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Adenosine-5′-*O*-(2-fluorodiphosphate) (ADPβF), an Analog of Adenosine-5′-phosphosulfate[1]

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Adenosine-5′-phosphosulfate (APS) and its 3′-phosphorylated metabolite PAPS (3′-phosphoadenosine-5′-phosphosulfate) play central roles in biological systems. Few analogs of APS or PAPS have been described, partially due to the instability of the phosphosulfate moiety. ADPβF, adenosine-5′-*O*-(2-fluorodiphosphate), an ADP derivative in which one of the nonbridge oxygens on the terminal phosphoryl group is replaced by fluorine, has been synthesized by reaction of AMP-morpholidate with bis(tributylammonium)fluorophosphate. In contrast to APS, ADPβF is stable at room temperature for at least a day from pH 3 to 10. ADPβF is an alternate substrate for adenosine phosphosulfate kinase (ATP : APS 3′-phosphotransferase) yielding the PAPS analog PADPβF. In the APS kinase reaction ADPβF has a K_m 7-fold greater than that of APS, and a V_{max} that is 9% that of APS. ADPβF is an alternate substrate for the reverse reaction of ATP sulfurylase (ATP : sulfate adenylyltransferase), yielding ATP and FPO_3 as products; ADPβF has a V_{max} that is 12% of the V_{max} with APS and a 1.4-fold higher K_m value than APS. ADPβF can conveniently be synthesized from ATP and FPO_3 in the presence of catalytic amounts of ATP sulfurylase and inorganic pyrophosphatase. ADPβF is a poor ADP analog for two enzymes tested: high concentrations did not inhibit pyruvate kinase or hexokinase, and ADPβF did not replace ADP in the reverse pyruvate kinase reaction. ADPβF, like APS, has one less charge than ADP at neutral pH. These results suggest that a major determinant in enzymatic discrimination between APS and ADP is the charge difference between the terminal sulfuryl and phosphoryl groups.

INTRODUCTION

Adenosine-5′-*O*-phosphosulfate (APS)[2] and its metabolite 3′-phosphoadenosine-5′-*O*-phosphosulfate (PAPS) are the major donors of —SO_3 (sulfuryl) groups in biological systems (*1, 2*). APS and PAPS are involved in processes ranging from the reduction of sulfur from the +6 oxidation state of SO_4^{2-} to the −2 oxidation state present in cysteine and methionine, to the sulfation of proteins

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[2] Abbreviations used: ADPβF, adenosine-5′-*O*-(2-fluorodiphosphate); ATPγF, adenosine-5′-*O*-(3-fluorotriphosphate); APS, adenosine 5′-phosphosulfate; FPLC, fast protein liquid chromotography, Hepes, 4-hydroxyethyl-1-piperazineethanesulfonic acid; PAPS, 3′-phosphoadenosine 5′-phosphosulfate; TLC, thin layer chromatography.

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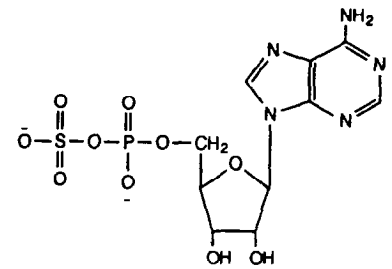
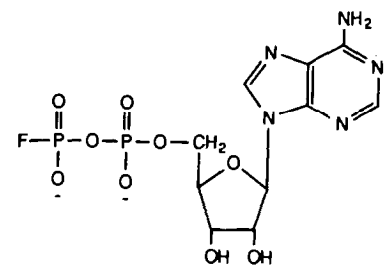


APS



ADPβF

FIG. 1. Structures of APS and ADPβF.

and numerous small molecules (*1*). Despite the importance of APS and PAPS in metabolism, few analogs of these compounds have been described, in part due to the lability of the phosphosulfate moiety. Recently analogs of APS and PAPS which contain a P—CH_2—S linkage have been reported and shown to be inhibitors of ATP sulfurylase and APS kinase (*3*, *4*). In view of the mixed successes of analogous P—CH_2—P containing nucleotides in studies of phosphoryl transfer enzymes (recently discussed in Ref. (*5*)), we have chosen an alternative approach which has yielded an alternate substrate for enzymes of APS metabolism. We report a simple synthesis of the ADP derivative adenosine-5′-*O*-(2-fluorodiphosphate) (ADPβF), in which one of the nonbridge oxygens on the terminal phosphoryl group is replaced by fluorine (Fig. 1). Preparations of fluorophosphate analogs of AMP (AMPF) and ATP (ATPγF) have been previously reported (*6*, *7*) but those compounds have seldom been used with enzymes. We describe experiments with several enzymes which show that ADPβF is an excellent APS analog, but a poor ADP analog. At neutral pH APS and ADPβF have one less charge than ADP, which may allow enzymes to discriminate among these very similar structures. ADPβF is much more stable than APS, which should facilitate its use in studies of enzymes, and will aid in the preparation of a variety of derivatives such as affinity labels.

MATERIALS AND METHODS

AMP-morpholidate was purchased from Sigma. Sodium fluorophosphate was purchased from Aldrich and converted to the tributylamine salt by passage of an aqueous solution through AG50W × 8 resin (Bio-Rad) in the H^+ form, followed by addition of tributylamine and drying *in vacuo*. ATPγF was synthesized by the diphenyl phosphorochloridate-mediated coupling of ADP and fluorophosphate as described by Haley and Yount (*6*).

The purity of all nucleotides was verified by anion-exchange fast protein liquid chromatography (FPLC) on a Mono-Q column with triethylamine bicarbonate gradients at pH 7.8, and by HPLC on a Waters SAX radial compression cartridge eluted with gradients of ammonium phosphate, pH 6.5. The identities of all nucleotides were ascertained by ^{31}P NMR spectra; spectra were obtained at 25°C on a Bruker AM300 spectrometer operating at 121.45 MHz. ^1H decoupling used the Waltz-16 sequence (*8*). Chemical shifts are referenced to external 85% H_3PO_4 with positive shifts designated as downfield from the reference.

Kinetic studies were performed at 21°C in 50 mM Hepes/KOH, 50 mM KCl, pH 8.0. Reactions were monitored using coupled assays which eventually resulted in the formation (or utilization) of NADH which was followed by absorbance changes at 340 nm.

APS kinase was purified from an overproducing strain of *Escherichia coli* as we described previously (*9*). Rabbit muscle pyruvate kinase, yeast ATP sulfurylase, yeast hexokinase, and other enzymes were purchased from Sigma and purified by FPLC gel filtration chromatography on Superose-12 in 50 mM Hepes/KOH, 50 mM KCl, 10% glycerol, pH 8.0.

Chemical synthesis of APS. APS was synthesized by the method of Baddiley *et al.* (*10*), as modified by Yount *et al.* (*11*). ADP was replaced by AMP in the reaction. The reaction mixture was chromatographed on Q-Sepharose (fast flow) using a linear gradient of 0 to 1 M triethylamine-bicarbonate, pH 7.8 (*8*).

Chemical Synthesis of ADPβF. ADPβF was synthesized by the reaction of 1 mmol AMP-morpholidate with 5 mmol of bis(tributylammonium)FPO_3 in 15 ml of dry pyridine. The reaction was incubated for 4 h at room temperature, then the solvent was removed under vacuum. The reaction mixture was dissolved in water and the pH was adjusted to 7.5. ADPβF was purified by chromatography on a DEAE-52 column (5 × 20 cm) with elution by a 2-liter gradient of 0.05 to 0.75 M triethylammonium bicarbonate, pH 7.5. The yield was ca. 25%. Longer reaction times did not improve the yield. A more convenient enzymatic synthesis is described in the Results section.

The proton-decoupled ^{31}P NMR spectrum of ADPβF agreed with that reported by Vogel and Bridger (*12*) who published data for an ADPβF sample that originated in Eckstein's laboratory: $\delta(\alpha\text{-P}) = -11.4$ ppm (doublet), $\delta(\beta\text{-P}) = -17.6$ ppm (doublet of doublets), $^3J_{\alpha\beta} = 20.1$ Hz, $^1J_{PF} = 934$ Hz; in the absence of ^1H decoupling each line of the −11.4 ppm doublet further broadened into a partially resolved triplet ($^3J_{PH} \approx 4$ Hz) due to coupling to the 5′ protons of the ribose. The synthetic route employed by the Eckstein laboratory is not clear, but they reported

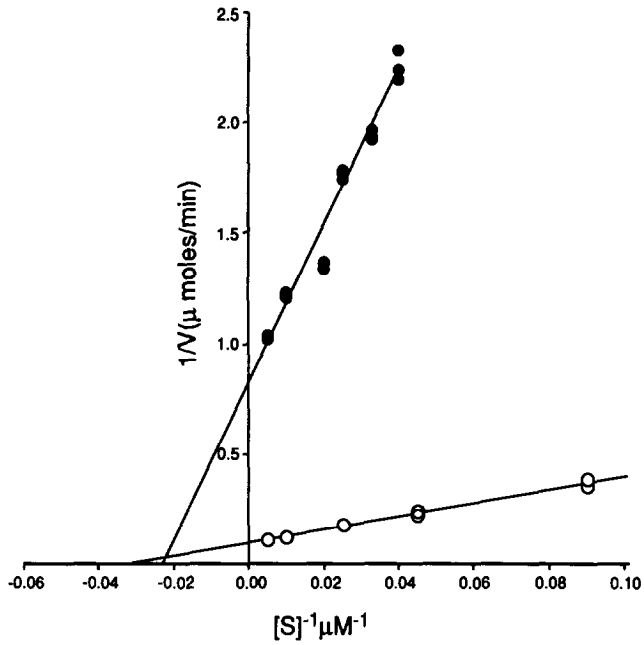


FIG. 2. Kinetic studies of the reverse ATP sulfurylase reaction with APS and ADPβF. Assays coupled ATP formation to NADH production using hexokinase and glucose-6-phosphate dehydrogenase. Solutions contained 0.5 mM PP_i, 1 mM $MgCl_2$, 2 mM glucose, 0.3 mM NAD in 50 mM Hepes/KOH, 50 mM KCl, pH 8.0.

obtaining GDPβF as a side product of the synthesis of GTPγF by the diphenyl phosphorochloridate-mediated coupling of GDP and FPO_3 (*13*).

RESULTS

A chemical synthesis of ADPβF is described in the Materials and Methods section. The stability of ADPβF was investigated by incubation at 25°C for 24 h at pH values between 3 and 10 (using 1 pH unit increments). There was no detectable decomposition (<10%) over this broad range, as monitored by anion-exchange HPLC. This contrasts with the extreme acid lability of APS (*14*).

ADPβF as an APS Analog

ATP sulfurylase. ADPβF is a substrate for the reverse reaction of yeast ATP sulfurylase (ATP: sulfate adenylyltransferase) yielding ATP and FPO_3 as products:

$$\text{ADP}\beta\text{F} + PP_i \leftrightarrows \text{ATP} + \text{FPO}_3 \qquad [1]$$

TABLE 1

Enzyme	K_m(ADPβF) μM	$\frac{K_m(\text{ADP}\beta\text{F})}{K_m(\text{APS})}$	$\frac{V_{max}(\text{ADP}\beta\text{F})}{V_{max}(\text{APS})}$
ATP sulfurylase	53	1.4	0.12
APS kinase	1.7	7.0	0.09

Note. Assays for APS kinase (0.8 ml) were carried out in 50 mM Hepes/KOH, 50 mM KCl, 1 mM ATP, 1 mM $MgCl_2$, 0.4 mM PEP, 0.3 mM NADH, pH 8.0. The assays were monitored at 340 nm to follow ADP-dependent conversion of NADH to NAD in the presence of 10 units each of pyruvate kinase, lactate dehydrogenase, and P1 nuclease. A radioactive TLC assay was also used as described in Reference 9; this monitored the formation of ^{14}C-ADP from ^{14}C-ATP; both assays gave equivalent results. ATP sulfurylase assays (0.8 ml) were carried out in 50 mM Hepes/KOH, 50 mM KCl, 0.5 mM PP_i, 1 mM $MgCl_2$, 2 mM glucose, 0.3 mM NAD, pH 8.0. To allow a spectrophotometric assay for ATP production 10 units each of hexokinase and glucose-6-phosphate dehydrogenase were included in the reaction and the ATP-dependent formation of NADH was monitored at 340 nm.

Steady state kinetic studies (Fig. 2 and Table 1) show that ADPβF is an excellent substitute for APS with a merely 1.4 fold higher K_m and only an ~eightfold decreased V_{max}.

Enzymatic synthesis of ADPβF. The reverse of Scheme [1] shown above provided an enzymatic method for the synthesis of ADPβF. In the presence of ATP sulfurylase and inorganic pyrophosphatase >95% of the ATP in a mixture of 5 mM ATP and 100 mM FPO_3 could be converted to ADPβF (in 50 mM Tris/HCl, 10 mM $MgCl_2$, pH 9.0, containing 0.1 u/ml ATP sulfurylase and 0.05 u/ml pyrophosphatase, incubated at 37°C for 24 h). This ADPβF is easily purified by ion-exchange chromatography using procedures linearly scaled down from those used for purification of the chemically synthesized ADPβF. The enzymatic method provides a convenient route to ADPβF and to isotopically labeled derivatives.

APS kinase. ADPβF is a substrate for APS kinase (ATP : APS 3′-phosphotransferase) yielding the PAPS analog 3′-phospho-ADPβF (PADPβF).

$$\text{ATP} + \text{ADP}\beta\text{F} \leftrightarrows \text{ADP} + \text{PADP}\beta\text{F} \qquad [2]$$

In the presence of equimolar ATP and ADPβF essentially quantitative product formation occurred, consistent with the equilibrium constant of 2×10^3 for APS phosphorylation (*9*). The product was isolated by ion-exchange chromatography (as described for the purification of ADPβF). Proton-decoupled ^{31}P NMR spectra of PADPβF at pH 9.0 showed a resonance at 3.9 ppm for the 3′ phosphate and slightly shifted signals for the other phosphorus nuclei: $\delta(5'\text{—}\alpha\text{—P}) = -11.5$ ppm, $\delta(5'\text{—}\beta\text{—P}) = -17.8$ ppm, $\delta(3'\text{—P}) = 3.9$ ppm, $^3J_{\alpha\beta} = 19.7$ Hz, $^1J_{PF} = 934$ Hz. The slight changes in the chemical shifts of the 5′ α and β phosphorus nuclei when ADPβF was converted to PADPβF were clearly seen when the

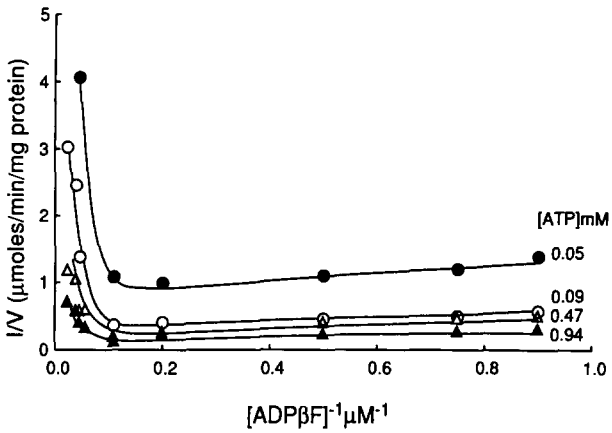


FIG. 3. Kinetic studies of the APS kinase reaction showing pronounced substrate inhibition by ADPβF at various concentrations of ATP. The reaction conditions are described in the legend to Table I.

phosphorylation reaction was followed by ^{31}P NMR spectroscopy. In the absence of proton decoupling, the 3.9 ppm resonance from PADPβF was a doublet ($^3J_{PH}$ = 7.2 Hz) and each line of the doublet at −11.5 ppm was broadened due to coupling to the two 5′ protons of the ribose moiety.

Steady state kinetic studies (Fig. 3 and Table 1) of ADPβF in the APS kinase reaction revealed pronounced substrate inhibition, as is observed with APS (*9*). Extrapolation of the data from noninhibitory concentrations showed a K_m of 1.7 μM, 7-fold greater than APS, and a V_{max} 9% of that obtained with APS. The K_m for ATP is 0.1 mM at the highest concentrations of ADPβF at which substrate inhibition is not observed; this K_m is 3-fold higher than the K_m for ATP when APS is the cosubstrate (*9*). Previous studies with APS have indicated that substrate inhibition results from a kinetic mechanism in which there is random substrate binding but the rate of product formation from the E · APS complex is ~100-fold slower than the rate of product formation from the E · ATP complex (*9*). There is no evidence for APS (or ADPβF) interacting at the site which binds ATP and the product ADP.

ADPβF as an ADP analog. We also tested ADPβF as an analog for ADP with two enzymes which catalyze phosphoryl transfer (Table 2). We included 50 mM $MgCl_2$ so that appreciable amounts of Mg(II)–ADPβF would be present (K_d = 40 mM (*12*)). Concentrations of ADPβF much greater than the K_m for ADP (0.2 mM (*15*)) did not detectably inhibit the reverse reaction catalyzed by pyruvate kinase nor did concentrations greater than the K_m for ATP (0.1 mM (*16*)) inhibit the hexokinase-catalyzed phosphorylation of glucose. ADPβF did not serve as a substrate for the reverse reaction catalyzed by pyruvate kinase (observed rate <1% of ADP at 10 mM ADPβF).

ADPβF with phosphatases. Incubation of 0.2 nmol ADPβF with 5 units of *E. coli* alkaline phosphatase did not result in any detectable degradation in 5 h at

TABLE 2

Enzyme	$\frac{V(ADP\beta F)}{V(ADP)}$	K_i (mM)
Pyruvate kinase	<0.01	>10
Hexokinase	N.D.	>5

Note. In inhibition experiments substrate nucleotides were present as 0.17 mM ADP (pyruvate kinase) or as 0.1 mM ATP (hexokinase), approximately the K_m value in each case (15, 16). The lower limit for the K_i values reflects the failure to observe inhibition at the reported concentration. Solutions contained 50 mM Hepes/KOH, 50 mM KCl, 50 mM $MgCl_2$, pH 8.0. N.D., not determined. The reactions were monitored at 340 nm to follow the formation of either NADH or NAD. The pyruvate kinase reaction also contained 10 units of lactate dehydrogenase, 0.3 mM NADH, and 0.4 mM PEP. The hexokinase reaction contained 10 units of glucose-6-phosphate dehydrogenase, 0.3 mM NAD and 2 mM glucose.

25°C, similar to the observation of Haley and Yount with ATPγF (*6*). However, incubation of ADPβF or ATPγF with calf intestinal alkaline phosphatase under the same conditions yielded P_i as the final phophorus containing product, consistent with the known ability of this enzyme to catalyze hydrolysis of FPO_3 to PO_4 and fluoride (*17*). ^{31}P NMR spectra showed the 3′-phosphoryl group of PADPβF was selectively removed by the 3′-nucleotidase activity of nuclease P1.

CONCLUSIONS

ADPβF appears to have substantial promise as an analog of APS. The extent of structural similarity of APS and ADPβF is unclear. A search of the Cambridge Structural Database (January 1991 release) indicated that no crystal structures of compounds which contain a P—O—S linkage have been reported (*18, 19*).

ADPβF radioactively labeled in the AMP moiety can be prepared enzymatically from radioactive ATP and unlabeled FPO_3 using ATP sulfurylase, inorganic pyrophosphatase and APS kinase. It should also be possible to prepare compounds labeled with ^{32}P in the $—PO_2F$ group by using the fluorokinase reaction catalyzed by pyruvate kinase to make $^{32}PO_3F$ from F^- and [γ-^{32}P]ATP (*20*), followed by ATP sulfurylase catalysis of $F^{32}PO_3$ reaction with ATP to yield [β-^{32}P]ADPβF. It remains to be seen whether PADPβF will be an alternate substrate for sulfotransferases, which would then transfer $—PO_2F^-$ instead of $—SO_3^-$. Recent studies indicate that several enzymes which catalyze phosphoryl ($—PO_3^{2-}$) transfer from ATP will also catalyze transfer of $—SO_3^-$ groups from adenosine-5′-sulfatopyrophosphate (ADP-sulfate) (*21, 22*), suggesting that the more conservative substitution of

$-PO_2F^-$ for $-SO_3^-$ may well be tolerated by sulfuryl transfer enzymes. Investigations of these potential reactions as well as efforts to synthesize derivatives such as (photo)affinity labels are now underway.

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