Mechanism for the Enzymatic Formation of 4-(β-D-Ribofuranosyl)aminobenzene 5′-Phosphate during the Biosynthesis of Methanopterin†

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Received December 16, 1997; Revised Manuscript Received June 5, 1998

ABSTRACT: A central step in the biosynthesis of the modified folate methanopterin is the condensation of *p*-aminobenzoic acid (*p*AB) and 5-phospho-α-D-ribosyl-1-pyrophosphate (PRPP) which produce 4-(β-D-ribofuranosyl)aminobenzene 5′-phosphate (β-RFA-P) [White, R. H. (1996) *Biochemistry 35*, 3447−3456]. This reaction, catalyzed by the enzyme β-RFA-P synthase, is unique among known phosphoribosyltransferases in that a decarboxylation of one of the substrates (*p*AB) occurs during the reaction and a *C*-riboside rather than an *N*-riboside is the product. In this work, the reaction catalyzed by the enzyme from *Methanosarcina thermophila* is shown to be analogous to other phosphoribosyltransferase reactions in that pyrophosphate is released as a product of the reaction, which is dependent upon magnesium ions. The molecular weight of the enzyme was estimated to be 65 000 using gel filtration chromatography, and the pH optimum was 4.8. Kinetic analysis indicated that the reaction involved a sequential pattern of substrate binding. Benzoic acid and several para-substituted benzoic acids inhibited β-RFA-P synthase activity, while aniline, 4-aminobenzamide, and the methyl ester of *p*AB did not, indicating that an ionized carboxylic group plays a role in the binding of *p*AB. The observation that the enzyme was not inhibited by carbonyl reagents and that 4-hydroxybenzoic acid served as an alternate substrate, producing 4-(β-D-ribofuranosyl)hydroxybenzene 5′-phosphate as the product, indicated that pyridoxal phosphate was not directly involved in the reaction mechanism. Incubation of the enzyme with PRPP and either *p*AB or 4-aminothiobenzoic acid in the presence of sodium cyanoborohydride led to the decreased production of β-RFA-P and the accumulation of a reduced form of the proposed cyclohexadienimine reaction intermediates. These compounds were characterized by their acid-catalyzed decomposition which produces β-D-ribofuranosylbenzene 5′-phosphate. On the basis of these results, a concerted mechanism is proposed for β-RFA-P synthase in which an S_N1-like reaction produces oxonium ion character at C-1 of PRPP which undergoes an ipso electrophilic aromatic substitution reaction at the carboxylic acid-bound carbon of *p*AB. Decarboxylation of the resulting cyclohexadienimine intermediate leads to the formation of β-RFA-P.

Phosphoribosyltransferases (PRTases)[1] are a group of enzymes involved in the biosynthesis of purine, pyrimidine, and pyridine nucleotides (*1*). In all of the reactions reported to date, with the exception of that of glutamine PRPP amidotransferase, an aromatic nitrogen of a purine, pyrimidine, or pyridine displaces the α-linked pyrophosphate of 5-phospho-α-D-ribosyl-1-pyrophosphate (PRPP) with the formation of a β-*N*-riboside monophosphate and inorganic pyrophosphate (PP_i). In the case of glutamine PRPP amidotransferase, an analogous reaction occurs but this time with the amide nitrogen of glutamine displacing the pyrophosphate of PRPP with the formation of β-D-5-phosphoribosyl-1-amine as the final product (*2*). Comparisons of the primary structures of the PRTases have revealed the conservation of only a short stretch of 12 or 13 homologous residues which is currently proposed to represent a PRPP binding motif (*3*, *4*). We now report the occurrence of a new type of PRTase which is involved in the biosynthesis of the C_1 carrier coenzyme methanopterin, one of the modified folates functioning in the methanogenic archaea (*5*, *6*). The reaction catalyzed by this PRTase, the condensation of 4-aminobenzoic acid (*p*AB) with PRPP which produces 4-(β-D-ribofuranosyl)aminobenzene 5′-phosphate (β-RFA-P), CO_2, and PP_i (Figure 1), represents the first enzymatic step in the incorporation of *p*AB into methanopterin. The reaction is mechanistically distinct from other those of PRTases in that a decarboxylation of one of the substrates, the *p*AB, occurs during the overall reaction, and

† This work was supported by National Science Foundation Grant MCB 963086.

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[1] Abbreviations: β-RFA-P, 4-(β-D-ribofuranosyl)aminobenzene 5′-phosphate; *p*AB, 4-aminobenzoic acid; PRPP, 5-phospho-α-D-ribosyl-1-pyrophosphate; P_i, inorganic phosphate; PP_i, pyrophosphate; β-RFH, 4-(β-D-ribofuranosyl)hydroxybenzene; β-RFH-P, 4-(β-D-ribofuranosyl)-hydroxybenzene 5′-phosphate; β-RFB, β-D-ribofuranosylbenzene; β-RFB-P, β-D-ribofuranosylbenzene 5′-phosphate; DTT, dithiothreitol; MES, 2-(4-morpholino)ethanesulfonic acid; MOPS, 3-(*N*-morpholino)propanesulfonic acid; TES, *N*-[tris(hydroxymethyl)methyl]-2-aminoethanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; PLP, pyridoxal phosphate; PRTase, phosphoribosyltransferase; GC−MS, gas chromatography−mass spectrometry.

S0006-2960(97)03086-9 CCC: $15.00
Published on Web 07/24/1998

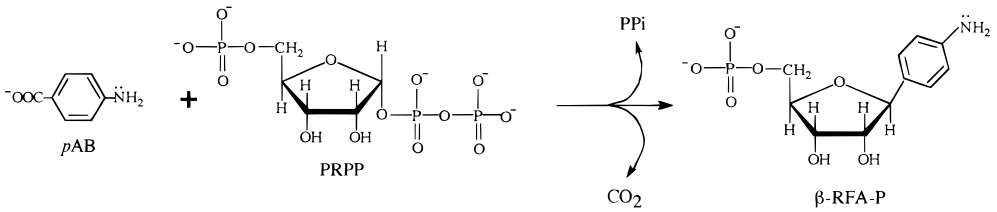

FIGURE 1: Reaction catalyzed by β-RFA-P synthase.

the product of the reaction is a *C*-riboside (*7*). Although many *C*-glycosides have been characterized in biological systems, this is the first confirmed example of PRPP being a reactant in the formation of such compounds.

In this paper, we report on the partial purification of β-RFA-P synthase from the methanogenic archaeon *Methanosarcina thermophila* and show that it has a native molecular weight of 65 000 and likely contains a bound pyridoxal phosphate. A possible mechanism of this enzyme based on inhibitor studies and alternate substrates and products generated by the enzymatic reaction is presented and discussed.

MATERIALS AND METHODS

Materials. 5-Phospho-α-D-ribosyl-1-pyrophosphate, 4-aminobenzoic acid, and pyridoxal phosphate were obtained from Sigma Chemical Co. Methoxylamine hydrochloride, 4-hydrazinobenzoic acid, phenylhydrazine, 2-aminobenzoic acid, 3-aminobenzoic acid, 4-aminobenzamide, 4-nitrobenzoyl chloride, sodium hydrosulfide, and methyl 4-aminobenzoate were obtained from Aldrich Chemical Co. 4-(Dimethylamino)benzoic acid was purchased from Fisher Scientific. 5-Aminopyrimidine-2-carboxylic acid was prepared as described by Krchnak and Arnold (*8*).

Bacterial Strain, Growth Conditions, and Preparation of Cell Extracts. *M. thermophila* strain TM-1 (*9*) was grown in the laboratory of J. G. Ferry as previously described (*10*). Cell extracts of *M. thermophila* were prepared by French Press lysis (*11*) in buffer containing 25 mM Tris (pH 7.4), 10 mM $MgCl_2$, 5% (v/v) glycerol, and 10 mM β-mercaptoethanol and were stored frozen under argon at −78 °C until they were needed. The protein concentrations of cell extracts typically ranged from 7 to 26 mg/mL.

Partial Purification of β-RFA-P Synthase. The purification of β-RFA-P synthase from *M. thermophila* was performed at ambient temperature (approximately 23 °C) aerobically in the presence of 2 mM dithiothreitol (DTT). Cell extract (7.5 mL) was applied to a Q-Sepharose column (2.5 cm × 15 cm) equilibrated with 25 mM Tris buffer (pH 7.4) containing 10 mM $MgCl_2$ and 2 mM DTT. The column was developed using a 0 to 0.6 M NaCl linear gradient in a volume of 360 mL at a flow rate of 5 mL/min. Fractions (10 mL each) eluting between 0.35 and 0.43 M NaCl contained the β-RFA-P synthase activity. All individual fractions were stored separately under argon at −78 °C until they were needed.

A Q-Sepharose fraction (9 mL) containing β-RFA-P synthase activity was filtered through a Centricon 100 concentrator (Amicon, Beverly, MA) to remove high-molecular weight proteins. The filtrate (flow-through) was centrifuged in a Centricon 50 concentrator, and the retained solution containing β-RFA-P synthase activity was diluted 10-fold with buffer A [50 mM TES (pH 6.8), 10 mM $MgCl_2$, 10% glycerol, and 2 mM DTT]. The sample was loaded onto a Mono Q 5/5 column (0.5 cm × 5 cm) (Pharmacia) equilibrated with the same buffer. The column was washed with 6 mL of 100 mM NaCl in buffer A. Proteins were separated with a 15 mL linear gradient of 100 to 130 mM NaCl in buffer A at a flow rate of 0.5 mL/min, and 1 mL fractions were collected. The β-RFA-P synthase activity eluted at about 110 mM NaCl.

Superose 12 Column Chromatography. One milliliter of a Q-Sepharose fraction containing β-RFA-P synthase activity was concentrated using a Centricon 30 microconcentrator (Amicon). The proteins were separated on a Superose 12 HR 10/30 gel filtration column (10 cm × 30 cm) (Pharmacia) at a flow rate of 0.5 mL/min. The column was equilibrated and eluted with 50 mM TES buffer (pH 6.8) containing 10 mM $MgCl_2$, 100 mM NaCl, and 2 mM DTT, and 0.5 mL fractions were collected. β-RFA-P synthase activity was measured in each fraction as described below.

Protein Determinations. Protein concentrations were determined using the Bio-Rad dye-binding assay (Hercules, CA) with bovine serum albumin as the standard.

Assay of β-RFA-P Synthase. The desired volume of enzyme solution to be assayed (up to 50 μL) was added to 50 mM TES buffer and 10 mM $MgCl_2$ (pH 6.8) to give a final volume of 100 μL. To this solution were added 7.5 μL of a 0.1 M *p*-aminobenzoic acid (*p*AB) solution and 1 mg of PRPP dissolved in 10 μL of the same buffer, unless otherwise indicated. This gave final concentrations of 6.4 mM *p*AB and 8.8 mM PRPP at the start of the incubation. The enzyme assay mixture was then incubated at 50 °C for periods of up to 3 h (the linear range of the assay), after which time the reaction was terminated by the addition of approximately 2 μL of 1 M citric acid to a pH of 3.6. The resulting mixture was then passed into a C_{18} column (0.5 cm × 3 cm) (preparative C_{18} bulk packing material; Waters Corp., Milford, MA) equilibrated with 0.1 M citrate buffer (pH 3.6). An additional 100 μL of the citrate buffer was used to transfer the remaining sample to the column. The column was then washed with 0.3 mL of the citrate buffer. The total effluent collect up to this point was fraction 1. Fractions of 0.8 mL were then collected using 0.1 M citrate buffer (pH 3.6) as the eluting solvent. Under these conditions, 90% of the β-RFA-P eluted in fraction 2. The *p*AB began to elute at fraction 5, and *p*AB was completely eluted from the column by washing the column with 5 mL of 50% methanol. The column was made ready for the next assay after washing with 5 mL of the citrate buffer.

The amount of β-RFA-P in the C_{18} column fractions was quantified by the Bratton−Marshall assay. This assay was carried out at 23 °C by adding, while the solution was being mixed, 0.1 mL of 6 M HCl to each 0.8 mL fraction, followed

by 0.1 mL of a 1.5% $NaNO_2$ solution. After 2 min, 0.1 mL of a solution of 7.5% ammonium sulfamate was added, while the solution was being mixed. After an additional 2 min, 0.3 mL of a 0.1% solution of *N*-(1-naphthyl)ethylenediamine dihydrochloride was added, and the solution was left to stand for 1 h at room temperature to complete the formation of the azo dye. The absorbance was then measured at 562 nm, and a molar extinction coefficient of 4.8×10^4 cm^{-1} M^{-1} was used for quantitation (*12*).

The pH optimum for β-RFA-P synthase activity was determined in the presence of 10 mM $MgCl_2$, 6.4 mM *p*AB, 4.4 mM PRPP, and a mixed buffer (40 mM acetate, 40 mM MES, and 40 mM MOPS) adjusted to pH values between 4.0 and 7.5 with NaOH or HCl. The β-RFA-P synthase activity was measured using the Bratton−Marshall assay as described above. For determination of kinetic constants, the assay contained the mixed buffer at pH 4.8 with 30 mM $MgCl_2$. The K_m for *p*AB was measured at a fixed PRPP concentration of 8.8 mM, and the K_m for PRPP was measured in the presence of 0.64 mM *p*AB. The K_m was determined by plotting the reaction velocity versus the substrate concentration and using a nonlinear least-squares program (EZ-Fit) to fit the data to the Michaelis−Menten equation, $v = (V_{max}[S])/(K_m + [S])$, where v is the reaction velocity and [S] is the substrate concentration (*13*).

Assay for Inhibitors of β-RFA-P Synthase. Assays for testing potential inhibitors of β-RFA-P synthase were performed as described above with 50 mM sodium acetate (pH 4.8) in place of 50 mM TES buffer (pH 6.8). Analogues of *p*AB (up to 5 mM final concentrations) were added to assays containing 85 μM *p*AB and 4.4 mM PRPP. The low *p*AB concentration was selected so that inhibition by *p*AB analogues could be observed. Inhibition by phosphate-containing compounds (up to 10 mM PP_i, phosphate, ribose 5-phosphate, or pyridoxol 5-phosphate) was measured in the presence of 6.4 mM pAB and 0.9 mM PRPP. Assays containing pyridoxal phosphate-modifying reagents (5 mM phenylhydrazine or 5 mM methoxylamine hydrochloride) were performed in the presence of 6.4 mM *p*AB and 4.4 mM PRPP. To test the effect of cysteine on β-RFA-P synthase activity, the enzyme was preincubated with 10 mM cysteine at room temperature for 15 min before measuring the activity in the presence of 6.4 mM *p*AB and 4.4 mM PRPP.

Treatment of the Enzyme with Sodium Borohydride or Cyanoborohydride. Solid sodium borohydride (approximately 1 mg) was added to a solution containing 125 mM bis-tris propane buffer (pH 7.5), 10 mM $MgCl_2$, and 10 μg of protein from a Q-Sepharose fraction in a final volume of 80 μL. The solution was incubated at room temperature for 15 min. To inactivate the remaining sodium borohydride, the pH of the solution was adjusted to 5.5 with the addition of 20 μL of 500 mM MES buffer (pH 5.5). The mixture was incubated at room temperature for an additional 15 min, and the amount of β-RFA-P synthase activity remaining in the sample was measured using the Bratton−Marshall assay. In a separate experiment, sodium borohydride was removed by passage of the sample through a 0.5 cm × 6 cm column of G-25−80 gel filtration medium (Sigma) equilibrated with 50 mM TES buffer (pH 6.8), 10 mM $MgCl_2$, and 100 mM NaCl. Assays for testing the effect of cyanoborohydride on enzyme activity contained 50 mM acetate (pH 4.8), 10 mM $MgCl_2$, 6.4 mM *p*AB, 4.4 mM PRPP, and 30 mM sodium cyanoborohydride.

Identification of PP_i as a Product of the β-RFA-P Synthase Reaction. [β-^{32}P]PRPP was synthesized enzymatically by incubating 0.15 unit of PRPP synthetase (Sigma, St. Louis, MO) for 45 min at 37 °C in 200 μL of a solution containing 50 mM TES buffer (pH 7.5), 3 mM ATP, 3 mM ribose 5-phosphate, 3 mM $MnCl_2$, 20 mM NaF, and 100 μCi of [γ-^{32}P]ATP (150 μCi/μL) (NEN, Boston, MA). Unreacted [γ-^{32}P]ATP was removed by passage of the reaction mixture through a 100 μL column (0.3 cm × 1.5 cm) containing a 1:1 mixture of Norit A decolorizing carbon (Fisher) and Hyflo Super Cel (Fisher). A portion of the [β-^{32}P]PRPP solution (12.5 μL) was added to a β-RFA-P synthase reaction solution (final volume of 50 μL) containing 50 mM acetate buffer (pH 4.8), 30 mM $MgCl_2$, 10 mM DTT, 6.4 mM pAB, 8.8 mM PRPP, and 27 μg of protein from a Q-Sepharose fraction. The reaction was terminated by adjusting the pH to 3.6 with 5 μL of 1 M citric acid. A portion of the β-RFA-P synthase reaction mixture (2 μL) was mixed with 1 μL of methanol and the mixture applied to a 20 cm × 20 cm polyethyleneimine−cellulose thin layer chromatography sheet (Fisher). The plate was developed (75 min) with 0.85 M KH_2PO_4 at pH 3.4 as described by Jensen et al. (*14*). Compounds containing ^{32}P were detected using a phosphorimager (Packard Instrument Co., Downers Grove, IL). To determine if β-RFA-P synthase catalyzed an exchange reaction between PRPP and PP_i in solution, the reaction was performed with 1 mM PP_i and [β-^{32}P]PRPP as described above, but in the absence of *p*AB. On the basis of the previously measured rates for the hydrolysis of PP_i and its metal complexes, no significant chemical hydrolysis of the PP_i should occur under the experimental conditions used (*15*).

Chemical Synthesis of 4-Aminothiobenzoic Acid. 4-Nitrobenzoyl chloride (1 mmol, 185 mg), ground to a fine powder, was added over a 2 min period to a solution of 2 mmol of sodium hydrosulfide dissolved in 2 mL of water being rapidly stirred. After 1 min, all of the material had reacted and dissolved to give a warm light yellow solution. After an additional 20 min at room temperature, 0.5 mL of 15 M aqueous ammonia was added to the stirred solution followed by 0.45 g of sodium dithionite from a freshly opened bottle. After a reaction period of 20 min, 88% formic acid was added to a pH of 2−3 and the resulting suspension was extracted three times with 2 mL portions of benzene. To the combined benzene layers was added an equal volume of hexane, and the sample was centrifuged to remove the precipitate. The resulting hexane/benzene solution was cooled to −20 °C to produce fine colorless needles of 4-aminothiobenzoic acid. The material dissolved in water had a λ_{max} of 312 nm with a shoulder at 264 nm. ^{1}HMR ($CDCl_3$): δ 7.73 (2H, d, $J = 8.85$ cps), 6.63 (2H, d, $J = 8.85$ cps), 4.2 (3H, broad band, NH_2 and SH). Mass spectra: M^+ *m/z* 153 with isotopic $M^+ + 2$ ion indicating the presence of one sulfur. Base peak $M^+ - SH = m/z$ 120.

Chemical Synthesis of 4-(β-D-Ribofuranosyl)hydroxybenzene (β-RFH) and Identification of 4-(β-D-Ribofuranosyl)-hydroxybenzene 5′-Phosphate (β-RFH-P) as an Alternate Product of the β-RFA-P Synthase Reaction. 2,5:3,4-*O*-Diisopropylidene-1-(4-aminophenyl)-D-ribitol (1−2 mg) was deprotected and cyclized by dissolution in 1 mL of 1 M HCl, heating at 100 °C for 15 min, and evaporation to dryness

with a stream of nitrogen gas (*15*). The resulting β-RFA was converted into β-RFH by the thermal decomposition of the diazonium salt of the β-RFA. Thus, the β-RFA was dissolved in 1 mL of 1 M H_2SO_4, and 100 μL of 1.5% $NaNO_2$ was added. After 5 min, 100 μL of 7.5% ammonium sulfamate was added, and the sample was heated at 100 °C for 10 min. After the solution cooled to room temperature, the β-RFH in the resulting sample was purified by column chromatography on a 0.5 cm × 4 cm C_{18} column using a water/methanol step elution gradient consisting of 2 × 2 mL fractions of 0, 5, 10, 20, 30, and 50% methanol in water. The elution was followed at the absorbance maximum of the sample at 272 nm, and the product eluted in the fraction containing 20% methanol in water. A portion of the purified sample (50 μg) was reacted overnight at room temperature with methylene chloride/trifluoroacetic anhydride to form the tetratrifluoroacetyl derivative which was analyzed by GC−MS under the same conditions used for the identification of β-RFA (*15*).

β-RFH-P was produced enzymatically by incubating β-RFA-P synthase (50 μg of protein) with 50 mM MES buffer (pH 6.0), 10 mM $MgCl_2$, 8.8 mM PRPP, and 9 mM 4-hydroxybenzoic acid in a final volume of 550 μL. To the incubated sample were added 200 μL of 0.1 M glycine buffer (pH 10.4) and 1 unit of *Escherichia coli* alkaline phosphatase. After 2 h at 37 °C, the sample was acidified to pH 1−2 with 1 M H_2SO_4, and the β-RFH was purified by C_{18} column chromatography under the same conditions used in the purification of the known compound. The C_{18} chromatographic fraction containing the enzymatically produced β-RFH showed an absorbance spectrum with an absorbance maximum of 274 nm in water at neutral pH; this is characteristic of a phenol. GC−MS of the tetratrifluoroacetyl derivative of the sample gave one peak with the same retention time and mass spectrum as that of the synthetic sample ($M^+ = m/z$ 610, $M^+ - CF_3COO^\bullet = m/z$ 497, $M^+ - CF_3COO^\bullet - CF_3COOH = m/z$ 383). The amount of β-RFH-P produced enzymatically was quantified using the Folin-Ciocalteu phenol reagent (Sigma) (*17*) with phenol as the standard.

Chemical Synthesis of β-D-Ribofuranosylbenzene (β-RFB) and Identification of β-D-Ribofuranosylbenzene 5′-Phosphate (β-RFB-P) as a Decomposition Product of the Reduced Cyclohexadienimine Reaction Intermediates. 2,5:3,4-*O*-Diisopropylidene-1-(4-aminophenyl)-D-ribitol was deprotected and cyclized as described above. After dissolution in 1 mL of 1 M H_2SO_4, 100 μL of 1.5% $NaNO_2$ was added. After 5 min, 100 μL of 7.5% ammonium sulfamate was added, the sample cooled to room temperature, and 50 μL of 50% hypophosphorous acid added. The sample was stored at 3 °C for 24 h. The sample was then purified by chromatography on a 0.5 cm × 4 cm C_{18} column as described above. The desired product eluted in the fractions containing 30% methanol in water was recovered by evaporation of the solvent with a stream of nitrogen gas. A portion of the sample (50 μg) was reacted overnight at room temperature with methylene chloride/trifluoroacetic anhydride to form the tritrifluoroacetyl derivative and analyzed by GC−MS under the same conditions used for the identification of β-RFA (*16*). β-RFB-P was produced by the acid-catalyzed decomposition of the enzymatically generated and sodium cyanoborohydride-reduced reaction intermediate as follows. β-RFA-P synthase (73 μg of protein) was incubated with 50 mM acetate buffer (pH 4.8), 10 mM $MgCl_2$, 8.8 mM PRPP, 2.5 mM 4-aminothiobenzoic acid or 2.5 mM *p*AB, and 30 mM sodium cyanoborohydride in a final volume of 600 μL. The solution was incubated at 50 °C for 3 h. The mixture was then acidified with 100 μL of 6 N HCl, heated at 100 °C for 15 min, and neutralized with 100 μL of 6 N NaOH. To the resulting sample were added 200 μL of 0.1 M glycine buffer (pH 10.4) and 1 unit of *E. coli* alkaline phosphatase. After 2 h at 37 °C, the sample was acidified to pH 1−2 with 1 M H_2SO_4, and the β-RFB was purified by C_{18} column chromatography under the same conditions used in the purification of the known compound. GC−MS of the tritrifluoroacetyl derivative of the sample gave a peak with the same retention time and mass spectrum as that of the synthetic sample ($M^+ = m/z$ 384, $M^+ - CF_3COO^\bullet = m/z$ 271, $M^+ - CF_3COO^\bullet - CF_3COOH = m/z$ 157).

Table 1: Partial Purification of β-RFA-P Synthase from *M. thermophila*

purification step	total activity (nmol of β-RFA-P produced h^{-1})	specific activity (nmol of β-RFA-P produced h^{-1} mg of protein^{-1})	yield (%)	fold purification
cell-free extract	2000	45	100	1
Q-Sepharose	590	440	30	10
Mono Q[a]	64	1600	3	35

[a] Samples were filtered through a Centricon 100 microconcentrator and concentrated in a Centricon 50 concentrator prior to application to the Mono Q column.

RESULTS

Enzyme Isolation and Partial Purification. Separation of a cell extract of *M. thermophila* on a Q-Sepharose column, followed by analysis for β-RFA-P synthase activity, showed the presence of one peak of activity eluting at about 0.4 M NaCl. The specific activity of this fraction (440 nmol of β-RFA-P produced h^{-1} mg of protein^{-1}) was 10-fold higher than the specific activity of the cell-free extract (45 nmol produced h^{-1} mg of protein^{-1}) (Table 1). The activity contained in this peak was stable to dialysis against aerobic buffer and incubation of the samples with the metal chelate, 8-hydroxyquinoline (0.2 mM). These results indicate that the enzyme was relatively stable in air and required no dissociable trace metal ions such as iron or cobalt ion for activity. During long-term storage and subsequent purification steps, 2 mM dithiothreitol and 10% (v/v) glycerol were found to stabilize the enzyme activity. Purification of β-RFA-P synthase either by a second ion-exchange step (Table 1) or by Superose 12 gel filtration chromatography (not shown) resulted in purification of the enzyme by only an additional ∼3-fold, but with an overall recovery of only 3% of the activity. The total amount of β-RFA-P synthase activity detected in *M. thermophila* extracts was relatively low, with 1 gram of cells yielding about 1800 nmol of β-RFA-P h^{-1}, which is equivalent to only 0.03 μmol min^{-1} or 0.03 standard unit of enzyme activity. The low amount of total activity in the cell extract, the protein instability, or the possibility that subunits of a multimeric protein may have been separated during purification could have contributed to the difficulty in further purifying the enzyme.

Effect of Magnesium and Manganese Ions and pH on β-RFA-P Synthase Activity. A common feature of phos-

phoribosyltransferase (PRTase) reactions is a requirement for magnesium ions, which combine with PRPP to produce the active form of the substrate, Mg^{2+}−PRPP. For this reason, 10 mM $MgCl_2$ was included in the β-RFA-P synthase assays. Decreasing the $MgCl_2$ concentration from 10 to 2 mM produced a 5-fold decrease in the reaction velocity, whereas increasing the concentration up to 20 mM doubled the rate of the reaction. Further increases up to a concentration of 50 mM, the highest tested, did not significantly increase the reaction rate. Similar results were obtained when Mn^{2+} was substituted for Mg^{2+}. When $MgCl_2$ was omitted from the assay and EDTA was added to chelate magnesium ions present in the enzyme solution, the activity decreased to 4% of the activity in the presence of 10 mM $MgCl_2$, demonstrating that like other PRTases, β-RFA-P synthase is dependent upon magnesium or manganese ions for activity.

The effect of pH on β-RFA-P synthase activity was determined using substrate concentrations that were greater than the K_m values. The optimal pH for the reaction was found to be 4.8, with a gradual decrease in activity observed at higher pH values and a sharp decline at lower values. To test whether the precipitous decline in activity at low pH was due to irreversible protein denaturation or to protonation of a substrate or amino acid side chain, the enzyme was incubated at pH 4.0, 4.8, or 5.9 for 30 min prior to measuring the activity at pH 4.8 in a 3 h assay. The activity of β-RFA-P synthase pretreated at pH 4.0 was only 48% of the activity for enzyme treated at either pH 4.8 or 5.9. This result indicates that the sharp decline in activity at pH values lower than 4.8 was due to enzyme inactivation. All additional experiments were carried out at pH 4.8, the optimum pH for the assay conditions used.

Identification of Pyrophosphate (PP_i) as a Product of the β-RFA-P Synthase Reaction. β-RFA-P was previously identified as one of the products of the enzyme-catalyzed reaction of *p*AB with PRPP in *M. thermophila*, and PP_i was proposed as the second product of the reaction (*16*). To verify that PP_i was a product of the β-RFA-P synthase reaction, the assay was performed in the presence of [β-^{32}P]-PRPP and the released radioactive PP_i identified by polyethyleneimine thin layer chromatography. PRPP is known to be unstable in aqueous solutions containing magnesium ions, and the nonenzymatic hydrolysis of PRPP producing PP_i upon heating has been reported (*18*). To correct for the rate of nonenzymatic PRPP hydrolysis during the reaction, [β-^{32}P]PRPP was incubated in the absence of *p*AB and enzyme at 50 °C for 90 min in a solution containing 50 mM acetate buffer (pH 4.8), 30 mM $MgCl_2$, and 10 mM DTT. A background level of [^{32}P]PP_i was detected, indicating that nonenzymatic hydrolysis of [β-^{32}P]PRPP to [^{32}P]PP_i had occurred during the incubation. The ratio of [^{32}P]PP_i to [β-^{32}P]PRPP under these conditions was 0.23. When *p*AB and the enzyme were added to the reaction mixture, the ratio of [^{32}P]PP_i to [β-^{32}P]PRPP increased by 60% to a value of 0.37. In contrast, when either *p*AB or the enzyme was omitted from the assay, the ratios of [^{32}P]PP_i to [β-^{32}P]PRPP were similar to the background level (0.23 and 0.25, respectively). The ratio of [^{32}P]P_i to [β-^{32}P]PRPP was the same in the presence or absence of *p*AB or β-RFA-P synthase, demonstrating that P_i was not a direct product of the β-RFA-P synthase reaction. These results demonstrate that the enzymatic production of PP_i from PRPP requires both *p*AB and β-RFA-P synthase, and that PP_i is a product of the β-RFA-P synthase reaction. The ratio of β-RFA-P produced to PP_i detected was 0.4. This difference in the amount of products detected is likely due to the high background level of nonenzymatically produced PP_i, which made it difficult to accurately determine the amount of PP_i produced during the enzymatic reaction. When β-RFA-P synthase was incubated with [β-^{32}P]PRPP and unlabeled PP_i in the absence of *p*AB, no exchange of radioactive label between [β-^{32}P]PRPP and -PP_i was detected (lower limit of detection, approximately 13 nmol of PP_i exchanged).

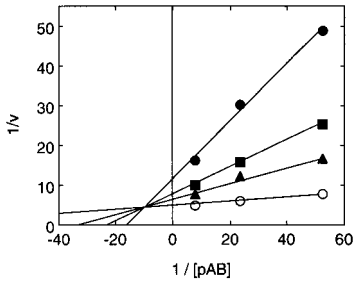

FIGURE 2: Plots of $1/v$ vs 1/[*p*AB] at four concentrations of PRPP. Enzyme assays were performed in 200 μL of solution containing 40 mM acetate, 40 mM MOPS, 40 mM MES (pH 4.8), 30 mM $MgCl_2$, *p*AB (0.019, 0.042, or 0.126 mM), 40 μL of a Q-Sepharose fraction (20 μg of protein), and PRPP at the following concentrations: 0.6 (●), 1.3 (■), 2.6 (▲), and 6.6 mM (○). 1/[*p*AB] is expressed in units of mM^{-1}, and $1/v$ is expressed in units of (μmol of β-RFA-P h^{-1} mg of $protein^{-1})^{-1}$.

Initial Velocity Kinetics. Plots of the reaction velocity versus the concentration of *p*AB at PRPP concentrations from 0.6 to 6.6 mM were found to follow typical Michaelis−Menten saturation kinetics. To determine whether the reaction involved a ping-pong mechanism versus a sequential pattern of substrate binding, the effect of *p*AB concentration on the reaction velocity was measured at four different concentrations of PRPP (Figure 2). In a double-reciprocal plot of the date, parallel lines indicated that the reaction is likely to involve a ping-pong mechanism, while intersecting lines are consistent with a sequential mechanism. The apparent K_m for *p*AB was estimated to be 58 ± 7 μM by a nonlinear least-squares computer fit of the data to the Michaelis−Menten equation. The concentration profile for PRPP exhibited substrate inhibition or complex formation above 10 mM, and thus, the apparent K_m for PRPP was estimated to be 3.6 ± 0.6 mM using PRPP concentrations below the inhibitory level. Reaction kinetics above 10 mM PRPP were complicated by the precipitation of an insoluble magnesium−PRPP complex. The intersecting lines in Figure 2 were taken to indicate that the substrate binding to β-RFA-P synthase followed a sequential and not a ping-pong mechanism.

Inhibitors and Alternate Substrates for the Enzyme. The binding specificity of β-RFA-P synthase was investigated by examining the effect of *p*AB analogues on enzyme activity (Table 2). The most potent inhibitor tested was 4-(methylamino)benzoic acid which, at a concentration of 5 mM, completely inhibited β-RFA-P synthesis in the presence of 85 μM *p*AB. Kinetic analysis indicated that 4-(methylamino)benzoic acid was a mixed type inhibitor of β-RFA-P synthesis versus *p*AB. The K_{is} for 4-(methylamino)benzoic acid (70 μM) was determined by plotting the slopes of the

Table 2: Effect of Inhibitors on β-RFA-P Synthase Activity

compound	β-RFA-P synthase activity remaining (% of control)[a]
*p*AB analogue (5 mM)[b]	
4-(methylamino)benzoic acid ($K_{is} = 70\ \mu M$)	0
4-hydroxybenzoic acid ($K_{is} = 590\ \mu M$)	12
4-bromobenzoic acid	40
4-methoxybenzoic acid	45
benzoic acid	69
4-aminothiobenzoic acid	76
4-(dimethylamino)benzoic acid	100
4-aminobenzamide	110
4-aminobenzoic acid methyl ester	110
aniline	110
sulfanilamide (*p*-aminobenzenesulfonamide)	94
phosphate-containing compound (inhibitor concentration)[c]	
pyrophosphate (5 mM)	16
phosphate (10 mM)	63
ribose 5-phosphate (10 mM)	80
pyridoxol 5-phosphate (2.5 mM)	79
reducing agent and pyridoxal phosphate modifying agent (inhibitor concentration)[d]	
sodium borohydride (160 mM)	0
sodium cyanoborohydride (30 mM)	36
phenylhydrazine (5 mM)	120
methoxylamine (5 mM)	120
cysteine (10 mM)	100

[a] The control activity was 50−200 nmol h^{-1} mg of $protein^{-1}$. The errors in the reported values are ±15% of the indicated number. [b] For the determination of the % of control assays of the *p*AB analogues, the assay contained 85 μM *p*AB, 4.4 mM PRPP, and 5 mM inhibitor. [c] For phosphate-containing compounds, the assay contained 0.9 mM PRPP, 6.4 mM *p*AB, and the concentration of inhibitor noted in parentheses. [d] The assay conditions for these compounds are described in Materials and Methods.

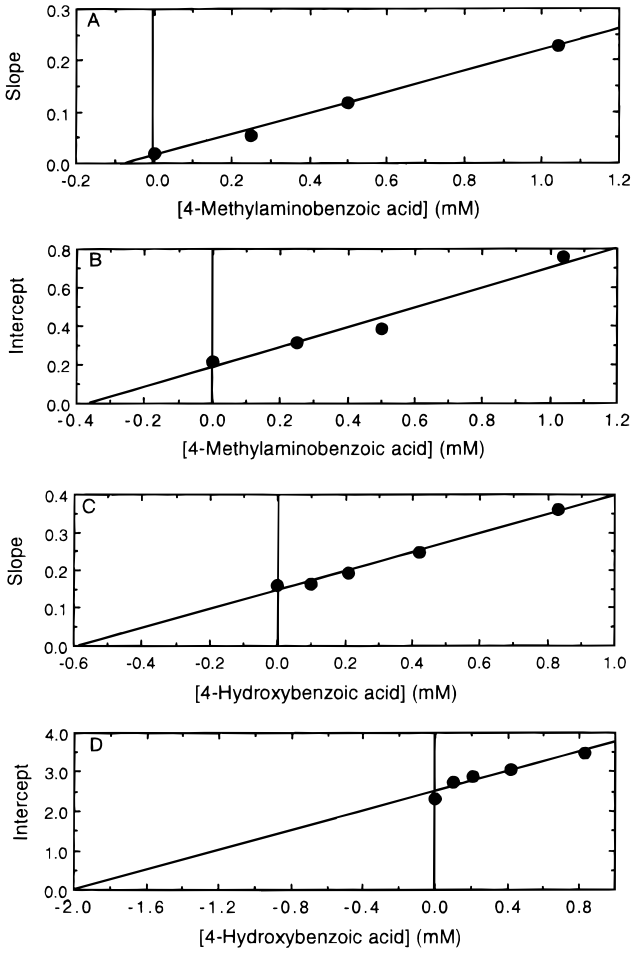

FIGURE 3: Replots for the determination of the K_{is}'s (A and C) and K_{ii}'s (B and D) for the inhibitors 4-(methylamino)benzoic acid (A and B) and 4-hydroxybenzoic acid (C and D).

lines from the double-reciprocal plots for the 4-(methylamino)benzoic acid inhibition versus the inhibitor concentration (Figure 3A). The K_{is} was obtained from the *x*-axis intercept. The K_{ii} was estimated to be 350 μM by plotting the *y*-intercept values from the double-reciprocal plots versus the inhibitor concentration (Figure 3B).

In the presence of 85 μM *p*AB, the substrate analogue 4-hydroxybenzoic acid (5 mM) inhibited β-RFA-P synthesis by 88%. Kinetic analysis indicated that 4-hydroxybenzoic acid was a linear mixed type inhibitor versus *p*AB with a K_{is} of 590 μM and a K_{ii} of 2.0 mM (Figure 3C,D). 4-Bromobenzoic acid, 4-methoxybenzoic acid, benzoic acid, and 4-aminothiobenzoic acid were all poor inhibitors of β-RFA-P synthesis (Table 2). Interestingly, although 4-(methylamino)benzoic acid was the most potent inhibitor studied, 4-(dimethylamino)benzoic acid, which contains only one additional methyl group, did not inhibit the reaction. 4-Hydroxybenzoic acid and 4-aminothiobenzoic acid not only were inhibitors of the *p*AB reaction but also functioned as alternate substrates for the enzyme. The product of the reaction with 4-hydroxybenzoic acid was identified as 4-(β-D-ribofuranosyl)hydroxybenzene 5′-phosphate (β-RFH-P) by gas chromatography−mass spectrometry of the tetratrifluoroacetyl derivative of the β-RFH produced after phosphatase cleavage of the reaction product. The enzyme produced 27 nmol of β-RFH-P h^{-1} mg of $protein^{-1}$ when it was incubated in the presence of 9 mM 4-hydroxybenzoic acid. In the case of 4-aminothiobenzoic acid (3 mM), the enzyme produced 74 nmol of β-RFA-P h^{-1} mg of $protein^{-1}$. The rates of production of these products for both of the alternate substrates were much lower than the rate observed in the presence of 0.6 mM *p*AB (400 nmol of β-RFA-P h^{-1} mg of $protein^{-1}$). Aniline, 4-aminobenzamide, and the methyl ester of *p*AB had no effect on the reaction rate (Table 2), demonstrating the importance of the negative charge of the carboxylate group for binding at the *p*AB site. Sulfanilamide was ineffective at inhibiting β-RFA-P synthase (Table 2), as were the following compounds at concentrations of up to 5 mM: 4-hydrazinobenzoic acid, 4-azidobenzoic acid, 2-aminobenzoic acid, 3-aminobenzoic acid, methoxylamine, carboxymethoxylamine, and 5-aminopyrimidine-2-carboxylic acid (not shown).

High concentrations (2.5−10 mM) of PP_i, P_i, ribose 5-phosphate, and pyridoxol 5-phosphate inhibited the β-RFA-P synthase reaction (Table 2). The nucleoside monophosphates AMP, CMP, GMP, and UMP were not inhibitors and did not replace PRPP as substrates for β-RFA-P synthase.

Evidence for Pyridoxal Phosphate (PLP) in the Enzyme. The UV spectrum of a Mono Q-purified sample showed a maximum absorbance at 416 nm, consistent with the presence of pyridoxal phosphate (PLP). The measured activity of the sample was not affected by incubation with 0.1 mM Fe^{3+} and 0.2 mM PLP. Reduction of the Mono Q-purified enzyme with sodium borohydride (1 mg in 100 μL of sample) completely destroyed the enzymatic activity as well as the

absorbance at 416 nm. These observations are all consistent with the enzyme being a PLP-dependent enzyme. Separation of the Mono Q-purified enzyme on a Superose 12 gel filtration column using the absorbance at 405 nm produced one major peak eluting at a molecular weight of 65 000, which also corresponded to the elution position of the enzymatic activity. Sodium borohydride reduction of the sample prior to Superose 12 separation produced one major peak displaying the typical fluorescence excitation and emission spectra of a reduced PLP enzyme ($\lambda_{max}^{excitation}$ = 330 nm and $\lambda_{max}^{emission}$ = 370 nm). This fluorescence was not present unless the sample was reduced with sodium borohydride prior to the separation. Separation of a sodium borohydride-reduced sample on the Superose 12 column in 6 M urea did not alter the elution position. Finally, reduction of the Mono Q-purified enzyme with NaB^3H_4, followed by separation with SDS−PAGE, showed the presence of a radioactive band with a molecular weight of approximately 64 000. These data are consistent with the enzyme being a monomer with a molecular weight of approximately 65 000 and possibly containing bound PLP which is required for catalytic activity. β-RFA-P synthase was not inhibited by carbonyl reagents at up to 5 mM, phenylhydrazine or methoxylamine hydrochloride, which are known to inhibit PLP-dependent enzymes (*19*). The carbonyl reagent and substrate analogue, 4-hydrazinobenzoic acid, was also found not to inhibit β-RFA-P synthase. Cysteine, a known inhibitor of a number of PLP enzymes (*20*), did not affect β-RFA-P synthase activity.

DISCUSSION

The enzyme catalyzing the formation of β-RFA-P and pyrophosphate (PP_i) from *p*AB and 5-phospho-α-D-ribosyl-1-pyrophosphate (PRPP) has been partially purified from *M. thermophila*. This reaction, which is an important step in the biosynthesis of the unique methanogen cofactor methanopterin, represents the first conclusive demonstration of the involvement of PRPP in the enzymatic formation of a *C*-glycoside (*21*). The enzyme was found to have a native molecular weight of 65 000 and may contain bound pyridoxal phosphate (PLP). The Mg^{2+} requirement for β-RFA-P synthase activity is consistent with the general observation, among PRPP-utilizing enzymes, that the active form of the substrate is the magnesium or dimagnesium salt of PRPP (*1*, *22*). The enzyme has a rather low pH optimum of 4.8.

Inhibitors of β-RFA-P Synthase. 4-(Methylamino)benzoic acid and 4-hydroxybenzoic acid were shown to be mixed inhibitors versus *p*AB of β-RFA-P synthesis, and 4-hydroxybenzoic acid was found to be an alternate substrate, producing 4-(β-D-ribofuranosyl)hydroxybenzene 5′-phosphate. The ability of 4-(methylamino)benzoic acid, 4-hydroxybenzoic acid, bromobenzoic acid, 4-methoxybenzoic acid, and benzoic acid to inhibit β-RFA-P synthase demonstrates that the enzyme can bind a number of different compounds that contain a para-substituted benzoic acid. Since aniline, 4-aminobenzamide, and the methyl ester of *p*AB did not inhibit β-RFA-P synthase activity, an ionized carboxylic group may play a critical role in the binding of *p*AB. When it is considered that the pK_a values for the carboxylic acid and amino groups of *p*AB are 4.7 and 2.1, respectively, this indicates that *p*AB occurs mostly as an anion at pH >4.7. Although sulfanilamide is structurally similar to *p*AB, this sulfonamide was not an inhibitor of β-RFA-P synthase. This finding establishes why the sulfonamides (sulfa drugs) are ineffective at inhibiting the growth of archaea that contain modified folates such as methanopterin (*23*). In bacteria, the sulfonamides function by blocking the coupling of *p*AB with 6-(hydroxymethyl)pterin pyrophosphate to form pteroic acid, the precursor of folic acid (*24*). Since this reaction is not involved in the biosynthesis of methanopterin, sulfonamides have no effect on the growth of methanogens.

Proposed Mechanism of β-RFA-P Synthase. Kinetic analysis of the β-RFA-P synthase reaction (Figure 3) indicates that the reaction occurs via a sequencial binding mechanism rather than via a ping-pong mechanism. The classical interpretation of binding predicts that a ternary complex, composed of the enzyme bound to both substrates (*p*AB and PRPP), must be formed before either of the products (β-RFA-P or PP_i) is released. The sequencial binding of substrates is commonly found in other PRTases (*1*). The inability of β-RFA-P synthase to catalyze an exchange reaction between [β-^{32}P]PRPP and PP_i in the absence of *p*AB is consistent with the requirement for ternary complex formation prior to PRPP hydrolysis.

Considering that PLP is known to catalyze the nonenzymatic decarboxylation of *p*AB (*25*) and is also a cofactor in the enzymatic decarboxylation of *p*AB to aniline (*26*), we were tempted to consider that PLP and *p*AB combined on the enzyme to form a Schiff base intermediate analogous to that characteristic of other PLP-dependent enzymes (*27*). This idea was supported by the observation that reduction of the enzyme with $NaBH_4$ not only led to the formation of an inactive enzyme but also produced a protein containing a bound fluorescently labeled group. The fluorescent excitation and emission maxima of this group were consistent with a reduced PLP bound to the protein via a lysine, as described for other PLP enzymes (*28*).

The enzymatic activity, however, was found not to be inhibited by a wide range of carbonyl reactive reagents (phenylhydrazine, methoxylamine, and cysteine) (Table 2) which are known to inhibit the activity of PLP-dependent enzymes because of their reaction with the Schiff base of the aldehyde group of the PLP (*19*). The enzyme was also not inhibited by the specifically designed substrate carbonyl reagent 4-hydrazinobenzoic acid, which should be both a substrate and a carbonyl reactive reagent. These observations, coupled with the fact that the enzyme catalyzed the formation of 4-(β-D-ribofuranosyl)hydroxybenzene 5′-phosphate (β-RFH-P) from PRPP and 4-hydroxybenzoic acid, which cannot form a Schiff base with PLP, indicate that Schiff base formation with the substrate is not required for the enzymatic reaction. That PLP may be required in another capacity is supported by the observations that pyridoxol 5-phosphate was an inhibitor of the reaction and that reduction of the protein with $NaBH_4$ destroyed the activity. These results are then consistent with PLP being a required cofactor for the enzymatic reaction but one where the aldehyde group is not chemically involved in the reaction mechanism. This situation is somewhat analogous to the involvement of PLP in phosphorylase *b* where the near-UV band can be attributed to either the hydrated aldehyde or a form of the bound PLP in which the intramolecular hydrogen bond between O-3′ and imine nitrogen cannot be formed (*29*). A key difference, however, is that phosphorylase *b*

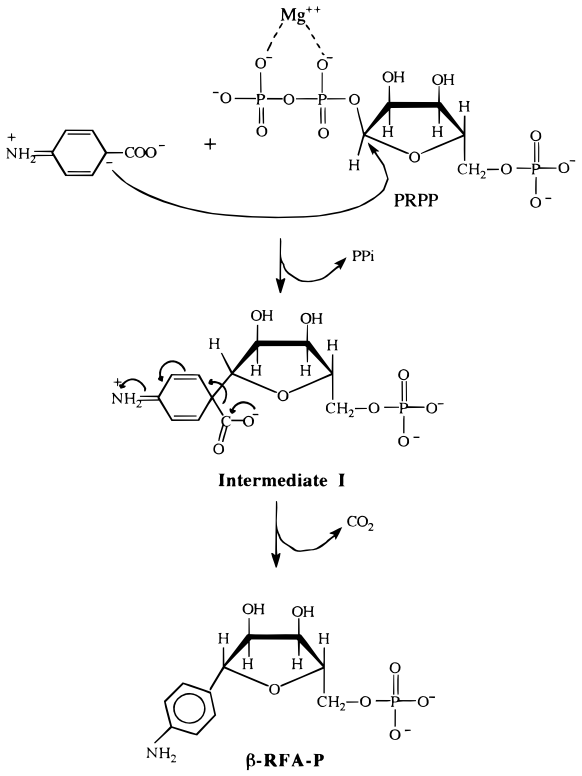

FIGURE 4: Proposed mechanism for the reaction of β-RFA-P synthase.

still retains its activity after reduction, whereas in the case of β-RFA-P synthase, the activity is decreased by $NaBH_4$ reduction.

The above data are consistent with two possible chemical mechanisms for β-RFA-P synthase. Both mechanisms lead to the formation of a common intermediate which then is decarboxylated to form β-RFA-P. The first mechanism is a direct transfer mechanism whereupon an enzyme-stabilized C-1 anionic intermediate of the *p*AB, serving as a nucleophile, undergoes an S_N2 reaction at C-1 of the PRPP with displacement of pyrophosphate. This mechanism is consistent with the observed inversion of the stereochemistry at C-1 of the ribose during the β-RFA-P synthase reaction and is the mechanism shown in Figure 4.

The second possibility is a two-step mechanism proceeding via an S_N1-like mechanism. In this mechanism, separation of the pyrophosphate from the PRPP would occur with the development of an carboxonium ion at C-1 of the PRPP. This S_N1-like mechanism has been proposed as the first step in the mechanism for other PRTases (*30*), where it is proposed that carboxylate residues in the enzyme are used to stabilize the carboxonium ion. Specific examples include glycosidase catalysis (*31*−*33*) and phosphorylase *b* (*29*). In the case of β-RFA-P synthase, it is also possible that the phosphate of the bound PLP could be used to stabilize the carboxonium ion. The resulting carboxonium ion would then participate in an electrophilic aromatic substitution reaction initiated with an ipso attack at C-1 of the *p*AB. This step would result in the formation of σ-complex arenium ion (intermediate I) which would be stabilized by the nonprotonated *p*-amino group. The fact that no exchange of the PP_i with the PRPP was observed indicates that all of these steps could proceed by a concerted process. The binding of the anionic carboxylate of the pAB in the same general area

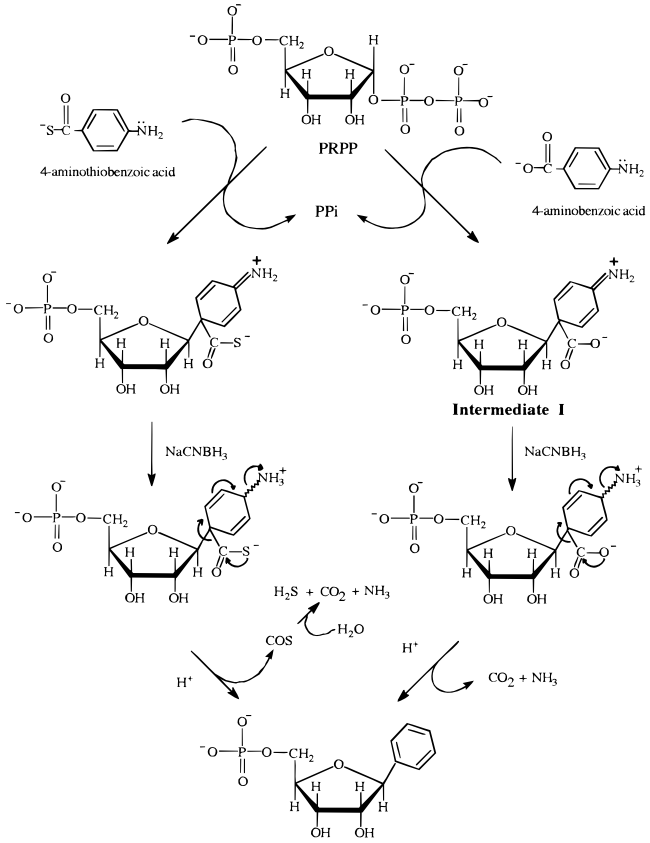

FIGURE 5: Trapping and characterization of the cyclohexadienimine intermediates produced from the reaction of PRPP with 4-aminothiobenzoic acid or *p*AB in the presence of cyanoborohydride.

as the anionic pyrophosphate of the PRPP could be explained by charge neutralization by the required Mg^{2+}. The presented data, however, do not allow for the distinction between these two possible mechanisms, leading to the formation of intermediate I.

Regardless of the exact mechanism for the formation of intermediate I, the final step in the reaction mechanism would involve the loss of CO_2 by the decarboxylation of the cyclohexadienimine intermediate I. Decarboxylations of such systems are very facile as documented by the observed rapid decarboxylation of 4-methyl-4-carboxy-2,5-cyclohexadien-1-one (*34*).

In an attempt to trap an analogue of this reaction intermediate I, 4-aminothiobenzoic acid was prepared and tested as a substrate for the reaction. 4-Aminothiobenzoic acid produced β-RFA-P as a product of the reaction but was a much poorer substrate than *p*AB. A possible explanation for this would be that the intermediate, being a thio acid, would undergo decarboxylation more slowly than the normal intermediate which is a carboxylic acid. As a result of this inability to decarboxylate, the concentration of this intermediate would build up as the reaction proceeds. Carrying out the reaction in the presence of sodium cyanoborohydride decreased the amount of the β-RFA-P formed when either *p*AB or 4-aminothiobenzoic acid was used as a substrate. This was attributed to reduction of the imine intermediates to the amino-containing compounds shown in Figure 5. These compounds, which are analogues of prephenic acid, would undergo acid-catalyzed decomposition to an aromatic ring as shown in Figure 5 (*35*, *36*). The involvement of such an

intermediate in the reaction, which may require a second enzyme for the decarboxylation step in the β-RFA-P synthase reaction, could account for our inability to purify this enzyme.

Several examples of organic reactions that follow this mechanism are known. The simplest example is the thermal protodecarboxylation of *p*AB to aniline (*37*). As expected, this reaction, as well as the thermal protodecarboxylation of other ortho- and para-substituted benzoic acids, was found to be greatly facilitated by the presence of an electron-donating para substitutent such as an amino, hydroxy, or methoxy group (*38*). Other examples, where the electrophilic species is a brominium ion instead of a proton, include the bromodesulfuration of methoxy-, amino-, and methylbenzenesulfonates (*39*) and the bromodecarboxylation of 3,5-dibromo-4-hydroxybenzoic acid (*40*). An example of an ipso carbonium ion attack at a carbon of the attached methyl group of a *p*-cresol is also known. Because of the inability of the methyl group to serve as a leaving group, the product of this reaction is a stable σ-complex in the form of a 4,4-disubstituted 2,5-cyclohexadienone (*41*). We are not aware of such a reaction occurring with an oxonium ion except for the biochemical example presented here.

ACKNOWLEDGMENT

We are grateful to Dr. James Greg Ferry for supplying *M. thermophila* cells, Birthe Borup for preparing Q-Sepharose fractions, Kim Harich for performing GC−MS studies, Gu Li for technical assistance, Dr. Peter J. Kennelly for assistance with the ^{32}P experiments, and Drs. Walter Niehaus and Constance Anderson for helpful discussions.

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BI973086Q