Mechanism of 4-(β-d-Ribofuranosyl)aminobenzene 5′-Phosphate Synthase, a Key Enzyme in the Methanopterin Biosynthetic Pathway

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The first committed step in methanopterin biosynthesis is catalyzed by 4-(β-d-ribofuranosyl)aminobenzene 5′-phosphate (RFA-P) synthase. Unlike all known phosphoribosyltransferases, β-RFA-P synthase catalyzes the unique formation of a C-riboside instead of an N-ribosyl group and the mechanistically related pyruvoyl cofactor has been strictly excluded.

Received for publication, June 9, 2004, and in revised form, July 12, 2004
Published, JBC Papers in Press, July 15, 2004, DOI 10.1074/jbc.M406442200

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The first committed step in methanopterin biosynthesis is catalyzed by 4-(β-d-ribofuranosyl)aminobenzene 5′-phosphate (RFA-P) synthase. This enzyme catalyzes the condensation between para-aminobenzoic acid (pABA) and 5-phospho-α-d-riboosyl-1-pyrophosphate (PRPP) to produce 4-(β-d-ribofuranosyl)aminobenzene 5′-phosphate (β-RFA-P), CO₂, and inorganic pyrophosphate (PPi) (1). This enzyme is a phosphoribosyltransferase and a decarboxylase and forms a C-riboside, which is unique among phosphoribosyltransferases and pABA-dependent enzymes. For example, in an early step in tetrahydrofolate biosynthesis, dihydropteroate synthase catalyzes a condensation between the amino group of pABA and dihydropterin pyrophosphate to generate dihydropteroate, eliminating PPi. Thus, β-RFA-P synthase and dihydropteroate synthase both use pABA as a substrate and produce PPi as product; however, the amino group is the nucleophile in dihydropteroate synthase, whereas the aromatic ring carbon 4 (C-4) is the nucleophile in β-RFA-P synthase (2, 3).

How does RFA-P synthase generate an electrostatic center at C-1 of PRPP? How does this enzyme poise ring-carbon 4 of pABA for nucleophilic attack on the C-1 of PRPP and activate the nucleophile? It was suggested that β-RFA-P synthase contains PLP, which is known to stabilize similar intermediates during decarboxylation or deamination of amino acids, and the partially purified enzyme appeared to contain a chromophore with properties similar to those of PLP (3). However, we show here that β-RFA-P synthase lacks PLP and apparently any other cofactor that would be capable of stabilizing negative charge developing during the reaction. Thus, all the interesting chemistry performed in this reaction appears to be promoted by the enzyme per se.

The study of β-RFA-P synthase is significant because it is an early step in the biosynthesis of tetrahydromethanopterin (H₄MPT), which is a modified folate that is of central importance in growth and energy metabolism of methanogens. H₄MPT is involved in multiple steps in methane formation as one carbon reactions involved in amino acid and nucleotide metabolism. Even though H₄MPT is found in Archaea and one class of Bacterium (e.g. Methylobacterium extorquens), the biosynthetic pathway for these two folates (folate and methanopterin) is different suggesting that they play different functional roles in physiology of the cell (4, 5). We are targeting this enzyme to inhibit specifically methanogenesis (6), which has the potential impact of reducing the levels of the greenhouse gas methane, which has doubled over the last 200 years.

In this paper, we report the cloning, overexpression, purification, and characterization of the steady-state mechanism of β-RFA-P synthase from the hyperthermophilic methanogenic
archaeon *Methanococcus jannaschii* and show that it lacks cofactors. We propose a mechanism for this reaction based on product inhibition studies and a rationale for how pABA is activated to perform a nucleophilic attack on PRPP. The reaction is believed to proceed via an oxy carb enium intermediate formed from PRPP that forms an adduct with the C-4 of ABA and isolated using a Shodex column developed first with a linear gradient from solution A to B for 30 min. Solution A contained 0.1 M ammonium formate, pH 3.0/acet onitrile (20/80), and solution B was composed of 0.5 M ammonium formate, pH 3.0/acet onitrile (80/20). The detector was set at 260 nm, and for the chromatography was performed at room temperature (22 °C). β-RFA-P eluted at a retention time of 12.5 min, while the retention times for pABA and PRPP were 22 and 26 min, respectively. β-RFA-P was identified and quantified with the Brattan-Marshall assay (3). In the control reaction lacking enzyme, no peak was observed at 12.5 min. The fractions containing β-RFA-P were collected and lyophilized, and the powder was stored at room temperature for further usage. To check the stability and purity of the β-RFA-P, the powder was dissolved in solution A and run by high pressure liquid chromatography, and only a single sharp peak was observed at 12.5 min.

**Cloning and Expression of the RFA-P Synthase Gene**—A BLAST search revealed that the *M. jannaschii* chromosomal DNA by the PCR using *Pfu* polymerase (Stratagene, La Jolla, CA). The commercially synthesized primers were 5′-CCTGAAGAGGGAGAATCATATGATATCCAAACCCATG-3′ and 5′-TCACCAATTITATGCCCACTATT-3′. The protocol used for cloning and overexpression was identical to the one employed for the gene with a tag at the N terminus. For the tag-less enzyme, the yield was lower (30%) than that for the His-tagged protein. For overexpression, E. coli Rosetta (DE3)pLysS strain was used because it provided a higher yield because of the presence of rare tRNAs required by MJ1427. The pure enzyme had a specific activity ranging from 200 to 250 nmol/min/mg protein.

**Purification of β-RFA-P Synthase**—β-RFA-P synthase from *M. jannaschii* was purified to homogeneity by column chromatography and heat treatment. Cells were suspended in lysis buffer (500 mM NaH2PO4, 300 mM NaCl, 10 mM imidazole, pH 8.0) and disrupted by sonication. The cell lysate was centrifuged at 15,000 × g for 2 h. The supernatant was loaded on a Ni-NTA column, which was washed with a buffer containing 50 mM NaH2PO4, 300 mM NaCl, and 20 mM imidazole, pH 8.0, to remove proteins that are nonspecifically attached to the resin. The His-tagged protein was eluted with buffer containing 50 mM NaH2PO4, 300 mM NaCl, and 150 mM imidazole, pH 8.0. Fractions with active enzyme were further purified by heat treatment at 70 °C in a water bath for 15 min. The turbid solution was again centrifuged at 10,000 × g for 30 min, and the supernatant was stored at −80 °C until needed.

**Assay for PLP and Pyruvolyt Progesticic Protein**—The protocol for removal of PLP from the enzyme involved treating the enzyme with 5 mM hydroxylamine for 24 and 72 h as described previously (7). To assay for pyruvate, HCl was added to adjust the pH of the enzyme solution to ~3.0. Then the solution was boiled for 1 h, and the pH was adjusted to pH 8.0, and lactate dehydrogenase and NADH were added to measure the amount of pyruvate formed by using the lactate dehydrogenase assay. The two conditions of the assay were 50 mM Tris-HCl, pH 7.6, 12.5 °C, and the reaction was followed spectrophotometrically at 340 nm for 5 min (8).

**Assay for β-RFA-P Synthase and Protein Determination**—Protein concentrations were determined by the Bradford assay with bovine serum albumin as standard (9). Proteins were separated by SDS-PAGE and stained with Coomassie Blue. β-RFA-P synthase activity was measured by following the release of 14CO2 from p-aminobenzamide with a radioactive label in the carboxyl group essentially as described earlier (6).

**Steady-state Kinetics of β-RFA-P Synthase**—Stock solutions of pABA (2.93, 5.86, 14.6, 29.3, 58.6, or 88 mM), 25 mM PPi, 10 mM NaHCO3, and 100 mM TES, 25 mM MgCl2, pH 8.0 were used. The CO2 concentration was estimated by the Henderson-Hasselbach equation taking into account the pH of the reaction mixture and the NaHCO3 concentration. The assays were performed in 50 mM Tris-HCl, pH 7.6, 12.5 °C, and the NaHCO3 concentration. The assays were performed for 3 h and were initiated by adding enzyme. In all the assays, the buffer was 100 mM TES, 25 mM MgCl2, pH 4.8. To determine the Michaelis parameters of β-RFA-P synthase for PRPP, the reaction mixture contained pABA (0.1, 0.2, 1, and 3 mM) and varying concentrations of pABA (0, 0.05, 0.1, 0.2, 0.5, and 1–3 mM) in 100 mM TES, 25 mM MgCl2, pH 4.8. To determine the Michaelis parameters of β-RFA-P synthase for PRPP, the reaction mixture contained pABA (0.1, 0.2, 1, and 3 mM) and varying concentrations of PRPP (0, 0.5, 2, 5, and 8 mM) in the same buffer as for pABA.

**Product Inhibition Studies**—To determine the type of inhibition and the inhibition constants for CO2, PPi, and RFAP, the assays were performed at fixed concentrations of each compound for each product in the presence of saturating concentrations of pABA under saturated and unsaturated conditions with respect to PRPP and varied PRPP concentrations under saturated and unsaturated concentrations for pABA. In one set of studies, RFAP (0.2 and 1 mM) was used to inhibit saturating PP conditions for pABA (5 mM) and varied PRPP at 0, 0.5, 1, 2, 5, and 8 mM. In another set of studies, RFAP (0.1 and 0.25 mM) was used to inhibit saturating PR conditions for pABA (25 mM) and varied PRPP (0, 0.05, 0.1, 0.5, 0.5, and 1–3 mM) in 100 mM TES, 25 mM MgCl2, pH 4.8. To determine the Michaelis parameters of β-RFA-P synthase for RFAP, the reaction mixture contained pABA (0.1, 0.2, 1, and 3 mM) and varying concentrations of RFAP (0, 0.5, 2, 5, and 8 mM) in the same buffer as for pABA.
Characterization and Kinetics of β-RFA-P Synthase

**TABLE I**

| Purification step           | Total volume | Enzyme activity | Protein concentration | Total protein | Specific activity | Total activity | Purification factor | Yield |
|----------------------------|--------------|-----------------|-----------------------|---------------|------------------|----------------|--------------------|-------|
| Cell extract               | 50           | 57              | 5.7                   | 183.05        | 10.0             | 2850           | 1                  | 100   |
| Ni-NTA column              | 10           | 214.57          | 2.6                   | 26            | 82.53            | 2145.78        | 8.25               | 75.29 |
| Heat treatment             | 9            | 198.66          | 0.8                   | 7.2           | 248.33           | 1787.97        | 24.83              | 62.73 |

* β-RFA-P synthase activity assays were measured at pH 4.5 for 3 h at 70 °C as described under “Experimental Procedures.”

RESULTS

The Bratton-Marshall assay (3) that was previously used to measure β-RFA-P synthase activity is quite cumbersome. Therefore, we have developed a rapid assay for the enzyme in which β-aminobenzoic acid with a radioactive label in the carboxyl group is reacted with PRPP. This assay facilitates purification and kinetic studies and could easily be adapted for rapid quench kinetic studies.

Cloning, Overexpression, and Purification of β-RFA-P Synthase from M. jannaschii—By using the methods described under “Experimental Procedures,” we successfully cloned and overexpressed the β-RFA-P synthase from M. jannaschii (MJ1427) gene in E. coli.

β-RFA-P synthase was purified to homogeneity by using a combination of column chromatography and heat treatment (Table I and Fig. 1). The specific activity for the enzyme in the cell extract of induced cells was 10.0 nmol of β-RFA-P produced per min/mg of protein, in agreement with the enzyme from *Arachaeoglobus fulgidus* (4). Overexpression of the protein was probed by using anti-His tag antibodies and antibodies raised against the pure protein, which stained a band around 37 kDa, indicating that this is the protein of interest.

The cell extract was loaded onto an Ni-NTA column, which was washed with 10 column volumes of Wash buffer (see under "Experimental Procedures"). β-RFA-P synthase (26 mg with a specific activity of 82.5 nmol min⁻¹ mg⁻¹) was eluted with a solution (Elution buffer) containing 150 mM imidazole. The active fractions were further purified by heat treatment at 70 °C for 15 min, which precipitated most *E. coli* proteins and yielded a pure protein (10 mg of protein per 1 liter of culture medium) with a specific activity of 248 nmol min⁻¹ mg⁻¹. The β-RFA-P synthase gene also has been cloned with a His tag at the C terminus (using a pQE60 expression vector from Qiagen, Valencia, CA) and with no tag (using the same vector as for the gene cloned with a His tag at N terminus), and the specific activities of the pure proteins were indistinguishable, suggesting that the His tag has no influence in the activity of the enzyme. However, in both cases, the levels of protein were lower than that those observed with the gene cloned into the pET 200 expression vector with a tag at N terminus (data not shown).

The recombinant enzyme from *M. jannaschii* has an optimum pH around 4.9, which is similar to the pH activity profiles of the enzymes from *A. fulgidus* and *Methanosarcina thermophila* (2, 3). The molecular mass of the native enzyme was estimated by gel filtration on a Sephadex G-50 molecular exclusion column to be 70 kDa, indicating that the protein is a homodimer containing two 36.2-kDa monomers (data not shown).

β-RFA-P Synthase Does Not Contain PLP or a Pyruvoyl Moiety as Cofactors to Perform Catalysis—The homogeneously purified and fully active enzyme was used to determine whether or not it contains any cofactors to perform catalysis. As described under the “Discussion,” it was proposed that β-RFA-P synthase might contain PLP (3), which is reasonable given the need to stabilize the build up of charge on the C-4 of pABA during the reaction. However, the UV-visible spectrum of the pure fully active *M. jannaschii* enzyme at concentration up to 50 μM showed no UV-visible or detectable peaks between 300 and 500 nm (Fig. 2), indicating the absence of PLP. If the enzyme sample shown in Fig. 2 contained PLP, there would be an absorption band at ~350 nm with an absorbance of 0.2. The
The activity of lactate dehydrogenase was 100 units/mg. The total volume of the assay was 1 ml and the buffer used was 50 mM Tris, pH 8.0. The experiments with enzyme have been repeated three times with protein from three different preparations, and the specific activity of the enzyme before acidic hydrolysis was 250 nmol/min/mg. The assays were run for 5 min.

| Assay composition | Amount of pyruvate expected | Amount of pyruvate measured based on NADH reduction |
|-------------------|-----------------------------|---------------------------------------------------|
| 2 l of lactate dehydrogenase | 100 | 98 |
| 300 μM NADH | 100 | 2 |
| 100 μM pyruvate | | |
| No lactate dehydrogenase | | |
| 300 μM NADH | 100 | 1.5 |
| 100 μM pyruvate | | |
| 2 μl of lactate dehydrogenase | 30 | 0 |
| 30 μM protein | | |
| No NADH | 30 | 0 |
| 30 μM protein | 100 | 0 |
| 2 μl of lactate dehydrogenase | | |
| No NADH | 100 | 0 |
| 100 μM pyruvate | | |

*Following treatment with hydroxylamine, β-RFA-P synthase was dialyzed for 2 days as described under “Experimental Procedures.”

The assays were conducted in absence or presence of added PLP, and the specific activities represent the average of two experiments.

**TABLE II**

**Correlation between PLP and β-RFA-P activity**

| Treatmenta | Specific activityb |
|------------|-------------------|
| None       | 150 ± 10 (−PLP)  |
| 5 mM NH₂OH, 4°C, 24 h | 160 ± 12 (+PLP) |
| 145 ± 15 (+PLP) | |
| 5 mM NH₂OH, 4°C, 72 h | 156 ± 14 (+PLP) |
| 148 ± 12 (+PLP) | |
| 161 ± 15 (+PLP) | |

The products were analyzed by anion exchange chromatography after treatment by RFA-P and finally PP_i.
last product to dissociate, PP\textsubscript{i} should bind to the free enzyme. To test the proposed mechanism further, isothermal titration calorimetry experiments were used to measure the dissociation constants for the predicted first substrate (PRPP) and last product released (PP\textsubscript{i}) (Table V). As expected, according to the just-described order of substrate/product binding and release, PRPP (K\textsubscript{D} = 2000 ± 150 \textmu M) and PP\textsubscript{i} (K\textsubscript{D} = 200 ± 20 \textmu M) bind to the free enzyme, whereas we were unable to find evidence for binding of pABA to the free enzyme at concentrations up to 1 mM. These results are consistent with the prediction of an Ordered mechanism in which PRPP binds first and PP\textsubscript{i} dissociates last and are inconsistent with a ping-pong mechanism or a Random Sequential mechanism.

**DISCUSSION**

The \(\beta\)-RFA-P synthase (MJ1427) from *M. jannaschii* has been actively overexpressed at a level of about 10 mg per liter of culture medium, which is sufficient for these and further mechanistic studies. Overexpression of this biosynthetic enzyme is important because it is in very low abundance in methanogenic cells; for example, the *M. thermophila* enzyme required a herculean 2,800-fold purification (4). We focused on this particular enzyme because *M. jannaschii* is a hyperthermophile, which often makes the proteins more stable than those of mesophilic or even moderately thermophilic organisms. **TABLE IV**

| Type of mechanism | Product inhibitor | A | B |
|-------------------|-------------------|---|---|
| Ordered Bi-Ter\textsuperscript{a} | P | MT | UC | MT | MT |
| Bi-Ter\textsuperscript{a} | Q | UC | UC | UC | UC |
| Ping-Pong | P | MT | C | C | MT |
| Bi-Ter\textsuperscript{a} | Q | UC | UC | MT | MT |
| Based on experiments | | | | |
| P | MT | UC | MT | MT | MT |
| Q | UC | UC | UC | UC | UC |
| R | C | C | MT | MT | MT |

\textsuperscript{a} From Segel (10).

**TABLE V**

| Kinetic and dissociation constants for \(\beta\)-RFA-P synthase |
|---------------------------------------------------------------|
| Kinetic constant | Value at pH 4.8 at 70 °C |
| \(k_{\text{cat}}\) | 0.23 s\textsuperscript{-1} |
| \(K_{\text{M}}\) PRPP | 1500 ± 300 \textmu M |
| \(K_{\text{M}}\) pABA | 150 ± 10 \textmu M |
| \(K_{\text{D}}\) PRPP\textsuperscript{a} | 2000 ± 150 \textmu M |
| \(K_{\text{D}}\) PP\textsubscript{i}\textsuperscript{a} | 200 ± 20 \textmu M |

\textsuperscript{a} \(K_{\text{D}}\) measurements have been performed using ITC experiments.
nisms. This property can become valuable in structural studies of an enzyme. The only other β-RFA-P synthase that has been actively overexpressed so far is from *A. fulgidus*. Although this organism is an archaeon, it is not a methanogen, and because one of our goals is to develop mechanism-based inhibitors to inhibit methanogenesis, we felt it was important to perform mechanistic studies on the enzyme from an organism that is as closely related as possible to ruminant methanogens. Correspondingly, we showed that one of the best inhibitors of the RFA-P synthases from *Methanothermobacter marburgensis* and the ruminant mesophilic methanogen *Methanobrevibacter smithii* (6) is equally effective with the *M. jannaschii* protein (data not shown). Thus, we expect that this particular enzyme will provide a useful model system for future structure-function studies of this unusual reaction. Perhaps the inability to express the *M. jannaschii* protein earlier (4) derives from the use of a TTG start codon, which could prevent *E. coli* from initiating translation, because TTG encodes a tryptophan instead of a methionine residue. This substitution of ATG by TTG is fairly common in Archaea (11). In order to express this gene in *E. coli*, we mutated this codon to an ATG for recognition by the *E. coli* translational machinery. Based on SDS-PAGE analysis, the purified protein exhibited a single band at 37 kDa and eluted in the molecular exclusion column at the position expected for a 70-kDa protein, indicating that the enzyme in solution is a dimer of identical subunits.

Based on three-dimensional modeling, it seems that the β-RFA-P synthase shares folding patterns with the GHMP kinase superfamily, which includes proteins that phosphorylate galactose, homoserine, mevalonate, and phosphomevalonate, rather than known and more functionally related N-riboside phosphoribosyltransferases, suggesting that N- and C-riboside proteases are more unrelated than initially believed. Based on the threading it seems that the monomer is composed of two domains with the active site located between the two domains (Fig. 4). By comparison with the homoserine kinase-active site (with ADP bound in the active site), we propose that the active site of β-RFA-P synthase is located in a cleft between the domains. This would be consistent with a conformational change during catalysis (see below). However, the model has to be validated by crystal structure and mutational analysis which are under way.

β-RFA-P synthase catalyzes the first committed step in the biosynthesis of the essential methanogenic cofactor, methanopterin. This is an unusual PRPP- and pABA-dependent reaction (Scheme 1). Unlike most pABA-dependent reactions, in which the amino group is a nucleophile, the C-4 group of the benzene ring of pABA is the nucleophile. The RFA-P synthase-catalyzed condensation of pABA and PRPP is unique among known phosphoribosyltransferases in that a decarboxylation of the substrate (pABA) occurs and a C-riboside is formed instead of an N-riboside. Given this unusual chemistry, one might expect the participation of a cofactor(s) to stabilize the build up of negative charge on the C-4 group of pABA and/or perhaps to promote formation of an oxocarbenium on PRPP. Correspondingly, based on inactivation of the partially purified β-RFA-P synthase from *M. thermophila* by sodium borohydride, the measurement of a fluorescence spectrum characteristic of reduced PLP, and the labeling of a protein the size of the synthase by radioactively labeled sodium borohydride, it was proposed that β-RFA-P synthase may contain PLP (3). Furthermore, β-RFA-P synthase is inhibited by pyridoxal phosphate, an analog of PLP (3), and nonenzymatic decarboxylation of pABA by PLP has been observed (16). However, β-RFA-P synthase is not inhibited by cysteine or by several carbonyl reactive reagents known to inactivate PLP-dependent enzymes (3). In addition, the partially purified β-RFA-P synthase from *A. fulgidus* showed no UV-visible absorbance at wavelengths characteristic of pyridoxal phosphate and a consensus pyridoxal phosphate-binding motif was not found in the amino acid sequence of β-RFA synthase. Thus, it was concluded that the PLP content remained ambiguous and that "further biochemical analysis will be necessary to determine whether pyridoxal phosphate is involved in the mechanism of β-RFA-P synthase" (4).

Here we have shown that the homogeneous fully active recombinant enzyme expressed in *E. coli* is unaffected by hydroxylamine under conditions that completely inactivate PLP-dependent cystathionine β-synthase (7) and lacks the characteristic UV-visible and fluorescence spectra of PLP-dependent enzymes. If the enzyme contained PLP, there should have been an absorption band centered at ~ 350 nm with an absorbance of 0.2 (usually 10% of the intensity of the peak at 280 nm) in Fig. 2. Thus, we are confident that β-RFA-P synthase does not contain any cofactor with an absorption spectrum in the region between 250 and 600 nm. One might hypothesize that the enzyme uses another nonchromogenic cofactor with a role similar to PLP, such as a bound pyruvyl cofactor, to perform catalysis; however, the inability to detect released pyruvate and lack of any evidence for processing of the enzyme strongly suggest that pyruvate is not formed in the active site of the enzyme. This information strongly indicates that this enzyme lacks any cofactor and focuses our attention...
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on catalytic mechanisms involving only the polypeptide backbone and its side chains.

Kinetic data summarized in Tables IV and V demonstrate that β-RFA-P synthase uses an Ordered Bi-Ter reaction mechanism with PRPP binding first, then pABA, followed by the ordered release of CO₂, then β-RFA-P, and finally PPᵢ. The following experimental observations are consistent with the proposed kinetic model. (a) The initial velocity and product inhibition studies are consistent with the proposed Bi-Ter mechanism. (b) Exchange reactions predicted by a ping-pong mechanism are not detected. (c) The decarboxylation of pABA has not been observed in the absence of PRPP suggesting that binding of pABA is preceded by binding of PRPP. (d) The dissociation constants for first substrate (Kₛ,PRPP = 2000 ± 200 μM) and last product released (Kₛ,PPᵢ = 200 ± 20 μM) are in agreement with the Kₘ and Kᵥ values obtained from the steady-state kinetics.

Given that the enzyme uses a Bi-Ter mechanism, why is PRPP required for decarboxylation of pABA? As in many PRPP-dependent enzymes (11), one possibility is that binding of the second substrate (pABA) induces a structural/conformational change that would bring the two substrates into proximity and the appropriate catalytic groups into position to induce decarboxylation and C–C bond formation. One important question is whether or not an oxocarbenium ion is formed, as in other phosphoribosyltransferases (11). If so, because the oxocarbenium would be one of the first intermediates in the reaction, PPᵢ must become trapped in a cavity in the protein after it is cleaved from PRPP, because PPᵢ is the last product released during the mechanism. One of the likely candidates for binding PPᵢ is Mg²⁺, which is required for β-RFA-P synthase activity (6) as well as that of other PRPP-dependent enzymes, such as the hypoxanthine-guanine phosphoribosyltransferases (17, 18). Retaining PPᵢ throughout the mechanism could be mechanistically important, because in other PRPP-dependent enzymes, the positive charge on the ribosyl group, which becomes a ribo-oxocarbenium ion, is stabilized by interactions with the oxygen atoms of the released PPᵢ (19).

Another mechanistic feature of the β-RFA-P synthase reaction that may be shared with other PRPP-dependent enzymes is the placement of the attacking nucleophile directly above the carbene carbon, coordinated with formation of the intermediate. This is important because the oxocarbenium carbon could potentially react with other groups in the active site. Furthermore, the structure of pABA in the transition state must be quite unstable. That the enzyme is inhibited by a number of N-alkyl benzoates, but not aniline or the methyl ester of pABA, indicates that the carboxylic acid moiety of pABA must be ionized for the reaction to occur, creating the apparent requirement for developing a negative charge at a carbon adjacent to a negatively charged carboxylate moiety.

Conclusion

The need for coordination of the reactive oxocarbenium intermediate and movement of the nucleophilic center on pABA above the C-1 position on the ribosyl group suggests that a conformational change might accompany binding of the substrates. The need for a conformational change is a feature of other phosphoribosyltransferases (12, 14) and is supported by our three-dimensional model, which places the active site in the cleft between the two lobes (or domains) so that closure of the two lobes could bring pABA and PRPP in close proximity in order to promote catalysis and to avoid unnecessary hydrolysis of PRPP. These movements could also place PPᵢ, in a position ideally suited for charge stabilization of the oxocarbenium, but distant (or sterically occluded) enough to prevent nucleophilic attack of PPᵢ on the ribo-oxocarbenium C-1, which would reform PPᵢ. Overall, the bipartite transition state proposed in Scheme 1, with the pABA ring carbon nucleophile building up negative charge and the reactive positively charged ribo-oxocarbenium ion, would require energetic stabilization via both intra-molecular and enzyme-transition state interactions. How the enzyme accomplishes this in the absence of cofactors is the subject of further studies.

Several other key questions remain to be answered. The role of Mg²⁺ in catalysis, not addressed here, may be to either assist in PRPP hydrolysis and/or to influence chemical steps. Although the formation of an oxocarbenium is the most attractive working hypothesis, it is important to test for this intermediate by primary and secondary kinetic isotope effects studies.

CONCLUSIONS

The gene encoding β-RFA-P synthase from a hyperthermophilic methanogen has been cloned and actively overexpressed, and its protein product has been purified to homogeneity. By catalyzing the first committed step in the biosynthesis of the essential cofactor methanopterin, this enzyme is a target for development of inhibitors of methanogenesis. The enzyme follows a strictly ordered Bi-Ter mechanism, which would support a reaction mechanism (Scheme 1) in which a negatively charged aromatic ring carbon 4 from pABA acts as a nucleophile to attack a positively charged ribo-oxocarbenium ion from PRPP to form a C-ribosyl-β-RFA-P synthase was shown to lack any chromogenic cofactor, and the presence of PLP and the mechanistically related pyruvoyl cofactor was strictly excluded. Product inhibition studies indicate an ordered Bi-Ter mechanism, which ensures that both substrates are present at the active site before either substrate undergoes chemical reaction. The details of how this enzyme accomplishes such a remarkable and unique reaction in the apparent absence of cofactors remain to be elucidated. The work also serves as a mechanistic underpinning for the development of inhibitors that may be used eventually to reduce methanogenesis in various anaerobic environments.

Acknowledgments—The titration calorimeter was provided by funds for the Biophysics Core of The Redox Biology Center through National Institutes of Health Grant P50GM33357. We are grateful for the helpful comments of James Takacs and Jess Miner during this research.

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J. Biol. Chem. 2004, 279:39389-39395.
doi: 10.1074/jbc.M406442200 originally published online July 15, 2004

Access the most updated version of this article at doi: 10.1074/jbc.M406442200

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