lack of this compensation process and the enzyme discriminates between purine and pyrimidine nucleotides.

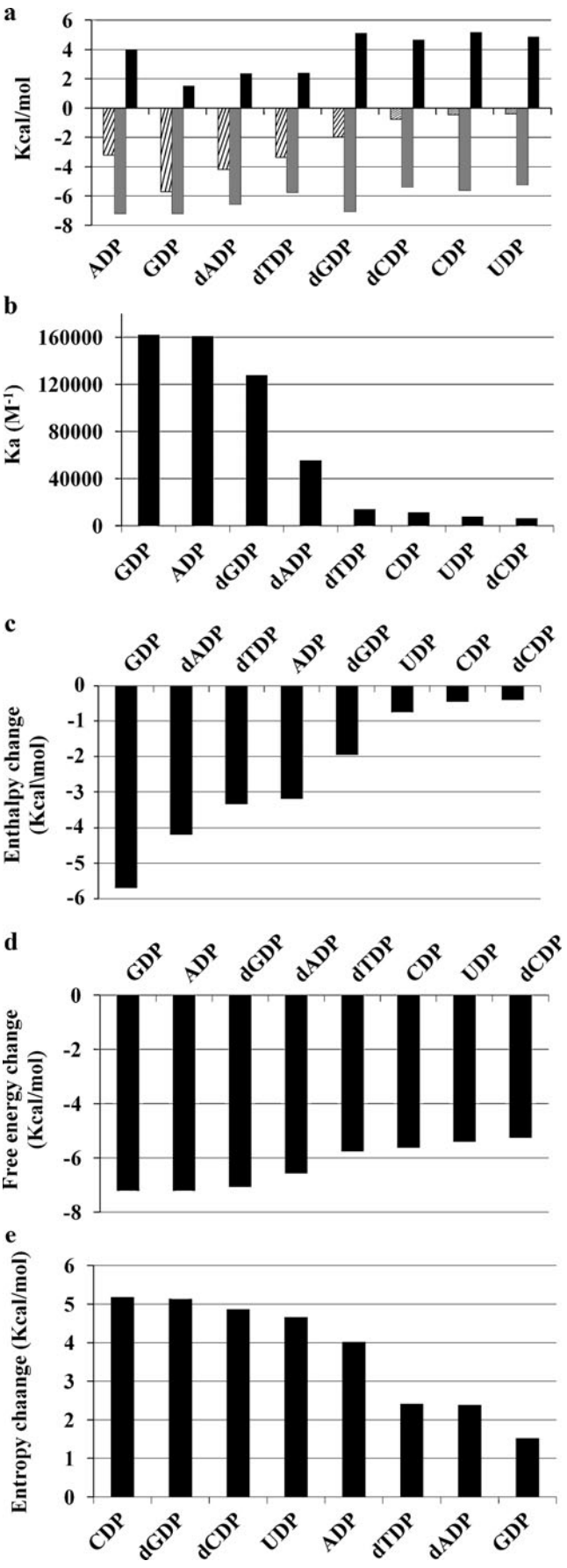

Fig. 3 Comparison of different binding parameters for the titration of PfNDK with different nucleotides. The figures show the thermodynamic signatures of different NTPs (Fig. 3a), the binding constant (Fig. 3b), enthalpy change (Fig. 3c), binding free energy (Fig. 3d) and entropy change (Fig. 3e) associated with the binding of different nucleotides

Modeling studies

Based on sequence alignment and phylogenic analysis, the nm23/nucleoside diphosphate family can be calssified into 2 subgroups, The group I genes encode highly homolgous proteins and possess the classic enzymic activity of a NDP kinase. This group includes NDP kinases A–D (Nm23H1 to -H4), which share 58 to 88% identity with each other. The *P. falciparum* nucleoside diphosphate kinase B is highly homologous with group I nm23 family genes. It encodes a protein that shares the common hexameric structure and nucleoside diphosphate kinase activity. It shares the common NDP kinase active site motif (NXXHG/ASD) with the four isofoms of this group.

We modeled the structure of the PfNDK in complex with various nucleotides, in order to understand the molecular basis of its affinity for the binding nucleotides. The comparison of the models with other known structures showed that the residues important for binding and activity are conserved. PfNDK shows the conserved residues for phosphoryl transfere H115, which is maintained in position by the conserved S117 and E126 residues (Fig. 4a). A conserved F57 maintains the role of aromatic stacking with purine and pyrimidine rings. The environment in which the phosphate groups bind was found to be conserved in the examined NDK structures. The ribose 3`OH makes proposed interaction with K9 and N112 (similar to other enzymes).

Discussion

NDK is a key enzyme in the control of cell energy and nucleotide metabolism; furthermore, it shares in the activation of various chemotherapeutic prodrugs. Currently little is known about the enzyme substrate specificity and enzyme-drug recognition forces. Furthermore, the design of new substrates and chemotherapeutics targeting of viral and other microbial enzymes requires accurate knowledge of the mode of compounds' interaction with the enzyme and the forces underlying this process. In addition, this process faces several obstacles including the lack of a suitable method to include all compounds in one practical method. So that, for determining the binding affinities of different nucleotides, we used ITC to obtain information that could help to determine the preferred nucleotides to PfNDK, estimate the order of the nucleotides binding affinities and quantify the thermodynamic parameters and the forces involved in the nucleotides binding to PfNDK.

Table 1 The thermodynamic constants obtained by isothermal titration calorimetry for the association of nucleotides with PfNDK. The one set of sites binding model is used for fitting the data. (n=3)

Substrate	Ka $M^{-1}*10^4$	K_d (μM)	ΔH (kcal/mol)	ΔG (kcal/mol)	$T\Delta S$ (kcal/mol)	ΔS (kcal/K mol)*10^{-3}
dADP	5.5	18	−4.2	−6.6	2.4	7.9
dGDP	12.8	7.8	−2	−7	5.1	16.9
ADP	16.1	6.2	−3.2	−7.2	4	13.2
GDP	16.2	6.2	−5.7	−7.3	1.5	5
CDP	1.1	87	−0.44	−5.6	5.2	17
dTDP	1.4	70.4	−3.3	−5.8	2.4	8
dCDP	0.8	125	−0.7	−5.4	4.6	15.5
UDP	0.63	158	−0.4	−5.2	4.8	16

During the past decades ITC is considered as a powerful tool or a specialist method for understanding molecular interactions; nowadays, ITC is becoming a method of choice for a wide range of applications (Garcia-Pino et al. 2007; Vieira et al. 2008; Freire 2009; Kulshina et al. 2009; Tzeng and Kalodimos 2009). During addition of ligand, it reacts with the macromolecule resulting in the absorption or release of heat, which can be measured by the microcalorimetry instrument. The produced heat is directly proportional to the fraction of bound ligand as well as to the degree of interaction between the interacting populations. The enthalpy change (ΔH) is the total heat produced or absorbed during titrations. Measurement of heat changes allow for accurate determination of the binding constant and a complete thermodynamic profile of the ligand-macromolecule interaction.

With the exception of thymidine diphosphate, the pyrimidine nucleotides showed lower binding affinity and less negative enthalpy compared with purines. Interestingly, the enthalpy-entropy compensation mechanism is not observed with pyrimidines. In this context, the binding affinity of ligands can be improved by overcoming the enthalpy-entropy compensations. Furthermore, strong enthalpy-entropy compensation indicates a higher magnitude of conformational changes, which is required for correct placement of active site residues facing the ligand. Thus, we expect that the binding of uridine and cytidine nucleotides is accompanied by freezing of the interacting partner accompanied by increased hydrophobic forces, lower conformational changes and less favorable enthalpy due to lower hydrogen bond formation.

The binding of pyrimidines (except TDP) is predicted to be entropically driven indicating a specialized reorder of water molecules around the binding pocket solvating the charged groups. The entropy is a measure of reacting system disorder in which the higher enthalpy indicates correct placement hydrogen bonding formation and decrease of system disorders. In this context, the binding of the less bulky pyrimidines is accompanied by more active site disorder indicated by the lower enthalpic term.

During hydrophobic interactions, the non-polar groups associates with each other missing contact with solvent.

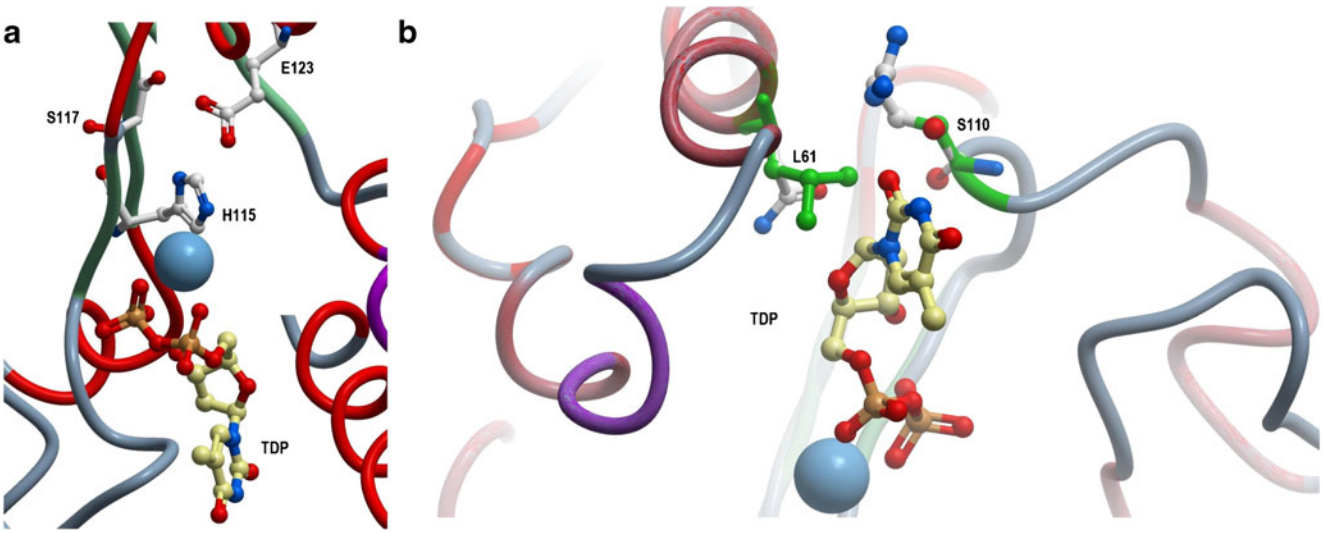

Fig. 4 Modeling of the active site of PfNDK showing the complex of the wild type enzyme with thymidine diphosphate. The model was created using ProtMod protein modeling server and visualized by Molsoft software. The template of modeling was based on the coordinates of PDB 3EVO. PfNDK residues are displayed in *green*

The predominance of hydrophobic interactions during macromolecule-ligand recognition is usually associated with small enthalpy changes and large positive (more favorable) entropy. The thermodynamic profiles of the binding of pyrimidines indicate that the hydrophobic forces are the major force governing their binding.

The binding of purine nucleotides was found to be in the low micromolar range with K_d of 6.2–18 μM. In addition, PfNDK showed preferential affinity to dTDP over all other pyrimidines. We predict that this finding concedes well with the high metabolic stress of the requirements for adenosine and thymidine nucleotides, which constitutes more than 80% of the plasmodium genome.

Enthalpic contribution ΔH reflects the strength of compound-target interactions, mainly due to hydrogen bond and van der Waals interaction. The greater exothermicity of purines binding over pyrimidines indicates more hydrogen bonding formations. The high binding enthalpy of all purines and thymidine nucleotides is indicative of strong electrostatic interaction with the binding pocket. The binding of pyrimidines is proposed to be less favorable than that of purines due to the overall less negative free energy change.

For purines, the binding to PfNDK is characterized by favorable enthalpic and entropic terms. Interestingly, both forces are thought to be equally contributing to the binding affinity. Therefore, the electrostatic forces combined with hydrophobic interactions contribute equally in purines recognition.

An interesting feature is that the specificity of PfNDK was not affected by the functional properties of the bound nucleotide. Thus, the presence or absence 2`OH of ribose was a little critical for determining the binding affinity. In conclusion, the size of nucleotide, rather than its functional properties, is the critical factor in ligand affinity.

At first, one could predict that the interaction of nucleotides with PfNDK especially with pyrimidine nucleotides is governed by hydrophobic forces (positive entropy). This suggests the presence of buried hydrophobic surfaces or release of water molecules upon ligand binding. In addition, examining the ligand binding cavities from several NDKs indicates the presence of a large hydrophobic interface. The binding pocket of hNM23H2 is almost formed of V116, L68, F64 (highly hydrophobic residues), H59, T98 (less hydrophobic residues) and R92, R109, K16 (partly hydrophobic). However, despite the large hydrophobic interface in which all nucleotides bind, some hydrogen bonds are crucial and specific for nucleotides bindings.

The low ΔH for pyrimidine nucleotides indicates reduction in the polar contacts with the enzyme. In spite of the high structural similarities between uridine, cytidine and thymidine nucleotides, the thymidine nucleotides thermodynamic profile was extremely different. The drastic increase in ΔH for TDP indicates important electroststic interactions with the ligand on the surface of the enzyme. Based on this feature, we could estimate that further interactions including sites other than the binding site can share the binding with thymidines. Indeed, if we realize the other functions of NDKs, the PfNDK can bind a thymidine rich region in plasmodium DNA. It is also worse mentioning that most of protein-DNA binding interactions are through electrostatic forces.

The direct titration of nucleotide triphosphates into NDKs cannot be measured by ITC because phosphorylation of the enzyme takes place followed by the formation of nucleotide diphosphates. Accordingly, the affinity of NDPs should be similar to that of the phosphorylated enzyme and that of NTPs to that of the free enzyme. To further elucidate the binding profiles of NTPs we titrated PfNDK with the nonhydrolysable ATP analogue β,γ-Imidoadenosine 5′-triphosphate (AMP-PNP). However, the enthalpy of binding was as low as −0.4 Kcal/mol. This may be due to the incorrect binding of AMP-PNP with the active site or the unusual bonds of AMP-PNP that support an unfavorable conformation for nucleotides binding.

The nucleoside diphosphate kinase structure was extensively studied in numerous microorganisms and fine details of enzyme activity, mechanism of phosphate transfer and the binding of various substrates was comprehensively investigated. In order to explain the reasons for the discrimination of PfNDK for different substrates we carried out the profile-profile alignment and fold recognition algorithm using one of the extensively studied structures of NDK (Mimivirus NDK). Models of NDK structures were created using all available structures of mimivirus NDK with various substrates. By modeling of the PfNDK structure, we can deduce the causes beyond the poor affinity for pyrimidine nucleotides. The affinity of mimivirus NDK for pyrimidine nucleotides is attributable to R107 and N62, which makes favorable interaction with the pyrimidine base and the oxygen (O_2) of the pyrimidine ring. In contrast, PfNDK contains S110 and L61 at these respective sites (Fig. 4b). We assume that this replacement provides unfavorable conditions for the binding of pyrimidine nucleotides with PfNDK. Interestingly, mutation of mimivirus residues to those similar to those found in the PfNDK model showed loss of specificity for pyrimidine nucleotides. The deoxypyrimidine nucleotides were barely visible in the structure and the nucleotides were less deeply buried in the active site than the native enzyme. These findings confirm our assumption for the poor specificity of PfNDK for pyrimidine nucleotides.

The absence of substrate specificity of cellular NDKs is a regular assumption of investigators based on the structural and biological roles of the enzyme. However, we provided in this report a model describing the specificity of PfNDK

for nucleotides, as the enzyme has poor affinity for CDP, UDP and dCDP. Similar finding was given by Jeudy et al. 2009 who found that mimivirus NDK has preferential affinity for deoxypyrimidine nucleotides.

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