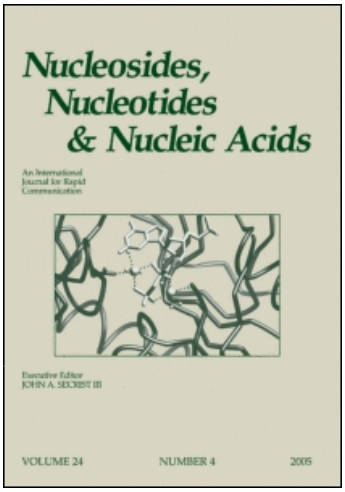

Nucleosides, Nucleotides and Nucleic Acids
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597286

Enzymatic Preparation of ^{32}P-Labeled β-L-2', 3'-dd-5' ATP and Its Use as a High-Affinity, Conformation-Specific Ligand for Labeling Adenylyl Cyclases

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To cite this Article Johnson, Roger A. , Shoshani, Ilana , Dessauer, Carmen and Gosselin, Gilles(1999) 'Enzymatic Preparation of ^{32}P-Labeled β-L-2', 3'-dd-5' ATP and Its Use as a High-Affinity, Conformation-Specific Ligand for Labeling Adenylyl Cyclases', Nucleosides, Nucleotides and Nucleic Acids, 18: 4, 839 — 842
To link to this Article: DOI: 10.1080/15257779908041575
URL: http://dx.doi.org/10.1080/15257779908041575

NUCLEOSIDES & NUCLEOTIDES, 18(4&5), 839-842 (1999)

ENZYMATIC PREPARATION OF ^{32}P-LABELED β-L-2',3'-dd-5'ATP AND ITS USE AS A HIGH-AFFINITY, CONFORMATION-SPECIFIC LIGAND FOR LABELING ADENYLYL CYCLASES

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Abstract: An enzymatic method was developed for the preparation of unlabeled and [β-^{32}P]-labeled β-L-2',3'-dd-5'ATP from the monophosphate with near quantitative yields. β-L-2',3'-dd-5'ATP was a competitive and potent inhibitor of adenylyl cyclases (IC_5 ~30 nM). Upon *uv*-irradiation β-L-2',3'-dd-[β-^{32}P]-5'ATP directly crosslinked to a chimeric construct of this enzyme. Data suggest that this is a pre-transition state inhibitor and contrasts with the equipotent 2',5'-dd-3'ATP, a post-transition state, noncompetitive inhibitor.

Adenylyl cyclases are a family of membrane-bound enzymes that catalyze the formation of adenosine 3':5'-monophosphate (cAMP) from 5'ATP. Among their many distinct regulatory mechanisms, it is their inhibition by adenine nucleoside 3'-phosphates via a domain referred to as the P-site which we find intriguing. It is a property of all known isoforms of mammalian adenylyl cyclases[1], save possibly the enzyme from sperm, and was originally so designated because of the increased inhibitory potency of ligands containing an intact purine[2]. The most potent inhibitors are adenine nucleoside 3'-polyphosphates[1-3], which inhibit via a dead-end, noncompetitive mechanism implying that they bind to the enzyme in the configuration for and at the leaving site of cAMP[4,5]. Whereas inhibition by P-site ligands has been well characterized biochemically and pharmacologically and potent and specific inhibitors of the enzyme have been synthesized, potent agents targeted to the substrate 5'ATP binding conformation have not been identified.

From a family of β-L-Ado-5'-phosphates we developed a facile enzymatic procedure for the preparation of unlabeled- and ^{32}P-labeled analogs of the most potent of these compounds, β-L-

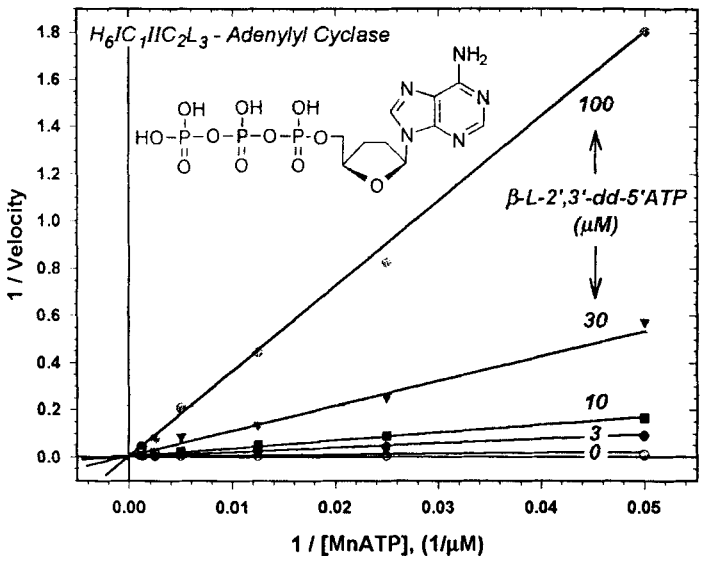

FIGURE 1: Double-reciprocal plot for inhibition of adenylyl cyclase by β-L-2',3'-dd-5'ATP. The chimeric construct $H_6IC_1IIC_2L_3$, comprising the C1 domain of the type I adenylyl cyclase linked with the C2 domain of the type II enzyme[7], was assayed in the presence of the indicated concentrations of substrate MnATP and β-L-2',3'-dd-5'ATP and 5 mM $MnCl_2$, fixed in excess of the 5'ATP concentration[4]. Units for velocity are nmols cAMP / (mg protein • min).

2',3'-dd-5'ATP. Unlabeled ligand was prepared from the corresponding 5'-monophosphate upon incubation with myokinase, creatine kinase, and creatine phosphate, with a spiking concentration of 5'ATP. Yields were quantitative. Labeled ligand was similarly prepared, but with a two-step incubation with myokinase and [γ-^{32}P]-5'ATP and then a subsequent phosphorylation reaction with creatine phosphate and creatine kinase. Overall yield was ~3 mC$_i$ of β-L-2',3'-dd-[β-^{32}P]-5'ATP from 10 mC$_i$ [γ-^{32}P]-5'ATP. Unlabeled and labeled ligands were purified by sequential ion exchange and ion-pairing reverse phase HPLC.

Inhibition of adenylyl cyclase by β-L-2',3'-dd-5'ATP was competitive with respective to substrate MnATP (Figure 1) and exhibited an IC_{50} ~30 nM with native enzyme from rat brain (not shown). This potency compares to that of 2',5'-dd-3'ATP (IC_{50}s ~40 nM), a non-competitive inhibitor of the enzyme[1,3,6]. Upon *uv*-irradiation β-L-2',3'-dd-[β-^{32}P]-5'ATP exhibited direct crosslinking to adenylyl cyclase (Figure 2). The best competitive displacement was noted with 2',5'-dd-3'ATP and 2',5'-dd-3'A_4P[6], potent P-site ligands, and 5'AP(CH$_2$)PP, a substrate analog,

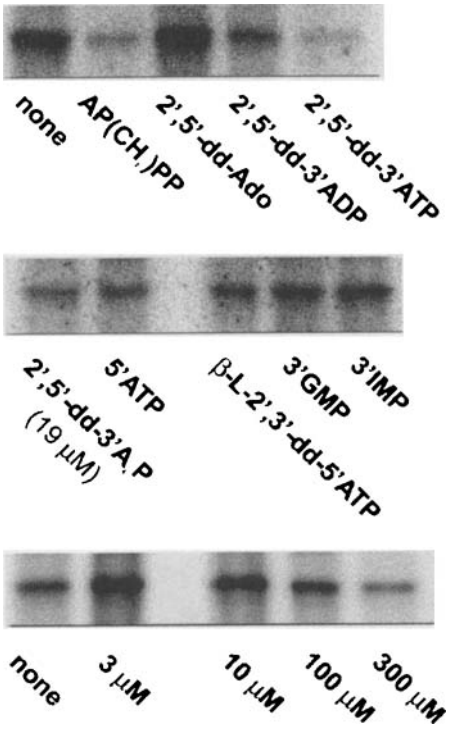

FIGURE 2: Direct photo-crosslinking of β-L-2',3'-dd-[β-^{32}P]-5'ATP. Enzyme was irradiated at 300 nm for 20 min in the presence of β-L-2',3'-dd-[β-^{32}P]-5'ATP and the indicated other ligands, in a reaction mixture containing 5 mM $MnCl_2$, 100 μM forskolin, and 0.5% acetone as sensitizing agent. The top two panels show the effects on labeling of the indicated additional unlabeled ligands, each at 300 μM, except as noted, and the bottom panel shows the effects of unlabeled β-L-2',3'-dd-5'ATP at increasing concentrations. Irradiated enzyme was isolated by denaturing polyacrylamide gel electrophoresis and the extent of crosslinking of ^{32}P-ligand was visualized by PhosphorImager techniques.

but not with 3'GMP, 3'IMP, or 2',5'-dd-Ado. In corroborating experiments β-L-2',3'-dd-[β-^{32}P]-5'ATP also labeled the enzyme specifically and reversibly in a reversible binding assay, in a manner consistent with an interaction with the catalytically active conformation of the enzyme. The data suggest that β-L-2',3'-dd-5'ATP, and 2',5'-dd-3'ATP interact with adenylyl cyclase at the same site, but with different enzyme conformations. In enzyme structure studies with these nucleotides conformational shifts occurring during catalysis may be determined.

ACKNOWLEDGMENT: This work was supported by NIH grant DK38828 to RAJ.

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