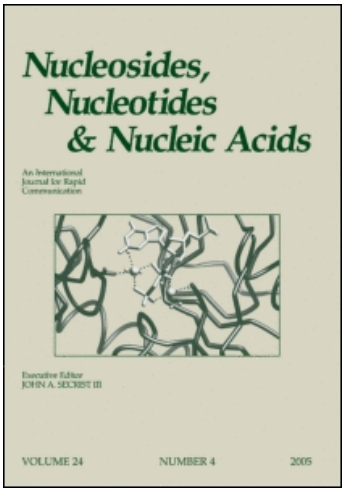

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Interactions of Potent Multisubstrate Analogue Inhibitors with Purine Nucleoside Phosphorylase from Calf Spleen—Kinetic and Spectrofluorimetric Studies

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ABSTRACT

Dissociation constants and stoichiometry of binding for interaction of trimeric calf spleen purine nucleoside phosphorylase with potent multisubstrate analogue inhibitors were studied by kinetic and spectrofluorimetric methods.

Key Words: Purine nucleoside phosphorylase; Multisubstrate analogue inhibitors; Stoichiometry; Fluorescence; Dissociation constants; Inhibition constants.

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INTRODUCTION

Trimeric purine nucleoside phosphorylase (PNP, E.C. 2.4.2.1.), found in mammals and some other organisms, catalyzes the reversible phosphorolysis of inosine, guanosine and their 6-oxo analogues, as follows: β-purine nucleoside + orthophosphate ⇔ purine base + α-D-pentose-1-phosphate. Potent selective inhibitors of PNPs are considered promising chemotherapeutic agents.[1] The mechanism of action of PNP still needs to be clarified and one of the important questions regards possible interactions of enzyme subunits. For trimeric calf spleen PNP negative cooperativity in binding of immucillins – transition state analogue inhibitors – was observed.[2] However, ground state analogues seem to bind uniformly to all three sites of this enzyme and non-Michaelis kinetics observed for some substrates seems to be the result of complex kinetic mechanism and not of the cooperativity between enzyme subunits.[3]

RESULTS AND DISCUSSION

We have tried to support the above hypothesis by studying interactions of the calf spleen PNP with potent ground-state analogue inhibitors, 9-(5,5-difluoro-5-phosphonopentyl)guanine (Danzin compound),[4] and recently synthesized (±)-cis-1,1-difluoro-2-(tetrahydro-3-piranozyl)ethylphosphonic acid with (hypoxanthine-9-yl)methyl aglycone[5] (Yokomatsu compound, see Fig. 1, right panel). Both were found to be very potent inhibitors of 7-methylguanosine (m^7Guo) phosphorolysis with apparent inhibition constants, K_i^{app}, in nM range. Moreover, inhibition is competitive vs. both substrates of the reaction, i.e., with nucleoside (m^7Guo) as variable substrate at constant phosphate (P_i) level, and with P_i as variable substrate at constant m^7Guo concentration. This indicates that inhibitors studied bind to both nucleoside and P_i binding sites, hence act as multisubstrate analogue inhibitors. K_i^{app} for both inhibitors determined at various constant P_i levels (from the range 0.025–2.0 mM) with m^7Guo as variable substrate, were found to decrease with decreasing P_i concentration (e.g., for Yokomatsu compound K_i^{app} was 2.7 ± 1.6 nM and 92 ± 34 nM for 0.025 mM and 2.0 mM P_i, respectively, and for Danzin compound K_i^{app} was 2.3 ± 0.1 nM and 6.9 ± 0.7 nM for 0.1 mM and 1.0 mM P_i, respectively). Extrapolation to zero P_i concentration (hyperbolic equation describing K_i^{app} vs. P_i dependence) leads to preliminary estimation of inhibition constants, K_i: ~2 nM and ~1 nM for Danzin and Yokomatsu compounds, respectively.

Fluorimetric titrations were carried out in the absence of P_i, with protein concentration in the range 70–300 nM. Virtually no protein fluorescence quenching due to ligand association is observed for Danzin compound, but binding of the second analogue leads to about 10% quenching allowing to determine the dissociation constant and binding stoichiometry. Titrations (see Fig. 1, left panel) are sufficiently described by the one-binding site model.[6] Dissociation constant K_d was roughly estimated to be ≤ 1 nM, consistent with preliminary values of inhibition constant K_i determined in kinetic experiments (see above). Estimated K_d value is small in comparison with enzyme concentration employed in titrations (70–270 nM).

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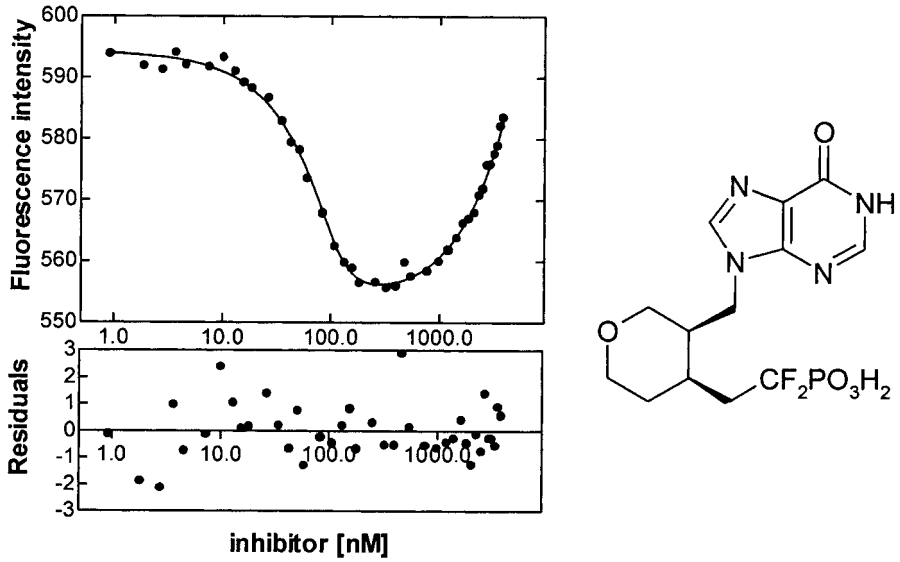

Figure 1. Effects of inhibitor (scheme shown above) on intrinsic fluorescence of calf spleen PNP. Upper panel: Typical titration (20 mM hepes buffer pH 7.0, 25°C, $\lambda_{exc} = 280$ nm, $\lambda_{obs} = 335$ nm, monomeric PNP concentration from UV spectra $[E_0] = 134$ nM). Equation for one-binding site model[6] (see below) was fitted to the data. Residuals are shown on the lower panel. $[E_{act}]$ found for this titration is 112 nM. Stoichiometry per enzyme trimer is calculated as $3[E_{act}]/[E_0]$, hence for this experiment is 2.5 ligand molecules bound per trimer. Equation fitted: $F([L_0]) = F_0 - (f_E - f_{EL} + f_L)\ \{[L_0]/2 + [E_{act}]/2 + K_d/2 - \text{sqrt}(([L_0] - [E_{act}] + K_d)^2 + 4[E_{act}]K_d)/2\} + L_0 f_L$ where K_d, dissociation constant, $[E_{act}]$, concentration of binding sites, $[L_0]$, total ligand concentration, f_E, f_L, f_{EL} fluorescence coefficients of free protein, free ligand and their complex. Fitted parameters: K_d, $[E_{act}]$, $f_E - f_{EL}$, f_L.

Therefore lower enzyme concentration should be used for more precise determination of K_d. Stoichiometry of binding, in several experiments with various enzyme concentration, was found to be in the range 2.2–2.9 inhibitor molecules bound per enzyme trimer, hence our data are most consistent with three non-interacting identical binding sites. This result supports hypothesis that in the case of calf spleen PNP only transition-state events occur at one site at a time, while reactant-state events, including binding of potent ground state inhibitors, can occur uniformly at all three sites of the enzyme[7] (see two related papers in this volume).

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