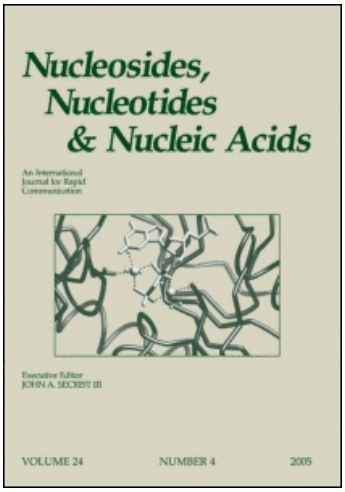

Nucleosides, Nucleotides and Nucleic Acids
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597286

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To cite this Article Wierzchowski, Jacek , Iwańska, Beata , Bzowska, Agnieszka and Shugar, David(2007) 'Interactions of Calf Spleen Purine Nucleoside Phosphorylase with Formycin B and its Aglycone—Spectroscopic and Kinetic Studies', Nucleosides, Nucleotides and Nucleic Acids, 26: 6, 849 — 854
To link to this Article: DOI: 10.1080/15257770701503993
URL: http://dx.doi.org/10.1080/15257770701503993

Nucleosides, Nucleotides, and Nucleic Acids, 26:849–854, 2007

ISSN: 1525-7770 print / 1532-2335 online
DOI: 10.1080/15257770701503993

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INTERACTIONS OF CALF SPLEEN PURINE NUCLEOSIDE PHOSPHORYLASE WITH FORMYCIN B AND ITS AGLYCONE—SPECTROSCOPIC AND KINETIC STUDIES

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□ *Phosphorolysis of 7-methylguanosine by calf spleen purine nucleoside phosphorylase (PNP) is weakly inhibited, uncompetitively, by Formycin B (FB) with $K_i = 100\ \mu M$ and more effectively by its aglycone (7KPP), IC_{50} 35–100 μM. In striking contrast, 7KPP inhibits the reverse reaction (synthesis of 8-azaguanosine from 8-azaguanine) competitively, with $K_i \sim 2$–4 μM. Formycin B forms only a weakly fluorescent complex with PNP, and 7KPP even less so, indicating that both ligands bind as the neutral, not anionic, forms. 7KPP is a rare example of a PNP non-substrate inhibitor of both the phosphorolytic and reverse synthetic pathways.*

Keywords Purine-nucleoside phosphorylase; Formycin B; inhibitor; fluorescence; ligand binding

INTRODUCTION

C-nucleosides are known inhibitors of both mammalian and bacterial[1,2] purine nucleoside phosphorylases (PNP, E.C. 2.4.2.1), which catalyze the reversible phosphorolysis of purine ribo- and 2'-deoxyribonucleosides, as follows:

$$\beta\text{- purine nucleoside} + \text{orthophosphate} \leftrightarrow \text{purine base} + \alpha\text{- D -pentose - 1 - phosphate.}$$

Interactions of calf spleen PNP with the tautomeric C-nucleoside Formycin B (FB, a structural analog of inosine) and its aglycone,

This work was supported by grants from the State Committee for Scientific Research (KBN), 3P04A 024 25, and from the Polish Ministry of Science and Higher Education, grant N301 003 31/0042.
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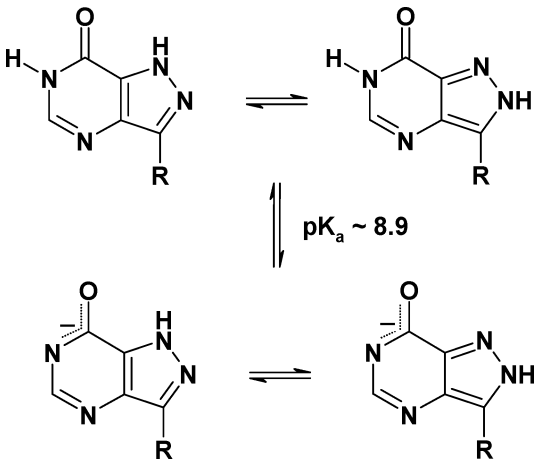
7-ketopyrazolo[4,3-d]pyrimidine (7KPP, see Scheme 1), were investigated by means of steady-state fluorescence spectroscopy and enzyme inhibition studies. Our goal was to obtain information concerning the reaction mechanism and/or inhibitor binding mode, complementing our previous results on fluorescent substrate binding.[3,4]



SCHEME 1 The neutral species of formycin B (R = ribose) and 7KPP (R = H), shown in their two most likely tautomeric and anionic forms (p$K_a \approx 8.9$).

EXPERIMENTAL

Calf spleen PNP (Sigma) was desalted and used as previously described.[5] FB, 7-methylguanosine (m^7Guo), 8-azaguanine (8-azaGua), and α-ribose-1-phosphate (R1P) were products of Sigma (USA). Syntheses of 7-ketopyrazolo[4,3-d]pyrimidine (7-KPP) and some 3-substituted derivatives were carried out according to known procedures at the University of Podlasie, Siedlce, Poland.

Interactions of FB and 7KPP with the enzyme were investigated by kinetic methods, using m^7Guo with phosphate, and 8-azaGua with R1P, as substrates for the phosphorolytic and synthetic reaction pathways, respectively. Both reactions are virtually irreversible and can be readily followed fluorimetrically or spectrophotometrically.[4] Advantage was taken of the fact that phosphorolysis of m^7Guo, but not Guo, follows Michaelis-Menten kinetics.[2]

The complex between PNP and FB was studied using steady-state fluorescence spectroscopy. Emission and excitation spectra were recorded, using semi-micro cuvettes with a 4-mm pathlength, and corrected for the inner filter effect. Difference spectra were additionally corrected for protein dilution.

TABLE 1 Inhibition of 7-methyguanosine phosphorolysis by 7KPP. Inhibitory constants (K_i) were calculated on the assumption of uncompetitive inhibition.

pH	Substrate concentration [μM]	IC_{50} [μM]	Hill coefficient (h)	K_i [μM]
6.0	32	$\sim$ 35	0.67	38
7.0	122	98	0.41	73
7.0	30	98	0.62	54
7.0	60	—	0.55	—
7.6	32	> 100	0.86	—
8.0	32	> 100	1.02	93

RESULTS AND DISCUSSION

Inhibition of Phosphorolysis of m^7Guo by FB and 7KPP

Phosphorolysis of m^7Guo was followed in 50 mM phosphate, using spectrophotometric detection.[1] Formycin B is known to be a moderate inhibitor of calf PNP, and a more potent one for the bacterial *(E. coli)* enzyme.[2] We have confirmed its moderate inhibitory activity and found it to be not fully competitive, as inferred from a Dixon plot (not shown), and calculation of K_i, 100 $\pm$ 20 μM (uncompetitive mode) is in agreement with previous data.[2]

Surprisingly, the aglycone 7KPP proved to be a moderate noncompetitive inhibitor of the phosphorolytic reaction pathway, with IC_{50} in the range 35 to above 100 μM, apparently independent of substrate concentration (Table 1), but dependent on pH. This inhibition exhibited negative cooperativity, especially pronounced at pH $\sim$ 6–7 (see Table 1, Hill coefficients). However, low solubility of the inhibitor precluded more precise investigations.

Inhibition of Enzymatic Synthesis of 8-Azaguanosine by 7KPP

Enzymatic synthesis of 8-azaGuo in a phosphate-free HEPES buffer, followed spectrophotometrically and/or fluorimetrically, was employed to evaluate inhibition constants for the FB aglycone in the reverse synthetic pathway. The reaction was carried out at various pH values, in the presence of $\sim$2 mM ribose-1-phosphate. The results given below indicate **competitive** inhibition, with K_i 2.0–4.2 μM. Other 3-substituted alkyl- and carboxy-derivatives of 7KPP exhibited weaker inhibitory effects (data not shown).

It should be noted that the phosphorolytic and synthetic reaction pathways are conducted under different conditions with respect to phosphate concentration, i.e., during phosphorolysis the enzyme is fully saturated with phosphate. Thus, the observed different modes of inhibition by nucleoside and purine analog may be related to the postulated regulatory role of the bound phosphate ligand.[2]

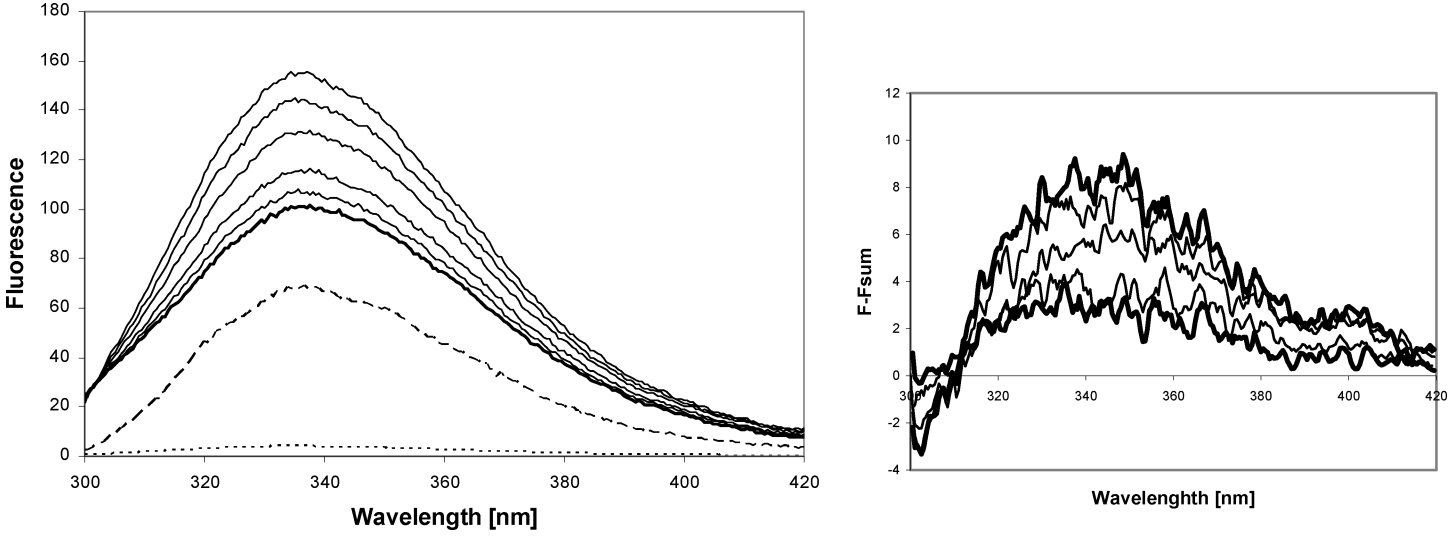


FIGURE 1 *Left panel:* Fluorimetric titration of calf spleen PNP, 5 μM, with formycin B, in the presence of 5 mM phosphate, at 25°C and pH = 7.0. Excitation was at 290 nm. The dotted line represents free neutral FB and the dashed line FB anion (both 5 μM). Protein fluorescence (lower solid curve) is normalized to 100 and the spectra corrected for the inner filter effect. *Right panel:* Difference spectra ($F–F_{sum}$), corrected for the inner filter effect and protein dilution; ligand concentrations in both panels were 4.7, 18.3, 43.2, 62, and 79.8 μM (from bottom to top).

TABLE 2 Inhibition of enzymatic synthesis of 8-azaguanosine by 7KPP.

pH	Substrate concentration [μM]	K_i [μM]	Hill coefficient (h)
6.5	50–250	2.8–3.5	1.02
7.3	5–50	2.0	nd*
7.9	5–50	3.6	0.95
8.2	5–50	3.7	nd
8.2	5–50	4.2	0.99

*Not determined.

Observation of the Complex Between PNP and FB

Formycin B is weakly fluorescent in neutral aqueous medium,[6] but elevation of the pH up to 10 leads to a significant increase in emission at $\sim$ 335 nm, due to deprotonation ($pK_a \sim 8.9$, see Figure 1 and Scheme 1). Similar effects are observed for 7KPP fluorescence (data not shown). Thus, this system allows a comparison of both enzyme-nucleoside and enzyme-purine analog complexes with respect to the ionic/tautomeric form of the bound ligand.

Fluorimetric titration of calf PNP with FB, conducted in the presence of 9 mM phosphate, at pH 7, indicated small, but reproducible, effects on the fluorescence emission (Figure 1) and fluorescence excitation spectra (not shown). This effect did not allow unequivocal identification of the tautomeric form of the bound ligand, as earlier achieved in binding of Formycin A by *E. coli PNP*,[6] but did eliminate the anionic form, which is much more intensely fluorescent, even in the unbound state (see above and Figure 1, dashed line).

A similar conclusion also applies to 7KPP, since there was no visible effect on the fluorescence of PNP, when titrated with 7KPP in the absence of phosphate (data not shown). This is in line with our previous data on the binding of 8-azapurine derivatives, including phosphonoalkoxy bisubstrate analog inhibitors, by calf spleen PNP, where only the neutral forms of the ligands were detected in the bound state.[3,4]

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