Bioorganic & Medicinal Chemistry 20 (2012) 6758–6769

Contents lists available at SciVerse ScienceDirect

Bioorganic & Medicinal Chemistry

journal homepage: www.elsevier.com/locate/bmc

Trimeric purine nucleoside phosphorylase: Exploring postulated one-third-of-the-sites binding in the transition state

Beata Wielgus-Kutrowska [a], Katarzyna Breer [a], Mariko Hashimoto [b], Sadao Hikishima [b], Tsutomu Yokomatsu [b], Marta Narczyk [a], Alicja Dyzma [a], Agnieszka Girstun [c], Krzysztof Staroń [c], Agnieszka Bzowska [a,*]

[a] Division of Biophysics, Institute of Experimental Physics, University of Warsaw, Żwirki i Wigury 93, 02-089 Warsaw, Poland
[b] School of Pharmacy, Tokyo University of Pharmacy and Life Sciences, 1432-1 Horinouchi, Hachioji, Tokyo 192-0392, Japan
[c] Department of Molecular Biology, Institute of Biochemistry, University of Warsaw, Miecznikowa 1, 02-096 Warsaw, Poland

ARTICLE INFO

Article history:
Received 13 August 2012
Accepted 24 August 2012
Available online 17 September 2012

Keywords:
One-third-of-the-sites
Enzyme catalysis
Enzyme inhibitors
Homooligomeric proteins
ITC
Negative cooperativity
Transition state
Spectroscopic methods

ABSTRACT

Transition-state analogue inhibitors, immucillins, were reported to bind to trimeric purine nucleoside phosphorylase (PNP) with the stoichiometry of one molecule per enzyme trimer [Miles, R. W.; Tyler, P. C.; Furneaux, R. H.; Bagdassarian, C. K.; Schramm, V. L. *Biochem*. **1998**, *37*, 8615]. In attempts to observe and better understand the nature of this phenomenon we have conducted calorimetric titrations of the recombinant calf PNP complexed with immucillin H. However, by striking contrast to the earlier reports, we have not observed negative cooperativity and we got the stoichiometry of three immucillin molecules per enzyme trimer. Similar results were obtained from fluorimetric titrations, and for other inhibitors bearing features of the transition state. However, we observed apparent cooperativity between enzyme subunits and apparent lower stoichiometry when we used the recombinant enzyme not fully purified from hypoxanthine, which is moped from *Escherichia coli* cells. Results presented here prove that one-third-of-the-sites binding does not occur for trimeric PNP, and give the highly probable explanation why previous experiments were interpreted in terms of this phenomenon.

1. Introduction

Mammalian purine nucleoside phosphorylase (PNP, E.C. 2.4.2.1) is a homotrimeric enzyme interesting as a drug target and as a model protein to study role of subunit interactions in catalysis. The lack of PNP activity in humans leads to selective immunodeficiency caused by the incorrect T-cell proliferation.[1,2] Therefore its inhibitors are in the centre of interest for their potential applications as selective immunosuppressive drugs and anticancer agents. For medicinal purposes, inhibitors need to be characterized by a dissociation constant (K_d) of 10 nM or lower,[2] which corresponds to the binding energy of 11 kcal/mol at 25 °C (at pseudo-standard concentrations of 1 mol/L). Up to now most of candidate inhibitors belonged to the negatively charged multisubstrate-analogues (phosphates and phosphonates), not able to effectively penetrate the cell membrane. Only transition-state analogues, immucillins (see Scheme 1), share both required features, that is are very potent and not charged at pH 7, and are therefore already in clinical trials.[3–5]

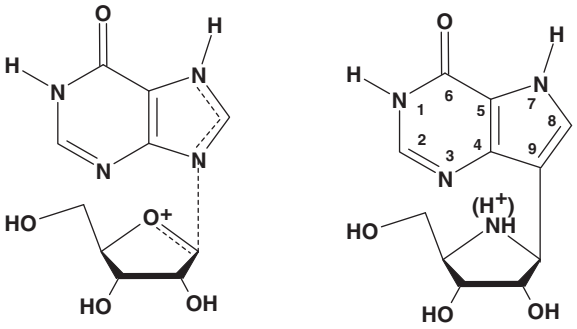
Immucillins were not only shown to be extremely strong inhibitors (with equilibrium dissociation constants in the pM range) but also postulated to cause complete inhibition of the homotrimeric enzyme at one mole of inhibitor per mole of enzyme trimer. This phenomenon was named one-third-of-the-sites binding.[6] However, although efforts were made, the postulated asymmetry in the structure and function of enzyme subunits was so far not confirmed. The crystal structure of PNP complexed with immucillin H or other ligands with some features of the transition state yielded either a molecule with the crystallographic 3-fold symmetry;[7] or a molecule with no structural differences between monomers in the trimer.[5,8]

Here we present detailed studies aimed to verify the one-third-of-the-sites binding complex of PNP with immucillin H with the help of isothermal titration calorimetry, fluorescence titrations

Abbreviations: DFPP-DG, 9-(5′,5′-difluoro-5′-phosphonopentyl)-9-deazaguanine; aza-DFPP-DG, 9-{[*N*-[3′′,3′′-difluoro-3′′-(diethylphosphono)propyl]amino]methyl}-9-deazaguanine; DFPP-G, 5′,5′-difluoro-5′-phosphonopentyl)-9-guanine; (*S*)-PMP-DAP, 2,6-diamino-(*S*)-9-[2-(phosphonomethoxy)-propyl]purine; (*S*)-PMP-Ada, 6-amino-(*S*)-9-[2-(phosphonomethoxy)propyl]purine; (DFTHPEP-H), (±)-*cis*-1,1-difluoro-2-(tetrahydro-3-piranozyl)ethylphosphonic acid with (hypoxanthine-9-yl)methyl aglycone; ImmH, immucillin H; Ino, inosine; m^{7}Guo, 7-methylguanosine; Hx, hypoxanthine; Gua, guanine; 9-deaza-Gua, 9-deazaguanine; PNP, purine nucleoside phosphorylase; K_d, dissociation constant; K_i^{app}, apparent inhibition constant; K_i^{eq}, inhibition constant in equilibrium; ITC, isothermal titration calorimetry.

* Corresponding author. Tel.: +48 22 554 0789; fax: +48 22 554 0771.
E-mail address: abzowska@biogeo.uw.edu.pl (A. Bzowska).

0968-0896/$ - see front matter © 2012 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.bmc.2012.08.045



Scheme 1. Inosine in the transition state of the phosphorolytic reaction catalysed by PNP (left) and immucillin H (right), transition-state analogue inhibitor with two features of the transition state: proton at purine position N7 and positive charge localized in the part of the ligand that occupies the pentose binding site and imitates the ribooxocarbenium ion of the natural substrate in the transition state. Immucillin has p*K* for protonation of the iminoribitol nitrogen around 7.0, so it may pass through the cell membrane as the non-charged molecule and may be later inside the cell protonated (as shown in this scheme) to give the cation strongly interacting with PNP.

and mutants engineered in order to obtain high accuracy fluorescence data. Since our results did not confirm the previous observations of the one-third-of-the-sites binding of immucillin H to PNP, we have synthesized other inhibitors with one or two of the transitions-state features, and subjected them as well to calorimetric and fluorimetric analysis. Our data prove that the one-third-of-the-sites binding does not occur for trimeric PNPs. We also provide plausible reasons why this phenomenon was postulated to interpret earlier results.[6,9,10]

2. Results and discussion

2.1. Synthesis of the new ligand with transition-state features

One-third-of-the-sites binding was postulated to occur only in the transition state of the trimeric PNP and the transition state was characterized by two features, namely protonated N7 position and ribooxocarbenium ion (Scheme 1). We have previously described the synthesis of DFPP-DG, (9-(5',5'-difluoro-5'-phosphonopentyl)-9-deazaguanine) with one feature of the transition state, that is with proton at purine position N7,[11] (Table 1 and Scheme 2). Here we report a new analogue 9-{[*N*-[3″,3″-difluoro-3″-(diethylphosphono)propyl]amino]methyl}-9-deazaguanine (inhibitor **6**, aza-DFPP-DG; Scheme 2 and 3) with two features of the transition state, that is also with the positive charge localized in the part of the ligand that occupies the ribose binding site and imitates the ribooxocarbenium ion. The inhibitor **6** was prepared from 9-iodo-9-deazaguanine derivative **1**, as shown in Scheme 3.[12] The palladium-catalyzed CO insertion of the compound **1** and subsequent quenching of the organometallic intermediate with tributyltin hydride gave 9-formyl-9-deazaguanine derivative **2** with 79% yield.[13] The 9-formyl derivative **2** was condensed with 4-amino-2,2-difluorophosphonate **4**. Compound **4** was prepared in situ from the corresponding azide **3** through catalytic hydrogenation over Pd-C in MeOH, by a reductive amination under the Woolhouse conditions.[14] This procedure gave a protective derivative of aza-DFPP-DG **5** with 62% yield. The removal of the ethyl protecting group and the dimethylaminomethylene group from **5** was achieved in concd HCl at reflux (16 h) to give aza-DFPP-DG (**6**) as a white solid with 70% yield.

2.2. Inhibitor properties of compounds with and without features of the transition state

Scheme 2 depicts the structures of all PNP inhibitors, with and without transition-state features, that were used in our studies.[15–19] Inhibitory properties of the ligands[20–23] are compared with those reported for immucillin H[6] (Table 1). Three analogues, DFPP-G, DFPP-DG and aza-DFPP-DG are, like immucillins, strong binding inhibitors with equilibrium inhibition constants, K_i^{eq} in the nanomolar range (0.72 ± 0.13 nM for DFPP-G, 0.085 ± 0.013 nM for DFPP-DG and 6.8 ± 0.4 nM for aza-DFPP-DG; Table 1). DFPP-DG with one feature of the transition state is the best inhibitor, and binds PNP with affinity comparable to immucillins H (equilibrium inhibition constants of 0.023 nM).[6] The above data show clear analogies between immucillins and DFPP-G, DFPP-DG and aza-DFPP-DG, and justify using them as reference compounds to study interactions of PNP with strongly binding ligands.

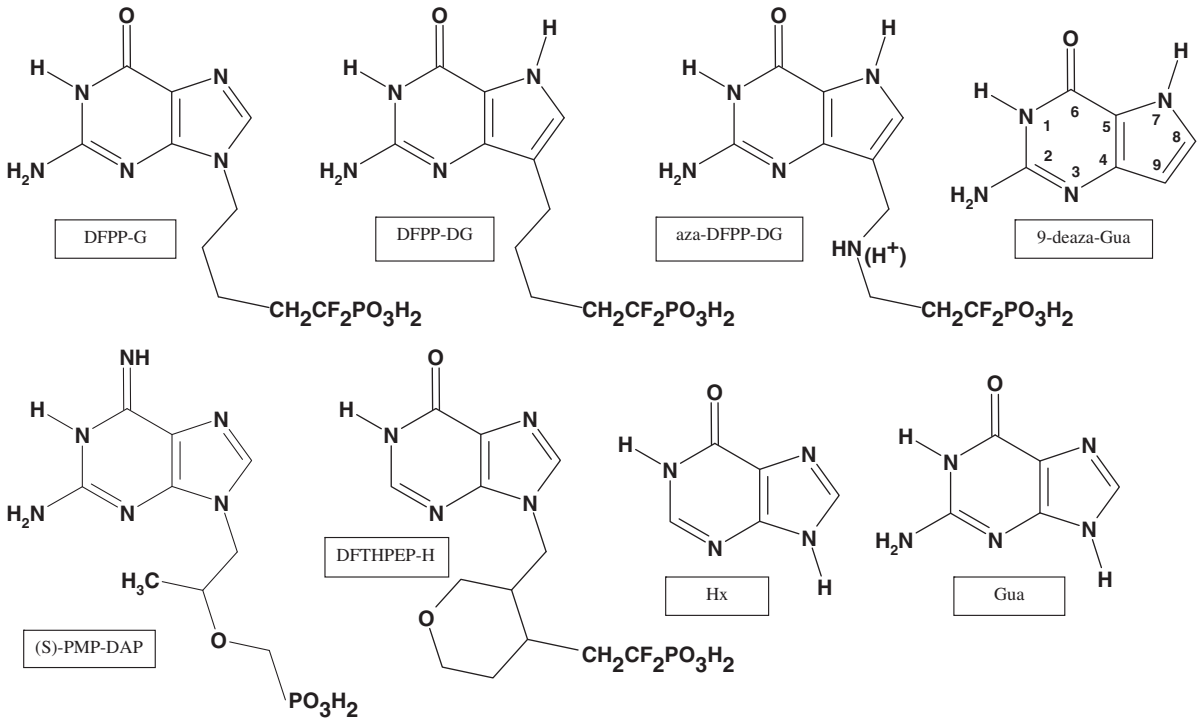
2.3. Conditions for postulated one-third-of-the-sites binding—calorimetric studies

One-third-of-the-sites binding of immucillin H was observed previously with the use of the ITC method.[10] In fact the phenomenon should be rather described as a very strong negative cooperativity since the apparent dissociation constants reported for the three binding sites of trimeric PNP and immucillin H were 23 pM, <5 mM and ~100 mM (calf spleen enzyme),[24] and later

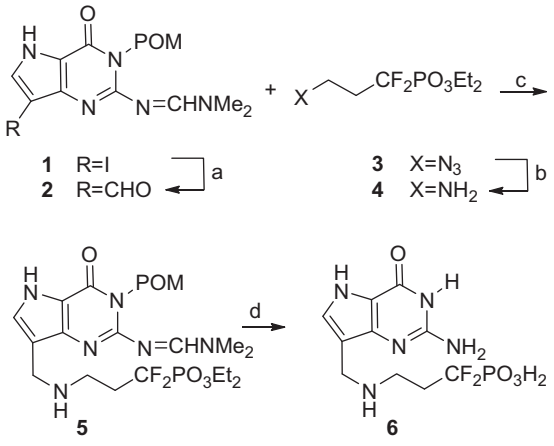
Table 1
Inhibitory properties vs calf PNP of compounds bearing one feature of the enzyme transition state (proton at position N7), two features (additionally positive charge on the pentose ring or its imitation) and with no such features. If not otherwise indicated data were obtained in 1 mM phosphate and 50 mM Hepes buffers pH 7.0 at 25 °C. BS is an abbreviation for the binding site

Compound		Features of the transition state		K_i^{eq} [nM]
Immucillin H	Two	N7-H	Positive charge in BS of pentose	0.023 ± 0.005[a]
aza-DFPP-DG	Two	N7-H	Positive charge in BS of pentose	6.8 ± 0.4
DFPP-DG	One	N7-H	—	0.085 ± 0.013[b]
DFPP-G	None	—	—	0.72 ± 0.13[b]
DFTHPEP-H	None	—	—	63 ± 16[c]
(*S*)-PMP-DAP	None	—	—	~6000[d]
Hx	One[e]	N7-H	—	2500[f]
Gua	One[e]	N7-H	—	4000[g]
9-deaza-Gua	One	N7-H	—	~7400[h]

[a] From,[6] in 50 mM phosphate, pH 7.7.
[b] From.[23]
[c] From.[21]
[d] From.[20]
[e] Only one tautomer, N7-H, has the feature of the transition state
[f] From,[35] in 50 mM phosphate
[g] From,[42] in 50 mM phosphate
[h] In 50 mM phosphate



Scheme 2. Structures of the PNP ligands used. Note the purine ring numbering, shown here for 9-deazaguanine, to point attention to proton at position N7 of the purine ring, one feature of the transition state. This feature is also present in the structure of DFPP-DG and aza-DFPP-DG. Guanine and hypoxanthine are mixture of N9-H (shown here) and N7-H tautomers (not shown). The second feature of the transition state, that is positive charge in the part of the ligand imitating the ribooxocarbenium ion of the substrate, is present only in the structure of aza-DFPP-DG.



Scheme 3. Preparation of aza-DFPP-DG. Conditions: (a) CO, $Pd_2(dba)_3$, PPh_3, Bu_3SnH, DMF, 60 °C; (b) H_2, Pd-C, MeOH; (c) $NaBH(OAc)_3$, AcOH, DCE; (d) concd HCl, reflux.

roughly estimated (since no model was found to describe the data properly) for the human recombinant enzyme to be 56 pM, 16 nM and 15 μM.[10]

Our calorimetric studies were done with immucillin H, aza-DFPP-DG, DFPP-DG, DFPP-G, (*S*)-PMP-DAP, hypoxanthine, guanine and 9-deazaguanine and recombinant PNP. Hypoxanthine and guanine were included in the study due to the controversy about their binding mode in the earlier reports. Hypoxanthine, but not guanine, were first published to be characterized by one-third -of-the-sites binding,[9] while a recent publication from the same group reports the single-site binding model also for hypoxanthine.[10] We have additionally included 9-deazaguanine, which does bear one feature of the transition state, by contrast with Hx and Gua, which have one feature of the transition state only in the N7-H tautomeric form (Scheme 2). As the published experiments for hypoxanthine were done in phosphate free buffer we have performed the experiments both in the phosphate-free media, and in the presence of 0.4–50 mM phosphate. Since one-third-of-the-sites binding phenomenon was previously detected for immucillin H and the non-recombinant PNP obtained from Sigma we have also performed the experiments either with immucillin H or with aza-DFPP-DG and the non-recombinant Sigma enzyme preparation. In all cases, in presence of phosphate and in the phosphate-free media, we determined the stoichiometry of three ligand molecules per enzyme trimer (Fig. 1 and Table 2). Neither the negative cooperativity nor the one-third-of-the-sites binding could have been observed.

2.4. Recombinant PNP with 'interacting binding sites'?

How can it then be explained that some experiments[6,9,10] suggested one-third-of-the-sites binding? As described in detail in Materials and Methods, the recombinant PNP used in the experiments reported here was not only purified on the ion exchange and gel-filtration columns but also dialyzed at 0.1 mg/ml (equal to a very low, 3 μM concentration of enzyme monomers), against 20 mM phosphate buffer. The last, rather unusual purification stage was applied to remove the hypoxanthine moped by the recombinant PNP from *Escherichia coli* cells, as a result of its extremely high overexpression.[25] When concentration of active sites is higher than dissociation constant almost all ligand molecules are bound. Since hypoxanthine binds with dissociation constant of ~1 μM (Table 2), dialysis is not effective at enzyme concentration substantially higher than ~1 μM. Phosphate buffer was used in dialysis because it was shown to improve the release of hypoxanthine.[26] If the non-dialyzed recombinant enzyme was used in further studies, or dialysis was conducted at a high enzyme concentration, hypoxanthine

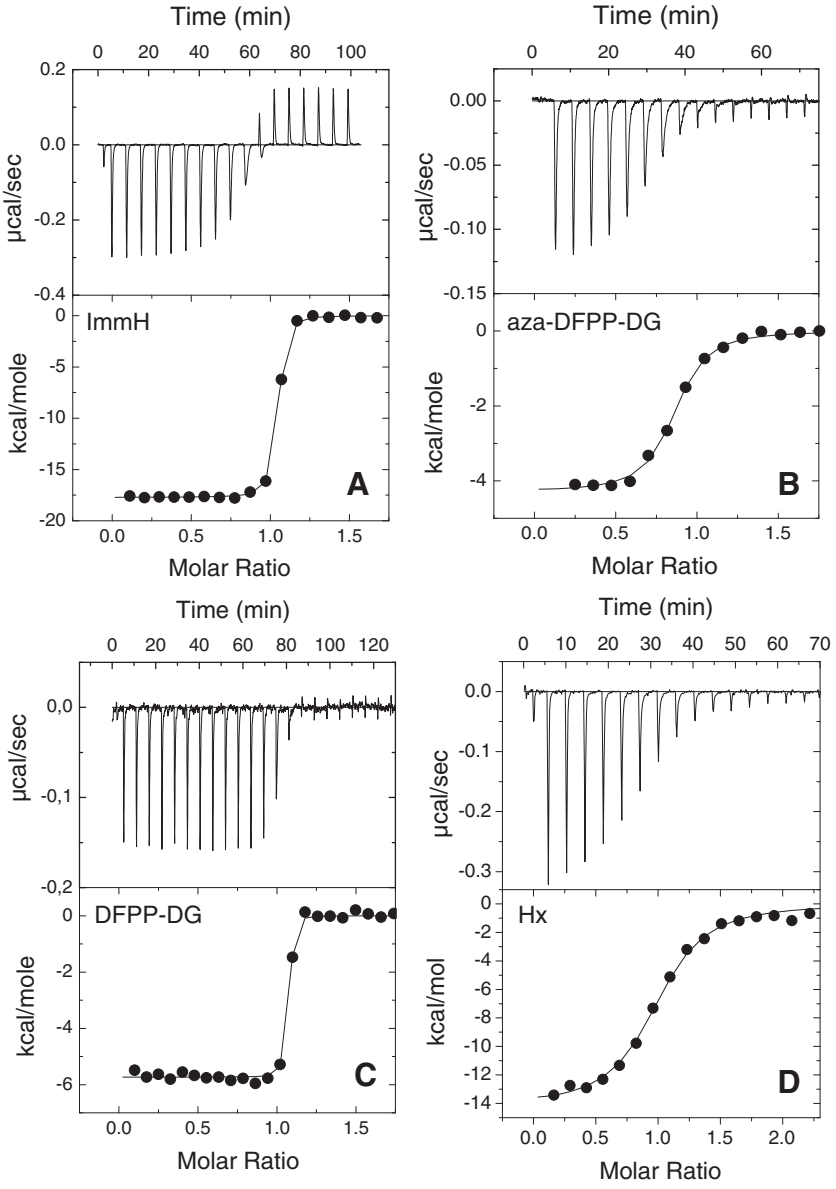


Figure 1. Isothermal calorimetric titrations in 20 mM Hepes buffer pH 7.0 (7.6 for ImmH), 25 °C of the recombinant calf PNP using ligands with one or two features of the transition state. (A) ImmH and 14.2 μM PNP (as a monomer) in the presence of 20 mM phosphate buffer (the positive peaks at the end of the titration are caused by a buffer dilution), (B) aza-DFPP-DG, 7.5 μM PNP, (C) DFPP-DG, 6.5 μM PNP (D) Hx, 31.3 μM PNP. The heat production per injection and the enthalpy per mole of injectant, measured as a function of the molar ratio of ligand versus PNP, are shown on the upper and lower part of each panel, respectively. Equation assuming one binding site model was fitted for all titrations.

was still present in the sample (around half of the sites were occupied) and the binding data are substantially biased. For very strong ligands, that may easily remove hypoxanthine from the active site, like DFPP-DG or immucillin H, either two binding sites or interacting binding sites model seems to be correct (Fig. 2, upper panels). However, when similar experiments are carried out for the hypoxanthine-free enzyme preparation, one binding site model correctly describes the experimental data, and stoichiometry of one ligand molecule per enzyme monomer is observed (Fig. 2, lower panels).

Binding of hypoxanthine is accompanied by a large negative change of the free enthalpy: $\Delta H = -14.7$ kcal/mol (Table 2). Therefore the replacement of this compound by other ligands with lower binding enthalpy, such as DFPP-DG ($\Delta H = -8.3$ kcal/mol, Table 2) yields positive heat signals, easier to detect and recognize (see Fig. 2, panel A). By contrast, binding of immucillin H generates even more heat than binding of hypoxanthine ($\Delta H = -20.3$ kcal/mol, Table 2). So replacement of Hx by ImmH is still exothermic, and only the shape of the titration curve is modified (Fig. 2, panel B), leading to the incorrect conclusion that binding cannot be described by the one binding site model.

When the ezyme preparation containing hypoxanthine was used in ITC titrations with ligands binding to PNP weaker than hypoxanthine one binding site model seemed to be correct but we obtained the stoichiometry lower than one ligand per enzyme monomer. This observation has a simple explanation. In the molar ratio typically used in the ITC studies the weak ligand is not able to remove hypoxanthine already bound to PNP but only binds to the empty, hypoxanthine-free sites. We have shown this effect for 9-deazaguanine to prove the above conclusion.[25] But before we discovered the problem of non-hypoxanthine-free PNP preparations we have ourselves also observed and reported lower stoichiometry for binding of hypoxanthine,[27] which we now know was most

Table 2
Binding and thermodynamic parameters obtained for calf PNP and ligands bearing none, one or two features of the transition state

Ligand	Method	Stoichiometry	K_d [M]	ΔG [kcal/mol]	ΔH [kcal/mol]	$T\Delta S$ [kcal/mol]
Hx	F[a]	0.98 ± 0.13	$12.5 \pm 2.1 \times 10^{-7}$	−8.0 ± 0.7	–	–
	ITC	1.07 ± 0.12	$6.8 \pm 1.2 \times 10^{-7}$	−8.3 ± 0.1	−14.7 ± 1.2[b]	6.4 ± 1.2
Gua	F	0.93 ± 0.10	$1.6 \pm 0.1 \times 10^{-7}$	9.3 ± 0.6	–	–
	ITC	1.02 ± 0.14	$1.2 \pm 0.1 \times 10^{-7}$	−9.4 ± 0.2	−16.2 ± 0.3	6.8 ± 0.3
9-deaza-Gua	F[c]	0.96 ± 0.10	$1.7 \pm 0.1 \times 10^{-7}$	9.2 ± 0.6	–	–
	ITC	0.88 ± 0.17	$1.6 \pm 0.1 \times 10^{-7}$	−9.3 ± 0.1	−11.7 ± 0.6	2.4 ± 0.6
DFPP-G[d]	ITC/F	1.13 ± 0.07	$4.6 \pm 1.1 \times 10^{-10}$	−12.7 ± 0.1	−1.6 ± 0.1	11.1 ± 0.2
DFPP-DG[e]	ITC/F	1.18 ± 0.13	$1.0 \pm 0.3 \times 10^{-10}$	−13.6 ± 0.2	−8.3 ± 0.2	5.3 ± 0.3
Aza-DFPP-DG	ITC/F	0.89 ± 0.11	$3.6 \pm 0.5 \times 10^{-9}$	−11.5 ± 0.1	−4.3 ± 0.1	7.2 ± 0.2
ImmH[f]	ITC	1.02 ± 0.12	$2.3 \pm 0.5 \times 10^{-11}$[g]	−14.5 ± 0.2	−20.3 ± 0.9	5.8 ± 0.9
	F[a,i]	0.98 ± 0.12	–	–	–	–
	F[h,i]	1.04 ± 0.18	–	–	–	–

Data were obtained from isothermal calorimetric titrations (ITC) and from fluorimetric titrations (F) using either standard approach or the competitive binding of weak and strong ligands (see Materials and Methods), or from combination of calorimetric and fluorimetric methods: K_d and ΔG from fluorimetric titrations, ΔH from calorimetric titrations (and $-T\Delta S$ determined as ΔG-ΔH). If not otherwise indicated experiments were done at 25 °C in 20 mM Hepes buffer pH 7.0.

[a] For Phe200Trp mutant.

[b] $\Delta H = -30.5$ kcal/mol was reported for the human PNP by Edwards et al.,[10] which differs markedly from −14.7 ± 1.2 kcal/mol determined here for calf PNP. However, Edwards et al.[10] obtained this value by fitting the binding curve for the ITC experiment yielding no low plateau region (see left panel of Fig. 7 in Edwards et al.),[10] hence this result is not very exact. The highest experimentally observed ΔH by Edwards et al.[10] is about −18.5 kcal/mol (Fig. 7, left panel),[10] while we for calf PNP observed the maximum value of −16.5 kcal/mol (from single experiment), so these data are in a fairly good agreement.

[c] From.[33]

[d] Dissociation constant and corresponding free energy change are from competitive binding approach titrations using guanine as a weak inhibitors and fluorimetric detection.

[e] Dissociation constant and corresponding free energy change are from competitive binding approach titrations using (*S*)-PMP-Ada as a weak inhibitors and fluorimetric detection.

[f] pH 7.7, 50 mM phosphate buffer.

[g] From,[6] dissociation constant was assumed to be equal to the inhibition constant in equilibrium.

[h] For Phe159Trp mutant.

[i] Dissociation constant enzyme concentration was too small to determine K_d accurately, but the goal of the experiment was to determine accurately binding stoichiometry.

probably due to the incorrect enzyme preparation used in our previous studies. In line with this conclusions Edwards et al. in their recent paper[10] reported that binding of hypoxanthine fits to an independent binding sites model but indicates a total binding stoichiometry of approximately two sites per trimer. This seems easy to explain if procedure that was used to remove hypoxanthine from the recombinant human PNP was not fully effective and some active sites were filled with hypoxanthine before titration has been started.

Here it should be recalled that Edwards et al.[10] were aware that PNP prepared by them contained 2 moles of hypoxanthine per mole of trimeric enzyme molecule, and conducted removal of hypoxanthine by incubation of the enzyme in 100 mM KH_2PO_4 containing 10% (w/v) charcoal for 5 min followed by centrifugation and filtration. However, it was not shown that this removal was fully successful. There are only two good ways we know up to now to show beyond the reasonable doubt that the enzyme is hypoxanthine-free. It is either to conduct an appropriate ITC titrations or, what we did in our previous study,[25] to denature the enzyme and check, for example by taking UV spectra, if the hypoxanthine was no released. By the appropriate ITC titrations we mean for example a titration with hypoxanthine yielding one hypoxanthine molecule bound per enzyme monomer, or a titration with a strong binding ligand with the enthalpy of binding different than enthalpy associated with binding of hypoxanthine. In the latter situation no deviation from one binding site model should be observed.

2.5. Hypoxanthine in the Sigma PNP?

The non-recombinant calf PNP is not available from Sigma anymore. In the non-recombinant Sigma PNP preparation, which we still have in our lab, we were not able to detect hypoxanthine. However, when we carefully examined UV spectra of the old samples that were used in our earlier ITC studies conducted in 1999 and 2000,[27] in some cases the 250 nm/280 nm absorbance ratio seems to be slightly elevated. We observed 250/280 ratio of 0.44, 0.47 and even 0.49, while for the hypoxanthine-free preparation the experimental value is 0.33, in agreement with the theoretically calculated value of 0.34, based on the absorbance of 3 tryptophan and 9 tyrosine residues present in the sequence of calf PNP.[28] This result might be the indication that hypoxanthine was present in some Sigma preparations used before. In agreement with this observation we have reported lower than one molecule per enzyme monomer stoichiometry for binding of Hx obtained when these enzyme preparations were used.[27] In agreement with this conclusion, stoichiometry for phosphate was found to be one molecule per monomer, again easy to understand since phosphate binds at a distinct site than Hx, and the ternary complex PNP/Hx/phosphate may be formed even if Hx is present.

2.6. PNP mutants exhibiting substantial fluorescent quench upon ligand binding

In the next step we decided to prove our unexpected ITC results by other experimental methods. Since PNP fluorescence is not very sensitive to binding of immucillin H and hypoxanthine, ligands for which one-third-of-the-sites binding was postulated, we have engineered mutants of the calf enzyme that respond strongly to binding of these ligands. Two mutants with tryptophan residues substituting Phe159 or Phe200 were prepared. Both of them showed near-normal kinetic properties, that is catalytic parameters and inhibition constants vs typical PNP substrates and inhibitors (Table 3).

Titrations of the Phe200Trp mutant with (*S*)-PMP-DAP is shown in Fig. 3, left panels. One binding site model is correct, dissociation constant obtained is 0.147 ± 0.012 μM, and stoichiometry is three ligand molecules per enzyme trimer. Thus Phe200Trp mutant binds (*S*)-PMP-DAP with the stoichiometry and dissociation constant similar to that previously reported for the wild type enzyme.[29] This mutant was used in the next experiments to confirm one to one binding stoichiometry for other ligands like hypoxanthine and ImmH.

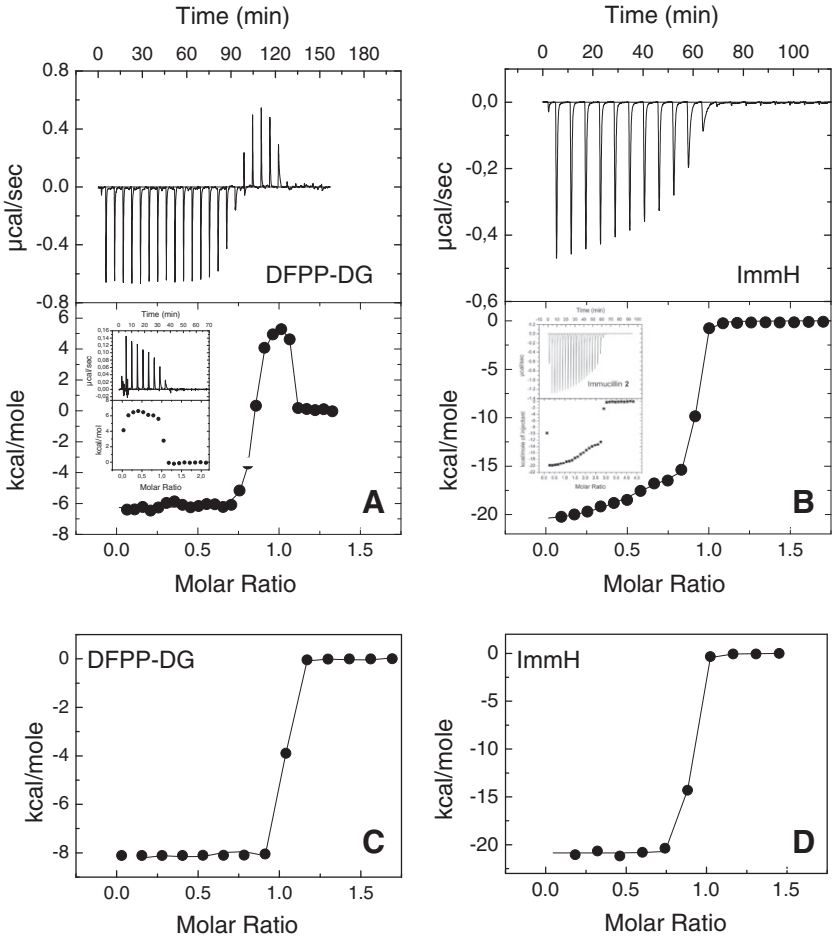


Figure 2. Isothermal calorimetric titrations, it means the enthalpy per mole of injectant, measured as a function of the molar ratio of ligand versus PNP (as a monomer) of the recombinant calf spleen PNP (A and B) purified using ion-exchange and site-exclusion chromatography (some active sites are filled with hypoxanthine), with DFPP-DG (panel A) in 20 mM Hepes buffer pH 7.0 at 20 °C and with ImmH (panel B) in 20 mM phosphate buffer pH 7.7, at 25 °C; and the non-recombinant calf PNP free from hypoxanthine, with DFPP-DG (panel C) in 20 mM Hepes buffer pH 7.0 at 25 °C and with ImmH (panel D) in 20 mM phosphate buffer pH 7.7, at 25 °C. In panels A the original data of the heat production per injection shows unusual endothermic and exothermic behaviour in the case of DFPP-DG binding to the PNP sample partially filled with Hx. Insert in panel A shows titration with DFPP-DG of the PNP sample fully filled with Hx (sample from the titration shown in panel D on Fig. 1). Please note also the similar shape of the curve from panel B with that shown in Figure 6 from Edwards et al.[10] (insert on panel B). Equation assuming two binding sites model was fitted to the titrations presented in (A) and (B), and one binding site model was fitted to the titrations shown in (C) and (D).

Table 3
Summary of kinetic and binding properties of the four Trp PNP mutants. Substrate properties were determined at 50 mM phosphate pH 7.0, 25 °C, and binding properties were determined at 25 °C in 50 mM phosphate pH 7.7 in the case of guanine, and 1 mM phosphate pH 7.0 for other inhibitors, temperature was 25 °C. K_i^{app} [nM] is an apparent inhibition constant observed by classical initial velocity method: since DFPP-DG is a slow binding inhibitor[23] this constant is higher than observed in equilibrium (Table 1)

Kinetic parameter	Wild type PNP	Phe159Trp	Phe200Trp
Inosine			
K_m [µM]	13.4 ± 0.9[b]	245 ± 12	326 ± 30
V_{max} [U/mg]	30.7 ± 0.5[b]	20.0 ± 0.3	19.2 ± 0.6
7-methylguanosine			
K_m [µM]	16.8 ± 1.2[b]	50 ± 4	70 ± 5
V_{max} [U/mg]	75.8 ± 3.5[b]	7.8 ± 0.3	13.7 ± 0.8
DFPP-DG (1 mM phosphate)			
K_i^{app} [nM]	6.9 ± 0.7	7.9 ± 0.5	16.1 ± 0.7
DFTHPEP-H (1 mM phosphate)			
K_i^{app} [nM]	63 ± 16	29.8 ± 1.8	42.7 ± 2.8
Gua (50 mM phosphate)			
K_d [µM]	0.9 ± 0.2	3.3 ± 1.5	8.2 ± 0.9

[b] Data from.[31]

2.7. One per monomer binding stoichiometry revealed by fluorimetric titrations

The ITC experiments were conducted at relatively high enzyme concentration since initially the goal was to find good conditions for crystallization of the PNP/immucillin H complex. To get results concerning stoichiometry of binding and dissociation constants at concentrations comparable to the previous studies reporting one-third-of-the sites binding[9] we repeated titrations with DFPP-G, DFPP-DG, aza-DFPP-DG, immucillin H, guanine, 9-deazaguanine and hypoxanthine using fluorimetric detection. This method is more sensitive than isothermal titration calorimetry, and thus much lower enzyme concentrations may be used. For the ligands that cause only a very small change in fluorescence signal of the wild type enzyme (Hx and immucillin H), Phe200Trp and Phe159Trp mutants were used. In addition in some cases, the simultaneous fitting to several titration traces obtained for various enzyme concentrations was performed as shown for hypoxanthine (Fig. 3, right panels), since this approach makes conclusions regarding binding model more reliable.[30] For very potent inhibitors, for which the lowest possible enzyme concentration is anyway too high to accurately determine the dissociation constant,

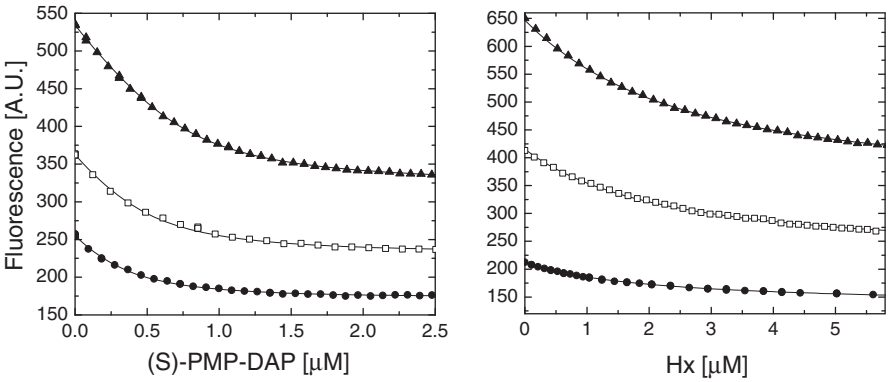


Figure 3. Fluorimetric titrations of Phe200Trp mutant by (*S*)-PMP-DAP (left) and hypoxanthine (right) in 20 mM Hepes buffer pH 7.0 at 25 °C; λ_{exc} = 290 nm, λ_{obs} = 340 nm. Three PNP concentrations (as a monomer): 0.6 μM (●), 0.88 μM (□), 1.23 μM (▲) and 0.52 μM (●), 1.02 μM (□), 1.59 μM (▲) in the case of (*S*)-PMP-DAP and Hx, respectively were used. Dissociation constants obtained by simultaneous fitting to several titration traces are 0.147 ± 0.012 μM for (*S*)-PMP-DAP and 1.25 ± 0.21 μM for Hx.

competitive binding assay was used (see Materials and Methods). For each ligand the best experimental approach was selected (wild type enzyme, Trp mutants, competitive binding and/or simultaneous fitting to more than one titration trace), which enabled very accurate determination of binding stoichiometry and dissociation constant.

Examples of such experiments are shown in Figures 3–6, and all results are summarized in Table 2. Fig. 3 (right panel) depicts titrations of various concentrations of Phe200Trp mutant with hypoxanthine analyzed simultaneously, and leading to K_d = 1.25 ± 0.21 μM and stoichiometry 0.98 ± 0.13. Fig. 4, left panel shows an example of the competitive binding approach: replacement of Gua by DFPP-G (yielding for DFPP-G K_d = 0.47 ± 0.10 nM and stoichiometry 1.38 ± 0.10). Similar experiment with replacement of Hx by DFPP-DG (data not shown) yielded for DFPP-DG K_d = 0.23 ± 0.06 nM and stoichiometry 0.86 ± 0.22. Right panel of Fig. 4 shows competition between DFPP-DG and (*S*)-PMP-Ada with simultaneous analysis of titration courses observed for removal of DFPP-DG from PNP/(*S*)-PMP-Ada complex analysed together, and the vice versa experiment, that is removal of DFPP-DG by (*S*)-PMP-Ada (not shown), leading to K_d = 0.86 ± 0.10 μM and 0.10 ± 0.03 nM for (*S*)-PMP-Ada and DFPP-DG, respectively. In Fig. 5 (upper panel) titration of wild type PNP with aza-DFPP-DG is presented. Fitting of the one binding site model gave K_d = 3.6 ± 0.5 nM and stoichiometry 0.89 ± 0.11. Titration of Phe200Trp mutant with guanine is shown in Fig. 6, and titrations of the wild type enzyme with this ligand (not shown) gave K_d = 0.16 ± 0.01 μM and stoichiometry 0.93 ± 0.10.

In none of these experiments one-third-of-the-sites binding was observed, one binding site model sufficiently good describes experimental data and stoichiometry of one ligand molecule per enzyme monomer was found for all ligands studied.

The final stoichiometry of binding of immucillin H to PNP was determined in the direct approach, without the necessity to fit any model to the data. For these experiments we have used the Phe200Trp and Phe159Trp mutants. Concentrations of binding sites for both mutants were determined with a very high accuracy by titrations with DFPP-DG, which binds very tightly, hence the titration curve has a stoichiometric character (see Fig. 5, middle panel for Phe200Trp mutant), and no fitting is necessary (dissociation constant cannot be determined with these approach). The enzyme from the same sample was then titrated with immucillin H (Fig. 5, lower panel). The same stoichiometry of binding of DFPP-DG and immucillin was obtained for both tryptophan mutants (data for Phe159Trp mutant not shown).

The final conclusion from all fluorimetric studies that we carried out is that one binding site model was sufficient to properly describe titration data and stoichiometry was one ligand molecule per one enzyme subunit.

2.8. Binding of immucillin to one active site does not block the binding to the remaining two sites of PNP

The binding of the transition-state inhibitor to one site of the PNP trimer was reported to prevent the inhibitor from binding to the remaining two sites.[6] The conclusion was later made slightly less definite, and it was described that immucillins bind trimeric PNP in a negatively cooperative manner so that binding to the first site hinders binding at the second and third sites.[10] Therefore we decided to check how the complex of the enzyme with one active

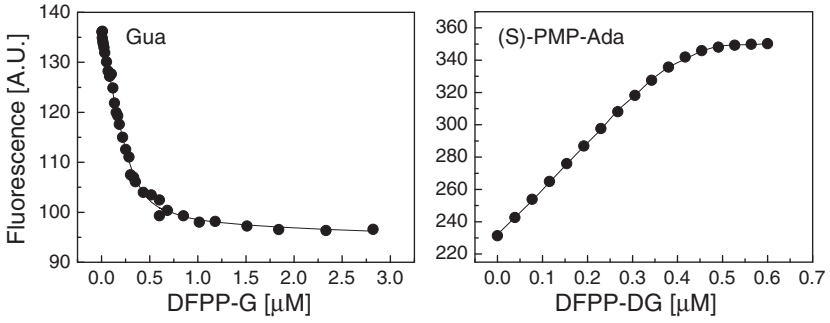


Figure 4. Determination of dissociation constants(20 mM Hepes buffer pH 7.0, 25 °C) for very potent ligands, using a competitive binding approach with fluorescence detection. Guanine (concentration 12 μM) was used as a weak ligand in the case of DFPP-G (left panel), and (*S*)-PMP-Ada (concentration 59.5 μM) in the case of DFPP-DG (right panel). PNP concentration (as a monomer) was 0.6–0.8 μM, λ_{exc} = 290 nm, λ_{obs} = 340 nm.

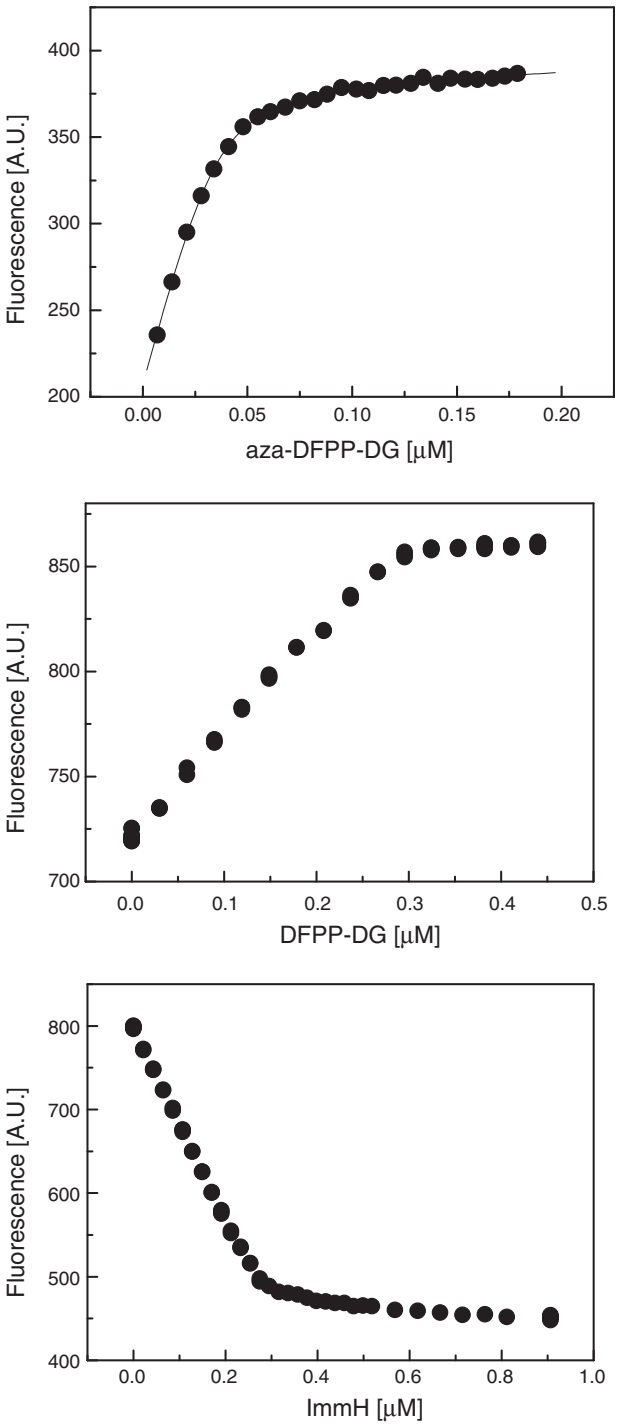


Figure 5. Fluorimetric titrations of the Phe200Trp mutant with DFPP-DG (middle panel), ImmH in the presence of 50 mM phosphate, (lower panel), and wild type PNP with aza-DFPP-DG (top panel). Enzyme concentration (as a monomer) was 0.27 μM, 0.31 μM and 0.05 μM in titration with DFPP-DG, ImmH, and aza-DFPP-DG, respectively; λ_{exc} = 290 nm, λ_{obs} = 353 nm. For very potent inhibitors, DGPP-DG and ImmH, even without fitting any model, due to stoichiometric character of the curve, binding stoichiometry of one ligand molecule per enzyme monomer is clearly visible. For aza-DFPP-DG fitting of the one-binding site model leads to K_d = 3.6 ± 0.5 nM and stoichiometry of.0.89 ± 0.11. Experiments is 50 mM Hepes pH 7.7 (for Phe200Trp mutant) and (20 mM Hepes pH 7.0 for wild type PNP) at 25 °C.

site per trimer filled with immucillin H binds guanine. Dissociation constant for guanine is much higher than that for immucillin. Thus if the hypothesis of Miles et al.[6] is true, guanine should not bind at all, because it should not exclude immucillin from the occupied

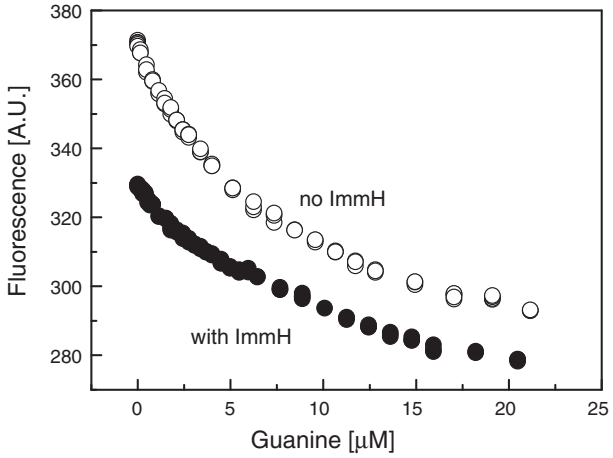


Figure 6. Fluorimetric titrations of the Phe200Trp mutant with guanine. PNP free form other ligands (o), in a concentration of 1.10 μM of binding sites titrated with guanine, PNP in a concentration of 1.10 μM of binding sites complexed with 0.37 μM ImmH, and then titrated with guanine (•). Experiments were conducted in 50 mM Hepes buffer pH 7.7 in the presence of 50 mM phosphate, at 25 °C λ_{exc} = 290 nm, λ_{obs} = 353 nm.

site, and binding to the remaining two sites as postulated should be blocked or at least hindered.

We have used the Phe200Trp and Phe159Trp mutants because binding of immucillin H with the wild-type enzyme cannot be followed fluorimetrically with a good accuracy (see above). Concentrations of binding sites for both mutants were determined by titrations with DFPP-DG, which yield titration curve with a stoichiometric character (Fig. 5). The enzyme sample of known binding site concentration was then complexed with amount of immucillin H required to fill one third of the binding sites. Next, this sample was titrated with guanine. As shown by the fluorescence change (Fig. 6), and the course of titration, the enzyme binds guanine similar as in the absence of immucillin H (Fig. 6). Hence, the presence of immucillin H at one site per trimer prevents binding of the inhibitor at this site of the PNP trimer but does not hinder its binding at the remaining two sites. We think that on the basis of the results presented here obtained for two ligands with the PNP transition-state analogue features (aza-DFPP-DG and ImmH) we may conclude that activity of PNP is not inhibited by binding of 1 mol of transition-state analogue inhibitors (including e.g., immucillin G[6]) to 1 mol of the trimeric PNP.

3. Conclusions

One-third-of-the-sites binding of the transition-state analogues was suggested for the trimeric purine nucleoside phosphorylase.[6,9,10] However, in the present study one-third-of-the-sites phenomenon was not detected for any of the ligands, including hypoxanthine and immucillin H, for which it was described,[9,10] and aza-DFPP-DG, which bears, like immucillins, two features characteristic for the proposed transition state. We postulate that this incorrect conclusion is most probably caused by the fact that the recombinant calf PNP purified by standard methods from the *E. coli* cells, is not ligand-free. If only routine purification steps are applied some of the active sites are filled with hypoxanthine. When such an enzyme preparation is used in inhibitor studies, depending on binding constants and enthalpies of the studied ligand when compared to hypoxanthine, lower binding stoichiometry and/or more complicated binding models may seem to be correct, which is certainly the case for immucillin. In contrast, titrations of the hypoxanthine-free enzyme with immucillin are consistent with one binding site model and stoichiometry of one

ligand molecule per enzyme monomer. This result was further confirmed by ITC titrations of the non-recombinant enzyme with immucillin H. Final proof of the simplified model was obtained from fluorimetric titrations of the Phe159Trp and Phe200Trp PNP mutants with immucillin H, when the complex formation was associated with the large quenching of the Trp fluorescence. In addition we have shown that binding of one immucillin H molecule per enzyme trimer does not block binding of guanine to the remaining two active sites.

On the basis of the results presented here, we conclude that transition-state analogue inhibitors of PNP, including immucillin H, bind with the same binding constant to all three active sites of trimeric PNP, and that the stoichiometry of binding is three ligand molecules per enzyme trimer. This finding however, does not change the fact that immucillins are powerful, strong binding and membrane permeable inhibitors of PNP with potential medical applications, as proven recently by their advanced clinical trials.[5]

4. Experimental

4.1. Chemicals

Recombinant calf PNP was obtained according to Breer et al.[31] As outlined elsewhere[25] PNP purified by the standard ion-exchange and gel-filtration chromatography is a mixture of the free enzyme and the complex with hypoxanthine, which was formed due to the high overexpression of PNP, and remains intact during the ion-exchange and size-exclusion purification steps. Therefore for the further studies hypoxanthine was removed by dialysis at 0.1 mg/ml (3 μM concentration of monomers) against 20 mM phosphate buffer, as described in Breer et al.[25] If phosphate-free conditions were necessary for the further experiment, second dialysis against 20 mM Hepes buffer pH 7.0 was done.

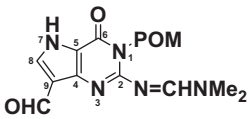
Commercially available purine nucleoside phosphorylase (PNP) from calf spleen (Sigma), suspension in 3.2 M ammonium sulphate, with specific activity vs Ino of approximately 15–28 U/mg was desalted as described previously to remove sulphate and phosphate.[26]

Inosine (Ino), Hepes (ultra pure), NA_2HPO_4, NaH_2PO_4 were products of Sigma or Fluka. Xanthine oxidase from buttermilk, suspension in 2.3 M ammonium sulphate (1 U/mg at 25 °C) was from Sigma. 7-Methylguanosine (m^7Guo) was prepared according to Jones and Robins.[32] 9-(5′,5′-Difluoro-5′-phosphonopentyl)-9-deazaguanine (DFPP-DG); 9-(5′,5′-difluoro-5′-phosphonopentyl)-guanine (DFPP-G); 6-amino-(*S*)-9-[2-(phosphonomethoxy)propyl] purine ((*S*)-PMP-Ada); 2,6-diamino-(*S*)-9-[2-(phosphonomethoxy) propyl]purine ((*S*)-PMP-DAP); (±)-*cis*-1,1-difluoro-2-(tetrahydro-3-piranozyl)ethylphosphonic acid with (hypoxanthine-9-yl)-methyl aglycone (DFTHPEP-H) and 9-deazaguanine (9-deaza-Gua) were synthesized as described previously.[15–19,33] All solutions were prepared with high-quality MilliQ water.

4.2. Synthesis of aza-DFPP-DG

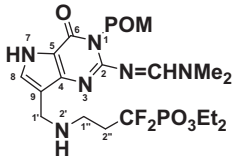
All reactions were carried out under a nitrogen atmosphere except stated otherwise. NMR data were obtained on a Bruker DPX 400 using $CDCl_3$, DMSO-d_6 or D_2O as a solvent. ^{13}C NMR (100 MHz) and ^{31}P NMR (162 MHz) were taken with broad-band ^1H decoupling. The chemical shift data for each signal on ^1H NMR (400 MHz) are expressed as relative ppm from $CHCl_3$ (δ 7.26), DMSO (δ 2.49) or H_2O (δ 4.79). The chemical shifts of ^{13}C are reported relative to $CDCl_3$ (δ 77.0), DMSO-d_6 (δ 39.5) or measured using 3-(trimethylsilyl)propionic acid sodium salt (TSPA) (δ 0.00) as an internal reference. The chemical shifts of ^{31}P are recorded relative to external 85% H_3PO_4. ^{19}F NMR spectra (376 MHz) were measured using benzotrifluoride (BTF) as an internal reference. Mass spectra were recorded on a VG Auto Spec. using electrospray ionization (ESI) techniques.

4.3. Synthesis of 2*N*-(*N*,*N*-dimethylaminomethylidene)-9-formyl-1*N*-(pivaloyloxy)methyl-9-deazaguanine (2)



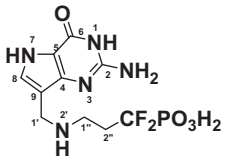
A solution of **1**[34] (3.6 g, 8.0 mmol), PPh_3 (1.70 g, 6.47 mmol) and $Pd_2(dba)_3$ (740 mg, 0.808 mmol) in DMF (97.6 mL) was heated at 60 °C under an atmosphere of carbon monoxide. Then, to this solution was added nBu$_3$SnH. After being stirred for 2 h, the volatile components of the mixture were removed in vacuo. The residue was purified by column chromatography on silica gel (1% MeOH–$CHCl_3$) to give **2** (2.27 g, 81%) as a white solid: mp 248–256 °C; ^1H NMR (400 MHz, $CDCl_3$) δ 11.15 (s, 1H, NH), 10.15 (s, 1H, CHO), 8.70 (s, 1H, $CHNMe_2$), 7.85 (d, 1H, 8-H, J = 3.6 Hz), 6.43 (s, 2H, $CH_2OC(O)^tBu$), 3.20, 2.09 (each s, 6H, $CHN(CH_3)_2$), 1.17 (s, 9H, ^tBu); ^{13}C NMR (100 MHz, $CDCl_3$) δ 184.87, 177.68, 157.62, 155.69, 145.09, 133.34 118.23, 115.56, 65.75, 41.35, 38.98, 35.34, 29.81, 27.20 (3 carbons); ESI-MS (LR) m/z 348 (MH^+); ESI-MS (HR) Calcd For $C_{16}H_{22}N_5O_3$ (MH^+): 348.1672, Found: 348.1666.

4.4. Synthesis of 9-{[*N*-[3′,3″-difluoro-3″-(diethylphosphono)-propyl]amino]methyl}-2*N*-(*N*,*N*-dimethylaminomethylidene)-1*N*-(pivaloyloxy)methyl-9-deazaguanine (5)



A solution of **3** (67 mg, 259 μmol) in MeOH (26 mL) over Pd-C (10%, cat) was stirred under an atmosphere of hydrogen for 1 h. The mixture was filtered through Celite pad and the filtrate was evaporated to give crude amine **4**. The amine **4** was too unstable to be purified and used for the next reaction without purification. To a stirred solution of the crude amine **4** (17 mg, 86 μmol) and aldehyde **2** (30 mg, 86.4 μmol) in CH_2ClCH_2Cl (426 μL) was added acetic acid (44.5 μL, 778 μmol). After being stirred for 30 min at room temperature, the mixture was treated with $NaBH(OAc)_3$ (55.0 mg, 259 μmol). The resulting mixture was stirred at the same temperature for 39 h and diluted with $CHCl_3$ and water. The separated aqueous layer was extracted with $CHCl_3$. The combined extracts were dried over $MgSO_4$ and evaporated. The residue was chromatographed on silica gel (5% MeOH–$CHCl_3$) to give **5** (30.3 mg, 62%) as a white solid: mp 125–130 °C; ^1H NMR (400 MHz, $CDCl_3$) δ 9.56 (s, 1H, NH), 8.54 (s, 1H, $CHNMe_2$), 7.15 (d, 1H, 8-H, J = 3.6 Hz), 6.36 (s, 2H, $CH_2OC(O)^tBu$), 4.25 (m, 4H, $OCH_2CH_3 \times 2$), 3.89 (s, 2H, 1′-H × 2), 3.16, 3.05 (each s, 6H, $CHN(CH_3)_2$), 3.02 (t, 2H, 1″-H × 2, $J_{1',2'}$ = 3.6 Hz), 2.39 (m, 2″-H × 2), 1.36 (t, 6H, $OCH_2CH_3 \times 2$, J = 7.1 Hz), 1.15 (s, 9H, ^tBu); ^{13}C NMR (100 MHz, $CDCl_3$) δ 177.77, 156.82, 155.39, 153.48, 143.33, 127.38, 120.68 (dt, $J_{C,P}$ = 216.3 Hz, $J_{C,F}$ = 261.3 Hz),114.62, 70.64, 65.93, 64.59 (d, $J_{C,P}$ = 7.0 Hz, 2 carbons), 43.23, 41.29 (m), 40.99, 38.87, 35.03, 34.01 (dt, $J_{C,P}$ = 14.3 Hz, $J_{C,F}$ = 20.4 Hz), 27.16 (3 carbons), 16.44 (d, $J_{C,P}$ = 5.4 Hz, 2 carbons); ^{19}F NMR (282 MHz, $CDCl_3$) δ −8.30 (dt, 2F, $J_{F,H}$ = 19.3 Hz, $J_{F,P}$ = 108.4 Hz); ^{31}P NMR (122 MHz, $CDCl_3$) δ 7.13 (t, 1P, $J_{P,F}$ = 108.4 Hz); ESI-MS (LR) m/z 563 (MH^+); ESI-MS (HR) Calcd. For $C_{23}H_{38}F_2N_6O_6P$ (MH^+): 563.2559, Found: 563.2546.

4.5. Synthesis of 9-{[*N*-[3″,3″-Difluoro-3″-(diethylphosphono)propyl]amino]methyl}-9-deazaguanine (6)



A solution of **5** (108 mg, 193 μmol) in concd HCl solution (12 mL) was refluxed for 16 h. The reaction mixture was diluted with water and washed with AcOEt. The water layer was concentrated and co-evaporated with EtOH. The resulting precipitate was suspended with water/MeOH (1: 1) and collected by filtration to give a solid. The solid was heated for 24 h at 40 °C in vacuo to give **6** (45.3 mg, 70%) as a pale brown solid: mp > 243 °C; ^{1}H NMR (400 MHz, D_2O, NaOD) δ 7.26 (s, 1H, 8-H), 3.79 (s, 2H, 1′-H × 2), 2.91 (m, 2H, 1″-H × 2), 2.25 (m, 2H, 2″-H × 2); ^{13}C NMR (100 MHz, D_2O, NaOD) δ 165.13, 159.96, 147.29, 130.33, 126.66 (dt, $J_{C,P}$ = 182.4 Hz, $J_{C,F}$ = 260.1 Hz), 116.68, 110.63, 44.43, 43.64, 35.47 (m); ^{19}F NMR(282 MHz, D_2O, NaOD) δ −47.58 (dt, 2F, $J_{F,H}$ = 20.9 Hz, $J_{F,P}$ = 85.8 Hz); ^{31}P NMR (122 MHz, D_2O, NaOD) δ 6.08 (t, 1P, $J_{P,F}$ = 85.8 Hz); ESI-MS (LR) *m*/*z* 338 (MH^+); ESI-MS (HR) Calcd For $C_{10}H_{15}F_2N_5O_4P$ (MH^+): 338.0830, Found: 338.0806; UV (λ_{max}, ε_{max}) water 270 nm, 7950 M^{-1} cm^{-1}; 231 nm, 14,400 M^{-1} cm^{-1}), pH 1.0 (270 nm, 10,150 M^{-1} cm^{-1}; 231 nm, 12,660 M^{-1} cm^{-1}), pH 7.0 (268 nm, 5720 M^{-1} cm^{-1}; 229 nm, 16,620 M^{-1} cm^{-1}), pH 13 (285 nm, 5100 M^{-1} cm^{-1}; 264 nm, 5530 M^{-1} cm^{-1}; 227 nm, 15,070 M^{-1} cm^{-1}).

4.6. Instrumentation

Kinetic and spectrophotometric measurements were carried out on a Uvikon 930 (Kontron, Austria) and Cary 100 (Varian, USA) spectrophotometers fitted with a thermostatically controlled cell compartment, using 10-, 5-, 2- or 1-mm path-length quartz cuvettes (Hellma, Germany). A Beckman model *Φ*300 pH-meter equipped with a combined semi-microelectrode and temperature sensor was used for pH determination. Fluorescence data were recorded on Perkin-Elmer LS-55 spectrofluorimeter (Norwalk, CT, USA) using 4 × 10-mm cuvettes with continuous mixing of solution. Calorimetric titrations in constant temperatures (ITC—isothermal titration calorimetry) were done using VP-ITC (MicroCal) calorimeter or MicroCal iTC-200 apparatus with the internal peltier temperature control. The sample volume was 1.45 ml in the case of VP-ITC and 0.2 ml in the case of iTC-200.

4.7. PNP and ligand concentrations

Concentrations of PNP and ligands were determined spectrophotometrically using the following extinction coefficients: $\varepsilon^{1\%}$ (280 nm) = 9.6 cm^{-1} for wild type calf PNP,[35] ε (268 nm) = 5 720 M^{-1} cm^{-1} for aza-DFPP-DG at pH 7.0, ε (260 nm) = 8500 M^{-1} cm^{-1} for m^7Guo at pH 7.0,[36] ε (273 nm) = 10,100 M^{-1} cm^{-1} for DFPP-DG at pH 7.0,[11] ε (246 nm) = 10,700 M^{-1} cm^{-1} for Gua, ε (252 nm) = 13,650 M^{-1} cm^{-1} for Guo, ε (250 nm) = 10,700 M^{-1} cm^{-1} for Hx, ε (249 nm) = 12,300 M^{-1} cm^{-1} for Ino, ε (253 nm)= 13,650 M^{-1} cm^{-1} for DFPP-G (as for guanosine),[37] ε (266 nm) = 6600 M^{-1} cm^{-1} for 9-deazaguanine,[33] ε (280 nm) = 10,400 M^{-1} cm^{-1} for (*S*)-PMP-DAP,[29] ε (249 nm) = 12,300 M^{-1} cm^{-1} for DFTHPEP-H (as for inosine).[37] Molar concentration of PNP is given always as monomers. In all calculations, the theoretical molecular mass of one monomer of the calf spleen enzyme based on its amino acid sequence was used, M_r = 32,093 Da (Swiss Prot entry P55859)[28] for the non-recombinant wild type enzyme, and the molecular mass from the ESI-MS, M_r = 31,654 Da for the recombinant wild type PNP.[31] For four Trp mutants extinction coefficient at 280 nm was calculated using the ProtParam[38] as 35,888 M^{-1} cm^{-1}.

4.8. Four Trp PNP mutants

The following primers were used: Phe159Trp 5′ GG TTT GGA GTT CGT TGG CCT GCC ATG TCT GAT GCC TAC 3′; Phe200Trp 5′ GG GGT CCC AAT TGG GAG ACT GTG GCA GAG TGT CGC 3′. Quick Change Mutagenesis system (Stratagene) was used according to the manufacturer instructions with one exception. The double concentration of deoxynucelosides were added, 0.4 mM, instead of suggested 0.2 mM. Vector pET28a(+):PNP was used. The mutations were confirmed by sequencing. Expression was done in *E. coli* BL21(DE3) cells. Four Trp mutants were purified using procedure previously described for wild type PNP[31] with later modifications to remove hypoxanthine.[25] In particular the final step of purification, in which hypoxanthine was removed, was the overnight dialysis of the PNP mutant sample in a concentration of about 2–3 μM against 20 mM phosphate buffer pH 7.0.

4.9. Enzymatic procedures

The specific activity of PNP was measured spectrophotometrically as described[26] with the use of the coupled assay, where inosine was the nucleoside substrate and the product (hypoxanthine) was oxidized by xanthine oxidase from buttermilk to form the final product of the coupled assay, uric acid.[34,39] Substrate and inhibitor properties were studied using the spectrophotometric method according to the previously published procedures.[23,26] Inhibition constants were determined with 7-methylguanosine as a substrate.

4.10. Concentration of active sites and true binding stoichiometry

To obtain a true binding stoichiometry it is essential to know concentration of active sites of the enzyme. Even in the electrophoretically homogenous enzyme preparation some part of molecules may be unable to bind ligands, hence lower apparent stoichiometry may be detected. We have shown before[29] that calf PNP with no activity vs Ino also does not bind multisubstrate-analogue inhibitor (*S*)-PMP-DAP. To show that it is also true for other ligands correlation between apparent stoichiometry (in ITC and fluorimetric titrations) and specific activity of the PNP sample vs Ino was determined for such ligands like guanine, 9-deazaguanine, (*S*)-PMP-Ada, DFPP-G, DFPP-DG and ImmH, For 43 studied cases high linear dependence of these two values was observed: correlation coefficient R^2 = 0.85 and no deviation from linearity in the runs tests. These results indicate that the part of the enzyme with no activity vs Ino has also no ability to bind these ligands. Since the maximal value of the specific activity, A_s observed for calf spleen PNP vs Ino is 34 U/mg[26,29] to obtain the true binding stoichiometry for the samples that showed specific activity lower than 34 U/mg the apparent stoichiometry was multiplied by the factor 34/A_s. This correction has of course a major influence on the calculated binding stoichiometry. We have not found in other published papers, including those describing one-third-of-the-sites binding,[6,9,10] that such a correction was introduced.

Of course it is very interesting to know what is the nature of the non-binding protein in the sample. From electrophoresis we know that this is PNP, but has no activity. We have just now started the project using the analytical ultracentrifugation to see if this lack of activity may be associated with the non-trimeric oligomeric state of the enzyme.

4.11. Calorimetric titrations

The amount of heat released or absorbed by the cell of the mirocalorimeter during formation of PNP-ligand complex was measured. PNP concentration (as monomer) was in the range 6.5–40 μM. Ligands, with the exception of guanine, were dissolved in the buffer used to dissolve protein. Guanine solution was prepared in water with addition of NaOH since guanine anion is much better soluble. Data were corrected by heat connected with addition of the ligand to the buffer. Titrations were done for the recombinant PNP and Sigma PNP, electrophoretically homogenous, with specific activity, A_s, vs Ino in the range 15–34 U/mg and 20–28 U/mg, respectively.

The ITC data obtained were evaluated, after subtraction of the heat of dilution of ligand, in terms of the following equation:

$$Q_t([L]) = n[E_o]\Delta HV\left(\frac{1}{2} + \frac{[L]}{2n[E_o]} + \frac{K_d}{2n[E_o]} - \frac{\sqrt{(1 + [L]/n[E_o] + K_d/n[E_o])^2 - 4[L]/n[E_o]}}{2}\right) \quad (1)$$

$[E_o]$ is the total PNP concentration from UV measurement (in terms of monomer), V is the cell volume, n—apparent binding stoichiometry; K_d is the dissociation constant and ΔH is the binding enthalpy. Using this equation, the total heat Q_t was fitted to the total titrant concentration added [L], via a non-linear least square minimization method. Fitted parameters are apparent binding stoichiometry n, the dissociation constant K_d and the enthalpy change ΔH.

The expression for the measured heat difference $\Delta Q_i = Q_i - Q_{i-1}$ is given by:

$$\Delta Q_i = Q_i + ((dV_i/2V)(Q_i + Q_{i+1})/2) - Q_{i-1} \quad (2)$$

where dV_i is the volume of titrant added to the solution in the calorimeter cell.

Calorimetric data were evaluated using the Origin 7.5 (MicroCal) program, which automatically corrected experimental results by the factors caused by dilution of the protein and of the ligand in the active volume of the sample in the course of titration. Three models implemented in the Origin 7.5 (MicroCal) program were initially taken into account: (a) identical independent sites, (b) two different kind of independent sites, (c) cooperating binding sites. But as outlined above the model (a) described by Eq. (1) was found to be sufficiently good to describe our data. True stoichiometry was determined from the apparent stoichiometry as described in section 4.10.

4.12. Fluorimetric titrations

If not otherwise indicated fluorimetric titrations were conducted in 50 mM Hepes buffer pH 7.0. Experiments were done in principle as described in our previous publications.[29] However, several improvements were introduced to be sure to detect cooperativity if this phenomenon exists, and do not interpret errors as a presence of the cooperativity.[23] In particular, ligands were dissolved not just in buffer but in buffer containing PNP in the same concentration as in the titration mixture present in a spectrofluorimetric cuvette. Hence, in both solutions used in the course of titration, that is in the mixture in a cuvette and in a ligand solution added to the cuvette, the same concentration of PNP was present. This prevents dilution of the PNP while ligand is added to the titration mixture, so no correction for dilution is necessary.

For very strong inhibitors (immucillin H, DFPP-DG) after each addition of the ligand to the cuvette fluorescence was monitored to reach the equilibrium, and only when it was reached next portion of the ligand was added.

Wild type Sigma PNP, recombinant wilt type PNP and two mutants, Phe159Trp and Phe200Trp were used in titrations. Excitation was at 290 nm and the observation wavelength was 340 nm or 353 nm.

Analysis was performed under the assumption that binding of each ligand molecule has the same influence on the enzyme intrinsic fluorescence. When ligand binding may be described sufficiently well by a single dissociation constant, K_d and K_d is comparable to the enzyme concentration, this leads to the following equation[41]:

$$F([L]) = F_o - (f_E - f_{EL} + f_L)\left(\frac{[L]}{2} + \frac{n[E_o]}{2} + \frac{K_d}{2} - \frac{\sqrt{([L] - n[E_o] + K_d)^2 + 4n[E_o]K_d}}{2}\right) + [L]f_L \quad (3)$$

Parameters f_E, f_L and f_{EL}, are fluorescence coefficients of free PNP, free ligand and PNP complexed with ligand, respectively, [L] is the total concentration of ligand, $F([L])$ is the fluorescence intensity observed for the ligand total concentration [L], n is binding stoichiometry and $[E_o]$ is the total concentration of the enzyme (in terms of monomer) from UV spectra measurement. Experimental data were fitted to Eq. (3), using non-linear regression analysis, obtaining values for four fitted parameters: K_d, n, f_L and $(f_E - f_{EL} + f_L)$. Program Origin 7.5 was used for fitting.

However, in most cases data were analysed using the DynaFit,[40] version 3 and 4 as described previously.[23] While analysing data with the DynaFit no analytical equation for binding is necessary since the program fits experimental binding data to an arbitrary molecular mechanism represented symbolically by a set of chemical equations. Therefore it is possible to test mechanisms for which no exact binding equation may be derived. The program is available by anonymous ftp via uwmml.pharmacy.wisc.edu and on the World Wide Web via http://uwmml.pharmacy.wisc.edu.

In most studied cases, to make the final conclusion regarding binding mechanism more reliable, three titrations with different receptor concentration were done (see for example Fig. 3). Two of the three courses of titrations were analysed simultaneously. In the next step all three were analysed together. Only if both approaches yielded the same parameters, the experiment was accepted.

True binding stoichiometry was determined as described above in section 4.10 and 4.11 for calorimetric titrations.

4.13. Competitive binding approach titrations

In the case of very potent ligands ITC and fluorimetric titrations gave reliable information according model of binding and binding stoichiometry but due to the fact that protein concentration was much higher that dissociation constant the later was not determined accurately. Hence, to determine dissociation constants of very potent inhibitors the method with competitive binding of weak and strong ligands was used,[41] which is described in detail in Supplementary data. The enzyme (0.3–1.0 μM of monomer) was previously mixed with about 50 μM or 10 μM of a weaker inhibitor (*S*)-PMP-Ada or Gua or, then titrated with potent inhibitor, for example DFPP-DG or DFPP-G, or vice versa, first complexed with strong inhibitor and then titrated with the weaker one. Examples of such experiments are shown in Fig. 4. The titrations were followed fluorimetrically. The equation describing competitive binding of two ligands characterized by a single dissociation constant for each ligand was fitted to the data using Origin 7.5 program and script (see Supplementary data) that allows to solve the analytical third power equation describing concentration of the free protein. K_d for weak ligands were kept constant during fitting. Molar fluorescence intensities for the protein and for the

buffer were determined in the separate experiments and were also kept constant in the course of fitting, and molar fluorescence intensities of ligands were determined from the ligands titrations into buffer.

Acknowledgements

Supported by the Polish Ministry of Science and Higher Education (grants N301 003 31/0042, N N301 044939 and BW-1724/BF). The authors thank very much Professor Vern Schramm and the Department of Biochemistry, Albert Einstein College of Medicine of Yeshiva University (Bronx, NY, USA) for the sample of immucillin H. We are also indebted to Mrs. Lucyna Magnowska for the excellent technical assistance.

Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmc.2012.08.045.

References and notes

1. Giblett, E. R.; Ammann, A. J.; Wara, D. W.; Sandman, R.; Diamond, L. K. *Lancet* **1975**, *1*, 1010.
2. Bzowska, A.; Kulikowska, E.; Shugar, D. *Pharmacol. Ther.* **2000**, *88*, 349.
3. Ravandi, F.; Gandhi, V. *Expert Opin. Investig. Drugs* **2006**, *15*, 1601.
4. Balakrishnan, K.; Nimmanapalli, R.; Ravandi, F.; Keating, M. J.; Gandhi, V. *Blood* **2006**, *108*, 2392.
5. Ho, M. C.; Rinaldo-Matthis, A.; Tyler, P. C.; Evans, G. B.; Clinch, K.; Almo, S. C.; Schramm, V. L. *Proc. Am. Chem. Soc.* **2010**, *107*, 4805.
6. Miles, R. W.; Tyler, P. C.; Furneaux, R. H.; Bagdassarian, C. K.; Schramm, V. L. *Biochemisty* **1998**, *37*, 8615.
7. Fedorov, A.; Shi, W.; Kicska, G.; Fedorov, E.; Tyler, P. C.; Furneaux, R. H.; Hanson, J. C.; Gainsford, G. J.; Larese, J. Z.; Schramm, V. L.; Almo, S. C. *Biochemistry* **2001**, *40*, 853.
8. Chojnowski, G.; Breer, K.; Narczyk, M.; Wielgus-Kutrowska, B.; Czapińska, H.; Hashimoto, M.; Hikishima, S.; Yokomatsu, T.; Bochtler, M.; Girstun, A.; Staroń, K.; Bzowska, A. *Biochem. Biophys. Res. Commun.* **2010**, *391*, 703–708.
9. Kline, P. C.; Schramm, V. L. *Biochemistry* **1992**, *31*, 5964.
10. Edwards, A. A.; Mason, J. M.; Clinch, K.; Tyler, P. C.; Evans, G. B.; Schramm, V. L. *Biochemistry* **2009**, *48*, 5226.
11. Hikishima, S.; Hashimoto, M.; Magnowska, L.; Bzowska, A.; Yokomatsu, T. *Bioorg. Med. Chem. Lett.* **2007**, *17*, 4173.
12. Hikishima, S.; Hashimoto, M.; Magnowska, L.; Bzowska, A.; Yokomatsu, T. *Bioorg. Med. Chem.* **2010**, *18*, 2275.
13. Klepper, F.; Jahn, E. M.; Hickmann, V.; Carell, T. *Angew. Chem., Int. Ed.* **2007**, *46*, 2325.
14. Clinch, K.; Evans, G. B.; Frohlich, R. F. G.; Furneaux, R. H.; Kelly, P. M.; Legentil, L.; Murkin, A. S.; Li, L.; Schramm, V. L.; Tyler, P. C.; Woolhouse, A. D. *J. Med. Chem.* **2009**, *52*, 1126.
15. Halazy, S.; Ehrhard, A.; Danzin, C. *J. Am. Chem. Soc.* **1991**, *113*, 315.
16. Yokomatsu, T.; Hayakawa, Y.; Kihara, T.; Koyanagi, S.; Soeda, S.; Shimeno, H.; Shibuya, S. *Bioorg. Med. Chem.* **2000**, *8*, 2571.
17. Rosenberg, I.; Holy, A.; Masojidkova, M. *Collect. Czech. Chem. Commun.* **1988**, *53*, 2753.
18. Šedivá, K.; Ananiev, A. V.; Votruba, I.; Holý, A. *Int. J. Purine Pyrimidine Res.* **1991**, *2*, 35.
19. Holý, A.; Dvořáková, H.; Masojídková, M. *Collect. Czech. Chem. Commun.* **1995**, *60*, 1390.
20. Kulikowska, E.; Bzowska, A.; Holy, A.; Magnowska, L.; Shugar, D. *Adv. Exp. Med. Biol.* **1998**, *431*, 747.
21. Iwanow, M.; Magnowska, L.; Yokomatsu, T.; Shibuya, S.; Bzowska, A. *Nucleosides Nucleotides Nucleic Acids.* **2003**, *22*, 1567.
22. Breer, K.; Wielgus-Kutrowska, B.; Hashimoto, M.; Hikishima, S.; Yokomatsu, T.; Szczepanowski, R.; Bochtler, M.; Girstun, A.; Staroń, K.; Bzowska, A. *Nucleic Acids Symp. Ser. (Oxf)* **2008**, *52*, 663.
23. Breer, K.; Glavas-Obrovac, L.; Suver, M.; Hikishima, S.; Hashimoto, M.; Yokomatsu, T.; Wielgus-Kutrowska, B.; Magnowska, L.; Bzowska, A. *FEBS J.* **2010**, *277*, 1747.
24. Wang, F.; Miles, R. W.; Kicska, G.; Nieves, E.; Schramm, L. V.; Angeletti, R. H. *Protein Sci.* **2000**, *9*, 1660.
25. Breer, K.; Wielgus-Kutrowska, B.; Girstun, A.; Staroń, K.; Hashimoto, M.; Hikishima, S.; Yokomatsu, T.; Bzowska, A. *Biochem. Biophys. Res. Commun.* **2010**, *391*, 1203.
26. Bzowska, A. *Biochim. Biophys. Acta* **2002**, *1596*, 293.
27. Wielgus-Kutrowska, B.; Holy, A.; Frank, J.; Koellner, G.; Bzowska, A. *Nucleosides Nucleotides Nucleic Acids* **2003**, *22*, 1695.
28. Bzowska, A.; Luić, M.; Schröder, W.; Shugar, D.; Saenger, W.; Koellner, G. *FEBS Lett.* **1995**, *367*, 214.
29. Bzowska, A.; Koellner, G.; Wielgus-Kutrowska, B.; Stroh, A.; Raszewski, G.; Holý, A.; Steiner, T.; Frank, J. *J. Mol. Biol.* **2004**, *342*, 1015.
30. Breer, K. PhD thesis, Warsaw University 2011.
31. Breer, K.; Girstun, A.; Wielgus-Kutrowska, B.; Staroń, K.; Bzowska, A. *Protein Expr. Purif.* **2008**, *61*, 122.
32. Jones, J. W.; Robins, R. K. *J. Am. Chem. Soc.* **1963**, *85*, 193.
33. Stępniak, K.; Žinić, B.; Wierzchowski, J.; Bzowska, A. *Nucleosides Nucleotides Nucleic Acids* **2007**, *26*, 841.
34. Burton, D. J.; Sprague, L. G. *Org. Chem.* **1989**, *54*, 613.
35. Stoeckler, J. D.; Agarwal, R. P.; Agarwal, K. C.; Parks, R. E., Jr. *Methods Enzymol.* **1978**, *51*, 530.
36. Kulikowska, E.; Bzowska, A.; Wierzchowski, J.; Shugar, D. *Biochim. Biophys. Acta* **1986**, *874*, 355.
37. Burton, K. In *Data for Biochemical Research*; Dawson, R. M. C., Elliott, D., Elliott, W. H., Jones, K. M., Eds.; Oxford University Press: Oxford, 1969; p 169.
38. Gasteiger, E.; Hoogland, C.; Gattiker, A.; Duvaud, S.; Wilkins, M. R.; Appel, R. D.; Bairoch, A. In *The Proteomics Protocols Handbook*; Walkier, John M., Ed.; Human Press: New York, 2005; p 571. PharmProt (http://expasy.org/tools/protparam.html).
39. Kalckar, H. M. *J. Biol. Chem.* **1947**, *167*, 429.
40. Kuzmic, P. *Anal. Biochem.* **1996**, *237*, 260.
41. Wang, Z. X. *FEBS Lett.* **1995**, *360*, 111.
42. Bzowska, A.; Kulikowska, E.; Darzynkiewicz, E.; Shugar, D. *J. Biol. Chem.* **1988**, *263*, 9212.