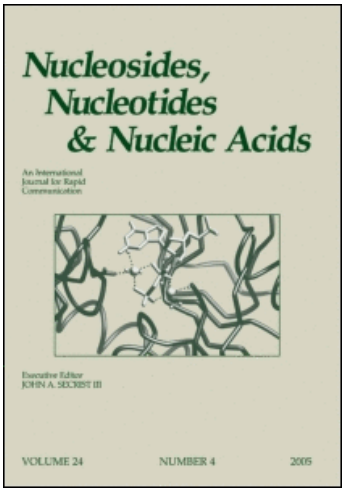

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AFFINITY OF (α-*P*-BORANO)-NTP ANALOGS TO RABBIT MUSCLE PYRUVATE KINASE

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▫ *The binding affinity of (α-P-borano) and other NTP analogs to rabbit muscle pyruvate kinase (PK) was investigated using a fluorescence quenching approach to obtain structure-activity relationships for substrate specificity of nucleotide analogs.*

Keywords Fluorescence Quenching, Pyruvate Kinase, Nucleotide Boranophosphate

INTRODUCTION

Most antiviral nucleoside analogues require stepwise phosphorylation to their respective triphosphates in order to exert their activity.[1,2] It was recently found that pyruvate kinase may be responsible for the last step of phosphorylation of 2′,3′-dideoxy- and acyclo-nucleoside diphosphates.[2] The ddNTPs with α-*P*-borano-substitution were shown to be better chain terminators for viral reverse transcriptases[3–5] and better substrates for human nucleoside diphosphate kinase[4] than parent ddNTPs. Here, we investigated the binding affinity of nucleoside triphosphate analogs to rabbit muscle PK. Quenching of intrinsic PK tryptophan fluorescence by added NTP analog was used to determine K_d values from the enzyme-substrate complex. A fluorescence quenching approach allowed us to quantitatively characterize each interaction and determine its importance for binding affinity.

NTPs and pyruvate kinase from rabbit skeletal muscle were purchased from Sigma. The synthesis and stereoisomer separation of (α-*P*-borano)-NTPs,[6] (α-*P*-borano)-dNTPs,[7] and (α-*P*-borano)-ddCTP[5] were published previously. The dissociation constants of NTP derivatives from the enzyme-ligand complex were measured by steady-state fluorescence quenching at 334 ± 3 nm upon excitation at 295 ± 2 nm. Equilibrium titrations were performed at 23 ± 1°C by sequentially

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adding 0.25–20 μL aliquots of a concentrated (20–100 mM) solution of NTP derivative to 1 μL of a mixture containing 50 mM HEPES (pH 7.5), 5% glycerol, 100 mM KCl, 0.03–0.1 mg/mL rabbit muscle PK, 5 mM $MgCl_2$, and 2 mM sodium oxalate.

Oxalate, a structural analogue of the enolate of pyruvate,[8] was used in the complex with the enzyme for determination of dissociative constants, because ATP binds with PK more tightly in the presence of oxalate, but the phosphoryl transfer reaction is considerably slower than in the presence of the natural substrate-pyruvate.[9] The percent of fluorescence quenching Q was calculated using the relationship:

$$Q = 100 \cdot (F_0 - F)/F_0 \tag{1}$$

where F and F_0 are the fluorescence intensities of PK in the presence and in the absence of a nucleotide derivative. For kinetic analysis, we assumed rapid equilibrium conditions in which the enzyme achieved an approximate binding equilibrium in the steady-state. Fluorescence quenching data were fit to the Hill equation:

$$\log Q'/(Q'_{max} - Q') = n \cdot \log[S]_{free} - \log K_d \tag{2}$$

where Q' is fluorescence quenching corrected for dilution and inner filter effects, Q'_{max} represents the maximal fluorescence quenching when nucleotide is present in a saturating concentration; n is the Hill constant representing the number of the substrate molecules binding to the enzyme in the reactive complex; K_d is the dissociation constant of the enzyme-substrate complex; $[S]_{free} = [S]_{total} - E_{total} \cdot (Q'/Q'_{max})$, where $[S]_{free}$ represents free substrate concentration, $[S]_{total}$ is total substrate concentration, $E_{total} \cdot (Q'/Q'_{max})$ is the concentration of the enzyme-substrate complex. The following equation describes the sequential binding of the substrate S to a tetrameric pyruvate kinase:

$$E_{f4} + nS \underset{-S}{\overset{K_{d1}}{\longleftrightarrow}} E_{f3}E_{b1} \cdot S \underset{-S}{\overset{K_{d2}}{\longleftrightarrow}} E_{f2}E_{b2} \cdot S_2 \underset{-S}{\overset{K_{d3}}{\longleftrightarrow}} E_{f1}E_{b3} \cdot S_3 \underset{-S}{\overset{K_{d4}}{\longleftrightarrow}} E_{b4} \cdot S4 \tag{3}$$

where E_f and E_b are free and bound with the substrate (S) subunit of the enzyme and K_{d1}, K_{d2}, K_{d3}, and K_{d4} are the respective equilibrium dissociation constants.

RESULTS AND DISCUSSIONS

Figure 1 shows the quenching curves for binding of some NTPs to the oxalate-$(Mg^{+2})_2$-PK complex with negative cooperativity. The structure of the NTP analog strongly affects the first binding mode at lower NTP concentrations but has a smaller effect on the second binding mode. To obtain structure-activity relationships for substrate specificity, we compared the K_{d1} values (Figure 2, Table 1) and

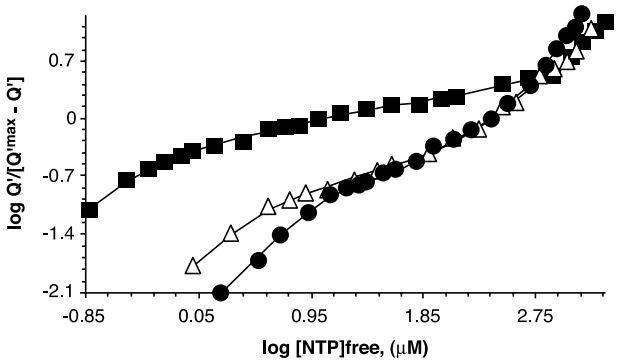

FIGURE 1 Hill plot for binding of ATP (■), GTP (Δ), and GDP(CH_2)P (●) with oxalate-(Mg^{+2})$_2$-PK complex. The curves represent the best fit of the data for each NTP.

the differences in free energy (ΔΔG1) levels (Table 1) for various enzyme-substrate complexes. The fluorescence of PK is quenched by 18% upon binding of ATP; the binding of GTP and especially CTP analogues resulted in a larger maximal quenching (Table 1).

Replacement of the adenine base by guanine or cytosine decreases the stability of the NTP-oxalate-(Mg^{+2})$_2$-PK complex by ~1.45 kcal/mol. Each 2′- or 3′- OH group contributes only 0.1–0.5 kcal/mol in stability of the enzyme-substrate complex, but the absence of both hydroxyls destabilizes the 2′,3′-ddNTP-PK complex by 1.1–1.6 kcal/mol. Replacement of the oxygen between β- and γ-phosphates by a methylene group destabilizes the GDP(CH_2)P-PK complex by 0.8–0.9 kcal/mol relative to the GTP-PK complex. The α-*P*-boranophosphate modification decreases the binding affinities of ribo- and 2′-deoxyribo NTPαBs by 0.2–0.6 kcal/mol, but it increases the binding affinity of 2′,3′-ddCTPαB analogues.

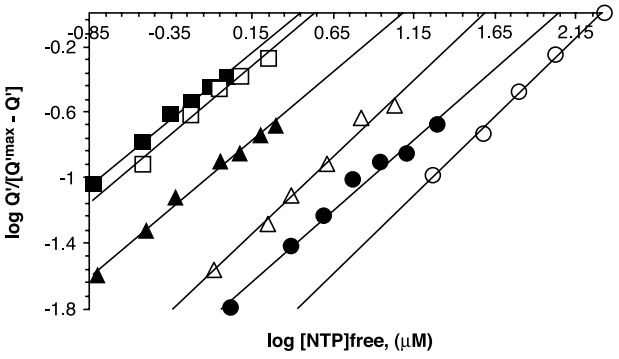

FIGURE 2 Hill plot for the first binding mode of various NTP derivatives to oxalate-(Mg^{+2})$_2$-PK complex: ATP (■); 3′-dATP (□); 2′-dATP (▲); 2′,3′-ddATP (Δ); 2′,3′-ddCTP (○); and the Rp-ddCTPαB isomer (●). The lines represent the best fit of the data. Interception of each line with the horizontal axis gives log K_{d1} value, and the slope gives the Hill coefficient n_1. Resulting kinetic constants are presented in Table 1.

TABLE 1 Dissociation Constants (Kd),[a] Hill Coefficients (n),[a] Maximal Values of Fluorescence Quenching (Q′max), and the Differences in Free Energy Levels ($\Delta\Delta G$) for Binding of NTP Derivatives with PK-$(Mg^{+2})_2$-Oxalate Complex

NTP	K_{d1} (μM)	n_1	K_{d2} (mM)	n_2	Q′max, (%)	$\Delta\Delta G_1$[c] (kcal/mol)
ATP	2.2 ± 0.3	0.90 ± 0.06	0.58 ± 0.1	1.2 ± 0.05	18	0
3′-dATP	2.7 ± 0.5	0.95 ± 0.03	—[b]	—[b]	18	0.1
2′-dATP	6.1 ± 1	0.95 ± 0.05	—[b]	—[b]	18	0.5
2′,3′-ddATP	35 ± 4.3	0.94 ± 0.06	—[b]	—[b]	18	1.63
CTP	26 ± 6.8	0.79 ± 0.07	0.86 ± 0.11	1.26 ± 0.1	42	1.45
2′-dCTP	31 ± 8.9	0.77 ± 0.04	2.3 ± 0.3	1.35 ± 0.2	52	1.56
Rp-dCTPαB	96 ± 16	0.91 ± 0.09	1.8 ± 0.3	1.4 ± 0.2	42	2.22
Sp-dCTPαB	78 ± 12	0.92 ± 0.09	0.9 ± 0.25	1.3 ± 0.1	41	2.1
2′,3′-ddCTP	170 ± 31	0.94 ± 0.06	3.2 ± 0.5	1.45 ± 0.2	41	2.56
Rp-ddCTPαB	66 ± 9	0.90 ± 0.06	3.3 ± 0.4	1.48 ± 0.1	38	2.0
Sp-ddCTPαB	69 ± 11	0.91 ± 0.09	3.5 ± 0.6	1.45 ± 0.2	42	2.03
GTP	26 ± 8	0.94 ± 0.05	0.97 ± 0.11	1.24 ± 0.1	43	1.45
GTPγS	38 ± 10	0.92 ± 0.11	1.4 ± 0.13	1.3 ± 0.1	36	1.68
Rp-GTPαB	53 ± 11	0.92 ± 0.02	—[b]	—[b]	36	1.87
Sp-GTPαB	71 ± 9	0.90 ± 0.05	—[b]	—[b]	36	2.04
2′-dGTP	43 ± 5.7	0.98 ± 0.04	1.6 ± 0.4	1.3 ± 0.06	38	1.75
Rp-dGTPαB	64 ± 8	0.93 ± 0.06	1.9 ± 0.4	1.4 ± 0.1	40	1.98
$GDP(CH_2)P$	113 ± 28	0.95 ± 0.11	2.5 ± 0.3	1.4 ± 0.1	46	2.32

[a]The kinetic constants were calculated from Eq. 1 for two linear parts of the kinetic curve as the average of three sets of experiments.

[b]Because those analogues had their own fluorescence (or fluorescent impurities), the K_{d2} and n_2 values were not determined accuratly.

[c]The differences of free energy levels between different enzyme-substrate complexes were calculated from the ratio of the corresponding equilibrium constants: $\Delta\Delta G_1 = 2.303 \cdot RT \cdot \log (K_{dt}^{ATP}/K_{d1}^{NTP})$, where R is the gas constant (1.987 cal/[mole · deg]) and T is the absolute temperature.

However, no significant stereospecificity for binding of rabbit muscle PK to the Rp- and Sp-stereoisomers of NTPαB, dNTPαB, and ddNTPαB was observed.

From the crystal structure data,[8] a comprehensive picture of the interactions of PK with ATP has been derived. The fluorescence quenching approach makes possible quantitative characterization of each interaction and determination of its importance for binding affinity.

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