Enzymatic Synthesis of Unlabeled and β.32P-labeled β-1,2’,3’-Dideoxyadenosine-5’-triphosphate as a Potent Inhibitor of Adenylyl Cyclases and Its Use as Reversible Binding Ligand*

(Received for publication, May 28, 1999, and in revised form, August 23, 1999)

Ilana Shoshani‡, Valérie Boudouš, Claire Pierraš, Gilles Gosselin§, and Roger A. Johnson¶

From the Department of Physiology and Biophysics, Health Sciences Center, State University of New York, Stony Brook, New York 11794-8661 and UMR CNRS-USTL 5625, Laboratoire de Chimie Organique Biomoléculaire de Synthèse, Université de Montpellier II, Sciences et Techniques du Languedoc, Place Eugene Bataillon, 34095 Montpellier, France

β-1,2’,3’-Dideoxyadenosine-5’-triphosphate (β-1,2’,3’-dd-5’-ATP) was prepared enzymatically from the corresponding monophosphate by the use of adenylyl kinase, creatine phosphate, and creatine kinase in a single step. The β.32P-labeled analog was prepared similarly, but in a two step reaction. β-1,2’,3’-dd-5’-ATP inhibited adenylyl cyclase from rat brain competitively with respect to substrate (5’-ATP-Mn2+) and exhibited an IC50 of 24 nM. The labeled ligand was used in the development of a reversible binding assay for adenylyl cyclases. Binding of β-1,2’,3’-dd-[β.32P]5’-ATP was saturable with increasing concentrations of ligand and increased in proportion to membrane protein, and was enhanced by Mn2+ to a greater extent than by Mg2+. Binding was displaced with adenine nucleotides known to be either competitive or noncompetitive inhibitors but not by agents known not to act on the cyclase, or by 3-isobutyl-1-methylxanthine, creatine phosphate, or creatine kinase. Binding was rapid, with a half-time for the on-rate <1.8 min and for the off-rate <0.8 min. The potency and mechanism of the inhibition of this ligand and the pattern of agents that displace binding suggest an interaction with adenylyl cyclase per se and to a configuration of the enzyme consistent with an interaction at the catalytic active site. The data suggest that this is a pretransition state inhibitor and contrasts with the equipotent 2’,5’-dd-3’-ATP, a post-transition state noncompetitive inhibitor.

Adenylyl cyclases (ATP-pyrophosphate lyase (cyclizing); E.C. 4.6.1.1.) are a family of membrane-bound enzymes that catalyze the formation of cAMP from 5’-ATP. From several lines of evidence, it has become clear that the adenylyl cyclase catalytic site exhibits specificity for the adenosine moiety, enhanced binding via substrate and inhibitor phosphate groups, and tolerance of modifications to the ribose (1–8). The impact of the phosphate groups, in terms of structure and electronic character, to interaction of nucleotides with adenylyl cyclases is evident in the observed catalytic efficacy of known substrates (2’,5’-d-5’-ATP > 5’-ATP > 5’-ATPγS1 > 5’-APP(NH)2p > 5’-APP(CH2)p)2, the competitive but weak inhibition by 5’-AP(CH2)p (6), and the enhanced inhibition via the so-called P-site3 of adenine nucleosides with progressively more phosphates added at the 3’-ribose position (1–4, 9–11). That the enzyme tolerates modifications at the ribose was evident in the earliest comparisons of inhibitors (1, 9–11) and was developed further in a characterization of P-site-mediated inhibition of several adenylyl cyclase isozymes (5). The adenine nucleoside 3’-polypophosphates are the most potent P-site ligands (1–4) and inhibit via a dead-end noncompetitive mechanism implying that they bind to the enzyme in the post-transition state configuration for and at the leaving site(s) of the products, cAMP and inorganic metal-PPi (12–14). 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 pretransition state configuration with which substrate interacts have not been identified.

β-1-Enantiomers of adenosine and its 5’-phosphorylated derivatives are modified through a rotation of the ribose relative to the orientations of adenosine and the 5’-phosphate and have proven useful as analogs of the naturally occurring β-5’-ATP in the study of viral enzymes and pharmacological characterizations of purinergic receptors (15–18). The attraction of these enantiomers for work with adenylyl cyclases is that the 3’-OH is on the opposite side of the ribose relative to the normal substrate and should place it too distant for the cyclizing reaction yielding cAMP to occur. The importance of the position of the 3’-OH group for effective product formation is obvious but its placement in the enzyme catalytic cleft becomes evident from studies of the enzyme reaction (12–14) and its structure (7, 8). Because of the possibility that such ligands would be competitive inhibitors of adenylyl cyclases, several β-1-adenosine 5’-phosphates were synthesized and their inhibition of a native adenylyl cyclase extracted from rat brain has been characterized. A facile enzymatic procedure was developed for the preparation of unlabeled- and β.32P-labeled analogs of the most potent of these compounds, which was then used for evaluating its reversible binding with the enzyme.

---

* This work was supported by a Grant DK38828 from the NIDDK, National Institutes of Health and an Innovative Technology grant from the Biotechnology Center of the State University of New York (to R. A. J.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† To whom reprint requests and correspondence should be sent. Tel.: 631-444-3040; Fax: 631-444-3432; E-mail: rjohnson@ccmail.sunysb.edu.

‡ The abbreviations used are: 3P-AγS, adenosine 5’-O-(thiotriphosphate); 2’, 5’-dd-Ado, 2’, 5’-dideoxyadenosine; 2’,5’-dd-3’-ADP, 2’,5’-dideoxyadenosine 3’-diphosphate; 2’,5’-dd-3’-ATP, 2’,5’-dideoxyadenosine 3’-triphosphate; 2’,5’-dd-3’-AAP, 2’,5’-dideoxyadenosine 3’-tetraphosphate; AP(CH2)p, adenosine 5’-(α,β-methylene)-triphosphate; TEA-HCl, triethanolamine-hydrochloride; TEA-HCO3, triethylammonium bicarbonate; FAB, fast atom bombardment; MS, mass spectrometry; HPLC, high pressure liquid chromatography.

¶ R. A. Johnson, unpublished observations.
**Scheme 1.**

**EXPERIMENTAL PROCEDURES**

Materials—2′,5′-dd-Ado, 2′,5′-dd-3′-ADP, 2′,5′-dd-3′-ATP, and 2′,5′-dd-5′-AMP were synthesized as described previously (2–4). AP(CH2)6PP, β,β-2′,3′-dd-5′-ATP, 3′-GMP, 3′-IMP, 5′-ATP, and Lubrol-PX, used in the preparation and assay of rat brain adenylyl cyclase, were from Sigma. [α-32P]5′-ATP (25 Ci/mmol) was from International Chemical and Nuclear Corp. and [γ-32P]5′-ATP (>3,000 Ci/mmol, as the tetra-(tritylthiophenylmethyl salt)) was from New England Nuclear Corp. Nitrocellulose membranes were from Millipore (type HA, 0.45 μm).

1H and 31P NMR spectra were recorded at ambient temperature with a Bruker AC 250 (250 MHz) or Bruker AC 400 (400 MHz) spectrometer. 1H NMR chemical shifts (6) are quoted in parts per million (ppm) referenced to the residual solvent peak (dimethyl sulfoxide (Me2SO-δ, 39.5) at 2.49 ppm) relative to tetramethylsilane. 31P chemical shifts are reported in ppm with phosphoric acid (H3PO4) as external reference. UV spectra were recorded on a Perkin-Elmer model 241 spectrophotometer. Optical rotations were measured in a 1-cm cell on a Perkin-Elmer model 241 spectropolarimeter.

**Chemical Synthesis of β,β-2′,3′-dd-5′-AMP (2)** and β,β-2′,3′-dd-5′-AMP (4). The stereochemical syntheses of β,β-adenosine (1) and β,β-2′,3′-dideoxyadenosine (3) have been previously described (15, 16, 19, 20).

To a solution of L-adenosine (1) (100 mg, 0.37 mmol) or β,β-2′,3′-dideoxyadenosine (3) (151 mg, 0.64 mmol), previously dried for several hours in high vacuum, and N,N,N′,N′-tetracarbamyl-1,8-naphthalenediamine (Proton Sponget, 1.5 eq) in triethyl phosphate (10 ml/mlmol), was added phosphorus oxychloride (3 eq) at 0°C, under argon. The reaction mixture was stirred at 0°C for 40 min, and then a cold TEA/CHO solution (pH 7.4, 0.24 ml) was added. The solvents were evaporated under reduced pressure (bath temperature being kept below 30°C), and the residue was purified by column chromatography on DEAE-Sephadex A-25, equilibrated in 10 mM TEA/CHO (1–500 mM). The appropriate fractions, containing the mono-phosphate derivative as a triethylammonium salt, were pooled, frozen, and lyophilized. The mononucleotides were converted to their sodium salt by passing an aqueous solution through a column of Dowex 50W-X2 (Na+ form). Lyophilization afforded β,β-2′-5′-AMP (2) (150 mg) and β,β-2′,3′-dd-5′-AMP (4) (240 mg) as white powders.

**β,β-Adenosine 5′-monophosphate (2)—**32P NMR (Me2SO-d6) 6 1.69 (s); 1H NMR (Me2SO-d6) 8 2.7–3.8 (m, 2H, 5-H and 5′-H), 4.9 (br s, 1H, 4′-H), 4.1–4.2 (m, 1H, 3′-H), 4.64 (1H, 2′-H, J2′,3′ = 5.5 Hz), 5.85 (1H, 1′-H, J1′,2′ = 5.6 Hz), 7.2 (br s, 2H, N3′), 8.09 and 8.43 (2H, 2-H, 3-H and 8-H); MS (FAB > 0, GT) m/e 346 (M-Na+), 368 (M-Na+), 370 (M-Na+2H), UV (H2O) λmax 226 nm, λmin 258 nm.

**β,β-2′,3′-Dideoxyadenosine 5′-monophosphate (4)—**31P NMR (D2O) 6 4.15 (s); 1H NMR (D2O) 8 1.99 and 2.11 (2H, 2′-H and 4′-H), 2.35 and 3.95 (5H, 5-H and 5′-H), 3.78 and 3.95 (5H, 5-H and 5′-H), 4.31 (1H, 1′-H, J1′,2′ = 3.1 and J2′,3′ = 5.7 Hz), 8.05 and 3.86 (2H, 2-H and 8-H); MS (FAB > 0, GT) m/e 338 (M+Na+, 360 (M+2Na+, 382 (M+3Na, MS (FAB > 0, GT) m/e 134 (B−1), 336 (M+Na+), 358 (M+2Na+), UV (H2O) λmax 228 nm, λmin 260 nm.

**Chemical Synthesis of β,β-5′-ATP (5)**—The preparation of β,β-adenosine-5′-triphosphate (5) was accomplished according to the Ludwig's method (22); Scheme 2). β,β-Adenosine (1) was reacted with phosphorus oxychloride in triethylphosphate to afford the phosphodichloridate intermediate. This compound was treated with a freshly prepared solution of bis-tri-n-butylammonium pyrophosphate (22) to give a cyclic trimetaphosphate derivative, which was hydrolyzed by addition of TEA/CHO. Purification of β,β-5′-ATP (5) was performed as described above for β,β-5′-AMP (2) and β,β-2′,3′-dd-5′-AMP (4).

Following a previously described procedure (23), a solution of tetrasodium pyrophosphate decahydrate (Sigma S-9515) (446 mg, 1.0 mol) in water was passed through Dowex 50W-X2 (20 ml; pyridine form). The collected fractions were concentrated to dryness and diluted with pyridine (30 ml) and tri-n-butylamine (1.0 ml). The homogeneous solution was evaporated to a syrup and co-evaporated with pyridine and toluene. The material was dissolved in N,N-dimethylformamide (2.0 ml) and tributylamine (0.20 ml) to give the anhydrous pyrophosphate solution, which was used later.

A cooled (0°C) solution of β,β-5′-adenosine (1) (53 mg, 0.20 mmol) in trimethylphosphate (0.5 ml) was treated with phosphorus oxychloride (27 μl, 0.26 mmol). The mixture was stirred at 0°C for 1.5 h, then the pyrophosphate solution was quickly added under vigorous stirring. 1 min later, the trimetaphosphate was hydrolyzed by adding 20 ml of cold TEA/CHO (1 M, pH 7.2), and the mixture was concentrated to dryness. The crude material was enriched by anion exchange chromatography (DEAE-Sephadex A-25; equilibrated in 1 mM TEA/CHO), which was developed with a linear gradient of TEA/CHO (1 mM to 1 M). A subsequent enrichment by preparative TLC (isopropanol/aqueous ammonia solution 20%/water, 11/7/2, v/v/v) was performed, followed by filtration through a Millex unity HV-0.45 μm, Millipore) to afford a solid material, which was again subjected to anion exchange chromatography as above. The appropriate fractions were combined, the solvent was evaporated under reduced pressure at room temperature, and the residue was lyophilized several times from water. The triphosphate derivative was converted to its sodium salt by passing an aqueous solution through a column of Dowex 50W-X2 (Na+ form). Lyophilization afforded β,β-5′-ATP (3, 45 mg) as a white powder.

**Enzymatic Preparation of Unlabeled and 32P-Labeled β,β-2′,3′-dd-5′-AMP and Adenylyl Cyclases**

Unlabeled and 32P-labeled β,β-2′,3′-dd-5′-AMP were prepared from β,β-2′,3′-dd-5′-AMP (4) by incubation with myokinase, creatine kinase, and creatine phosphate, with a less than stoichiometric concentration of 5′-ATP. This 5′-ATP is used by myokinase in the primary phosphorylation of β,β-3′-dd-5′-AMP but is then regained from creatine phosphate by the action of creatine kinase. Although concentrations of 5′-ATP > 100 μM will allow the total conversion of β,β-2′,3′-dd-5′-AMP to β,β-2′,3′-dd-5′-ATP to occur more rapidly, separation of substrates and products becomes more difficult. The typical phosphorylation of 1 mM β,β-2′,3′-dd-5′-AMP was achieved with a reaction mixture containing 100 μM 5′-ATP, 100 μg of myokinase/ml, 100 μg of creatine kinase/ml, 5 mM creatine phosphate, 10 mM MgCl2, and 50 mM triethanolamine-HCl in

*The phosphorylation of β,β-3′-dd-5′-AMP to the corresponding 5′-triphosphate was also catalyzed by myokinase (100 μg/ml), phosphono-oxalpyruvate (5 mM), and pyruvate kinase (100 μg/ml).*
small amount of an unknown labeled material eluted later than the myokinase. Middle panel, distribution of $^{32}$P after incubation of $\beta$-l-2',3'-dd-$^{32}$P[5]-ATP with myokinase overnight at 30 °C. Bottom panel, distribution of $^{32}$P after the second incubation, with creatine phosphate and creatine kinase overnight at 30 °C. The retention time of an internal standard of $\beta$-l-2',3'-dd-5'-ATP is indicated by the arrow.

4.4 ml overnight at 30 °C; effective reaction times were determined in other experiments. The resulting $\beta$-l-2',3'-dd-5'-ATP was separated from the starting 5'-monophosphate and 5'-ATP by reverse phase HPLC (see Fig. 1, described below). The column (Beckman Ultrasphere; 5 μm, 4.6 × 250 mm) was developed with a linear gradient from a buffer containing 100 mM triethylammonium bicarbonate, pH 8, to a buffer containing 0.9 M triethylammonium bicarbonate, pH 5.5 and 10% methanol. The appropriate fractions were pooled, triethylammonium bicarbonate was removed by repetitive roto-evaporation from HPLC grade methanol, the dried product was resuspended in water, and then stored at −80 °C. Under these reaction conditions there is a quantitative conversion of $\beta$-l-2',3'-dd-5'-ATP to the corresponding 5'-triphosphate and the only losses incurred are those associated with purification of product.

Labeled ligand was prepared similarly, but with a two-step incubation, first with myokinase and [γ-32P]P5'-ATP and then with creatine phosphate and creatine kinase. Because of the enzymes and [γ-32P]P5'-ATP used for this preparation, the $\beta$-l-2',3'-dd-5'-ATP becomes labeled in the β-phosphate. In a typical reaction 10 mCi of [γ-32P]P5'-ATP was incubated in a reaction mixture containing 300 μM $\beta$-l-2',3'-dd-5'-AMP, 1 mg myokinase/ml, 10 mM MgCl₂, and 50 mM triethanolamine·HCl overnight at 30 °C in a volume of 680 μl. To inactivate the myokinase, the sample was placed in a boiling water bath for 10 min. It was then cooled on ice for 5 min and creatine phosphate and creatine kinase were added to final concentrations of 10 mM and 1 mg/ml, respectively, to a final volume of 800 μl. The reaction was allowed to proceed overnight at 30 °C. Substrate and product nucleotides were purified by reverse phase HPLC as used before for the unlabeled ligand above, [32P]P from [γ-32P]P5'-ATP (Fig. 1, top panel) was effectively transferred to $\beta$-l-2',3'-dd-5'-AMP to yield $\beta$-l-2',3'-dd-[32P]P5'-AMP (Fig. 1, middle panel). A small amount of an unknown labeled material eluted later than the 5'-diphosphate, suggesting that some 5'-triphosphate was also formed.

It is important that the triethylammonium bicarbonate be made fresh for the day of use. Over time, oxidation products of triethylamine will form and the loss of CO₂ causes an increase in pH, and methanol will result in substantial shifts in retention times.

Quantifying Nucleotides by HPLC—Unlabeled nucleotides were quantified after HPLC as areas under peaks determined with a Waters 996 photodiode array detector and the accompanying Millennium software (v.2.10). Radioactivity of HPLC column eluates was quantified with a Flow-Count Detector from Bioscan (Washington, D.C.) with the accompanying Laura software (GC Ram version 1.4a) from LabLogic (Sheffield, U.K.).

Adenylyl Cyclase Preparation and Assay—Adenylyl cyclase was prepared as a detergent-dispersed extract from rat brain as described previously (1, 24). Activity was assayed in a reaction mixture containing 50 mM HEPES buffer, pH 7.5, 5 mM MnCl₂, 100 mM forskolin, 0.1 mM [α-32P]P5'-ATP (2 × 10⁵ to 10⁶ cpm), in a volume of 100 μl, for 15 min at 30 °C. The reaction was started by the addition of [α-32P]P5'-ATP and was ended by the addition of zinc acetate and sodium carbonate (24). [32P]PAMP was purified by sequential chromatography on Dowex 50 and alumina and was quantified in a scintillation spectrometer by Chenrenkov radiation. Inhibition kinetics were evaluated as described previously (25) with variable concentrations of substrate Mn²⁺-5'-ATP and free cation held fixed in excess of the 5'-ATP concentration.

RESULTS

Adenine Nucleotide Isomers and Inhibition of Adenylyl Cyclase—Product formation from the normal substrate, β-l-5'-ATP, was effectively blocked by its l-isomer, but notably at a concentration significantly lower than that of the substrate (Fig. 2); β-l-5'-ATP exhibited an IC₅₀ ~ 3 μM in the presence of 100 μM substrate. This suggested that the β-l-configuration of the nucleotide interacted particularly well with the enzyme nucleotide binding site. By comparison β-l-5'-AMP exhibited an inhibitory potency comparable to that of the naturally occurring β-l-5'-AMP (Fig. 2 and Table 1), implying that the addition of phosphate groups may influence the orientation of the ribose moiety in the binding site. The observation that $\beta$-l-2',3'-dd-[32P]P5'-AMP was more potent than $\beta$-l-5'-AMP, how-
ever, suggested that this unnatural orientation of the 2'- and 3'-hydroxyl groups of β-L-5'-AMP impaired its interaction with the enzyme and that their removal from β-L-5'-ATP would be expected also to increase inhibitory potency. This was borne out for both β-L- and β-S-isomers of 2',3'-dd-5'-AMP (Fig. 2 and Table I). Consistent with the apparent affinity of β-L-5'-ATP being greater than that of the naturally occurring β-s-isomer, β-L-2',3'-dd-5'-AMP was >30-fold more potent than β-S-2',3'-dd-5'-AMP. With an IC<sub>50</sub> value of ~24 nM, β-L-2',3'-dd-5'-AMP exhibited an inhibitory potency comparable to that of 2',5'-dd-5'-AMP (IC<sub>50</sub> ~40 nM), although it was not as potent as the corresponding 3'-tetraphosphate (Fig. 2 and Table I) (4). With such low IC<sub>50</sub> values, enzyme concentration becomes an important consideration. Although the exact concentration of adenyl cyclase used in experiments with the rat brain preparation was not known, it was estimated to be approximately 0.9 nM, with the assumptions of a mass of 116 kDa and a specific activity of 7 μmol/min/mg of protein for the purified type I adenyl cyclase (26, 27). This would suggest that the IC<sub>50</sub> values are probably good approximations.

Inasmuch as P-site ligands, including 2',5'-dd-3'-ATP and 2',5'-dd-3'-AMP, are noncompetitive inhibitors of adenyl cyclases (2–4), it was of interest to establish the mechanism of interaction of the 1'-5' ATP enantiomers. For β-L-2',3'-dd-5'-AMP, β-L-2',3'-dd-5'-ATP (Fig. 3), and β-L-5'-ATP (not shown) inhibition of the rat brain adenyl cyclase was competitive with respect to substrate Mn<sup>2+</sup>-ATP. In contrast, β-S-2',3'-dd-5'-AMP (not shown) exhibited a noncompetitive inhibitory pattern akin to that seen with P-site ligands (1–4, 13, 14). Thus, 2',5'-dd-5'-AMP and β-L-2',3'-dd-5'-AMP are ATP isomers that inhibit adenyl cyclases with comparable potencies in the nanomolar range but with different mechanisms for inhibition.

**Filtration Binding Assay for Adenyl Cyclases**—The high affinity of β-L-2',3'-dd-5'-ATP and the fact that we could readily synthesize it as a 32P-labeled ligand suggested the possibility that it could be used in a reversible binding assay for adenyl cyclases. In early experiments we noted that in the absence of enzyme, components of the incubation buffer significantly affected the apparently nonspecific binding of β-L-2',3'-dd-[β-32P]5'-ATP to nitrocellulose membranes (Table II). At pH 8.2 and 50 mM both HEPES and TEA-HCO<sub>3</sub> resulted in substantial binding of this ligand, but this was reduced at the lower pH of 7.5. Sodium phosphate, even at pH 7.5 and a lower concentration (10 mM) caused a more obviously elevated nonspecific binding. Because of this effect of phosphate, TEA-HCl, at pH 7.5, was used in subsequent binding assays.

**Binding of β-L-2',3'-dd-[β-32P]5'-ATP to adenyl cyclase** was dependent on enzyme concentration (not shown) and was a saturable process (Fig. 4) under conditions with which equilibrium was rapidly reached (Fig. 5). The average half-time for binding was <1.8 min, as best as could be determined under these conditions and with these procedures, with maximum binding being achieved within approximately 10 min. When 30 mM unlabeled β-L-2',3'-dd-5'-ATP was added at 30 min as indicated, 32P-labeled ligand was rapidly displaced, with a half-time <0.8 min, as best as could be measured here (Fig. 5).
Fig. 4. Saturation binding of β-L-2',3'-dd-[β-32P]5'-ATP. Rat brain adenylyl cyclase (61 μg/ml) was incubated with the indicated concentrations of β-L-2',3'-dd-[β-32P]5'-ATP (~80 cpm/fmol) in a reaction mixture containing 50 mM TEA-Cl, pH 7.5, 5 mM MgCl2, 1 mM 3-isobutyl-1-methylxanthine, 2 mM creatine phosphate, 100 μg of creatine kinase/ml, 100 μM forskolin, and β-L-2',3'-dd-[β-32P]5'-ATP (413,700 cpm) in a volume of 50 μl for 15 min at 30 °C. Samples were filtered through nitrocellulose membranes and then were washed twice with a 1-ml solution of 20 mM TEA-Cl, pH 7.5, and 5 mM MgCl2. Filter membranes were allowed to air dry and then bound ligand was quantified by Cherenkov radiation of dried filters in a liquid scintillation spectrometer. Total ligand was determined as for Fig. 4. The dotted lines indicate the concentration of unlabeled ligand eliciting a 50% reduction in bound ligand; i.e., ~27 nM. Nonspecific binding was determined in the presence of 100 μM 5'-ATP (~3,100 cpm) and was subtracted to get the values shown here. Regression analysis gave the amount of 32P-labeled ligand present as ~1 pmol. With the assumption of a 40% counting efficiency this gives a nucleotide specific activity of ~460 Ci/mmol. Inset, Scatchard plot of binding curve. Two regression lines depict two phases of binding with maximal binding values of 21.6 and 47.4 fmol/μg protein.

Fig. 5. Time course for binding of β-L-2',3'-dd-[β-32P]5'-ATP to and displacement from rat brain adenylyl cyclase. Enzyme (61 μg/ml; ~0.5 nM) was incubated in a volume of 1,100 μl and at the indicated times 50-μl aliquots were taken; otherwise reaction conditions and sample handling were as for Fig. 6. Total initial β-L-2',3'-dd-[β-32P]5'-ATP was 567,000 cpm/50-μl aliquot. Incubation conditions were as follows: with 32P-labeled ligand alone (control, ●); in the presence of 30 μM unlabeled ligand added at zero time (—•—); or with 32P-labeled ligand for 30 min and then 30 μM unlabeled ligand was added after 30 min (—□—), as indicated.

Clearly both on and off rates were rapid and these estimates can only be rough approximations. The concentration of unlabeled ligand used here was >1,000-fold greater than its IC50 value and sufficient to displace virtually all labeled ligand from these high affinity binding sites (cf. Table I and Figs. 6 and 7).

Displacement of β-L-2',3'-dd-[β-32P]5'-ATP occurred with concentrations of unlabeled ligand consistent with the sensitivity of the enzyme to inhibition by this ligand (cf. Figs. 2 and 6) and allowed estimation of ligand-specific radioactivity to be ~460 Ci/mmol.
Cy/ml in this experiment. From the Scatchard plot (Fig. 6, inset) a concave upwards plot resulted that may be resolved into two lines yielding maximal binding of 21.6 fmol/µg protein and 47.4 fmol/µg protein. With the assumptions stated above for the activity of the pure enzyme, a binding of 21.6 fmol/µg protein, or 1.3 nmol/liter in this experiment, compares with the estimated enzyme concentration of 0.9 nM. From saturation and displacement data the higher affinity site yielded an estimated Kd value of ~16 nM.

The concentration-dependent displacement suggested that the 32P-labeled ligand was binding to adenylyl cyclase and was consistent with the behavior of β-l-2',3'-dd-5'-ATP expected from enzyme inhibition studies (cf. Table I and Figs. 2, 3, and 6). This is supported by the competition displacement pattern observed with several nucleosides and nucleotides (Fig. 7). Not surprisingly, the most effective displacement was achieved with substrate 5'-ATP and with the competitive inhibitors β-l-2',3'-dd-5'-ATP and 5'-AP(CH2)2PP. The lesser effectiveness of 2',5'-dideoxyadenosine and the corresponding 3'-polyphosphates was somewhat unexpected, because these are believed to bind in the catalytic cleft as well, albeit with a different configuration of the enzyme. But the increasingly effective displacement with the addition of 3'-phosphates was consistent with the increasing potency of these ligands to inhibit the enzyme (Table I). The lack of effect of 3'-GMP or 3'-IMP was expected, because these nucleotides are known not to have any effect on adenylyl cyclase activity. The acyclic adenine derivatives, PMEApp and PMEAp(NH)p, which are comparably effective inhibitors of catalysis (IC50 values of ~170 and ~180 nM, respectively (28)), also displaced binding of β-l-2',3'-dd-[β-32P]5'-ATP but with different efficacies.

**DISCUSSION**

The β-l-adenosine 5'-phosphate ligands presented here represent a new class of potent inhibitors of one of the most important enzymes involved in mediating transmembrane signal transduction. These compounds considerably extend the number and type of compounds known to inhibit the adenylyl cyclase family of enzymes. Our earlier studies focused on inhibition by P-site ligands (1–5, 13), which exhibit classical non-competitive inhibition, with the most potent compounds in this series being 2',5'-dd-3'-ATP (IC50 ~40 nM) and its corresponding 3'-tetraphosphate (IC50 ~7.4 nM) (Table I). This inhibition is believed to be via a dead-end mechanism in which inhibitor binds to the leaving configuration for products. The studies in which this was most clearly shown used 2'-3'-AMP as inhibitor and implied the participation of inorganic pyrophosphate, formed with cAMP from 5'-ATP by the enzyme (14). Presumably the 3'-triphosphate or 3'-tetraphosphate, which are also noncompetitive inhibitors, bring their own PPi to the catalytic cleft and do not need additional PPi. By contrast, the β-l-adenosine 5'-polyphosphates, and the most potent of these (β-l-2',3'-dd-5'-ATP; IC50 ~24 nM) in particular, are competitive inhibitors. Thus, we have described two classes of inhibitory nucleotides for adenylyl cyclases. One is a post-transition state noncompetitive inhibitor (2',5'-dd-3'-ATP) and the other is a comparably potent, pretransition state, competitive inhibitor (β-l-2',3'-dd-5'-ATP). The former is viewed as interacting with the enzyme configuration from which cAMP and PPi leave and the latter binds to the same site and configuration with which substrate 5'-ATP interacts (Fig. 8). Structures of adenylyl cyclase with 2',5'-dd-3'-ATP and β-l-2',3'-dd-5'-ATP bound at this site should allow the visualization of the transitions that occur during the catalytic cycle and are presently being developed with the VC1-1IC, chimeric enzyme complex (29).

An often useful characteristic of adenine nucleotides is that they can be readily labeled with 32P or 33P. This and the enzymatic selectivities of myokinase and creatine kinase allowed the ready synthesis of both unlabeled and 32P-labeled forms of β-l-2',3'-dd-5'-ATP from the corresponding monophosphate. Because the initial labeling step is from [γ-32P]5'-ATP with myokinase, the label appears in the β-phosphate position of the triphosphate. Moreover, because [γ-32P]P5'-ATP is readily purchased or synthesized (30) at specific activities of 3,000–6,000 Ci/ml, it should be possible to prepare β-l-2',3'-dd-[β-32P]5'-ATP routinely with a comparable specific radioactivity, although values we have obtained to date have been in the range of 400–1,000 Ci/ml.

The availability of β-l-2',3'-dd-[β-32P]5'-ATP makes several experiments with adenylyl cyclases possible that heretofore have not been possible. One that was explored in this paper was the development of a filtration binding assay for adenylyl cyclase.6 Consistent with expectations β-l-2',3'-dd-[β-32P]5'-ATP exhibited a rapid, specific, and freely reversible binding to adenylyl cyclase. The interaction was consistent with the concentrations of the unlabeled nucleotide to inhibit adenylyl cyclase and with an interaction with the catalytically active conformation of the enzyme. The Scatchard (31) plot suggests more than one binding affinity, though the basis of this is not known. Obvious possibilities include negative cooperativity, the presence of multiple adenylyl cyclase isozymes in this detergent extract from rat brain, or mistaken assumptions regarding the estimation of specific radioactivity of the 32P-labeled ligand. Nonetheless, the competitive displacement by other nucleotides suggested further that both 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, fully consistent with enzyme structures solved with these ligands. In principal, this ligand should prove useful for quantitative estimations of enzyme levels, for characterizing the catalytic domains of adenylyl cyclase isozymes, and in screening applications for the identification and quantifying of other ligands which may interact with this domain.

REFERENCES

1. Johnson, R. A., Yeung, S.-M. H., Stühmer, D., Bushfield, M., and Shoshani, I. (1990) *Mol. Pharmacol.* 35, 681–688

2. Desaubry, L., Shoshani, I., and Johnson, R. A. (1996) *J. Biol. Chem.* 271, 2380–2385

3. Desaubry, L., Shoshani, I., and Johnson, R. A. (1996) *J. Biol. Chem.* 271, 14029–14034

4. Desaubry, L., and Johnson, R. A. (1998) *J. Biol. Chem.* 273, 24972–24977

5. Johnson, R. A., Desaubry, L., Bianchi, G., Shoshani, I., Lyons, E., Jr., Taussig, J.

---

6 The use of β-l-2',3'-dd-[β-32P]5'-ATP for direct photolytic cross-linking to the substrate-binding configuration of the enzyme has also been explored and will be presented elsewhere. The principal conclusions of those experiments corroborate those seen here in the reversible binding assay.
R., Watson, P. A., Cali, J. J., Krupinski, J., Pieroni, J. P., and Iyengar, R. (1997) J. Biol. Chem. 272, 8962–8966
6. Dessauer, C. W., Scully, T. T., and Gilman, A. G. (1997) J. Biol. Chem. 272, 22272–22277
7. Zhang, G., Liu, Y., Ruoho, A. E., and Hurley, J. H. (1997) Nature 386, 247–253
8. Tesmer, J. J. G., Sunahara, R. K., Gilman, A. G., and Sprang, S. R. (1997) Science 278, 1907–1916
9. Weinryb, I., and Michel, I. M. (1974) Biochim. Biophys. Acta 334, 218–225
10. Haslam, R. J., Davidson, M. M. L., and Desjardins, J. V. (1978) Biochem. J. 176, 83–95
11. Wolff, J., Londos, C., and Cooper, D. M. F. (1981) Adv. Cyclic Nucleotide Res. 14, 199–214
12. Florio, V. A., and Ross, E. M. (1983) Mol. Pharmacol. 24, 195–202
13. Johnson, R. A., and Shoshani, I. (1990) J. Biol. Chem. 265, 11595–11600
14. Dessauer, C., andGilman, A. G. (1997) J. Biol. Chem. 272, 27787–27795
15. Bolon, P. J., Wang, P., Chu, C. K., Gosselin, G., Boudou, V., Pierra, C., Mathé, C., Imbach, J.-L., Faraj, A., el Alasou, M. A., Sommadossi, J.-P., Pai, S. B., Zhu, Y.-L., Lin, J.-S., Cheng Y.-C., and Schinazi, R. F. (1996) Bioorg. Med. Chem. Lett. 6, 1657–1662
16. Pierra, C. (1997) Analogues nucleosidiques et pronucleotides inedits d’anomerie β et d’enantiomerie non naturelle L: Synthèse et propriétés antivirales de dérivés de l’adénosine, de la 5-chlorouridine et de la 5-chlorocytidine. Ph.D. dissertation, Université Montpellier II, Montpellier, France
17. Welford, L. A., Cusack, N. J., and Hourani, S. M. O. (1986) Eur. J. Pharmacol. 129, 217–224
18. Welford, L. A., Cusack, N. J., and Hourani, S. M. O. (1987) Eur. J. Pharmacol. 141, 123–130
19. Boudou, V. (1997) Synthèse et Propriétés Biologiques de β-Pentofuranonucléosides de l’Adénine d’Enantiomérisation Non Naturelle L: Influence des Substituants et des Configurations en position(s) 2’ et/ou 3’. Ph.D. dissertation, Université Montpellier II, Montpellier, France
20. Boudou, V., Gosselin, G., and Imbach, J.-L. (1999) Nucleosides Nucleotides 18, 607–609
21. Mansuri, M. M., Starrett J. E., Jr., Ghazzouli, I., Hitchcock, M. J. M., Sterrycki, R. Z., Brankovan, V., Lin, T.-S., August, E. M., Prusoff, W. H., and Sommadossi, J.-P. (1989) J. Med. Chem. 32, 461–466
22. Ludwig, J. (1981) Acta Biochem. Biophys. Acad. Sci. Hung. 16, 131–133
23. Moffatt, J. G. (1964) Can. J. Chem. 42, 599–604
24. Johnson, R. A., Alvarez, R., and Salomon, Y. (1994) Methods Enzymol. 238, 31–36
25. Garbers, D. L., and Johnson, R. A. (1975) J. Biol. Chem. 250, 8449–8456
26. Pfeuffer, E., Müllner, S., and Pfeuffer, T. (1985) EMBO J. 4, 3675–3679
27. Smigel, M. D. (1986) J. Biol. Chem. 261, 1976–1982
28. Shoshani, I., Laux, W. H. G., Perigaud, C., Gosselin, G., and Johnson, R. A. (1999) J. Biol. Chem. 274, 34742–34744
29. Tesmer, J. J. G., Sunahara, R. K., Johnson, R. A., Gosselin, G., Gilman, A. G., and Sprang, S. R. (1999) Science 285, 756–760
30. Walseth, T. F., and Johnson, R. A. (1979) Biochim. Biophys. Acta 562, 11–31
31. Scatchard, G. (1949) Ann. N. Y. Acad. Sci. 51, 660–672
Enzymatic Synthesis of Unlabeled and $\beta^{32}$P-labeled $\beta$-l-2′,3′-Dideoxyadenosine-5′-triphosphate as a Potent Inhibitor of Adenylyl Cyclases and Its Use as Reversible Binding Ligand

Ilana Shoshani, Valérie Boudou, Claire Pierra, Gilles Gosselin and Roger A. Johnson

J. Biol. Chem. 1999, 274:34735-34741.
doi: 10.1074/jbc.274.49.34735

Access the most updated version of this article at http://www.jbc.org/content/274/49/34735

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 29 references, 15 of which can be accessed free at http://www.jbc.org/content/274/49/34735.full.html#ref-list-1