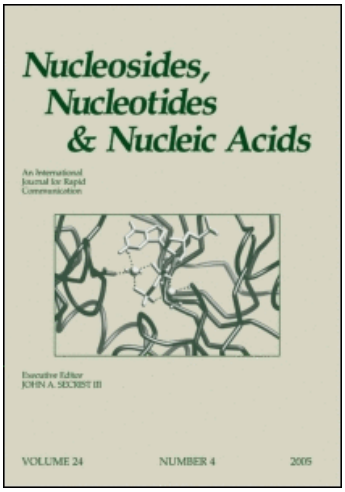

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Kinetics of Binding of Multisubstrate Analogue Inhibitor (2-Amino-9-[2-(Phosphonomethoxy)Ethyl]-6-Sulfanylpurine) with Trimeric Purine Nucleoside Phosphorylase

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KINETICS OF BINDING OF MULTISUBSTRATE ANALOGUE INHIBITOR (2-AMINO-9-[2-(PHOSPHONOMETHOXY)ETHYL]-6-SULFANYLPURINE) WITH TRIMERIC PURINE NUCLEOSIDE PHOSPHORYLASE

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□ *Complex formation of multisubstrate analogue inhibitor—2-amino-9-[2-(phosphonomethoxy)-ethyl]-6-sulfanylpurine (PME-6-thio-Gua) with trimeric purine nucleoside phosphorylase from* Cellulomonas *sp. was investigated using a stopped-flow spectrofluorimetric approach. Results obtained indicate that, in contrast to binding of guanine, i.e., the transition-state conformation trapping ligand, for which binding at each active site is followed by the enzyme conformational change, association of the ground-state analogue PME-6-thio-Gua is a one-step process.*

Keywords Binding; PME-6-thio-Gua; multisubstrate analogue inhibitor

INTRODUCTION

Homotrimeric purine nucleoside phosphorylase (PNP, EC 2.4.2.1.) catalyses the reaction: 6-keto β-purine nucleoside + orthophosphate $\leftrightarrow$ purine base + α-D-pentose-1-phosphate. Potent, selective inhibitors of PNP are considered potential immunosuppressive and antiparasitic agents.[1]

Trimeric PNPs bind some of their ligands, e.g., hypoxanthine (Hx) and guanine (Gua), extremely tightly, especially in the absence of orthophosphate,[2–4] and this is one of the reasons of the non-Michaelis kinetics of these enzymes.[3] After phosphorolysis of some nucleosides (e.g., Ino and Guo) and releasing of the first product, pentose-1-phoshate, the

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enzyme, in a binary complex with the respective purine base product (Hx or Gua), is trapped in the transition state conformation, and presence of phosphate is necessary to relax this state.[2] In line with these findings, our previous stopped-flow studies have documented that binding of Gua by each of the trimeric calf spleen PNP active sites is a two-step process, it means association of the ligand is followed by the enzyme conformational change.[5] Moreover, interactions between three active sites occur,[5] in agreement with cooperativity between subunits documented for the transition state events.[6] In this study the kinetics of binding of a ground-state multisubstrate analogue inhibitor, 2-amino-9-[2-(phosphono-methoxy)ethyl]-6-sulfanylpurine (PME-6-thio-Gua) to trimeric PNP from *Cellulomonas* sp. was investigated using a stopped-flow method. The purpose is to check if the two-step interacting site model is necessary to describe complex formation of trimeric PNPs also with their ground-state ligands.

MATERIALS AND METHODS

The commercial PNP from *Cellulomonas* sp. (Toyobo Co., Japan) was purified on a BioCad station (PerSeptive Biosystems) by ion-exchange chromatography as descrbed.[4] PME-6-thio-Gua was synthesized according to Holy et al.[7] All other chemicals were from Sigma. Concentration of PNP and the ligand was determined spectrophotometrically using the following extinction coefficients: $\varepsilon^{1\%}_{280\,\mathrm{nm}} = 5.9$[4] and $\varepsilon_{341\,\mathrm{nm}}$ (pH 7.0) = 23 800 $\mathrm{M^{-1}cm^{-1}}$.[8] PNP concentration was expressed as monomer concentration.

Emission of PNP/PME-6-thio-Gua complexes was excited at 290 nm (slit widths = 0.5 mm = 2.32 nm), and their fluorescence was monitored using a cut-off filter (320 nm). Extinction and emission path lengths in the stopped-flow cell were 2 and 10 mm, respectively. The investigated association reactions consisted of mixing equal volumes of solutions of PNP and of the ligand, both in 20 mM Hepes buffer pH 7.0, 20°C, with ionic strength adjusted with KCl to 50 mM. The concentration of the protein was 0.4 μM (in the stopped-flow syringe). Concentrations of the ligand varied between 0.4 and 52 μM (8 experiments in total, conducted in duplicate). Data was analyzed according to various association models using home-written software.[5]

RESULTS AND DISCUSSION

Kinetic traces, resulting from the decrease of the enzyme intrinsic fluorescence accompanying binding of various concentration of PME-6-thio-Gua to PNP (Figure 1), were simultaneously fit by numerical algorithms which predict time dependence of the fluorescence of the mixture. Two models of the complex formation, each treating PNP molecule as a trimer

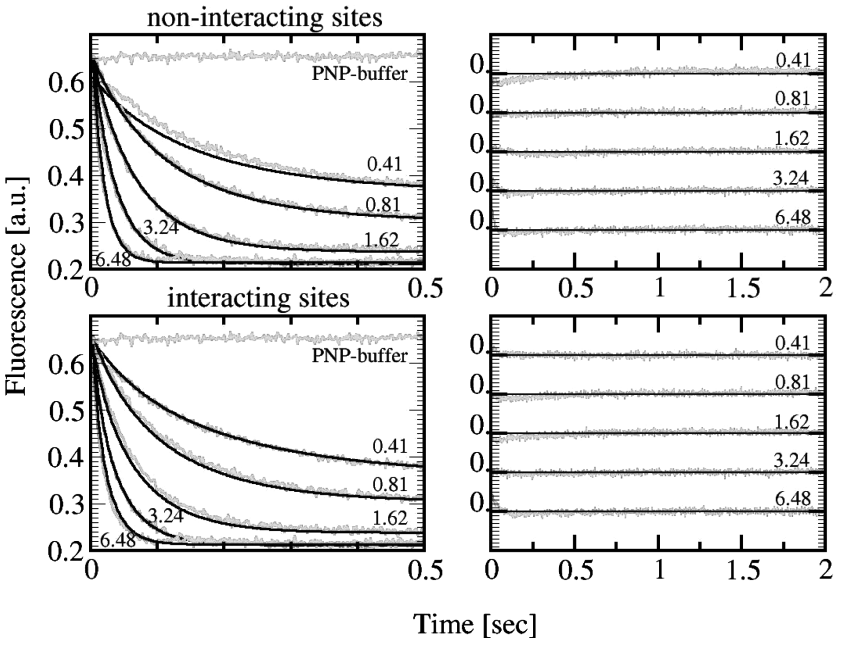

FIGURE 1 Results of fitting kinetic traces obtained after mixing of solutions of PNP and PME-6-thio-Gua at pH 7.0, using one-step binding models with non-interacting binding sites (model 1A, upper left panel) and interacting binding sites (model 1B, lower left panel). Graphs of residuals are shown on right panels. Concentrations of the ligand in the stopped-flow spectrometer syringe are given in μM. Only the first 0.5 seconds of the traces are shown for better illustration of differences between models 1A and 1B. For the clarity, traces for the three highest ligand concentrations were omitted. Small ticks on the vertical axis of all four panels correspond to the same value of the measured signal [0.01 Volt].

with three identical binding sites, were assumed. The first model (1) considers binding by each of the three binding sites of PNP as a one-step process, and the second model (2) assumes a two-step association process by each site (binding followed by the enzyme conformational change). In addition for each model independent (models 1A, 2A) and interacting (1B, 2B) binding sites were taken into account. Figure 2 presents the two-step binding model, while its upper row corresponds to the one-step binding mechanism. Parameters obtained from fitting all four models to kinetic traces from two independent experiments are summarized in Table 1 (description of the rate constants is shown in Figure 2).

These results show that, in contrast to the transition-state analogue guanine,[3] the one-step binding mechanism of PME-6-thio-Gua by each of PNP subunits is sufficient to explain experimental data. Attempts to fit two-step models do not improve substantially the agreement with the experiment and result in unreliable values of the parameters (e.g., some of the rate constants are too high to be measured by stopped-flow technique). Mean values of association and dissociation rate constants, resulting from fitting of one-step model, for individual (non-interacting) sites are

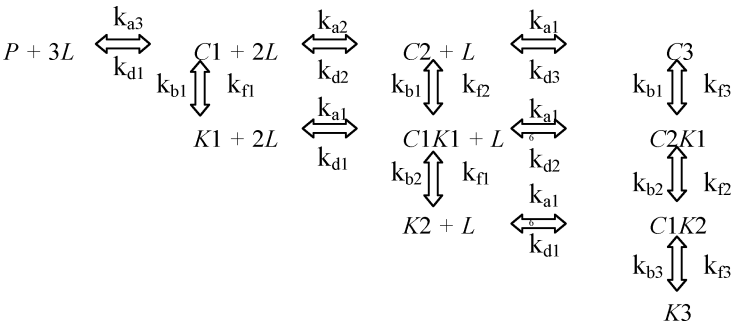

FIGURE 2 Reaction scheme describing binding of a ligand to trimeric PNP consistent with two-step model with interacting binding sites (2B). *P* is the free trimeric PNP molecule, *L* is the free ligand, *C*I is the complex of protein with I ligand molecules (I = 1, 2, or 3) with all binding sites not undergoing conformational change, *K*I is the respective complex with all binding sites after conformational change, *C*IKJ is the complex with I+J ligand molecules with I and J binding sites before and after conformational change, respectively. Model 2A, which assumes no conformational change of the enzyme and interactions between active sites, is just the upper horizontal line of the Figure. In models 1A and 2A with independent (not-interacting) binding sites appropriate rate constants are related $k_{a1}:k_{a2}:k_{a3} = k_{d1}:k_{d2}:k_{d3} = 1:2:3$ and $k_{a1}:k_{a2}:k_{a3} = k_{d1}:k_{d2}:k_{d3} = k_{f1}:k_{f2}:k_{f3} = k_{b1}:k_{b2}:k_{b3} = 1:2:3$, respectively. These relations are a direct result of the probability of association of one ligand molecule by the protein having 1, 2, or 3 empty binding sites, or of the probability of releasing of one ligand molecule from the protein with 1, 2, or 3 occupied sites.

$19 \pm 2.6\ \mu M^{-1}s^{-1}$ and $2.03 \pm 0.23\ s^{-1}$, respectively. These leads to a dissociation constant $K_d = 0.11 \pm 0.02\ \mu M^{-1}$ in agreement with our previous steady-state measurement $K_d = 0.12 \pm 0.01\ \mu M^{-1}$,[9] supporting the validity of the applied model of association.

Attempts to include interactions between binding sites, i.e., fitting 9 independent parameters instead of 3, improves the fits only slightly or not at all (see root-mean-square deviation, RMSD, for experiments 2 and 1, respectively). Moreover, it is not clear at this moment if the rate constants obtained from such fits are physically meaningful. However, interacting binding sites model could not be excluded at this stage since it gives better residual plots (Figure 1, right panels). Namely, for the lowest ligand concentration trace (0.41 μM) for non-interacting sites model, residual plots show that experimental points are not randomly scattered above and below the horizontal axis (upper right panel). In addition, RMSD for this concentration is almost twice that for each of the remaining traces (data not shown). This is not the case for the interacting-sites model (lower right panel).

The data obtained with interacting sites indicate that binding of the second ligand molecule is characterized by significantly increased association and dissociation rate constants, resulting in a higher affinity constant. Clarification of these issues requires further study involving more than one ground-state analogue ligand.

TABLE 1 Parameters obtained from fitting four various models to the two sets of the stopped-flow kinetic data (experiment 1 and 2) of association of PME-6-thio-Gua to *Cellulomonas* sp. trimeric PNP. The fluorescence intensities of the free PNP and PME-6thioGua were obtained from independent measurements. See text and Figure 2 for description of models and of the rate constants. Fluorescence intensities of different forms of the complex are given as relative to the measured signal of the free PNP solution. RMSD values are given in units of the signal measured in the experiments, i.e., volts

	Experiment 1				Experiment 2			
	Model				Model			
Parameter [units]	1A	1B	2A	2B	1A	1B	2A	2B
k_{a1} [$\mu M^{-1}s^{-1}$]	17.1	10.7	17.2	0.84	20.9	15.7	21.6	14.4
k_{a2} [$\mu M^{-1}s^{-1}$]	34.2	50.6	34.4	11.7	41.7	55.0	43.3	112
k_{a3} [$\mu M^{-1}s^{-1}$]	51.3	31.2	51.6	28.1	62.2	81.4	64.9	50.9
k_{d1} [s^{-1}]	1.87	1.74	3.06	1.93	2.19	8.64	1.86	4.54
k_{d2} [s^{-1}]	3.74	3.09	6.11	4.12	4.39	0.64	3.73	1.76
k_{d3} [s^{-1}]	5.61	9.03	9.17	18.1	6.58	4.74	5.59	7.07
k_{f1} [s^{-1}]	—	—	720	34.3	—	—	3556	79.4
k_{f2} [s^{-1}]	—	—	1440	5.02	—	—	7312	272
k_{f3} [s^{-1}]	—	—	2160	2.58	—	—	10969	243
k_{b1} [s^{-1}]	—	—	1022	92.1	—	—	8660	70.4
k_{b2} [s^{-1}]	—	—	2045	26453	—	—	17321	256
k_{b3} [s^{-1}]	—	—	3067	28650	—	—	25981	41550
F_{C1} [rel.]	0.746	0.490	0.745	0.386	0.756	0.640	0.755	0.025
F_{C2} [rel.]	0.491	0.494	0.489	0.284	0.512	0.610	0.509	0.525
F_{C3} [rel.]	0.237	0.190	0.234	0.081	0.268	0.280	0.264	0.169
F_{K1} [rel.]	—	—	0.745	0.812	—	—	0.755	1.214
F_{K2} [rel.]	—	—	0.489	0.174	—	—	0.509	0.587
F_{K3} [rel.]	—	—	0.234	0.068	—	—	0.264	0.458
F_{K1C1} [rel.]	—	—	0.489	0.151	—	—	0.509	0.659
F_{K2C2} [rel.]	—	—	0.234	0.061	—	—	0.264	0.265
F_{K3C3} [rel.]	—	—	0.234	0.048	—	—	0.264	0.326
RMSD [a.u.]	0.0517	0.0537	0.0513	0.0511	0.0543	0.0447	0.0553	0.0428

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