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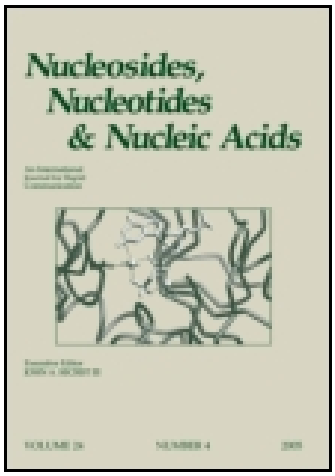

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SPECTROSCOPIC AND KINETIC STUDIES OF INTERACTIONS OF CALF SPLEEN PURINE NUCLEOSIDE PHOSPHORYLASE WITH 8-AZAGUANINE, AND ITS 9-(2-PHOSPHONYLMETHOXYETHYL) DERIVATIVE

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SPECTROSCOPIC AND KINETIC STUDIES OF INTERACTIONS OF CALF SPLEEN PURINE NUCLEOSIDE PHOSPHORYLASE WITH 8-AZAGUANINE, AND ITS 9-(2-PHOSPHONYLMETHOXYETHYL) DERIVATIVE

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▫ *Spectroscopic and kinetic studies of interactions of calf spleen purine nucleoside phosphorylase with 8-azaguanine, an excellent fluorescent/fluorogenic substrate for the synthetic pathway of the reaction, and its 9-(2-phosphonylmethoxyethyl) derivative, a bisubstrate analogue inhibitor, were carried out. The goal was to clarify the catalytic mechanism of the enzymatic reaction by identification of ionic/tautomeric forms of these ligands in the complex with PNP.*

Keywords Purine Nucleoside Phosphorylase, Catalytic Mechanism, Enzyme-Ligand Complexes, 8-Azaguanine, Fluorescence

INTRODUCTION

Purine nucleoside phosphorylase (PNP, E.C. 2.4.2.1), is a potential target for antimicrobial, antileukemic and antiparasitic therapies.[1] The enzyme catalyzes a reversible phosphorolysis of purine ribo-and 2′-deoxyribonucleosides, as follows:

$$\beta\text{–D–purine nucleoside} + \text{orthophosphate} \Longleftrightarrow$$
$$\text{purine base} + \alpha\text{–D–pentose–1–phosphate}$$

The reaction mechanism involves an oxocarbenium intermediate in the transition state,[2] but controversy remains regarding protonation of the purine N(7),

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and, consequently, the endpoint of the reaction. If, as postulated by Fedorov et al.,[2] N(7) is protonated, the product purine must be released as the N(7)H tautomer. But, in the absence of protonation, the purine must adopt either an anionic form,[3] or be neutralized by proton transfer from the second product, ribose-1-phosphate.[4]

We here apply fluorescence spectroscopy to analyze complexes of calf spleen PNP with 8-azaguanine (8-azaG, Scheme 1), a fluorescent substrate for the reverse synthetic pathway of the reaction,[5,6] and its 9-(2-phosphonylmethoxyethyl) derivative[7] (PME-azaG, see Scheme 1), a bisubstrate analogue inhibitor,[8] with the aim to identify tautomeric/ionic structures of the bound ligands, using methodology similar to that applied previously to bacterial PNP.[9]

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EXPERIMENTAL

Calf spleen PNP from Sigma (18–34 units/mg), following desalting, was only minimally contaminated with phosphate (≤ 1 μM), and PNP concentration was determined from the extinction of 9.6 cm^{-1} at 280 nm for a 1% solution.[10] 9-(2-Phosphonylmethoxyethyl)-8-azaguanine (PME-azaG) was synthesized by Holý et al.[7] Ribose-1-phosphate (R1P), 7-methylguanosine (m^7Guo), 8-azaG, and xanthine oxidase were products of Sigma. All other chemicals were of the highest purity available. Concentrations of stock solutions of 8-azaG, PME-azaG, and m^7Guo were determined spectrophotometrically[5,6,10]

Spectrophotometric measurements were carried out with Kontron (Austria) Uvikon-922, Uvikon-930, or Cary 3E (Varian) instruments, and fluorescence with a Perkin-Elmer LS-50 spectrofluorimeter (Norwalk, CT), all equipped with thermostatically controlled cell compartments. In fluorimetric measurements the spectral bandwidths were 2–5 nm for the entrance slit, and 3–15 nm for the exit slit. Fluorimetric titrations were performed and analyzed as elsewhere described.[6,11,12]

Kinetic Procedures

With m^7Guo and 8-azaG as substrates, direct spectrophotometric assays were based on differences in molar extinctions between substrates and products: $\Delta\varepsilon$ = 4 600 $M^{-1}cm^{-1}$ at 260 nm at pH 7.0 for m^7Guo[10] and $\Delta\varepsilon$ = 6 500 $M^{-1}cm^{-1}$ at 260 nm and pH 7.0 for 8-azaG. For 8-azaG also fluorimetric method was employed.[5,6] The initial velocity data were fitted to the generalized kinetic equation:

$$\frac{V_{\max}}{V} = 1 + \frac{K_{m1}}{[S_1]} + \frac{K_{m2}}{[S_2]} + \frac{K_{12}}{[S_1][S_2]} \quad (1)$$

Inhibition of the enzyme by the bisubstrate analogue inhibitor PME-azaG was analyzed assuming a double competitive model (i.e., assuming only a binary enzyme-inhibitor complex):

$$\frac{V_{\max}}{V} = 1 + \frac{K_{m1}}{[S_1]} + \frac{K_{m2}}{[S_2]} + \frac{K_{12}}{[S_1][S_2]}\left(1 + \frac{[I]}{K_i}\right) \quad (2)$$

where [I] is the inhibitor concentration and K_i the inhibition constant. With one of the substrates (S_2) at constant concentration, it is possible to determine the apparent inhibition constants, K_i^{app}, by measuring initial rates with varying concentrations of the second substrate (S_1) and the inhibitor. The thermodynamic value of the inhibition constant K_i is related to K_i^{app} by:

$$K_i^{app} = K_i\left(1 + \frac{[S_2]}{K_{m2}}\right) \quad (3)$$

RESULTS AND DISCUSSION

8-AzaG is a good substrate for the calf PNP in the reverse, synthetic pathway.[5] Kinetic parameters for this reaction, recently re-evaluated using desalted calf enzyme and phosphate-free buffers[6] are highly pH-dependent, the K_m increasing from 90 μM at pH 5.9 to ~700 μM at pH 8, with virtually constant V_{max}. This behavior, contrasting with that of guanine, indicates that the monoanionic form of 8-azaG, predominating at pH > 7 (pK_a 6.5), is not a substrate for the enzyme.

The increased acidity of 8-azapurines, relative to the parent purines, results from high acidity of the triazole proton.[13] Therefore, the monoanionic form of 8-azaG bears a negative charge on the five-membered ring (Scheme 1), like that postulated in the Erion mechanism of PNP catalysis.[3] We conclude that inability of this form to react with PNP testifies against the model, and in favour a model postulating neutral purine as the substrate.

Neutral 8-azaG exist as a mixture of several tautomeric forms (Scheme 1), with N(9)H predominating in aqueous medium.[5] The compound is strongly fluorescent at ~390 nm, but its fluorescence excitation spectrum is red-shifted by ~20 nm relative to the UV absorption, indicating that this emission comes from a minor tautomer; i.e., N(8)H or N(7)H. To resolve which of these forms dominates when bound to the enzyme we examined fluorescence of the PNP/8-azaG complex.

Fluorimetric titration of calf PNP with 8-azaG with λ_{exc} 315 nm shows a strongly fluorescent complex with λ_{max} ~370 nm, with dissociation constants K_d of 90 ± 5 μM at pH 7.0, and ~450 μM at pH 7.7.[6] With excitation at 285 nm (Figure 1, left panel) significant quenching of protein fluorescence is additionally observed. The fluorescence excitation difference spectra, monitored with λ_{obs} 370 nm, exhibit a maximum at ~280 nm, which we interpret as a result of energy transfer from tryptophan to the ligand. But the entire spectrum is clearly red-shifted relative to both UV absorbance and fluorescence excitation of the free 8-azaG (Figure 1). It is also clearly distinct from that of the monoanion. These observations question both the N(9)H tautomer and the monoanion as the principal forms of 8-azaG in the complex with PNP.

PME-8azaG has been shown previously to bind to the calf PNP, leading to a highly fluorescent complex.[14] We selected this compound because of its fixed

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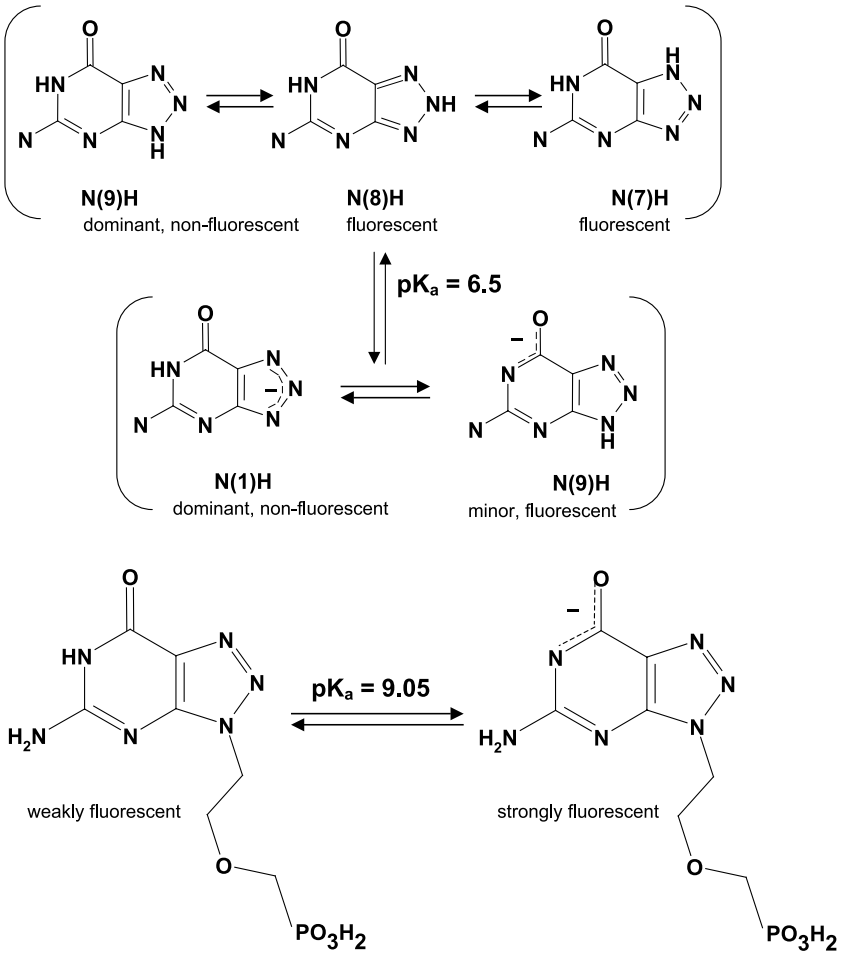

SCHEME 1

tautomeric structure, which is spectrally similar to the N(9)H form of 8-azaG (Scheme 1).

Inhibition of phosphorolysis by PME-8azaG was studied at pH 7.0, with m^7Guo as a substrate reasonably following Michaelis-Menten kinetics.[10] Dixon plots displayed a competitive mode of inhibition (not shown), but, in accordance with Eq. 3, plots of K_i^{app} *vs.* fixed substrate concentrations were linear in the concentration range of 0.1–5 mM for phosphate (P_i) and 20–300 μM for m^7Guo, giving intercepts (K_i) of (0.70 ± 0.28) μM for P_i, and (0.98 ± 0.21) μM for m^7Guo as fixed substrates, respectively.[6] In turn, we examined inhibition of the reverse synthetic reaction by PME-azaG, with 8-azaG and R1P as substrates, in phosphate-free buffer. As for the phosphorolytic pathway, an apparently competitive inhibition was observed, and the K_i^{app} values were markedly dependent on the second (fixed) substrate concentration. The calculated values of K_i = (1.9 ± 0.3) μM, obtained

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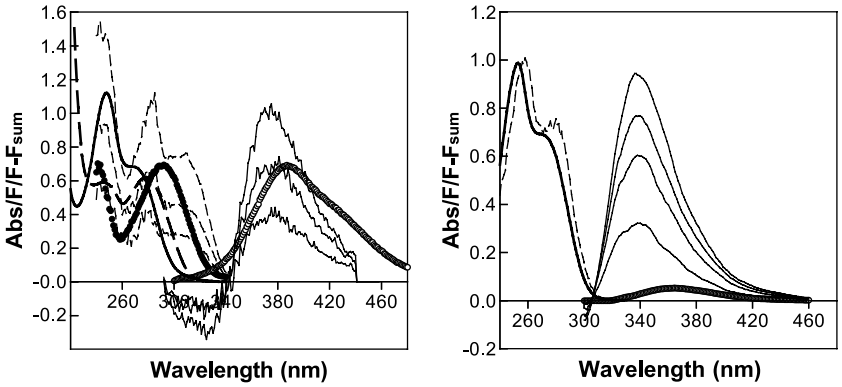

FIGURE 1 Left panel: Comparison of fluorescence excitation (- - -) and emission (—) difference spectra, on titration of calf PNP with 8-azaG at pH = 6.15, with absorption (– –) and fluorescence excitation (● ● ●) and emission (○ ○ ○) spectra of the neutral form of 8-azaG, and absorption spectrum (– –) of the monoanion of 8-azaG. Active site concentration is 5.2 μM, and ligand concentrations (— , bottom to top) are 2.7, 5.5, and 10.9 μM. Conditions: λ_{exc} 285 nm, λ_{obs} 370 nm; spectral resolution 3/5 nm. Right panel: Fluorescence difference emission (—) and excitation (- - -) spectra of PNP/PME-azaG complex at pH = 7.0. Active site concentration is 6.5 μM, and ligand concentrations (— , from bottom to top) are 2.3, 4.5, 5.9, 21.5 μM. Conditions: λ_{exc} 285 nm, λ_{obs} 340 nm; spectral resolution 3/5 nm. Absorption (– –) and fluorescence emission (○ ○ ○) spectra of PME-azaG are shown for comparison.

assuming a "double-competitive" model of inhibition (Eqs. 2 and 3), was independent of the fixed substrate concentration, consistent with competition of PME-azaG *vs.* both 8-azaG and R1P.

PME-azaG emits weakly at pH 7.0, but strongly as an anion (pK_a 9.0). Fluorimetric titration of PNP with PME-azaG at pH 7.0 gave K_d = (0.6 ± 0.3) μM, in agreement with kinetic results, and ligand emission at 340 nm increased about 10-fold upon binding (Figure 1, right panel). At first sight, this might be interpreted as preferential binding of the fluorescent anionic form. But the UV difference spectrum recorded during titration clearly does not resemble the UV spectral difference between the neutral and anionic species of PME-azaG.[6] Furthermore, the difference excitation spectrum resembles the UV spectrum of the neutral ligand (Figure 1), with ∼5 nm red shift, and clearly differs from that of the anion (not shown). We conclude that the ligand remains in the neutral form in the complex, and that enhancement of the emission of this form is observed. This neutral species is spectrally distinct from that of the bound 8-azaG, confirming that the latter does not adopt the N(9)H form.

The spectral properties of PNP/8-azaG and PNP/PME-azaG complexes, and in particular the previously unpublished fluorescence excitation difference spectra, support our conclusion[6] that 8-azaG is bound to PNP as a neutral N(7)H or N(8)H tautomer, and therefore question model of catalysis in which the purine base is bound to the enzyme as an anion. This model was based in part on the properties of the fluorescent PNP-guanine complex, interpreted as evidence of dissociation of Gua upon binding.[15] But the observed fluorescence of Gua complexed with PNP

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may also originate from the neutral N(7)H form, which is known to emit intensely in rigid glasses at 140–160 K.[16] The reported strong binding of purines by PNP in the absence of phosphate[10,17] may lead to sufficient rigidity to enhance fluorescence yield of the bound N(7)H tautomer.

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