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BINDING OF SUBSTRATES BY PURINE NUCLEOSIDE PHOSPHORYLASE (PNP) FROM *CELLULOMONAS SP.* - KINETIC AND SPECTROFLUORIMETRIC STUDIES.

B. Wielgus-Kutrowska[1], J. Tebbe[2], J.Wierzchowski[3], D. Shugar[1], W. Saenger[2], G. Koellner[2] and A. Bzowska[1*]

[1]Department of Biophysics, Institute of Experimental Physics, University of Warsaw, Żwirki i Wigury 93, 02 089 Warsaw, Poland; [2]Institute für Kristallographie, Freie Universität Berlin, Takustraβe 6, D-14195 Berlin, Germany; [3]Department of Organic Chemistry, Pedagogical University, 3 Maja 54, 08-110 Siedlce, Poland

ABSTRACT: Dissociation constants and stoichiometry of binding for interaction of *Cellulomonas sp.* purine nucleoside phosphorylase with its substrates: inosine/guanosine, orthophosphate, guanine/hypoxanthine and D-ribose-1-phosphate were studied by kinetic and spectrofluorimetric methods.

Purine nucleoside phosphorylase (PNP, E.C. 2.4.2.1.) from *Cellulomonas sp.* catalyzes the reversible phosphorolysis of inosine (Ino) and guanosine (Guo), and some analogues, as follows: β-purine nucleoside + orthophosphate ⇔ purine base + α-D-pentose-1-phosphate. Adenosine is not a substrate, but is a competitive inhibitor of Ino phosphorolysis[1]. Hence *Cellulomonas* PNP, although trimeric, cannot be assigned to one of the two main classes of PNPs: "high-molecular weight" hexameric bacterial enzymes, with broad specificity towards nucleosides[2,3], and "low-molecular weight" trimeric, mainly mammalian, PNPs specific for both binding and catalysis of 6-oxopurine nucleosides[4,5].

Commercial *Cellulomonas* PNP (Toyobo, Japan) was purified as previously described[1]. Michaelis constants (K_m) and maximal velocities (V_{max}) were determined for both substrates, i.e. for Ino/Guo and orthophosphate (P_i). K_m is 43 and 11 μM for Ino and Guo, respectively, and the Hill coefficient is 1. Kinetic plots for P_i were not linear, suggesting negative cooperativity or two classes of binding sites (as observed for both

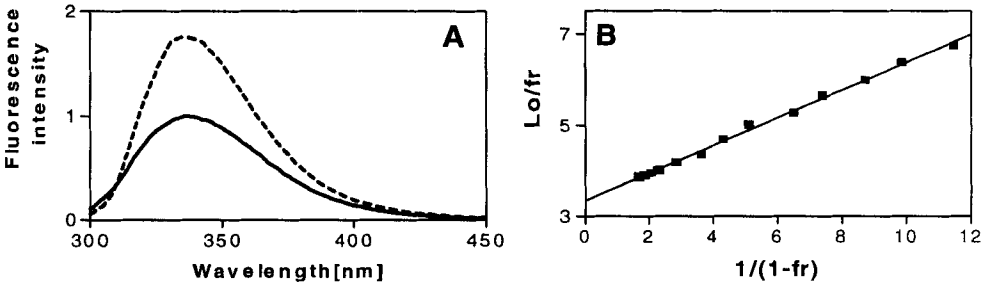

Figure 1: **A** – Fluorescence spectra (λ_{exc} = 290 nm) of PNP from *Cellulomonas sp.* in 50 mM Hepes buffer pH=7(——), and (– –) with 8 μM Gua (saturating concentration); **B** – Fluorimetric titration (λ_{obs} = 340 nm) of *Cellulomonas sp.* (trimer E_0, 1.05 μM) with Gua (2.3 – 6.2 μM) expressed as: $[L_0]/fr = K_d/(1-f_r)+N[E_0]$, where L_0 is total concentration of Gua; $f_r = (F_0-F)/(F_0-F_\infty)$; F, F_0 and F_∞ are the fluorescence intensities in the presence, absence and saturating concentration of Gua; K_d, dissociation constant; N, number of binding sites per PNP trimer.

"low-" and "high-"molecular weight PNPs). Formation of binary complexes of *Cellulomonas* PNP with guanine and hypoxanthine (Gua, Hx), as well as P_i and D-ribose-1-phosphate (rib-1-P), is accompanied by a marked increase of the intrinsic fluorescence of the protein, most pronounced for Gua (Fig. 1A). Dissociation constants and stoichiometry of binding were determined by fluorimetric titration of PNP in the presence of Hx, Gua, P_i and 1-rib-P. Gua and Hx bind with K_d = 0.40 ± 0.05 μM and 2.0 ± 0.5 μM , and N = 1.07 ± 0.05 and 0.9 ± 0.23, respectively. Hence three molecules are bound per enzyme trimer (Fig. 1B). Assuming there is one binding site per monomer, $F(L_0) = F_0-L_0(F_0-F_\infty)/(K_d+L_0)$, titration with rib-1-P gives K_d = 60 ± 4 μM. For P_i, in line with kinetic results suggesting negative cooperativity, a model with two binding constants, or two classes of binding sites, $F(L_0) = F_0-(F_0-F_\infty)[N_1L_0/(K_{d1}+L_0)+N_2L_0/(K_{d2}+L_0)]$, gave the best fit. Low- and high- affinity binding sites have K_d values in the mM and μM range, respectively. (Supported by Polish Committee for Scientific Research (KBN grant 6 P04A 062 09) and, in part, by the Howard Hughes Medical Institute (HHMI 75195-543401).

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