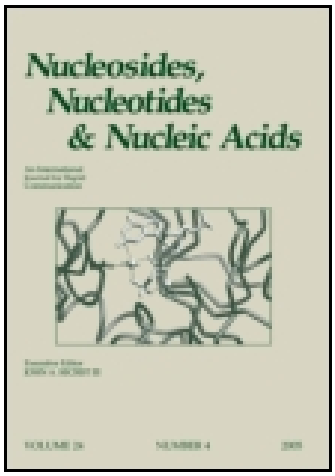

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Beata Wielgus-Kutrowska [a] & Agnieszka Bzowska [a]
[a] Department of Biophysics , Institute of Experimental Physics, University of Warsaw , Warsaw, Poland
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KINETIC PROPERTIES OF *CELLULOMONAS* SP. PURINE NUCLEOSIDE PHOSPHORYLASE WITH TYPICAL AND NON-TYPICAL SUBSTRATES: IMPLICATIONS FOR THE REACTION MECHANISM

Beata Wielgus-Kutrowska and Agnieszka Bzowska ▫ *Department of Biophysics, Institute of Experimental Physics, University of Warsaw, Warsaw, Poland*

▫ *Phosphorolysis catalyzed by Cellulomonas sp. PNP with typical nucleoside substrate, inosine (Ino), and non-typical 7-methylguanosine (m^7Guo), with either nucleoside or phosphate (P_i) as the varied substrate, kinetics of the reverse synthetic reaction with guanine (Gua) and ribose-1-phosphate (R1P) as the varied substrates, and product inhibition patterns of synthetic and phosphorolytic reaction pathways were studied by steady-state kinetic methods. It is concluded that, like for mammalian trimeric PNP, complex kinetic characteristics observed for Cellulomonas enzyme results from simultaneous occurrence of three phenomena. These are sequential but random, not ordered binding of substrates, tight binding of one substrate purine bases, leading to the circumstances that for such substrates (products) rapid-equilibrium assumptions do not hold, and a dual role of P_i, a substrate, and also a reaction modifier that helps to release a tightly bound purine base.*

Keywords Purine Nucleoside Phosphorylase, Catalytic Mechanism, Enzyme Kinetics, Non-Michaelis-Menten Kinetics

INTRODUCTION

Purine nucleoside phosphorylase (PNP, E.C. 2.4.2.1) is a ubiquitous enzyme of the purine salvage metabolic pathway. In eukaryotes and in some bacteria trimeric enzyme is present which catalyses the reversible phosphorolysis of 6-oxo-purine ribo- and 2′-deoxyribonucleosides, as follows: β-D-purine nucleoside + orthophosphate ⟺ purine base + α-D-pentose-1-phosphate. Hexameric PNP of prokaryotic origin accepts also 6-amino-purine and their nucleosides as substrates. Both classes of PNPs are important targets for chemotherapy. Potent inhibitors of human and parasitic PNPs are considered potential immunosuppressive and antiparasitic agents, respectively, while broad specificity of some prokaryotic phosphorylases

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Address correspondence to Agnieszka Bzowska, Department of Biophysics, Institute of Experimental Physics, University of Warsaw, 93 Zwirki & Wigury St., Warsaw 02-089, Poland; E-mail: abzowska@biogeo.uw.edu.pl

makes them ideal candidates for gene therapy of some tumours and also a tool for enzymatic synthesis of nucleosides.[1]

The mechanism of action of PNP still needs to be clarified and one of the important questions regards possible interactions of enzyme subunits upon substrate binding and in the process of catalysis. PNPs isolated from various sources show complex kinetic characteristics which could indicate cooperativity between monomers forming the entire enzyme.[1] In fact, for hexameric *E. coli* PNP it was demonstrated by X-ray studies in the crystal and fluorescence studies in solution that binding of phosphate and nucleoside is associated with negative cooperativity.[2,3] By contrast, for trimeric mammalian PNPs negative cooperativity was documented only for transition-state events.[4–6] Ground-state analogues seem to bind uniformly to all three sites of calf spleen PNP[7,8] 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.[7] Here we extend our studies in solution for trimeric, bacterial origin PNP (isolated from *Cellulomonas* sp.), and show by kinetic methods that the above conclusion is valid not only for mammalian, but also for bacterial trimeric PNP.

MATERIALS AND METHODS

Materials

Hepes (ultra pure), Na_2HPO_4, NaH_2PO_4, α-D-ribose-1-phosphate (R1P), inosine (Ino), guanosine (Guo), 7-methylguanosine (m^7Guo), guanine (Gua), hypoxanthine (Hx), and other chemicals were products of Sigma or Fluka. All solutions were prepared with high-quality MiliQ water. Commercially available PNP from *Cellulomonas* sp. (Toyobo Co., Japan) was purified on a BioCad station (PerSeptive Biosystems) by ion-exchange chromatography as described[9] to a maximal final specific activity of 125 U/mg. Xanthine oxidase (XO) from buttermilk (1 U/mg at 25°C) was purchased from Sigma.

Concentrations

Concentration of Gua, nucleosides and PNP were determined spectrophotometrically from their molar extinction coefficients at pH 7.0: Gua ε_{246} = 10 700 M^{-1} cm^{-1}, Ino ε_{249} = 12,300 $M^{-1}cm^{-1}$, m^7Guo ε_{260} = 8,500 $M^{-1}cm^{-1}$, Guo $\varepsilon_{252,5}$ = 13,650 $M^{-1}cm^{-1}$; PNP ε_{280} = 9.6 cm^{-1} for a 1% solution.[7] The concentrations of ligands that show no absorbance in the UV-VIS region (phosphate, P_i, and R1P) were calculated from their molecular mass.

Apparatus

Spectral data were collected on a Kontron (Switzerland) Uvikon 930. A Beckman model Ö300 pH-meter, was used for pH determinations. Data were analyzed using the PC GraphPad Prism 3.0 (GraphPad Software Inc.) and Origin 6.0 (Microcal) computer software packages.

Kinetic Procedures

The specific activity of PNP was measured spectrophotometrically with the use of Kalckar's method, where inosine was the substrate and the product, hypoxanthine (Hx), was oxidized by xanthine oxidase to form uric acid,[10] $\Delta\varepsilon = 9{,}600\ M^{-1}cm^{-1}$ for 300 nm. With Guo and m^7Guo as substrates a direct assay was employed based on marked differences in extinction coefficients of these nucleosides and products of their phosphorolysis: $\Delta\varepsilon = 4{,}600\ M^{-1}cm^{-1}$ at 260 nm at pH 7.0 for m^7Guo and $\Delta\varepsilon = 5{,}500\ M^{-1}cm^{-1}$ at 258 nm for Guo.[7]

The reaction mixtures contained 50 mM Hepes pH 7.0, both substrates (nucleoside and phosphate for the measurements of the phosphorolytic reaction, or purine base and ribose-1-phosphate for the measurements of the synthetic reaction). In the case of Ino phosphorolysis coupled assay was used, so xanthine oxidase was also present, while for product inhibition studies the appropriate product was included in the reaction mixture. Cuvettes with 10 mm, 2 mm, or 1 mm path length were used. The reaction was started by the addition of PNP. The initial velocity of the reactions was calculated by the linear regression of absorbance versus time.

Lineweaver-Burk transformation was used to analyze kinetics of phosphorolysis and synthesis, and product inhibition data.[11] Eqs. 1 and 2 were fitted to kinetic data of phosphorolysis obtained over a broad concentration range of a variable substrate. Statistically significant decrease in the sum of residuals, judged by the Snedecor's F test at a 95% confidence level, residual plots, and P value from the runs test were used to determine whether the data differ significantly from the equation selected and to discriminate between possible models.

RESULTS

Kinetics of phosphorolysis and synthesis for Ino, P_i, R1P, and Gua were measured for a variable concentration of one substrate and for four different, constant concentrations of a second substrate. For these conditions linear double reciprocal plots were obtained (but see below). The crossing of straight lines observed for phosphorolysis indicate the sequential mechanism, while the parallelism of lines for synthesis surprisingly suggest the ping-pong mechanism for this reaction direction.[11]

To verify these contradictory results product inhibition pattern and apparent inhibition constants were determined for the phosphorolytic and synthetic reactions with Ino and P_i, and Gua and R1P as the varied substrates and respective products. Gua was a competitive inhibitor for P_i and for Ino as the varied substrates, with K_i in μM range (2.5 ± 0.5 μM and 3.6 ± 0.6 μM, respectively). R1P causes mixed inhibition, with K_i in μM range for P_i (25 ± 5 μM; 320 ± 40 mM) and in mM range for Ino (7.5 ± 0.5 mM; 10.2 ± 0.4 mM) as the varied substrates. The synthesis reaction was inhibited much weaker by the products (Guo and P_i), either with K_i in mM range or, in one case, no inhibition was observed (for Gua as the varied substrate and Guo as the inhibitor). Inhibition of R1P by Guo (K_i ~0.8 mM), and

Gua by P_i (K_i 4.7 ± 1.0 mM) is uncompetitive, while of R1P by P_i is competitive (K_i = 2.5 ± 0.8 mM). The inhibition pattern described above does not agree with any Bi–Bi reaction mechanism.[12]

In the next step the phosphorolytic reaction of inosine and 7-methylguanosine was investigated over a broad concentration range of a varied substrate (P_i, Figure 1, and nucleoside, not shown), and, in the case of Ino as a varied substrate, at various levels of a co-substrate. Kinetics (initial velocity, v_o versus variable substrate concentration, c_o) is clearly non-hyperbolic for some substrates. Kinetic data was therefore analyzed either by the rational function of degree 1:1 (Michaelis-Menten equation):

$$v_o(c_o) = \frac{V_{\max} c_o}{c_o + K_m} \tag{1}$$

or by the rational function of degree 2:2, which is the mathematical expression describing several reaction mechanisms (interpretation of constants i, j, l, and m depends on the mechanism):[11]

$$v_o(c_o) = \frac{jc_o + ic_o^2}{1 + mc_o + lc_o^2} \tag{2}$$

The Michaelis-Menten model is sufficient for phosphate (P_i) as the varied substrate with 200 μM of m^7Gua (Figure 1, panel B) and for Ino as the varied substrate with 0.5 mM and 50 mM of P_i (not shown). However, for the P_i as the

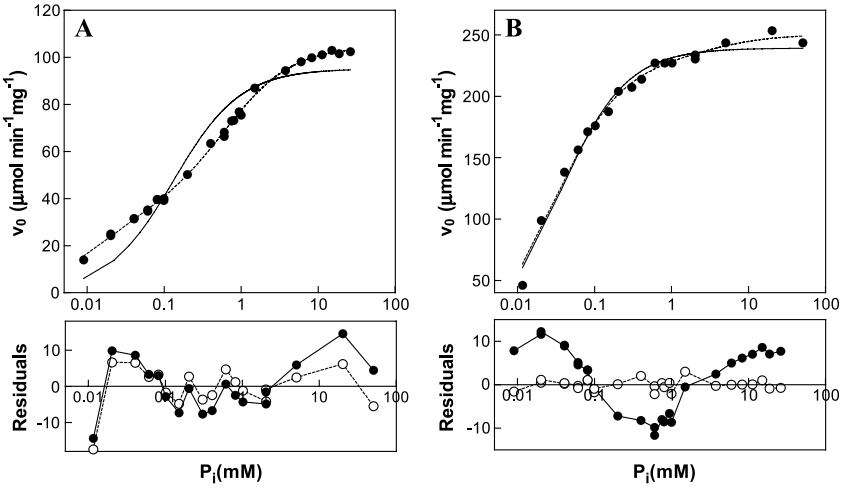

FIGURE 1 Kinetics of phosphorolysis of Ino (A) and m^7Guo (B) studied with P_i as variable substrate. Reactions were conducted at 25°C in 50 mM Hepes buffer pH 7.0 and followed by the coupled XOassay for Ino[10] and direct assay for m^7Guo.[15] In both cases, Michaelis-Menten equation and rational function of degree 2:2 were fitted (solid and broken lines, respectively, see Eqs. 1 and 2). Residuals for both fits are shown as closed and open symbols for Michaelis-Menten and 2:2 equation, respectively.

varied substrate with 1 mM Ino (Figure 1, panel A), for Ino as the varied substrate with 0.05 mM P_i, and for m^7Guo with 50 mM P_i (not shown), the kinetic data could only be described by the rational function of degree 2:2.

DISCUSSION

All experiments described above indicate that the reaction catalyzed by *Cellulomonas* PNP, similar like the catalytic process for mammalian trimeric PNPs,[7] cannot be sufficiently described by the Michaelis-Menten kinetic model. However, deviations from this model are pronounced stronger or weaker and vary markedly for various substrates. Hence, interpretation of kinetic data on the basis of Michaelis-Menten equation is in principle incorrect and may lead to puzzling and contradictory conclusions like those presented in the first two paragraphs of the Results. The kinetic data for *Cellulomonas* PNP could only be described by the rational function of degree 2:2., i.e., by the model with quadratic terms in c_o, which is characteristic for several reaction mechanisms, e.g., allosteric interactions between enzyme subunits but also steady-state random systems.[11] Steady-state kinetic data alone can not distinguish between these two mechanisms. However, data presented here, together with our earlier results[13,14] obtained by means of fluorimetric titrations and protective effects of ligands on the thermal inactivation of the enzyme point to the steady-state random system. We have shown: 1) formation of binary complexes with the enzyme for all substrates (purine base, R1P, P_i, and nucleoside), 2) stoichiometry of three molecules bound per enzyme trimer for several ground-state ligands, and 3) potent binding of some purines like Gua and Hx, (but not 7-methylguanine), in the absence of P_i, and less potent binding when P_i is present.[13,14] Therefore, it seems that for *Cellulomonas* sp. PNP, like for mammalian trimeric PNPs,[7] complex kinetic characteristics results from simultaneous occurrence of three phenomena. These are: 1) sequential but random, not ordered binding of substrates, 2) tight binding of one substrate purine bases, e.g., Gua and Hx, leading to the circumstances that for such substrates (products) rapid-equilibrium assumptions do not hold, and 3) a dual role of P_i—a substrate and also a reaction modifier that helps to release a tightly bound purine base. Under these assumptions and limitations all puzzling results can be interpreted.

For example the rational function of degree 2:2 in general should be used to describe kinetic data obtained for PNP from *Cellulomonas* sp. However, in some cases when 1) either one substrate is present in saturating concentration and randomness of the reaction disappears (phosphorolysis of Ino with 50 mM and 0.5 mM P_i) or 2) binding of product is not potent (e.g., for 7-methylguanine) and rapid-equilibrium assumptions hold, kinetics simplifies to the one described by the Michaelis-Menten equation.

Also the occurrence of the seemingly parallel lines for synthesis of Gua pointing to the ping-pong mechanism, which for sure is not valid for PNP,[1] is intelligible. Some sequential systems are known to appear as to be ping-pong,

among others a non-rapid equilibrium random system where the rate constants for release of substrates are lower than the maximal velocity.[11] In fact, release of Gua is very slow especially when there is no phosphate, which helps in this process.

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