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Stereospecificity, substrate, and inhibitory properties of nucleoside diphosphate analogs for creatine and pyruvate kinases

Charlotta K. Wennefors, Mikhail I. Dobrikov, Zhihong Xu, Ping Li [1], Barbara Ramsay Shaw *

Department of Chemistry, French Family Science Center, Duke University, 124 Science Drive, Durham, NC 27708-0354, USA

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ABSTRACT

Antiviral α-*P*-borano substituted NTPs are promising chain terminators targeting HIV reverse transcriptase (RT). Activation of antiviral nucleoside diphosphates (NDPs) to NTPs may be carried out by pyruvate kinase (PK) and creatine kinase (CK). Herein, are presented the effects of nucleobase, ribose, and α-phosphate substitutions on substrate specificities of CK and PK. Both enzymes showed two binding modes and negative cooperativity with respect to substrate binding. The stereospecificity and inhibition of ADP phosphorylation by α-*P*-borano substituted NDP (NDPαB) stereoisomers were also investigated. The *Sp*-ADPαB isomer was a 70-fold better substrate for CK than the *Rp* isomer, whereas PK preferred the *Rp* isomer of NDPαBs. For CK, the *Sp*-ADPαB isomer was a competitive inhibitor; for PK, the *Rp*-ADPαB isomer was a poor competitive inhibitor and the *Sp*-ADPαB isomer was a poor non-competitive inhibitor. Taken together, these data suggest that, although the *Rp*-NDPαB isomer would be minimally phosphorylated by CK or PK, it should not inhibit either enzyme.

1. Introduction

Nucleoside analogs targeting retroviral reverse transcriptases (RTs) are an important class of antiviral agents [1–4]. The need for these analogs to undergo metabolic conversion to be biologically active [1,4,5] has lead to problems such as poor phosphorylation by intracellular kinases [3,5,6] and undesired metabolic effects [7,8]. Another disadvantage of existing nucleoside analogs is the eventual emergence of drug resistance [4,9–12]. It has been demonstrated that α-*P*-borano modifications in clinically relevant dideoxy NTPs (ddNTPs) such as AZT, d4T [2], ddA [4,10], and acycloT [13] improve their incorporation into viral DNA by wildtype HIV-1 RT [2], and even more so by mutant drug resistant HIV-1 RTs [4,10] and MMLV RT [13,14]. Furthermore, after incorporation into viral DNA, the *Rp* isomers of α-*P*-borano substituted AZT-TP and d4T-TP have demonstrated increased stability toward the ATP-dependent repair mechanism that contributes to drug resistance [2,4,10]. Thus α-*P*-borano nucleotide analogs are promising antiviral candidates for selectively targeting mutant RTs.

In boranophosphates analogs, one of the non-bridging oxygens on the α-phosphate of nucleoside mono- (NMP), di- (NDP), or triphosphates (NTPs) is replaced by a borane $-BH_3^-$ group (Fig. 1) [15–26].

The borane group is isoelectronic with oxygen in normal phosphate, isolobal with sulfur in phosphorothioates, and isosteric with the methyl group in methylphosphonates [15–24]. Whereas the boranophosphates share the same net charge and geometry about the phosphorus as unmodified phosphates, the boranophosphates have a longer P–B bond than the P–O bond in normal phosphate (1.91 versus 1.51 Å), a reduced tendency to coordinate metal ions or form H bonds, and an altered polarity [27]. The lower electronegativity of boron (2.04) than the oxygen (3.44) in a phosphodiester bond [27,28] gives reason to the observation that α-*P*-borano phosphate groups accelerate the incorporation of chain terminators into viral DNA [2,4,10,14,26,29], resulting in the increased potency of these drugs. Ultimately, however, the potency of these nucleotide analogs as antiviral drugs is highly dependent on their phosphorylation by host cellular kinases. To circumvent one or two steps involved in the activation of ddNTPαBs, prodrugs of ddNMPαB and ddNDPαB were recently developed [13,30,31]. Despite these efforts, the last step in the phosphorylation of

Abbreviations: CK, creatine kinase; PCr, creatine phosphate; PK, pyruvate kinase; PeP, phosphoenolpyruvate; NDPK, nucleoside diphosphate kinase; TSAC, transition state analog complex; TS, transition state; N, nucleoside; NDP, nucleoside diphosphate; NTP, nucleoside triphosphate; dNTP, deoxynucleoside triphosphate; ddNTP, 2′,3′-dideoxynucleoside triphosphate; ddNTPαB, α-*P*-borano substituted 2′,3′-dideoxynucleoside triphosphate; ATPαB, α-*P*-borano substituted ATP; ATPαS, α-*P*-thio substituted ATP; AZT, 3′-deoxy-3′-azidothymidine; d4T, 2′,3′-dideoxythymidine; ddA, 2′,3′-dideoxyadenosine, acycloT, 1-(2-hydroxyethoxymethyl)-thymine; HIV, human immunodeficiency virus; NRTI, nucleoside reverse transcriptase inhibitor; HPLC, high performance liquid chromatography.

* Corresponding author. Fax: +1 919 660 1605.
E-mail address: barbara.r.shaw@duke.edu (B.R. Shaw).
[1] Present address: Department of Chemistry, Massachusetts Institute of Technology, 77 Massachusetts Avenue, Cambridge, MA 02139, USA.

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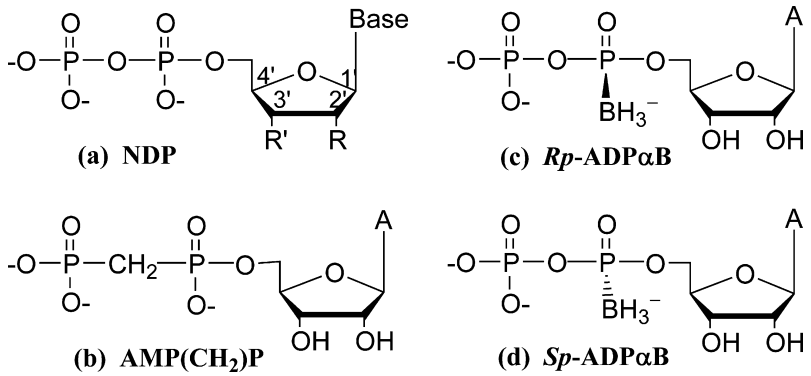

Fig. 1. Nucleoside diphosphate (NDP) ribose and α-*P* modifications. NDP: R = OH, R′ = OH; 2′-dNDP: R = H, R′ = OH; 3′-dNDP: R = OH, R′ = H; 2′,3′-ddNDP: R = H, R′ = H. α-*P* modifications: α,β-AMP(CH_2)P (bottom left). The *Rp*-ADPαB isomer (top right), and the *Sp*-ADPαB isomer (bottom right).

nucleoside analog diphosphates to their respective triphosphates remains largely unexplored [5].

Previous studies revealed that nucleoside diphosphate kinase (NDPK), a major enzyme for NDP phosphorylation in cells, phosphorylates AZT-DP, d4T-DP, and ddC-DP poorly [1,3,5,32]. Krishnan and co-workers evaluated the roles of creatine kinase (CK), 3-phosphoglycerate kinase (PGK), and pyruvate kinase (PK) in NDP analog phosphorylation and proposed that the specificity of the kinases toward the antiviral ddNDPs is dependent on both the configuration of the analog (L or D) and the presence of a 3′-hydroxyl group in the sugar moiety [5]. Furthermore, the researchers suggest that CK and PK may be responsible for the last phosphorylation step of 2′,3′-dideoxy- and acyclo-nucleoside diphosphates *in vivo* [5].

Previous studies by Meyer and co-workers indicated that α-*P*-borano substitutions on AZT-DP and d4T-DP enhance efficiency of phosphorylation by NDPK by 10-fold [2]. Although the catalytic efficiency was enhanced by the borano, it was still 1000-fold less than for the parent NDPs [2], suggesting that other kinases, perhaps CK or PK, might be more efficient enzymes for the phosphorylation of ddNDP to ddNTP. CK and PK have opposite stereospecificity for binding and phosphorylation of the *Rp* and *Sp* isomers of α-*P*-thio substituted NDPs [33]. It is therefore likely that the *Rp*- and *Sp*-stereoisomers of α-*P*-borano substituted NDPs will be recognized distinctly by these enzymes. Thus, it is of interest to study the effects of the NDPαB stereoisomers on phosphorylation and substrate specificity of CK and PK.

CK is endogenous to muscle tissues that require large energy fluxes [34,35] as well as to non-muscle cells [36]. The dimeric enzyme catalyzes the reversible reaction between ADP and phosphocreatine or ATP and creatine. PK is a tetrameric enzyme involved in the glycolytic pathway by catalyzing the transfer of a phosphate group from phosphoenolpyruvate to ADP, to yield pyruvate and ATP. The kinetic schemes for both CK and PK are initiated by direct binding of ADP and creatine phosphate (for CK) or phosphoenolpyruvate (for PK) to the enzyme, subsequent formation of the transition state involving a conformational change of the enzyme, followed by a rate limiting phosphate transfer reaction, and finally product release (Scheme 1).

In the present study, the binding affinities of the substrate analogs used in this study were determined by a fluorescence quenching assay using equilibrium titration. A transition state analog complex (TSAC) was evaluated to determine the affinity of the NDP substrate for the transition state (Fig. 2). For both CK and PK, the TSAC is formed when a nitrate ion, NO_3^- occupies the position of the transferable phosphate during the transition state of the enzyme catalyzed reaction as demonstrated previously for ADP [37–42].

Herein, we report the specificity of CK and PK toward nine different nucleobase, ribose, and α-phosphate substituted NDPs and the effect of the α-*P*-borano modification on the phosphorylation of NDPs (refer to Fig. 1). Substrate properties, inhibitory properties, and stereochemical effects of α-*P*-borano nucleoside diphosphates for CK and PK are also presented.

2. Materials and methods

2.1. Materials

Creatine, $NaNO_3$, Hepes, Bicine, KCl, sodium oxalate, glycerol, tryptophan methyl ester hydrochloride, creatine phosphate and nucleoside diphosphates (including AMP(CH_2)P) and triphosphates were obtained from Sigma–Aldrich. KOH was obtained from Fisher. $MgCl_2$ was obtained from Ambion. Rabbit muscle creatine kinase and rabbit muscle pyruvate kinase were purchased as desiccated powders from Roche. ADPαB and GDPαB were synthesized, purified, and the isomers were separated by HPLC as published previously [43].

2.2. Stock solutions

Concentrations of NDPs in 70 mM Hepes (pH 7) were determined by Cary UV–vis spectrophotometer and the purity was determined by Varian HPLC. For CK studies the direct binding (DB) buffer [38] was adjusted to pH 8.3 and contained 50 mM Bicine, 50 mM KCl, and 5 mM $MgCl_2$; the transition state analog complex (TSAC) binding buffer [38] was adjusted to pH 8.3 and contained 50 mM Bicine, 5 mM $MgCl_2$, 50 mM $NaNO_3$, and 20 mM creatine. For PK studies the direct binding buffer was adjusted to pH 7.5 and contained 50 mM Hepes, 5% glycerol, 5 mM $MgCl_2$, 2 mM sodium oxalate, and 100 mM KCl. The TSAC binding buffer was adjusted to pH 7.5 and contained 50 mM Hepes, 5% glycerol, 5 mM $MgCl_2$, 2 mM sodium oxalate, 50 mM KCl, and

$$\mathrm{E{:}PX + ADP} \overset{K_d^{ADP}}{\rightleftharpoons} \mathrm{E{:}PX{:}ADP} \overset{K'^{ADP}_d}{\rightleftharpoons} \left[\mathrm{E{:}PX{:}ADP}\right]^{\ddagger} \overset{k_p}{\rightleftharpoons} \mathrm{E{:}X{:}ATP} \overset{K_d^{ATP}}{\rightleftharpoons} \mathrm{E + X + ATP}$$

Scheme 1. CK and PK kinetic scheme. ADP and phosphate donor binds to the enzyme. A conformational change of the enzyme forming the transition state is followed by the rate limiting phosphate transfer, followed by product release. E = CK or PK, PX = creatine phosphate (for CK) or phosphoenolpyruvate (for PK), X = creatine (for CK) or pyruvate (for PK), ADP = adenosine diphosphate, and ATP = adenosine triphosphate.