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Phosphorothioate Analogues of 5-Phosphoribosyl 1-Diphosphate: Synthesis, Purification, and Partial Characterization†

Geoffrey W. Smithers‡ and William J. O'Sullivan*

ABSTRACT: The 1-phosphorothioate analogues of 5-phosphoribosyl 1-diphosphate (P-Rib-PP) have been prepared enzymatically, in reactions catalyzed by P-Rib-PP synthetase from *Salmonella typhimurium*. 5-Phosphoribosyl 1-*O*-(2-thiodiphosphate) (P-Rib-PPβS) was synthesized from ribose 5-phosphate (Rib-5-P) and the Mg^{2+} complex of adenosine 5′-*O*-(3-thiotriphosphate). The S_P and R_P diastereomers of 5-phosphoribosyl 1-*O*-(1-thiodiphosphate) (P-Rib-PPαS) were synthesized from Rib-5-P and the Mg^{2+} complex of adenosine 5′-*O*-(2-thiotriphosphate) (ATPβS) (S_P diastereomer, Δ-configuration) and the Cd^{2+} complex of ATPβS (R_P diastereomer, Δ-configuration), respectively. The strategy for the synthesis and stereochemical assignment of the P-Rib-PPαS diastereomers was based on the specificity of P-Rib-PP synthetase for the (Δ)-β,γ-bidentate metal–nucleotide substrate and the stereochemical course of the synthetase reaction, leading to inversion of configuration at the P_β atom of the nucleotide [Li, T. M., Mildvan, A. S., & Switzer, R. L. (1978) *J. Biol. Chem. 253*, 3918–3923], and the known configurations of the Mg^{2+} and Cd^{2+} β,γ-bidentate complexes of the ATPβS diastereomers [Jaffe, E. K., & Cohn, M. (1979) *J. Biol. Chem. 254*, 10839–10845]. The P-Rib-PP analogues were purified by gradient elution from DEAE-Sephadex and characterized by chemical analysis and ^{31}P nuclear magnetic resonance [Smithers, G. W., & O'Sullivan, W. J. (1984) *Biochemistry* (following paper in this issue)]. A preliminary account of their interaction with human brain hypoxanthine phosphoribosyltransferase and yeast orotate phosphoribosyltransferase (OPRTase) is described. The substrate activity of the analogues with OPRTase formed the basis for a specific and convenient assay based on the release of $^{14}CO_2$ from [*carboxy*-^{14}C]orotate in the presence of excess orotidylate decarboxylase, analogous to the procedure used in the determination of the parent compound. The potential application of the P-Rib-PP analogues as probes of the mechanistic basis of the phosphoribosyltransferase enzymes is discussed.

Phosphorothioate analogues of the adenosine nucleotides, particularly ATP, in which a nonbridging oxygen atom is replaced with sulfur have proved highly rewarding as probes of the mechanistic basis of enzyme-catalyzed phosphoryl group transfer reactions and other ATP-dependent processes (Eckstein, 1979, 1980, 1983; Knowles, 1980; Eckstein et al., 1982; Cohn, 1982). Important applications have included (1) studies of the chelate configuration of the metal–nucleotide substrate species at the active site of various kinases (Eckstein, 1979; Cohn, 1982) based on the preferential ligation of Cd^{2+} ions to sulfur and Mg^{2+} ions to oxygen in the phosphorothioates (Jaffe & Cohn, 1978a, 1979) and (2) the elucidation of the mechanism and stereochemical course of phosphoryl group transfer reactions, usually in combination with the ^{17}O and ^{18}O isotopes (Knowles, 1980; Eckstein et al., 1982; Frey et al., 1982; Tsai, 1982).

An extension of such applications to a study of the phosphoribosyltransferases would yield insight into the mechanism of the reactions catalyzed by this group of enzymes. Such reactions involve the transfer of the 5-phosphoribose moiety from P-Rib-PP[1] to various nitrogenous acceptor molecules and, like the kinase reactions, are absolutely dependent upon the presence of a divalent cation, usually Mg^{2+} (Musick, 1981). This requirement reflects, at least in part, the formation of a complex (or complexes) between the metal ion and the phosphooxyanion moieties of P-Rib-PP (Thompson et al., 1978; Smithers & O'Sullivan, 1982). As has been the experience with the diastereomers of ATPαS and ATPβS (Jaffe & Cohn, 1978a, 1979; Cohn, 1982), suitable sulfur-substituted analogues of P-Rib-PP should have direct application as probes of the "metal–P-Rib-PP" structure at the active site of the phosphoribosyltransferase enzymes.

† From the School of Biochemistry, University of New South Wales, Kensington, New South Wales 2033, Australia. *Received November 1, 1983.* This work was supported, in part, by financial assistance from the Australian Research Grants Scheme.

‡ Supported by a Commonwealth postgraduate research scholarship. Present address: Department of Biochemistry and Biophysics, University of Pennsylvania School of Medicine, Philadelphia, PA 19104.

[1] Abbreviations: P-Rib-PP, 5-phosphoribosyl 1-diphosphate; ATPαS, adenosine 5′-*O*-(1-thiotriphosphate); ATPβS, adenosine 5′-*O*-(2-thiotriphosphate); P-Rib-PPβS, 5-phosphoribosyl 1-*O*-(2-thiodiphosphate); ATPγS, adenosine 5′-*O*-(3-thiotriphosphate); P-Rib-PPαS, 5-phosphoribosyl 1-*O*-(1-thiodiphosphate); HPRTase, hypoxanthine phosphoribosyltransferase; OPRTase, orotate phosphoribosyltransferase; NMR, nuclear magnetic resonance; ADPβS, adenosine 5′-*O*-(2-thiodiphosphate); Mes, 4-morpholineethanesulfonic acid; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; PEI, poly(ethylenimine); DEAE, diethylaminoethyl; ODCase, orotidylate decarboxylase; EDTA, ethylenediaminetetraacetic acid; HPLC, high-performance liquid chromatography; TLC, thin-layer chromatography; P_i, inorganic phosphate; P-Rib-PP(S), 1-phosphorothioate analogue of P-Rib-PP; SPP_i, thiopyrophosphate.

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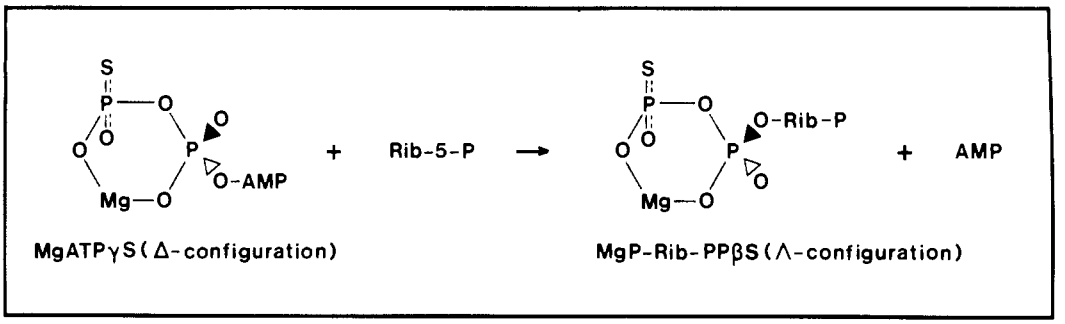

FIGURE 1: Synthesis of P-Rib-PPβS from Rib-5-P and the Mg^{2+} complex of ATPγS in the reaction catalyzed by P-Rib-PP synthetase from *S. typhimurium*. The predicted structural specificity and stereochemical course of the reaction were based on the known preference of the enzyme for only one diastereomer (Δ) of the substitution-inert $Co(NH_3)_4ATP$ complex and inversion of configuration at the P_β atom during the catalytic process (Li et al., 1978) (cf. Figure 2). "Solid" and "open" bonds at the asymmetric center represent atoms protruding above and below the plane of the metal–chelate ring, respectively. Charges have been excluded for the sake of clarity.

We report here the synthesis of P-Rib-PPβS from ATPγS and the diastereomers of P-Rib-PPαS from the ATPβS diastereomers in reactions catalyzed by P-Rib-PP synthetase isolated from *Salmonella typhimurium*.[2] Partial characterization of the purified analogues is also described, including a preliminary account of their interaction with human brain HPRTase and yeast OPRTase. A detailed ^{31}P NMR study of P-Rib-PPβS and the S_P diastereomer of P-Rib-PPαS is presented in the following paper (Smithers & O'Sullivan, 1984a).

Experimental Procedures

Chemicals. The lithium salts of ATPγS and ADPβS and phosphoenolpyruvate (Na salt) were purchased from Boehringer-Mannheim. Lithium potassium acetyl phosphate, Mes, bovine serum albumin, 2-mercaptoethanol, unsubstituted adenosine nucleotides, and the sodium salts of P-Rib-PP and Rib-5-P were obtained from Sigma Chemical Co. Ultrapure-grade $CdCl_2$ (99.999%), analytical-grade $MgCl_2$ crystals, and Hepes (Ultrol grade) were supplied by Aldrich Chemical Co., Merck, and Calbiochem-Behring, respectively. [8-^{14}C]Hypoxanthine and [*carboxy*-^{14}C]orotic acid were purchased from the Radiochemical Centre and New England Nuclear Corp., respectively.

PEI–cellulose plates (20 × 20 cm; Macherey-Nagel and Co.) were washed in 4 M formic acid–NaOH, pH 3.40, followed by methanol–water (1:1 v/v) before use. DEAE-Trisacryl M and DEAE-Sephadex (A-25), purchased from LKB and Pharmacia Fine Chemicals, respectively, were converted to their HCO_3^- forms according to the manufacturer's instructions. Activated charcoal (Sigma Chemical Co.) was acid washed prior to use (Zimmerman, 1963). Sephadex G-25 (medium) was from Pharmacia Fine Chemicals. Other chemicals were analytical reagent grade obtained from local suppliers and were used without further purification. Water was deionized and glass distilled.

Enzyme Preparations. P-Rib-PP synthetase from *S. typhimurium* (strain SU 422) was a gift from the laboratory of Dr. R. L. Switzer (University of Illinois, Urbana, IL). The enzyme was purified to a specific activity of ~100 units/mg (~6 mg/mL) (Switzer & Gibson, 1978) and was free of any nonspecific phosphatase activity (R. L. Switzer, personal communication). Aliquots (0.5 mL) were lyophilized, transported to Australia by air, and reconstituted with 50 mM potassium phosphate, pH 7.5. The preparation was rapidly frozen in liquid nitrogen and stored at −70 °C until required for use. Enzyme activity after reconstitution varied from 70 to 90% that of the original preparation.

Pyruvate kinase (rabbit muscle) and acetate kinase (*Escherichia coli*) were obtained from Boehringer-Mannheim, and a partially purified preparation of yeast OPRTase and ODCase was from Sigma Chemical Co. HPRTase was purified from human brain to apparent electrophoretic homogeneity (Smithers & O'Sullivan, 1984b). To remove P-Rib-PP, a mixture (1 mL) of the enzyme (10 μg) and bovine serum albumin (1 mg) was chromatographed on a column (1.6 × 26 cm) of Sephadex G-25 equilibrated with 10 mM Mes–KOH, 100 mM KCl, 1 mM 2-mercaptoethanol, and 0.1 mM EDTA, pH 6.0.

Preparation of ATPγS, ADPβS, and the Diastereomers of ATPβS. Commercial preparations of ATPγS and ADPβS were routinely found to contain up to 30% of contaminants, primarily AMP and ADP, as determined by HPLC on a Whatman Partisil 10-SAX ion-exchange column (Smithers & O'Sullivan, 1979) and by PEI–cellulose TLC (2 M formic acid–NaOH, pH 3.40) (Randerath & Randerath, 1964). Both analogues were purified prior to use by elution (50 mL/h) from a column (1.6 × 46 cm) of DEAE-Trisacryl M at ~5 °C, with a 2600 mL linear gradient of NH_4HCO_3 (0–0.25 M) (Smithers, 1983). Chemical purity of the preparations was >97%, as assessed by HPLC.

A mixture of the diastereomers of ATPβS, enriched in the S_P form (80%), was prepared from ADPβS and phosphoenolpyruvate in the reaction catalyzed by pyruvate kinase (Jaffe & Cohn, 1978a). The reaction was terminated with EDTA (15 mM) and the product purified as described above for ATPγS. Chemical purity of the preparation was >95%, as assessed by HPLC.

The R_P diastereomer of ATPβS was prepared from ADPβS and acetyl phosphate in the reaction catalyzed by acetate kinase (Jaffe & Cohn, 1978b). The product was purified as described above for ATPγS. Chemical purity of the preparation was ~90%, as estimated by PEI–cellulose TLC.

Solutions of the purified ATP analogues (pH ~7) were extracted with 1% (w/v) 8-hydroxyquinoline in chloroform to remove trace contaminating metal ions (Brown et al., 1973) and stored at −70 °C until required. Concentrations were determined at pH 2 (10 mM HCl) by using the extinction coefficient (15 000 M^{-1} cm^{-1} at 259 nm) reported by Goody & Eckstein (1971).

Synthesis of P-Rib-PPβS. The synthesis of P-Rib-PPβS from Rib-5-P and the Mg^{2+} complex of ATPγS is illustrated in Figure 1. The reaction mixture contained triethanolamine hydrochloride, pH 8.5 (75 mM), potassium phosphate, pH 8.5 (50 mM), $MgCl_2$ (5 mM), Rib-5-P (10 mM), ATPγS (2.5 mM), and P-Rib-PP synthetase (1.5 mg) in a final volume of

[2] Synthesis of the related analogue 5-(thiophospho)ribosyl 1-diphosphate from ribose 5-(thiophosphate) and ATP has been described (Murray et al., 1969).

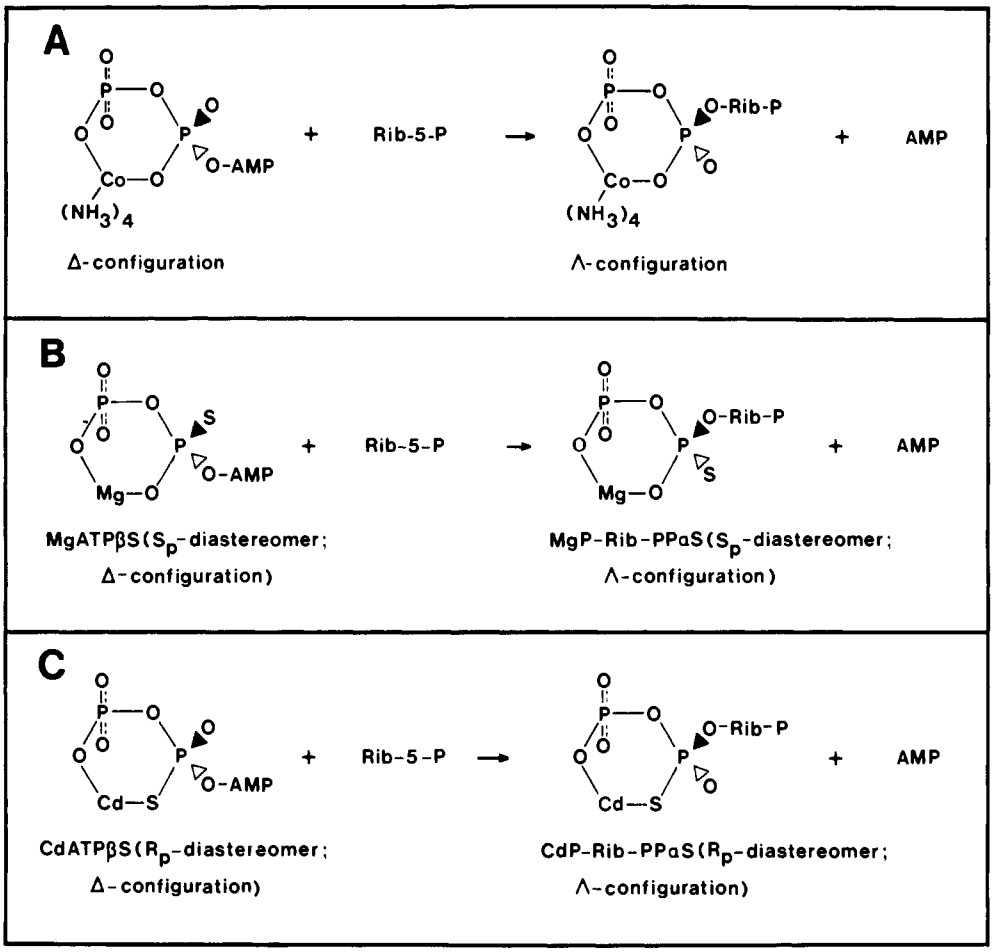

FIGURE 2: Synthesis of the P-Rib-PPαS diastereomers from Rib-5-P and the diastereomers of ATPβS in reactions catalyzed by P-Rib-PP synthetase from *S. typhimurium*. On the basis of the specificity of the enzyme for only one diastereomer (Δ) of the substitution-inert $Co(NH_3)_4ATP$ complex and inversion of configuration at the P_β atom during the catalytic process (Li et al., 1978) (A) and the stereochemistry of the ATPβS diastereomers (Jaffe & Cohn, 1978a), this figure depicts the synthesis of the Mg^{2+} complex of P-Rib-PPαS (S_P diastereomer, Λ-configuration) from the Mg^{2+} complex of ATPβS (S_P diastereomer, Δ-configuration) (B) and of the Cd^{2+} complex of P-Rib-PPαS (R_P diastereomer, Λ-configuration) from the Cd^{2+} complex of ATPβS (R_P diastereomer, Δ-configuration) (C). Although these reactions proceed with inversion of the metal-chelate configuration (Δ → Λ), the stereochemical descriptor of the chiral phosphorus atom does not change ($S_P \rightarrow S_P$; $R_P \rightarrow R_P$). "Solid" and "open" bonds at the asymmetric center represent atoms protruding above and below the plane of the chelate ring, respectively. Charges have been excluded for the sake of clarity.

5.0 mL. (Potassium phosphate was added immediately before the enzyme in order to avoid the formation of a precipitate, presumably $MgHPO_4$.)[3] The reactants were incubated at 37 °C and the progress of the reaction monitored by TLC (5-μL aliquots) on PEI–cellulose (2 M formic acid–NaOH, pH 3.40) by following the appearance of AMP, which was located under ultraviolet light. Almost quantitative conversion (>80%) of ATPγS ($R_f \sim 0$) into AMP ($R_f \sim 0.85$) was noted after 2 h, and the reaction was terminated by the addition of 1 mL of 100 mM EDTA, pH 8.0. Nucleotides and protein were removed by adding an equal volume of a 25% (w/v) aqueous charcoal suspension (Gibson et al., 1982). Following centrifugation at 3000*g* for 10 min, the supernatant was chromatographed on a column (1.6 × 26 cm) of Sephadex G-25 (medium), equilibrated and eluted with 50 mM NH_4HCO_3, pH 7.4. Eluate fractions (~3 mL) containing P-Rib-PPβS (detected as described below) were combined and diluted to 50 mL. This solution was adjusted to pH 7.0 (dilute HCl) and stored frozen (−70 °C) prior to purification.

Synthesis of the Diastereomers of P-Rib-PPαS. The synthesis of P-Rib-PPαS (S_P diastereomer) from Rib-5-P and the Mg^{2+} complex of ATPβS (S_P diastereomer, Δ-configuration) and of P-Rib-PPαS (R_P diastereomer) from Rib-5-P and the Cd^{2+} complex of ATPβS (R_P diastereomer, Δ-configuration) is illustrated in Figure 2.[4]

The S_P diastereomer of P-Rib-PPαS was synthesized as described above for P-Rib-PPβS, with the exception that ATPβS (enriched in the S_P diastereomer) (2.2 mM) replaced ATPγS in the reaction mixture. Progress of the reaction was monitored by PEI–cellulose TLC, as described for P-Rib-PPβS. Following incubation of the reactants at 37 °C for 3 h, approximately 30% conversion of ATPβS ($R_f \sim 0$) into AMP ($R_f \sim 0.85$) was noted, with some decomposition into ADPβS ($R_f \sim 0.10$). The reaction was terminated and the mixture treated as described above for P-Rib-PPβS.

The reaction mixture for the synthesis of the R_P diastereomer of P-Rib-PPαS contained triethanolamine hydrochloride, pH 8.5 (75 mM), potassium phosphate, pH 8.5 (50 mM), $CdCl_2$ (3 mM), $MgCl_2$ (2 mM), Rib-5-P (10 mM), ATPβS (R_P diastereomer) (3.8 mM), 2-mercaptoethanol (5

[3] P-Rib-PP synthetase from *S. typhimurium* has an absolute requirement for P_i, which appears to act as a stabilizer and activator of the enzyme (Switzer, 1969).

[4] The chelate configurations of chiral metal–ligand complexes have been designated by using the screw-sense nomenclature (Δ or Λ) recommended by Merritt et al. (1978). This notation readily identifies similar spatial structure without the necessity for reference to the individual atoms of the complex.

mM), and P-Rib-PP synthetase (3 mg) in a final volume of 5.0 mL.[5] Progress of the reaction was monitored by PEI–cellulose TLC, as described for P-Rib-PPβS. Following incubation of the reactants at 37 °C for 6 h, minimal conversion (<10%) of ATPβS ($R_f \sim 0$) into AMP ($R_f \sim 0.85$) was noted, with substantial decomposition of the triphosphate into ADPβS ($R_f \sim 0.10$). The reaction was terminated and the mixture treated as described above for P-Rib-PPβS.

Purification of the P-Rib-PP Analogues. The analogues were purified by elution (60 mL/h) from a column (2.6 × 36 cm) of DEAE-Sephadex (A-25, HCO_3^- form) at ~5 °C. The treated reaction mixtures were applied to the column, previously equilibrated with distilled water, and elution was continued with distilled water (~100 mL) followed by 0.1 M NH_4HCO_3, pH 7.4 (~250 mL). Column eluates in both cases were discarded. The P-Rib-PP analogues were eluted by applying a 1000-mL linear gradient of NH_4HCO_3, pH 7.4 (0.1–0.5 M). Fractions containing 6.5 mL were collected.

The analogues were detected by using a coupled enzyme assay based on the P-Rib-PP(S)-dependent release of $^{14}CO_2$ from [*carboxy*-^{14}C]orotic acid, in the presence of a divalent cation and yeast OPRTase and ODCase. The procedure was analogous to that used in estimating P-Rib-PP (Smithers & O'Sullivan, 1979), with the following exceptions. The reaction mixture described was altered to include [*carboxy*-^{14}C]orotic acid (0.1 mM, 10000–20000 dpm/nmol), 3.5×10^{-3} unit of yeast OPRTase–ODCase (capable of converting 3.5 nmol of orotic acid and P-Rib-PP into UMP and CO_2 in 1 min), and P-Rib-PP(S) (0.1–20 μM) in place of P-Rib-PP. In the assay of P-Rib-PPβS and P-Rib-PPαS (S_P diastereomer), the incubation time was 2 h, while in the assay of P-Rib-PPαS (R_P diastereomer) $CdCl_2$ (5 mM) replaced $MgCl_2$, the concentration of 2-mercaptoethanol was increased to 100 mM, and the incubation time was 3 h. Fractions containing the P-Rib-PP analogues were combined and the solvent and NH_4HCO_3 removed by lyophilization. The purified analogues were stored frozen in solution (pH ~7, 0.2-mL aliquots) at −70 °C until required.

Purity of the P-Rib-PP Analogues. Total phosphorus was determined on ashed samples by using the ascorbate–molybdate procedure of Ames & Dubin (1960), and total ribose was estimated by the orcinol procedure (Albaum & Umbreit, 1947). Ultraviolet absorption spectra (210–300 nm) were recorded on neutral aqueous samples.

Substrate Activity of the P-Rib-PP Analogues. The activity of human brain HPRTase was determined by measuring the amount of [8-^{14}C]IMP formed from [8-^{14}C]hypoxanthine, following the separation of the compounds by PEI–cellulose TLC (Smithers & O'Sullivan, 1984b). The enzyme preparation free of P-Rib-PP was used as the HPRTase source and P-Rib-PP(S) (0.05–0.3 mM) replaced P-Rib-PP in the reaction mixture.

Yeast OPRTase activity was determined by measuring liberated $^{14}CO_2$ following the conversion of [*carboxy*-^{14}C]orotic acid into UMP in the presence of excess ODCase (Fox et al., 1971) with P-Rib-PP(S) (0.02–0.3 mM) instead of P-Rib-PP in the reaction mixture. For assays carried out in the presence of $CdCl_2$, 2-mercaptoethanol (10 mM) was included in the reaction mixture and the metal ion concentration never exceeded the P-Rib-PP(S) concentration, in order to prevent $Cd(OH)_2$ formation and other effects of the free cation. All assays were carried out at pH 8.0 (50 mM Hepes–KOH) and 30 °C.

Results and Discussion

Preparation of the P-Rib-PP Analogues. Reactions catalyzed by P-Rib-PP synthetase from *S. typhimurium*, leading to the synthesis of P-Rib-PPβS and the diastereomers of P-Rib-PPαS are depicted in Figures 1 and 2, respectively. Enzymatic synthesis of these analogues was preferred to a possible chemical approach [e.g., Tener & Khorana (1958)] since (1) improved yields would be anticipated, (2) P-Rib-PP synthetase retains catalytic activity with sulfur-substituted substrates, as demonstrated in particular for ribose 5-(thiophosphate) (Murray et al., 1969) and ATPαS (Gibson & Switzer, 1980), and (3) the stereospecificity of the *S. typhimurium* enzyme and the stereochemical course of the reaction were known (Li et al., 1978) (Figure 2A). The latter was an important consideration in designing the synthesis of the P-Rib-PPαS diastereomers and also facilitated their stereochemical assignment (Figure 2B,C).

P-Rib-PP synthetase from *S. typhimurium* is highly stereospecific for only one diastereomer (Δ) of the β,γ-bidentate substitution-inert $Co(NH_3)_4ATP$ complex (Li et al., 1978), a configuration opposite to that (Λ) preferred by yeast hexokinase (Cornelius & Cleland, 1978) (Figure 2A). The synthetase also catalyzed an inversion at the chiral P_β atom of the complex, resulting in a product with opposite configuration (Λ) at the chiral $1P_\alpha$ atom (Figure 2A). Moreover, Jaffe & Cohn (1978a, 1979) have demonstrated that the strict stereospecificity of the hexokinase reaction, originally observed for the $Co(NH_3)_4ATP$ diastereomers, also applies to the ATPβS diastereomers, in a divalent cation dependent manner. These workers argued that the active substrate species (MgATPβS, R_P diastereomer; CdATPβS, S_P diastereomer) must have the Λ-configuration and the inactive species (MgATPβS, S_P diastereomer; CdATPβS, R_P diastereomer) the Δ-configuration, since the source of asymmetry for both ATPβS and $Co(NH_3)_4ATP$ resides in the configuration about the P_β atom. Since P-Rib-PP synthetase also reacts asymmetrically with the $Co(NH_3)_4ATP$ complex (Figure 2A), this enzyme would be expected to behave similarly with the ATPβS diastereomers. Thus, on the basis of the known specificity of the enzyme and the stereochemical course of the catalytic process (Figure 2A), P-Rib-PPαS (S_P diastereomer) was synthesized from Rib-5-P and the Mg^{2+} complex of ATPβS (S_P diastereomer, Δ-configuration) (Figure 2B) and P-Rib-PPαS (R_P diastereomer) was synthesized from Rib-5-P and the Cd^{2+} complex of ATPβS (R_P diastereomer, Δ-configuration) (Figure 2C).

The progress of reactions catalyzed by P-Rib-PP synthetase was conveniently monitored by following the appearance of AMP, the common product. The intensity of the ultraviolet-absorbing AMP spot served as a qualitative measure of the yield of each analogue (see Experimental Procedures). ATPγS formed an effective substrate for P-Rib-PP synthetase, leading to an almost quantitative yield of P-Rib-PPβS. However, much lower rates were observed with the ATPβS analogues, restricting the yields of the two P-Rib-PPαS diastereomers. Increased incubation times partially overcame this problem, but considerable hydrolysis of the ATPβS diastereomers into

[5] P-Rib-PP synthetase from *S. typhimurium* has a dual metal ion requirement (Li et al., 1978), both nucleotide and enzyme bound. Since the Cd^{2+} concentration never exceeded the ATPβS concentration, in order to prevent $Cd(OH)_2$ formation and other effects of the free cation, Mg^{2+} functions as the enzyme-bound activator (Switzer, 1969). The presence of this cation should not interfere with the stereospecificity of the reaction, since MgATPβS (R_P) has the inactive Λ-configuration (Jaffe & Cohn, 1978a, 1979) and, on the basis of the ability of Cd^{2+} to form complexes 2–3 orders of magnitude tighter than Mg^{2+} (Sillén & Martell, 1964), the ATPβS should be present primarily as the Cd^{2+} chelate (Δ-configuration).

ADPβS during the course of these reactions also appeared to contribute to the reduced yields of the P-Rib-PPαS analogues. The relative lability of ATPβS was expected, since the equilibrium of reactions involving phosphorothioates favors the formation of a molecule containing a terminal thiophosphate group (ADPβS) from one containing an internal thiophosphate group (ATPβS) (Cohn, 1982). Unfortunately, such a process results in both the removal of the P-Rib-PP synthetase substrate and the generation of ADPβS, a potential inhibitor of the enzyme [cf. Switzer & Sogin (1973) and Gibson et al. (1982)]. Problems associated with the preparation of the P-Rib-PPαS analogues were highlighted in the synthesis of the R_P diastereomer from the Cd^{2+} complex of ATPβS (R_P diastereomer) (Figure 2C). Although Cd^{2+} is known to activate the P-Rib-PP synthetase reaction, the rate of pyrophosphoryl group transfer from ATP in the presence of this cation is only ~10% of that measured in the presence of Mg^{2+} (Gibson & Switzer, 1980). This necessitated very lengthy incubation times for the synthesis of P-Rib-PPαS (R_P diastereomer). Substantial decomposition of ATPβS occurred during the course of the reaction, a process that appears to be accelerated by Cd^{2+} and related divalent cations (E. K. Jaffe, personal communication).

The P-Rib-PP analogues were purified by gradient elution from DEAE-Sephadex. Column profiles depicting their separation from contaminating Rib-5-P and P_i are illustrated in Figure 3. As expected, the P-Rib-PP analogues were retarded on the column, eluting significantly later than the parent compound, which probably reflects the lowered pK_a values associated with thiophosphate groups (Jaffe & Cohn, 1978b; Smithers & O'Sullivan, 1984a). Supporting evidence for this conclusion comes from the analogous chromatographic behavior observed for the phosphorothioate analogues of ADP and ATP, when compared with their respective parent molecules (Smithers, 1983). Although the structure of the P-Rib-PPαS diastereomers differs only in the configuration about the $1P_\alpha$ atom, these analogues could be resolved by DEAE-Sephadex chromatography (compare parts B and C of Figure 3). Such behavior is similar to the recently reported separation of the GTPαS diastereomers by an analogous chromatographic procedure (Connolly et al., 1982) and suggests that this phenomenon may be a common feature of diastereomeric phosphorothioates.

On the basis of the initial concentration of the appropriate ATP phosphorothioate, the estimated overall yield of P-Rib-PPβS, following synthesis and purification, was ~75% (~9.5 μmol), while the yields of the S_P and R_P diastereomers of P-Rib-PPαS were ~20% (~2 μmol) and ~1% (~0.2 μmol), respectively.

Chemical purity of the P-Rib-PP analogues was assessed by chemical analysis and ultraviolet spectroscopy. The purified analogues contained ribose and phosphorus in a molar ratio similar to that measured for the purified parent compound and approaching the theoretical value of 1.00:3.00: viz., P-Rib-PPβS, 1.00:3.02; P-Rib-PPαS (S_P diastereomer), 1.00:3.07; P-Rib-PPαS (R_P diastereomer), 1.00:3.10; P-Rib-PP, 1.00:3.11. Contamination of the analogues by nucleotide material (OD_{260}) and residual protein (OD_{280} and OD_{215}) was negligible. The chemical purity of P-Rib-PPβS and P-Rib-PPαS (S_P diastereomer) and the diastereomeric purity of the latter analogue were also assessed by ^{31}P NMR (Smithers & O'Sullivan, 1984a). On the basis of the known effect of sulfur substitution on ^{31}P chemical shifts and spin–spin coupling constants (Eckstein & Goody, 1976; Jaffe & Cohn, 1978b), this technique also confirmed the location of the thiophosphate group in both P-Rib-PPβS and P-Rib-PPαS.

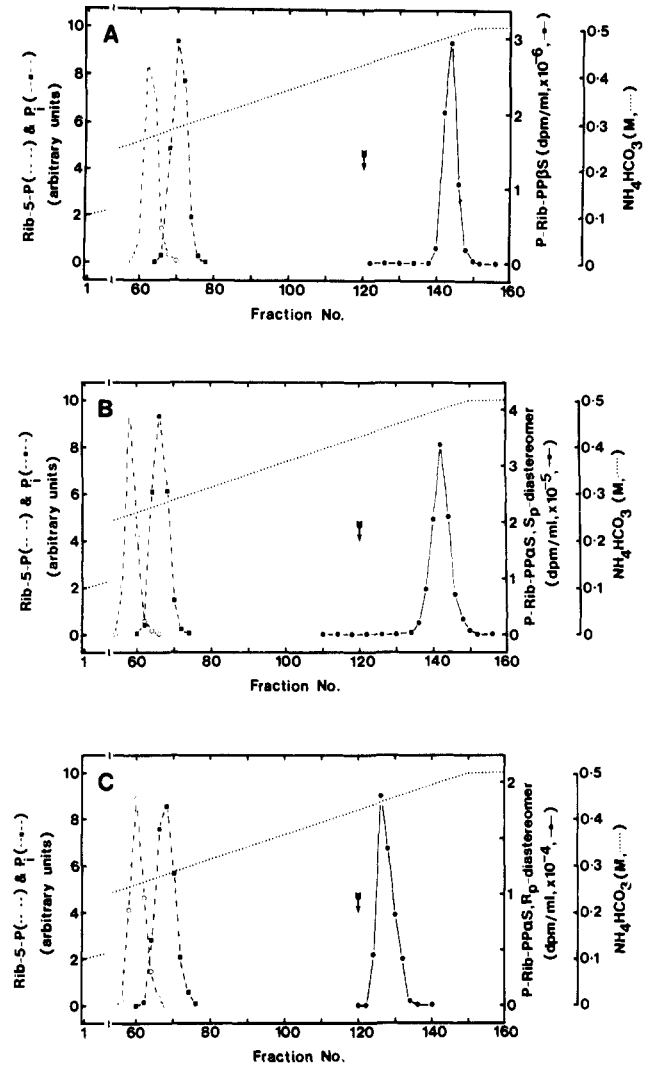

FIGURE 3: Column profiles depicting the purification of P-Rib-PPβS (A), P-Rib-PPαS (S_P diastereomer) (B), and P-Rib-PPαS (R_P diastereomer) (C) achieved by the gradient elution of a column (2.6 × 36 cm) of DEAE-Sephadex (A-25) with NH_4HCO_3, pH 7.4. The P-Rib-PP analogues were prepared as described in the text and illustrated in Figures 1 and 2. The treated reaction mixtures were applied to the column, which was equilibrated and initially developed with distilled water (~100 mL). Elution was continued with 0.1 M NH_4HCO_3, pH 7.4 (~250 mL), followed by a 1000-mL linear gradient of NH_4HCO_3, pH 7.4 (0.1–0.5 M). Fractions contained 6.5 mL. The P-Rib-PP analogues were detected by using an enzymatic assay based on the P-Rib-PP(S)-dependent release of $^{14}CO_2$ from [*carboxy*-^{14}C]orotic acid in the presence of Mg^{2+} (P-Rib-PPβS and the S_P diastereomer of P-Rib-PPαS) or Cd^{2+} (R_P diastereomer of P-Rib-PPαS) and yeast OPRTase and ODCase, as described in the text. Authentic samples of Rib-5-P [(○) 2.5 mg] and P_i [(■) 5 mg] were chromatographed separately in order to establish elution positions. The point of elution of P-Rib-PP, chromatographed separately under identical conditions, is indicated by the arrow (↓).

Substrate Activity of the P-Rib-PP Analogues. A preliminary assessment of the ability of the P-Rib-PP analogues to act as alternative substrates to P-Rib-PP in the reactions catalyzed by human brain HPRTase and yeast OPRTase was carried out. In the presence of Mg^{2+}, P-Rib-PPβS and P-Rib-PPαS (S_P diastereomer) proved to be effective phosphoribose donors in both reactions.[6] Although the estimates were

[6] Under the assay conditions, detectable activity represents the recovery of >1500 dpm (above background) in the radiolabeled assay product.

only qualitative, these two analogues gave activities of approximately 30 and 5–10%, respectively, of that seen with P-Rib-PP under standard assay conditions. While OPRTase activity could not be detected with the R_P diastereomer of P-Rib-PPαS,[7] this analogue was found to serve as a substrate when Cd^{2+} was used as the activating metal ion (see also Figure 3C).

Relatively poor yields in the synthesis of the P-Rib-PPαS diastereomers, particularly the R_P form, restricted a more extensive study at this stage. Nevertheless, the demonstrated activity of the analogues in the HPRTase, OPRTase, and, more recently, the quinolinate phosphoribosyltransferase (Kunjara et al., 1983) reactions illustrates their potential as probes of the phosphoribosyltransferase enzymes. The reversal of stereospecificity for the P-Rib-PPαS diastereomers, upon changing the activating metal ion from Mg^{2+} to Cd^{2+} in the yeast OPRTase reaction, is analogous to the divalent cation dependent stereospecificity reported for the ATPαS and ATPβS diastereomers in a number of kinase reactions (Cohn, 1982). Extrapolation of the interpretations drawn in the kinase studies would suggest that the (Λ)-α,β-bidentate metal–P-Rib-PP complex (Figure 2B,C) forms the active substrate for yeast OPRTase and that the metal ion remains liganded to the $1P_\alpha$ moiety during the catalytic process. Such interpretations are based upon the preference of Mg^{2+} for oxygen and Cd^{2+} for sulfur at the thiophosphate group of the P-Rib-PPαS diastereomers, a rationale already established for the ATPβS diastereomers in the study of the hexokinase-catalyzed reaction (Jaffe & Cohn, 1978a, 1979).

The activity of the P-Rib-PP analogues with OPRTase formed the basis for a specific and sensitive radiochemical assay for these analogues. The method was directly adapted from that used in the determination of the parent compound (May & Krooth, 1976; Tax & Veerkamp, 1977) and was based on the P-Rib-PP(S)-dependent release of $^{14}CO_2$ from [*carboxy*-^{14}C]orotic acid in the presence of a divalent cation and excess ODCase. Although the lower limit of detection of this assay was ~0.5 nmol, the sensitivity would be improved by favorably displacing the equilibrium of the reaction sequence with yeast inorganic pyrophosphatase, for which SPP_i is an active substrate (Webb & Trentham, 1980).

In conclusion, the use of the P-Rib-PP analogues as probes of the stereoselectivity of the phosphoribosyltransferase enzymes and, in particular, the chelate configuration of the active metal complex of P-Rib-PP (as described above for OPRTase), represents the most immediate and potentially rewarding application of these analogues. Similar studies involving quinolinate phosphoribosyltransferase (Kunjara et al., 1983) and amido phosphoribosyltransferase are in progress and will be reported elsewhere.

Acknowledgments

We are grateful to Dr. Robert L. Switzer and members of his laboratory, particularly Dr. Katherine J. Gibson and Simon Rosenzweig, for providing the purified P-Rib-PP synthetase and information regarding its use. Studies on the stability of the lyophilized enzyme preparation were also carried out in Dr. Switzer's laboratory.

Registry No. P-Rib-PPβS, 91385-22-7; Rib-5-P, 4300-28-1; Mg–ATPγS, 75625-12-6; (*S*)-P-Rib-PPαS, 91389-14-9; (*R*)-P-Rib-PPαS, 91465-65-5; Mg–(*S*)-ATPβS, 72052-07-4; Cd–(*R*)-ATPβS, 72052-15-4; P-Rib-PP synthetase, 9015-83-2; HPRTase, 9016-12-0; OPRTase, 9030-25-5; phosphoribosyltransferase, 9076-94-2.

[7] Enzyme activity could not be detected, even in the presence of 3 times as much enzyme (9×10^{-3} unit) and an incubation time of 3 h.

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Phosphorothioate Analogues of 5-Phosphoribosyl 1-Diphosphate: ^{31}P Nuclear Magnetic Resonance Study†

Geoffrey W. Smithers‡ and William J. O'Sullivan*

ABSTRACT: ^{31}P nuclear magnetic resonance (NMR) has been used to study the 1-phosphorothioate analogues of 5-phosphoribosyl 1-diphosphate (P-Rib-PP). Comparison of the proton-decoupled spectra of 5-phosphoribosyl 1-*O*-(2-thiodiphosphate) (P-Rib-PPβS) and the S_P diastereomer of 5-phosphoribosyl 1-*O*-(1-thiodiphosphate) (P-Rib-PPαS) with the parent molecule revealed a characteristic large downfield chemical shift change for the resonance signal associated with the thiophosphate group ($\Delta\delta \sim 40$–50 ppm) and an increase in the magnitude of the phosphate–thiophosphate spin–spin coupling constant ($\Delta J_{\alpha\beta} \sim 10$ Hz). Both these changes are consistent with the observed effects of sulfur substitution on the behavior of the adenosine nucleotides, particularly ADP [Jaffe, E. K., & Cohn, M. (1978) *Biochemistry 17*, 652–657]. High-field ^{31}P NMR has also been used to demonstrate the diastereomeric purity of P-Rib-PPαS (S_P diastereomer) and the greater lability of this analogue when compared with both P-Rib-PPβS and P-Rib-PP. Sulfur substitution was found to cause a large decrease in the apparent pK_a associated with the thiophosphate moiety of P-Rib-PPβS (Δp$K_a \sim 1.4$ units) and also to enhance the sensitivity of the thiophosphate chemical shift to protonation and, in particular, to Mg^{2+} binding, compared with P-Rib-PP. The potential application of the phosphorothioate analogues as probes of the reactions catalyzed by the phosphoribosyltransferase enzymes is discussed.

The utility of ^{31}P NMR[1] in studies of phosphorus-containing molecules of biological importance and their interaction with diamagnetic metal ions, particularly Mg^{2+} (Tran-Dinh et al., 1975; Tran-Dinh & Neumann, 1977; Tran-Dinh & Roux, 1977; Nageswara Rao & Cohn, 1977; Bock, 1980; Bishop et al., 1981; Schliselfeld et al., 1982; Vogel & Bridger, 1982; Smithers & O'Sullivan, 1982), and also as a probe of enzyme-catalyzed reaction mechanisms and the structure and dynamics of enzyme–substrate complexes (Cohn & Nageswara Rao, 1979) has been well documented.

Although these applications of the technique appear to suffer from some limitations (Jaffe & Cohn, 1978a; Vogel & Bridger, 1982; Smithers & O'Sullivan, 1982), ^{31}P NMR has proved particularly rewarding in several studies involving phosphorothioate analogues. In a pioneering study, Jaffe & Cohn (1978a) elucidated differences in ^{31}P chemical shifts, spin–spin coupling constants, and apparent pK_a values between the phosphorothioate analogues of the adenosine nucleotides and their respective parent molecules. Of these differences, the change in pK_a upon sulfur substitution appears to provide a potential tool for probing the nature of metal–substrate–enzyme complexes where the pK_a of the bound metal–substrate differs significantly from that of the free complex (Jaffe & Cohn, 1978a; Cohn & Nageswara Rao, 1979). More recent applications include the direct determination of the equilibrium constants of phosphoryl group transfer reactions (Jaffe & Cohn, 1980; Lerman & Cohn, 1980) and studies of the coordination structure of free and enzyme-bound metal–ATP and metal–ADP complexes (Jaffe & Cohn, 1978b). ^{31}P NMR has also proved useful in distinguishing the diastereomers of several phosphorothioate analogues (Jaffe & Cohn, 1978a; Connolly et al., 1982; Orr et al., 1982). The technique thus provides a direct, nondestructive measure of diastereomeric purity and also the stereospecificity of enzymatic reactions involving these analogues [e.g., Orr et al. (1982)].

In the preceding paper (Smithers & O'Sullivan, 1984), we described the preparation and partial characterization of the 1-phosphorothioate analogues of P-Rib-PP. We present here the results of a ^{31}P NMR study of P-Rib-PPβS and the S_P diastereomer of P-Rib-PPαS. [Unfortunately, low yields in

† From the School of Biochemistry, University of New South Wales, Kensington, New South Wales 2033, Australia. *Received November 1, 1983.* This work was supported, in part, by financial assistance from the Australian Research Grants Scheme.

‡ Supported by a Commonwealth postgraduate research scholarship. Present address: Department of Biochemistry and Biophysics, University of Pennsylvania School of Medicine, Philadelphia, PA 19104.

[1] Abbreviations: NMR, nuclear magnetic resonance; P-Rib-PP, 5-phosphoribosyl 1-diphosphate (the designations 5P and 1PP are used to denote the 5-phosphate and 1-diphosphate groups, respectively; $1P_\alpha$ indicates the phosphorus atom of the diphosphate group closest to the anomeric carbon of the ribose ring and $1P_\beta$ is the successively remote phosphorus atom); P-Rib-PPβS, 5-phosphoribosyl 1-*O*-(2-thiodiphosphate); P-Rib-PPαS, 5-phosphoribosyl 1-*O*-(1-thiodiphosphate); $J_{\alpha\beta}$, spin–spin coupling constant between the P_α and P_β nuclei; Rib-5-P, ribose 5-phosphate; SPP_i, thiopyrophosphate; δ, chemical shift (parts per million from 85% H_3PO_4); *R*, $[Mg]_T/[\text{phosphorylated ligand}]_T$; ADP$\beta$S, adenosine 5′-*O*-(2-thiodiphosphate); P_i, inorganic phosphate.

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