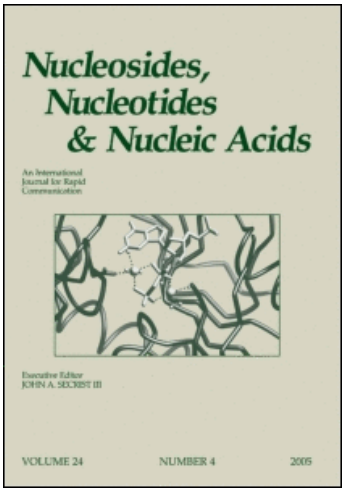

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Fluorescence studies of calf spleen purine nucleoside phosphorylase (PNP) complexes with guanine and 9-deazaguanine

Katarzyna Stępniak[a]; Biserka Žinić[b]; Jacek Wierzchowski[c]; Agnieszka Bzowska[a]
[a] Department of Biophysics, Institute of Experimental Physics, Warsaw University, Warsaw, Poland [b] Division of Organic Chemistry and Biochemistry, Rudjer Bošković Institute, Zagreb, Croatia [c] Department of Biophysics, University of Warmia and Mazury, Olsztyn, Poland

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FLUORESCENCE STUDIES OF CALF SPLEEN PURINE NUCLEOSIDE PHOSPHORYLASE (PNP) COMPLEXES WITH GUANINE AND 9-DEAZAGUANINE

Katarzyna Stępniak □ *Department of Biophysics, Institute of Experimental Physics, Warsaw University, Warsaw, Poland*

Biserka Žinić □ *Division of Organic Chemistry and Biochemistry, Rudjer Bošković Institute, Zagreb, Croatia*

Jacek Wierzchowski □ *Department of Biophysics, University of Warmia and Mazury, Olsztyn, Poland*

Agnieszka Bzowska □ *Department of Biophysics, Institute of Experimental Physics, Warsaw University, Warsaw, Poland*

□ *Interactions of trimeric calf spleen purine nucleoside phosphorylase (PNP) with guanine (Gua) and its analogue, 9-deazaguanine (9-deaza-Gua), were studied by means of the steady-state fluorescence. The aim was to test the hypothesis that the enzyme stabilizes the anionic form of purine, inferred previously from the unusual increase of fluorescence observed after binding of guanine by calf spleen PNP. We have found that the dissociation constants obtained form titration experiments are in fact pH-independent in the range 7.0-10.25 for both PNP/Gua and PNP/9-deaza-Gua complexes. In particular, at pH 7.0 we found* $K_d = 0.12 \pm 0.02\ \mu M$ *for Gua and* $0.16 \pm 0.01\ \mu M$ *for 9-deaza-Gua, while at the conditions where there is more than 40% of the anionic form the respective values were* $K_d = 0.15 \pm 0.01\ \mu M$ *for Gua (pH 9.0) and* $0.25 \pm 0.02\ \mu M$ *for 9-deaza-Gua (pH 10.25). Hence, the enzyme does not prefer binding of anionic forms of these ligands in respect to the neutral ones. This result questions the involvement of the anionic forms in the reaction catalyzed by trimeric PNPs, and contradicts the hypothesis of a strong hydrogen bond formation between the enzyme Asn 243 residue and the purine N(7) position.*

Keywords PNP; guanine; 9-deazaguanine; fluorescence

INTRODUCTION

Purine nucleoside phosphorylase (PNP, E.C. 2.4.2.1) fulfills the role of the regulation factor to the nucleoside level, DNA synthesis, and T-cell proliferation in the organism.[1] For these reasons the inhibitors of the enzyme

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

are of interest for clinical studies. One of them, immucilin H (BCX 1777), the strong inhibitor of human PNP, is currently tested as an immunopressive drug for T-type cell leukemia.[2]

PNP conducts the reversible reaction:

β-purine nucleoside + orthophosphate $\leftrightarrow$ purine base + α-D-pentose-1-phosphate.

The equilibrium of this reaction favors nucleoside synthesis over phosphorolysis so the enzyme can be used for the effective purine nucleoside synthesis.[1] Homotrimeric and homohexameric PNPs are known and substrate specificity depends on enzyme source and subunit composition. While homotirmeric PNP are highly specific for 6-ketopurine nucleosides, homohexameric phosphorylases show much broader specificity.[1]

Calf spleen PNP, a homotrimer, is characterized by complicated kinetics caused by the dual regulatory and substrate role of phosphate and strong binding of some of the purine bases resulting in their rate-limiting dissociation from the active site.[3] It serves as a model for studying trimeric, including human, PNPs. The details of the molecular mechanism of the reaction catalyzed by phosphorylases are not fully known, especially for trimeric PNPs. In particular the tautomeric/ionic form of purine compound stabilized by the active side of the enzyme, and the reasons of preference of 6-oxopurines over 6-aminopurines remain unclear.[1]

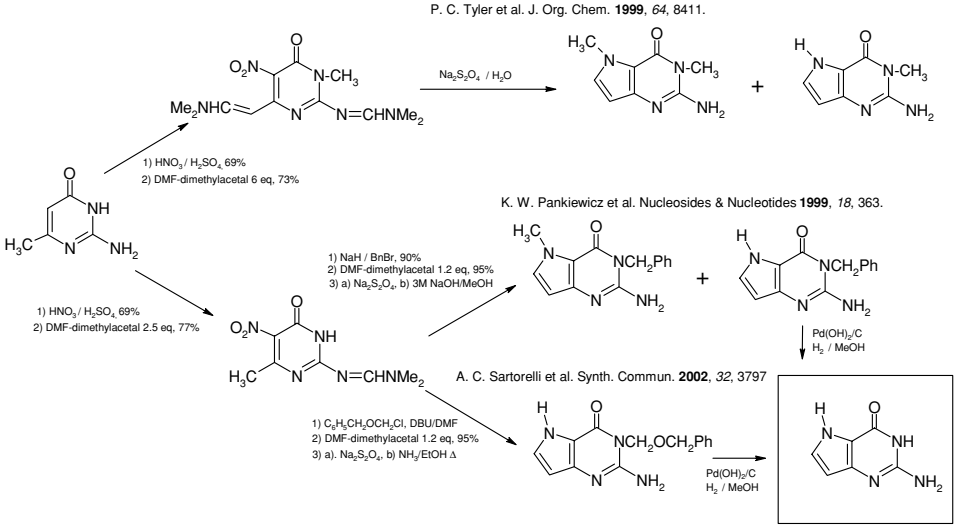
One of the existing hypotheses concerning catalytic mechanism for trimeric PNPs was based on mutation experiments of the human enzyme.[4] and the previous suggestion that the N(1) fluorescent anionic form of Gua, formed in pH above 9, is stabilized in the neutral pH by the calf spleen PNP active site.[5] This mechanism postulates deprotonation of either N(1)-H[5] or N(9)-H[4] of the purine ring of the substrate upon its binding with the enzyme and/or in the transition state. In the second case the negative charge would be dislocated between N(9) and N(7) positions, and stabilized by the Asn243 hydrogen bond donation to the purine position N(7).[4] It, therefore, implies that the enzyme prefers binding of the anionic form of the purine base. However, this mechanism is not fully consistent with the crystallographic findings for trimeric PNP from *Cellulomonas sp.*, where Asn246 (Asn243 in mammalian PNPs) does not interact with the purine base directly, but via a water molecule. In the reaction catalyzed by this enzyme the crucial role was therefore attributed to Glu 204, which was proposed to stabilize the negative charge at the purine position O(6) by stabilizing a rare enolate anionic form of the base.[6] The following proton transfer from ribose-1-phosphate leads to the release of the neutral purine base. The mechanism proposed for *Cellulomonas sp.* PNP was later claimed to be correct also for human[7] and *Thermus thermophilus* phosphorylases.[8] The other alternative mechanism, based on the idea of acid-catalyzed hydrolysis of purine nucleosides, assumes protonation of the purine ring N(7) at the transition state.[9] After the glycosidic bond cleavage the neutral form of

the purine is released. Since ther is no acidic residue that could be responsible for the proton transfer occuring in this mechanism it was proposed that the proton could be transfered form the bulk solvent via a chain of the immobilized water molecules, which are present in the active site of the enzyme.

In order to distinguish between the proposed mechanism, in the studies described here we analyze interactions of calf spleen PNP with its natural substrate, guanine, and its analogue, 9-deazaguanine, with the aim to answer the question which ionic form, anion or neutral species, is bound in the enzyme active site.

MATERIALS AND METHODS

Calf spleen PNP and guanine (Gua) were purchased from Sigma. 9-Deazaguanine (9-deaza-Gua) was synthesized as described in literature procedure (Scheme 1).[15–17] The protein and ligand concentrations were determined spectrophotometrically from their extinction coefficients: 1% PNP solution $\varepsilon(278\ \text{nm}) = 9.6\ \text{cm}^{-1}$, Gua neutral form $\varepsilon(246\ \text{nm}) = 10\,700\ \text{M}^{-1}\ \text{cm}^{-1}$, 9-deaza-Gua neutral form $\varepsilon(266\text{nm}) = 6\,600\ \text{M}^{-1}\ \text{cm}^{-1}$. The fluorimetric titrations with Gua were done in 50 mM HEPES buffer at pH 7.0 and 8.0, and in 50 mM HEBPS buffer at pH 8.0, 9.0, and 9.2. The measurements for 9-deaza-Gua were carried out in 50 mM HEPES pH 7.0 and in 50 mM CAPSO pH 9.0, 9.6, 10.0, and 10.25. The loss in the enzyme activity during the experiments under the non-physiological conditions did not exceed 15%. The enzyme concentration was in the range 0.3–1.5 μM



SCHEME 1

and the ligand concentration was in the range 80 pM to 20 μM for Gua, and 120 pM to 15 μM for 9-deaza-Gua. All titration experiments were conducted at 25°C, in the absence of phosphate. The results were corrected for dilution of the sample during titrations and for the inner filter effect. The model assuming one type of non-interacting binding sites was fitted to the data.[10]

The emission spectra were recorded for 0.5–6 μM Gua and 9-deaza-Gua concentrations. The complex solutions were prepared by mixing 6 μM Gua with 1.5 μM PNP, 2 μM Gua with 0.6 μM of the enzyme monomer, 10.6 μM 9-deaza-Gua with 5.7 μM of the protein, and 3.5 μM. 9-deaza-Gua with 0.5 μM PNP. In these conditions about 90% of the enzyme active sites are occupied. Somewhat higher concentrations were used for recording absorption spectra, the lower for fluorescence measurements. The spectra of the PNP-ligand complex were calculated by subtraction from the PNP/ligand complex spectra the spectra of free PNP and that of unbound Gua or 9-deaza-Gua. The appropriate concentrations were determined from dissociation constant values obtained form titration experiments and the enzyme activity versus inosine.

RESULTS AND DISSCUSSION

Binding of Gua and 9-deaza-Gua by calf spleen PNP causes about 170–180% fluorescence increase of the mixture (Figure 1), as already noted by Proter[2] for PNP/Gua complex. This behaviour is exceptional among natural PNP substrates like phosphate or hypoxanthine, where only some

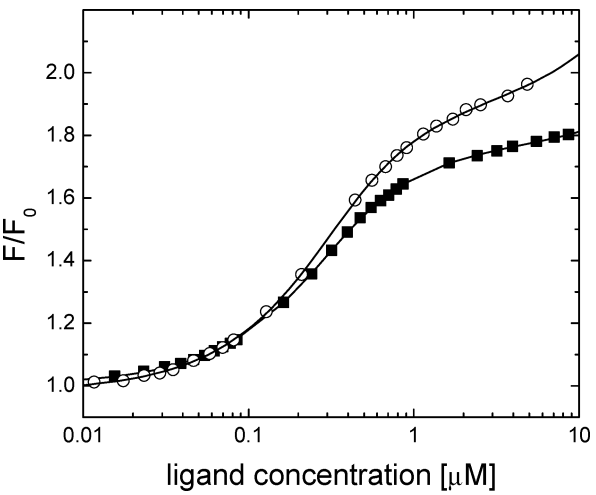


FIGURE 1 Fluorescence titrations of calf spleen PNP by Gua (squares) and 9-deaza-Gua (circles) at pH 7.0 in 50 mM HEPES buffer. The enzyme concentration was 0.5 μM. Excitation and observation wavelengths were 290 nm and 340 nm.

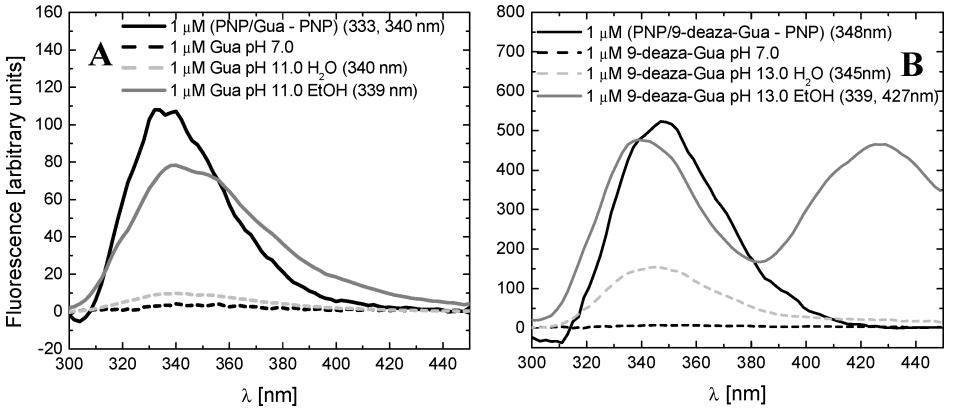


FIGURE 2 Fluorescence emission spectra of free ligands and fluorescence spectra of calf spleen PNP complexed with: (A) guanine, (B) 9-deaza-guanine. The neutral ligand form and spectra of the PNP/Gua and PNP/9-deaza-Gua complexes were recorded in 50 mM HEPES pH 7.0 (black dashed line). The spectra of anionic ligand forms were recorded in water (light gray dashed line) or in 95% ethanol (dark gray solid line) at pH 11 (1 mM NaOH) and at pH 13 (100 mM NaOH) for Gua and 9-deaza-Gua, respectively. The excitation wavelengths for emission spectra were 290 nm for Gua and 285 nm for 9-deaza-Gua. The spectra of the calf spleen PNP/ligand complex (black solid line) were calculated by subtraction from the recorded PNP/ligand complex spectra the spectra of free PNP and that of unbounded guanine or 9-deaza-Gua. All ligand spectra were calibrated to the concentration of 1 μM. The spectra maxima are given in brackets.

fluorescence quenching is observed.[3,5] The unbound Gua and 9-deaza-Gua virtually do not fluorescence at pH 7.0 (Figure 2), thus, the non typical raise of the fluorescence intensity of the PNP-ligand mixture can be most likely explained by formation of a complex with fluorescent tautomeric or ionic form of the purine ring in the PNP active site.

Both Gua and 9-deaza-Gua undergo dissiociation of N(1)-H proton at alkaline environment, with pK_a of 9.3 and 10.3, respectively. The anionic fluorescence in aqueous medium is in both cases much higher comparing to that of the neutral forms of the compounds (Figure 2). Furthermore, in the nonpolar environment (e.g., ethanolic), which can be a good approximation of the protein neighborhood of a purine compound at the enzyme active site, the anion emission increases over 7-fold for Gua (Figure 2A) and about 3-fold for 9-deaza-Gua (Figure 2B).

The comparison of recorded free Gua and 9-deaza-Gua emission spectra in alkaline ethanolic solution with the spectra of the protein-ligand complex revealed that the emission maxima and spectral distribution of these were not identical (Figure 2). Even more pronounced differences can be observed in the excitation spectra of the complex, which are red-shifted in respect to the anionic absorption and to protein spectra (data not shown). In addition, the significant differences between absorption spectra of PNP/ligand complex and the arithmetic sum of the enzyme and ligand spectra (at the same concentrations as were previously used for the

complex formation) were observed (not shown). We conclude that spectral data do not necessarily confim bonding of the anionic species of both Gua and 9-deazaGua to the protein active site, and the observed fluorescence increase may be explained by shifting of the tautomeric equilibrium towards the N(7)-H species, as previously demonstrated for PNP/8-azaguanine complex.[11]

The fluorimetric tiltration curves obtained for both ligands were satisfactorily well described by the model of identical noninteracting active sites. The equilibrium between free and occupied enzyme is characterized by one dissociation constant, which at pH 7.0 is $K_d = 0.12 \pm 0.02$ μM for Gua and $K_d = 0.16 \pm 0.01$ μM for 9-deaza-Gua (Figure 1). Similar titrations were conducted at pH ranges 7.0–9.2 for Gua and 7.0–10.25 for 9-deazaGua, which cover <1–44% (Gua) and <1–47% (9-deaza-Gua) of the ligand anion form contribution in solution. We did not observe any marked K_d change over the examined pH range, for example, dissociation constants obtained at pH 9.2 for Gua is 0.15 ± 0.01 μM, while at pH 10.25 for 9-deazaGua we found $K_d = 0.25 \pm 0.02$ μM. We once again conclude, on this basis, that the bovine PNP does not prefer binding N(1) anionic form of the purine compound over the neutral one.

Our fluorescence studies presented here show unequivocally that highly fluorescent purine species, which is formed after binding of Gua to the PNP is not the N(1) anion and that the enzyme does not preffer binding of the anion of purine base (Gua, 9-deaza-Gua). These results are in agreement with similar experiments conducted for interactions of calf spleen PNP with formycin B and its aglycone,[12] and with some fluorescent 8-azapurine analogues,[11] and for *Cellulomonas sp.* phosphorylase interactions with other ligands[13] and consequently supports two other postulated mechanisms. The unique high fluorescence of the bounded guanine can be explained differently, not by the preferential binding of the naturally occurying anionic guanine form with the negative charge localized at the position N(1) of the base. One possible explanation could be the assumption that the rare enolic form of the guanine anion is highly fluorescent. Alternatively, the strong emission may come from the neutral N(7)-H form of Gua immobilized in the enzyme active site, since it was shown previously that this form is fluorescent in rigid glasses.[14]

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