p-Aminobenzoate Synthesis in *Escherichia coli*: Kinetic and Mechanistic Characterization of the Amidotransferase PabA†

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ABSTRACT: *p*-Aminobenzoic acid (PABA) is an important precursor in the bacterial biosynthetic pathway for folate enzymes. This biosynthesis requires three separate proteins: PabA, PabB, and PabC. Together PabA and PabB convert glutamine and chorismate to glutamate and 4-amino-4-deoxychorismate. This aminochorismate is subsequently transformed to PABA by PabC. In this study, PabA from *Escherichia coli* has been purified to homogeneity from an overproducing construct and found to have no detectable glutaminase activity until addition of the *E. coli* PabB subunit. PabB forms a 1:1 complex with PabA to yield a glutaminase k_{cat} of 17 min^{-1}. The addition of chorismate, the substrate of PabB, induces a 2-fold increase of k_{cat} as well as a 3-fold increase of K_m for glutamine. The PabA/PabB complex has $K_d < 10^{-8}$ M but does not form a stable complex isolable by gel filtration. Studies with the glutamine affinity label diazooxonorleucine (DON) reveal it is an inactivator of the glutaminase activity of the PabA/PabB complex, but DON does not alkylate and inactivate PabA alone. Similarly, while isolated PabA shows no tendency to form a glutamyl–enzyme intermediate, the PabA/PabB complex forms a covalent intermediate with [^{14}C]glutamine on PabA that accumulates to 0.56 mol/mol in hydrolytic turnover. PabA is thus a conditional glutaminase, activated by 1:1 complexation with PabB.

In microorganisms and plants, the dihydroaromatic compound chorismate serves as a branch point for pathways leading to important aromatic products. This includes phenylalanine and tyrosine, coenzyme Q, enterobactin (via isochorismate), tryptophan (via anthranilate), and folic acids (via *p*-aminobenzoate). *p*-Aminobenzoic acid (PABA)[1] is formed from chorismic acid and glutamine in the reaction catalyzed by the three subunits of PABA synthase: PabA, PabB, and PabC. Mechanistic and structural similarities (Walsh et al., 1990) have been noted among the three enzymes isochorismate synthase, anthranilate synthase (subunit I), and *p*-aminobenzoate synthase B (PabB), which convert chorismate, respectively, to isochorismate (Liu et al., 1990), anthranilate (Zalkin & Hwang, 1971), and 4-amino-4-deoxychorismate (Ye et al., 1990; Anderson et al., 1991). In particular, the homology between anthranilate synthase, yielding *o*-aminobenzoate (anthranilate), and PABA synthase, yielding *p*-aminobenzoate, from chorismate and cosubstrate glutamine has been noted for some years, since the cloning of PabA (Kaplan & Nichols, 1983) and PabB (Goncharoff & Nichols, 1984) and detection of homology to the anthranilate synthase II and anthranilate synthase I genes, respectively. While PabB and AS I encode chorismate-aminating enzymes of ≈50 kDa, the PabA and AS II genes encode ≈20-kDa proteins with anticipated glutaminase activity.

While anthranilate synthase component purification and characterization has been relatively well advanced, the PABA synthase biochemistry has lagged behind due to inability to detect and purify active enzyme from crude extracts. Overproduction of PabA and PabB revealed some unusual features, including the requirement for a third protein (Nichols et al., 1989), now known as PabC (Green & Nichols, 1991), for production of *p*-aminobenzoate. We previously purified PabB to homogeneity and confirmed that it can use NH_3 to aminate chorismate (Ye et al., 1990) but by itself produces the dihydroaromatic intermediate 4-amino-4-deoxychorismate (Anderson et al., 1991). PabC is required for the elimination of pyruvate and aromatization to yield PABA (Nichols et al., 1989; Ye et al., 1990), and it has now been cloned and purified to near homogeneity (Green & Nichols, 1991).

The third component of PABA synthase, PabA, has not been previously studied as a pure protein, and while one might expect it to be just another glutaminase subunit, initial studies from Nichols had indicated that PabA separated from PabB by dialysis membrane could not convert glutamine to glutamate (Nichols et al., 1989; Green & Nichols, 1991). In this study we have purified PabA to homogeneity and confirmed that it is a conditional glutaminase, requiring PabB in stoichiometric complex before displaying any enzymatic activity.

MATERIALS AND METHODS

Chorismate as barium salt, isopropyl β-D-thiogalactopyranoside (IPTG), 3-acetylpyridine adenine dinucleotide (APAD), 6-diazo-5-oxo-L-norleucine (DON), and L-glutamate dehydrogenase (GlDH; EC 1.4.1.3) were purchased from Sigma. [U-^{14}C]-L-Glutamine (285 mCi/mmol) was purchased from Amersham.

PabB was purified from the overproducer *Escherichia coli* XA90/pNPB and PabC from *E. coli* K12 (Ye et al., 1990). *Salmonella typhimurium* anthranilate synthase component I was prepared from the *trpE* overproducer CB90/pSTG3 (gift from R. Bauerle). The purification followed the published procedure (Bauerle et al., 1987; Caligiuri & Bauerle, 1991). *Serratia marcescens* anthranilate synthase component I was prepared from the overproducer XA90/pHNA-1 (gift from J. Liu, Harvard Medical School). The enzyme was purified according to Zalkin (1985).

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[1] Abbreviations. APAD, 3-acetylpyridine adenine dinucleotide; AS, anthranilate synthase; BSA, bovine serum albumine; DON, 6-diazo-5-oxo-L-norleucine; GlDH, L-glutamate dehydrogenase; IPTG, isopropyl β-D-thiogalactopyranoside; PABA, *p*-aminobenzoate; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate; TLC, thin-layer chromatography.

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Purification of PabA. A 1-L culture of *E. coli* XA90/pNPA (Ye et al., 1990) in 2×TY medium containing 100 μg/mL ampicillin was incubated at 37 °C until the OD_{595} reached 0.6–0.7. IPTG was added to a final concentration of 1 mM, and the incubation was continued for an additional 5 h. The cells were harvested by centrifugation at 5000 rpm for 10 min. All the following steps were carried out at 4 °C. The cell pellet was resuspended in 10 mL of 100 mM Tris-HCl buffer (pH 7.4) containing 1 mM EDTA and 30 mM 2-mercaptoethanol and then passed through a French pressure cell twice at 18 000 psi. The lysate was centrifuged at 15 000 rpm for 20 min. The protein was precipitated with ammonium sulfate (15–40% saturation), and the pellet was dissolved in 5 mL of buffer A [50 mM Tris-HCl (pH 7.4) containing 10 mM 2-mercaptoethanol, 5 mM $MgCl_2$, 1 mM EDTA, and 5% glycerol]. The protein solution was loaded on an Ultrogel AcA54 gel filtration column (2.6 × 100 cm) and eluted with buffer A. The active fractions were combined. The protein was >95% pure at this stage. PabA was dialyzed against 100 volumes of 20 mM Tris-HCl (pH 7.4) containing 50% glycerol. The enzyme was kept at −20 °C.

Enzyme Assays. The amidotransferase activity of PabA was measured by three independent methods. One method, used during the purification, was to detect the formation of PABA by its fluorescence (Huang & Gibson, 1970). A second method used for determining the pH optimum of PabA and the activity of PabA by itself was to monitor by thin-layer chromatography (TLC) the transformation of [^{14}C]glutamine into [^{14}C]glutamic acid. Finally, for all kinetic studies, the initial velocity was measured by detecting the formation of L-glutamate with an L-glutamate dehydrogenase-coupled assay (Nagano et al., 1970).

Fluorescence Assay. A 200-μL assay mixture contained 50 mM Tris-HCl (pH 7.4), 5 mM $MgCl_2$, 50 mM L-Gln, 1 mM chorismate, and various concentrations of PabB and PabC. Following the addition of PabA, the mixture was incubated at 37 °C for 30 min, acidified with 20 μL of 2 N HCl, and extracted with 1 mL of ethyl acetate. The concentration of PABA was determined by measuring the fluorescence of the organic solution at 290 nm (excitation)/340 nm (emission) at 20 °C.

[^{14}C]Glutamine Assay. In a 25-μL assay of 100 mM triethanolamine of desired pH (9.0 for routine assays), 15–60 pmol of PabA (and 1.5 equiv of PabB if needed) was incubated at room temperature with 28 μM [^{14}C]Gln (6.27×10^5 dpm/nmol). A 3-μL aliquot of the assay mixture was loaded on a cellulose-coated TLC sheet. After elution (methanol–water 8:2), the migration areas of glutamine and glutamic acid were located by autoradiography of the TLC. The radioactive spots were cut and transferred to scintillation vials containing 10 mL of scintillation cocktail (Atomlight, DuPont). The vials were shaken until the cellulose coating was in suspension. The dpm results were corrected to take into account a 5% impurity present in the glutamine which comigrated with glutamic acid.

Glutamate Dehydrogenase-Coupled Assay. (*a*) *In the Absence of Chorismate.* A 1-mL assay mixture contained 100 mM triethanolamine (pH 9.0), 1.25 mM L-glutamine, 4 mM APAD, 20 units of GlDH, and 5 mM $MgCl_2$. PabA and 1.5 equiv of PabB were added to the assay before incubation at 25 °C. The reduction of APAD was followed at 364 nm, taking the molar extinction coefficient equal to 9100 (Nagano et al., 1970). The initial velocity was determined after 2 min to avoid a lag period.

(*b*) *In the Presence of Chorismate.* For the determination of some steady-state kinetic constants, the 1-mL assay mixture contained 100 mM triethanolamine (pH 9.0), 0.2–2 mM L-glutamine, 4 mM APAD, 20 units of GIDH, 800 μM chorismate, and when needed 5 mM $MgCl_2$. PabA (0.1 nmol) and PabB (0.15 nmol) were added to the assay before incubation at 25 °C. The reduction of APAD was followed as described above. When present in the assay, magnesium ions allowed PabB to be under turnover conditions and to use nascent ammonia and chorismate to produce 4-amino-4-deoxychorismate.

Inactivation by DON. PabA (1 nmol) (and 1.5 nmol of PabB if present) was incubated with various amounts of DON (6–200 μM) and L-glutamine (0, 200, 500 μM) in a 200-μL assay mixture containing 100 mM triethanolamine (pH 9.0) and 5 mM $MgCl_2$. At the indicated times, the reaction was quenched by dilution to 1 mL in the glutamate dehydrogenase-coupled assay buffer [100 mM triethanolamine (pH 9.0), 4 mM APAD, 20 units of GlDH, 5 mM $MgCl_2$, and 50 mM glutamine; Gln/DON ratio >1000]. The remaining activity was determined as described above.

Isolation of a Covalent Intermediate between PabA/PabB and [^{14}C]Glutamine. The intermediate was formed by incubating at room temperature 0.22 nmol of PabA (and in some experiments 1.5 equiv of PabB) with 7.5 nmol of [U-^{14}C]glutamine (specific activity 5.85×10^5 dpm/nmol) in a 100-μL assay containing 100 mM triethanolamine (pH 9.0) and the desired concentration of L-glutamine. The mixture was diluted after 30 s in 1 mL of a 8% TCA solution, and 100 μL of BSA (10 mg/mL) was added. After 2 min, to allow the proteins to precipitate, the solution was filtered under vacuum on a 2.5-cm nitrocellulose filter (Schleicher and Schuell, 0.45-μm porosity). The filtrate was washed with 50 mL of 1 N HCl. The filter was transferred to a 20-mL scintillation vial containing 10 mL of scintillation cocktail (Atomlight, DuPont). After the vial was shaken for 30 min, the ^{14}C activity was measured.

Gel Filtration of the Intermediate between PabA/PabB and [^{14}C]Glutamine. PabA (10 nmol) and PabB (10 nmol) were incubated with 81 μM [^{14}C]glutamine (5.85×10^5 dpm/nmol) in 28 mM triethanolamine (pH 9.0) at 0 °C in a total volume of 100 μL. After 30 s, the acylenzyme intermediate was quenched by addition of 22 μL of 0.5 M sodium acetate (pH 4.0) containing 5% SDS. The assay mixture was chromatographed on a Sephadex G-100-120 column (36 × 1.6 cm) equilibrated with 1% SDS in 100 mM sodium acetate (pH 4.0). The column was eluted with the same buffer at a flow rate of 0.6 mL/min at room temperature. From the 280-μL fractions collected, 170 μL was used for counting the radioactivity after dilution in 6 mL of scintillation cocktail and 50 μL was used to determine the protein composition after a 14% SDS–PAGE.

Gel Filtration of the PabA/PabB Complex. Aliquots (100 μL) of different mixtures of PabA and PabB (21.3 nmol/4.3 nmol, 15 nmol/7.5 nmol, 7.5 nmol/11 nmol) were loaded on a Superose 12 HR10/30 FPLC column (Pharmacia LKB Biotechnology Inc.) equilibrated with 100 mM triethanolamine (pH 9.0), 5 mM EDTA, and 5 mM glutamine when needed. The proteins were eluted at a flow rate of 0.4 mL/min with the same buffer and detected by their UV absorption at 280 nm. The elution volumes of PabA and PabB alone were used to identify the peaks. Fractions of 200 μL were collected when a peak was detected. The composition of these fractions was analyzed by SDS–PAGE.

RESULTS

PabA Production and Purification. Upon IPTG induction, a 1-L culture of freshly transformed XA90/pNPA gave 115 mg of pure enzyme, which represents 15% of the total soluble

Table I: Purification of *E. coli* PabA from XA90/pNPA Cells

purification step	protein (mg)[a]	total act. (units)[b]	sp act. (units/mg)	yield (%)	purifcatn (*x*-fold)
crude extract	790	283 000	359	100	1.0
15–40% ammonium sulfate precipitation	295	86 700	291	31	0.8
gel filtration column	115	88 300	775	31	2.2

[a] Protein concentration was determined by the Bradford protein assay (Bio-Rad) using BSA as standard. [b] One unit is the amount of enzyme required to produce 1 nmol of glutamic acid/min.

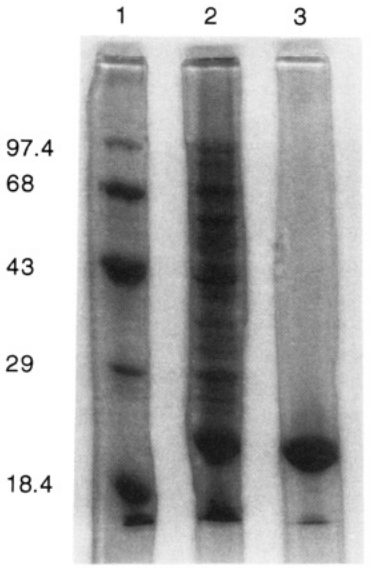

FIGURE 1: SDS–polyacrylamide gel electrophoresis of PabA (11% running gel): lane 1, molecular weight standards; lane 2, crude extract; lane 3, protein after gel filtration.

proteins. But a 30-fold decrease of the overproduction was observed when 1-month-old single colonies of XA90/pNPA were used. Thus, purifications proceeded from freshly transformed single colonies.

The low molecular mass of PabA (21 kDa) and its abundance allowed a ready purification to homogeneity in two steps. First, a 15–40% ammonium sulfate precipitation removed two-thirds of the proteins, among them a 30-kDa protein which otherwise coeluted with PabA on a gel filtration column. Then PabA was purified by gel filtration to apparent homogeneity (>95%, estimated on a 11% SDS–polyacrylamide gel). The purification is summarized in Table I. The activity of PabA in the crude extract and in the 15–40% ammonium sulfate pellet was determined after dialysis in gel filtration buffer A. Although PabA was pure after gel filtration, the purification factor by specific activity assay was ca. 3-fold lower than one might have expected. This does not seem due to a fast inactivation of the enzyme in the early stages of the purification, as indicated by the 56% stoichiometry of the glutamyl–PabA intermediate (vide infra). But the removal of other glutaminases present in the crude extract may also explain the large decrease of total glutaminase activity after the ammonium sulfate precipitation.

The identity of purified PabA was confirmed by two methods: its 21-kDa molecular mass was determined by polyacrylamide gel electrophoresis in denaturing conditions (Figure 1), and its N-terminal sequence Met-Ile-Leu-Leu-Ile was found to conform to the published *E. coli* sequence prediction (Kaplan & Nichols, 1983).

A 10% loss of activity per day for PabA was observed when the enzyme was kept at 4 °C in the gel filtration buffer. No degradation products of finite molecular size were detected by SDS–PAGE, suggesting that the inactivation was due to

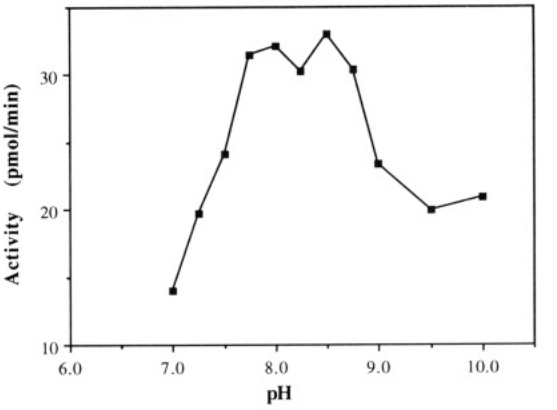

FIGURE 2: pH dependence of the PabA/PabB glutaminase activity. PabA (15 pmol) and PabB (22 pmol) were incubated for 4 min at room temperature with 28 μM [^{14}C]Gln (6.27×10^5 dpm/nmol) in 100 mM triethanolamine at pH varying from 7.0 to 10.0. The amount of [^{14}C]glutamic acid formed in the reaction was determined as described under Materials and Methods.

conformational or chemical modifications of PabA. The stability of the enzyme was greatly increased when kept at –20 °C in a 20 mM Tris-HCl buffer (pH 7.4) containing 50% glycerol. After an initial 5–10% loss of activity during the first week, the activity remained stable for at least 1 month.

Catalytic Activity of PabA. Preliminary studies had indicated that the amidotransferase activity of PabA may occur only when PabA and PabB were interacting directly (Nichols et al., 1989; Green & Nichols, 1991). In an experiment carried out by Nichols, purified PabA had been separated from purified PabB by a dialysis membrane in the presence of glutamine, Mg^{2+}, chorismate, and crude PabC. In these conditions, glutamine was not hydrolyzed and subsequently no accumulation of PABA was detected. But in a control experiment, when PabA and PabB were in the same compartment, PABA was produced in significant amount. This result has been validated in these studies. When several preparations of purified PabA were assayed for glutaminase activity, none was detected. Addition of purified *E. coli* PabB led to acquisition of glutaminase activity by PabA.

A quantitative study of this interaction was undertaken. The initial velocity of PabA in the presence of PabB was determined at room temperature by monitoring the formation of L-glutamate in a coupled assay with L-glutamate dehydrogenase. The use of APAD, an analogue of NAD^+, allowed displacement of the unfavorable equilibrium of GlDH toward the consumption of glutamate. A lag period of 1 min was consistently observed before the initial velocity was stable, but this was not caused by a slow reassociation of the two enzymes PabA and PabB. Preincubation experiments of PabA and PabB before assay had no effect on the activity, suggesting that the reassociation process is completed in less than 15 s (data not shown). The assays were carried out at pH 9.0, where the coupled PabA/PabB–GlDH assay exhibited the highest activity. But it is noteworthy that the transformation of [^{14}C]glutamine into [^{14}C]glutamic acid by PabA/PabB only (monitored by TLC) at pH varying from 7.0 to 10.0 reached a maximum between pH 7.75 and 8.75 (Figure 2). The PabB-dependent glutaminase activity of PabA of 810 nmol min^{-1} mg^{-1} PabA corresponds to a glutaminase k_{cat} of 17 min^{-1}.

The titration of a constant amount of freshly purified PabA (typically 1 nmol) with increasing amounts of pure PabB is shown in Figure 3. A plateau of activity was reached after the addition of 1 equiv of PabB, consistent with formation of a 1:1 stoichiometric complex.

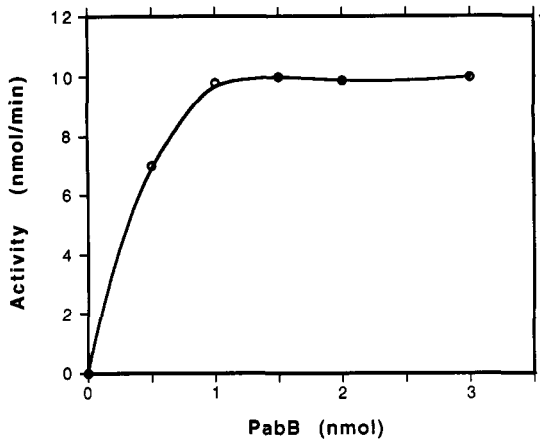

FIGURE 3: Activation of PabA glutaminase activity in the presence of PabB. The L-glutamate dehydrogenase-coupled assay (see Materials and Methods) contained 1 nmol of PabA and increasing amounts of PabB in 1 mL of 100 mM triethanolamine (pH 9.0), 1.25 mM L-glutamine, 5 mM $MgCl_2$, 4 mM APAD, and 20 units of GlDH.

As noted above, with the GlDH-coupled assay, no measureable glutaminase activity was detected when PabA was incubated without PabB. In order to evaluate a minimum value for the ratio of PabA activity with and without PabB, the kinetics of the transformation of [^{14}C]glutamine to [^{14}C]-glutamic acid in the presence of PabA/PabB or PabA alone was followed by TLC. Although 11.5% of the glutamine was transformed per minute by 59 pmol of PabA complexed with PabB, less than 18% was converted in 21 h by 510 pmol of PabA itself. It may thus be estimated that PabA alone is at least 6700-fold less active than PabA and PabB together.

It is known that PABA synthase and anthranilate synthase are functionally and structurally related enzymes. In several species such as *Bacillus* and *Acinetobacter*, a unique glutaminase subunit is shared by PABA synthase and anthranilate synthase (Kane & O'Brien, 1975; Sawula & Crawford, 1973). In *Escherichia coli*, the identity at the amino acid level is 44% between the PabA and anthranilate synthase component II (AS II) glutaminase subunits (Kaplan & Nichols, 1983) and 26% between PabB and anthranilate synthase component I (AS I) (Goncharoff & Nichols, 1984). To test for interchangeability of PabB and AS I, the activation of PabA glutaminase activity by AS I was investigated with pure *Salmonella typhimurium* and *Serratia marcescens* AS I. In both cases no glutaminase activity was detected with the GlDH-coupled assay, even when molar ratios of AS I/PabA up to 30 were used. The *S. typhimurium* enzyme was chosen because it is easily obtained from an overproducer strain and mimics the behavior of *E. coli* AS I: a 90% identity was found between these two enzymes (Yanofsky & VanCleemput, 1982), and a hybrid *S. typhimurium* AS I/*E. coli* AS II complex exhibited the same activity as the *E. coli* AS I/AS II complex (Ito, 1969). But the *trpG*-encoded *E. coli* or *S. typhimurium* AS II are fused to the *trpD*-encoded anthranilate phosphoribosyltransferase, the second enzyme of the tryptophan pathway (Henderson et al., 1970), which distinguishes *E. coli* anthranilate synthase complex from PABA synthase complex. On the contrary, *S. marcescens* AS II is unfused (Zalkin & Hwang, 1971) and the AS complex could be structurally close to the PABA synthase complex. This explains the decision of testing a possible activation of PabA by *S. marcescens* AS I.

Steady-State Kinetics of the Glutaminase Activity of the PabA/PabB Complex. Given the absolute dependence of PabA activity on the presence of PabB, the glutaminase steady-state kinetic parameters were measured with or without the presence of chorismate, one of the substrates of PabB, and magnesium ions, which are also necessary for PabB activity (Ye et al., 1990). When both chorismate and magnesium ions were present in the assay, PabB was catalytically active (Ye et al., 1990; Anderson et al., 1991). Nascent ammonia produced by PabA was used for the conversion of chorismate to 4-amino-4-deoxychorismate. The results are summarized in Table II.

Table II: Steady-State Kinetic Parameters for PabA/PabB Glutaminase Activity[a]

	K_m (μM)		k_{cat} (min^{-1})		k_{cat}/K_m	
ligand[b]	$-Mg^{2+}$	$+Mg^{2+}$ [c]	$-Mg^{2+}$	$+Mg^{2+}$ [c]	$-Mg^{2+}$	$+Mg^{2+}$ [c]
–chorismate	333	335	17.4	20.1	0.052	0.060
+chorismate	725	960	42.3	39.2	0.058	0.041

[a] The activity of 1 nmol of PabA with a saturating amount of PabB (1.5 nmol) was measured at room temperature using the GlDH-coupled assay in 100 mM triethanolamine (pH 9.0). The results are the means of values obtained from Lineweaver–Burk, Eadie–Hofstee, and Hanes–Woolf plots. [b] Determined with saturating concentration of chorismate (800 μM). [c] Determined with 5 mM $MgCl_2$.

The presence of 800 μM chorismate in the assay clearly induced a 2-fold increase in PabA k_{cat} (from 19 to 40 min^{-1}), but magnesium ions had no significant effect on the glutaminase turnover number. The K_m value for Gln was increased 2.2-fold when chorismate alone was added and 2.9-fold when both chorismate and magnesium were present. But magnesium ions alone did not affect the Gln K_m. As PabB was not under turnover conditions in the absence of Mg^{2+}, these data indicated that the higher specific activity of the PabA glutaminase was linked to conformational changes in the PabA/PabB complex rather than to the consumption by PabB of nascent ammonia. The influence of the chorismate concentration on glutaminase k_{cat} was studied at 50 mM glutamine (50 K_m in the assay conditions) in the presence of magnesium ions, which allowed PabB to be under turnover conditions. A plateau was reached around 100 μM chorismate, but 50% activation was already obtained with 5 μM chorismate. These values were low compared to the K_m of PabB for chorismate: 67 μM for PabB alone in the presence of 50 mM ammonium sulfate (Ye et al., 1990) and 130 μM for PabB in the presence of 3 equiv of PabA and 50 mM glutamine (Roux and Walsh, unpublished results).

Stability of the PabA/PabB Complex. To attempt determination of the dissociation constant K_d between the two components PabA and PabB, the activity of PabA/PabB (1:1 mixture) was measured when the concentration of enzymes was lowered. The plot of log (activity) vs log ([PabA]) should have a slope of 1 for [PabA] $\gg K_d$ and a slope of 2 for [PabA] $\ll K_d$. In the range of 10^{-7}–10^{-6} M, the slope was found equal to 1.06, indicating that the dissociation constant is lower than 10^{-8} M. Lower concentrations could not be studied due to lower limits of detection of glutaminase activity.

Despite this limit for the dissociation constant, the PabA/PabB complex was completely separated on a gel filtration column. Different ratios of PabA (50–200 μM) and PabB (50–100 μM) were loaded on a Superose 12 column (Pharmacia). The dilution occurring during the gel filtration may be estimated at 20-fold. The peaks corresponding to pure PabB (51 kDa) and pure PabA (21 kDa) were well resolved, but no peak corresponding to a PabA/PabB complex was detected. The analysis by SDS–PAGE of fractions collected during the elution of the enzymes showed unequivocally the total separation of PabA and PabB. The addition of 5 mM glutamine in the sample and in the running buffer did not improve the stability of the complex during the gel filtration.

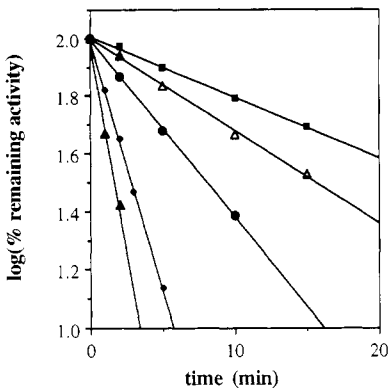

FIGURE 4: Inactivation of PabA/PabB by DON. PabA (1 nmol) and PabB (1.5 nmol) were preincubated at room temperature (pH 9.0) in the presence of 6 (■), 10 (△), 20 (●), 50 (◆) and 100 μM (▲) DON. The residual glutaminase activity was determined as described under Materials and Methods.

Inactivation by DON. DON, a diazo ketone analogue of glutamine, has been shown to be an irreversible alkylating inhibitor of glutaminases such as anthranilate synthase component II (Goto et al., 1976) and CAD glutaminase (Chaparian & Evans, 1991) when it is preincubated with those enzymes.

Preliminary studies showed a time-dependent loss of activity of the PabA/PabB complex after preincubation with 250 μM DON at room temperature over a period of 15 min. In the same conditions, PabB or PabA alone showed no measurable inhibition. The remaining activity of the preincubated enzymes was determined with the GlDH-coupled assay using 50 mM L-glutamine, which provided at least a glutamine/DON ratio of 1000. It should be noted that no loss of activity of PabA/PabB was detected after standing for 1 h at room temperature in the buffer used for the study of the inactivation by DON.

The time-dependent inhibition of PabA/PabB was then further investigated with concentrations of DON ranging from 6 to 200 μM. The presence of 200 or 500 μM glutamine during the preincubation between DON and the enzymes induced a decrease of the inactivation. This could be the result of DON and glutamine both competing for the active site of the glutaminase, although some conformational changes in the PabA/PabB complex induced by the binding of glutamine might explain this protection against inactivation by DON. The semilogarithmic plot of the remaining activity vs time showed pseudo-first-order kinetics during the first 5–10 min. The time course of the inactivation without protection by glutamine is shown in Figure 4. The half-inactivation times, τ, were determined from these plots. If a complex between DON and PabA/PabB is formed prior to the inactivation, as it is suggested by the protection by glutamine, the relationship τ vs 1/[DON] is given by eq 1, where K_s is the dissociation

$$\tau = TK_i(1 + [\mathrm{Gln}]/K_s)/[\mathrm{DON}] + T \qquad (1)$$

constant for glutamine, K_i the inactivation constant for DON, and T the time required for half-inhibition at infinite concentration of DON (Meloche, 1967). For each concentration of glutamine, the replot τ vs 1/[DON] is shown in Figure 5. A second replot of the slope of the curve (τ vs 1/[DON]) against [Gln] gave a value of K_s = 500 μM. But the value of K_i was difficult to evaluate precisely due to the uncertainty on the determination of the y-intercept, T (0.04–0.9 min). By use of the results obtained when glutamine was omitted from the preincubation reaction, a value of K_i = 210 μM for DON was calculated.

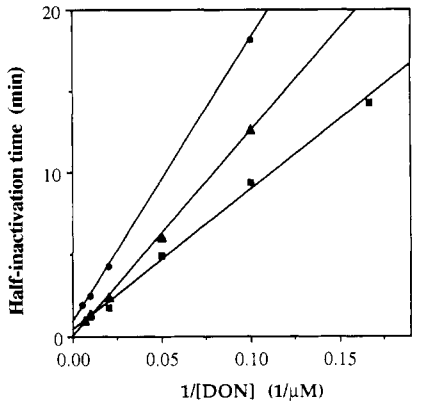

FIGURE 5: Kinetics of the inactivation of PabA/PabB by DON and competition with L-glutamine. The conditions are described under Materials and Methods. Reaction: (■) without glutamine, (▲) with 200 μM glutamine, and (●) with 500 μM glutamine.

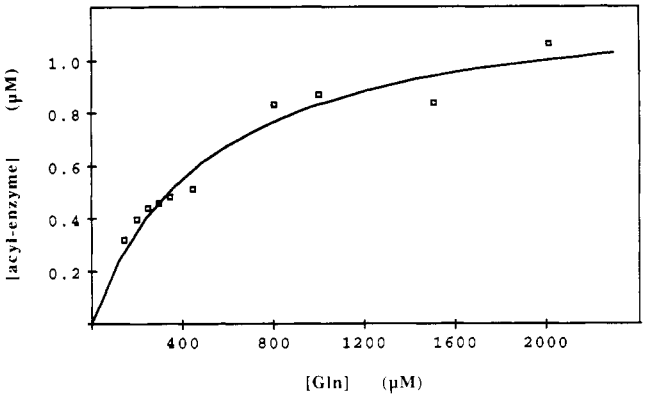

FIGURE 6: Effect of glutamine on the steady-state concentration of the intermediate between PabA/PabB and [^{14}C]glutamine. In a 100-μL assay, 0.22 nmol of PabA and 0.33 nmol of pabB were incubated for 30 s at pH 9.0 with increasing concentrations of glutamine containing a constant amount (7.5 nmol) of [^{14}C]glutamine (specific activity 5.85 × 10^5 dpm/nmol). The quantification of the covalent intermediate was determined as described under Materials and Methods.

A slow time-dependent inactivation of 1%/min was observed over a period of at least 10 min when PabA alone was preincubated with 1 mM DON. When the concentration of DON was increased to 10 mM, a 15% inactivation occured in less than 1 min, followed by a slower time-dependent inactivation of 1%/min. If PabA/PabB had been incubated with such concentrations of DON, the half-inactivation time τ would have been 0.48 (1 mM DON) and 0.41 min (10 mM DON) as derived from eq 1 with K_i = 210 μM and T = 0.4 min. The inactivation observed with PabA alone was at least 100-fold slower than in the presence of PabB. These results indicated that DON reacted only very slowly at the active site of the glutaminase PabA when this enzyme was not associated with PabB.

Isolation of a Covalent Glutamyl Intermediate in the PabA/PabB Complex. The *trpG*-amidotransferase family, which includes enzymes such as anthranilate synthase, *p*-aminobenzoate synthase, carbamyl phosphate synthetase, and CTP synthetase, is characterized by three highly conserved DNA regions (Amuro et al., 1985). In the case of *S. marcescens* anthranilate synthase II, one of these regions contains a cysteine residue that participates in the formation of a γ-glutamyl thioester intermediate during the hydrolysis of glutamine (Paluh et al., 1985). It has been suggested that the same catalytic mechanism may apply to the different amidotransferases (Mei & Zalkin, 1989). The existence and isolation of a covalent CAD glutaminase–γ-glutamyl intermediate has

recently been reported (Chaparian & Evans, 1991).

The existence of such a γ-glutamyl intermediate was investigated in the case of PabA/PabB. This intermediate was trapped by quenching the reaction of PabA/PabB complex and [^{14}C]glutamine with trichloroacetic acid (TCA). The steady-state concentration of the acylenzyme was reached after a 30-s incubation at room temperature in a triethanolamine (pH 9.0) buffer. The use of a high glutamine/enzyme ratio (70–900) ensured a constant glutamine concentration during the incubation. After denaturation of the protein with TCA, the precipitate was isolated by filtration and washed with 1 N HCl, and the radioactivity was counted. This experiment was carried out with increasing concentrations of glutamine in order to determine the steady-state concentration of intermediate at saturating concentration of glutamine (Figure 6). A nonlinear least squares fit gave a value of 0.56 mol of acylenzyme/mol of PabA/PabB complex at saturating concentration of glutamine. If the following kinetic mechanism is assumed

$$\mathrm{Gln} + \mathrm{E} \overset{K_s}{\rightleftharpoons} \mathrm{E{\cdot}S} \overset{k_2}{\rightarrow} \mathrm{E{-}S} \overset{k_3}{\rightarrow} \mathrm{E} + \mathrm{P}$$

the initial velocity is given by $v = k_3[\text{acylenzyme}] = k_{cat}[E_T][Gln]/(K_m + [Gln])$, where $k_{cat} = k_2k_3/(k_2 + k_3)$, $K_m = K_sk_3/(k_2 + k_3)$, and $[E_T]$ is the total enzyme present. In this case, the least squares analysis of the data gave a K_m value of 475 μM and $k_{cat}/k_3 = 0.56$. A combination of these results led to the determination of $K_s = 1080$ μM. The values were not too different from the K_m and K_s values found when the initial velocity of the reaction was measured with the GlDH-coupled assay ($K_m = 333$ μM, $K_s = 500$ μM). It should be noted that these results implied also that the breakdown of the acylenzyme intermediate was marginally the rate-limiting step ($k_3 = 0.8k_2$).

Finally, no measurable concentration of acylenzyme intermediate was detected after incubation of PabA alone with [^{14}C]glutamine. Assuming an amount of intermediate equal to the noise in the experiment, the concentration of PabA–glutamyl intermediate was estimated to be at least 100-fold lower than the concentration obtained with PabA/PabB complex.

As glutaminase activity was only detected when PabA was complexed with PabB, the direct role of PabA in the hydrolysis of glutamine was addressed by determining which protein contained the [^{14}C]-γ-glutamyl group by chromatography of the trapped PabA/PabB–γ-glutamyl intermediate. Once the steady-state concentration of [^{14}C]acylenzyme was reached, the proteins were denatured, without precipitation, by addition of SDS to 1% at pH 4.0, followed by a gel filtration on a Sephadex G-100-120 column equilibrated with 100 mM sodium acetate (pH 4.0) containing 1% SDS. The composition of fractions collected during the gel filtration was analyzed by SDS–PAGE. As shown in Figure 7B, the maximum concentration for PabB and PabA was respectively at fraction 30 and fraction 38. The radioactivity distribution followed the concentration of PabA (maximum activity at fraction 39, Figure 7A), indicating PabA to be the labeled protein. It should be noted that the same radioactivity ($^1/_4$ of the maximum) was detected in fraction 30 (highest concentration of PabB) and in fraction 51 (no PabB detected by SDS–PAGE), ruling out the possibility that PabB was labeled by [^{14}C]glutamine. Free [^{14}C]glutamine eluted in a peak well separated from the proteins (fractions 180–260). Thus, the glutaminase active site where a covalent bond is formed with a γ-glutamyl residue appeared to be located on PabA, as expected due to the homology between PabA and other *trpG*-encoded proteins such as *S. marcescens* anthranilate synthase component II, whose role as glutaminase had been fully demonstrated (Zalkin & Hwang, 1971; Paluh et al., 1985).

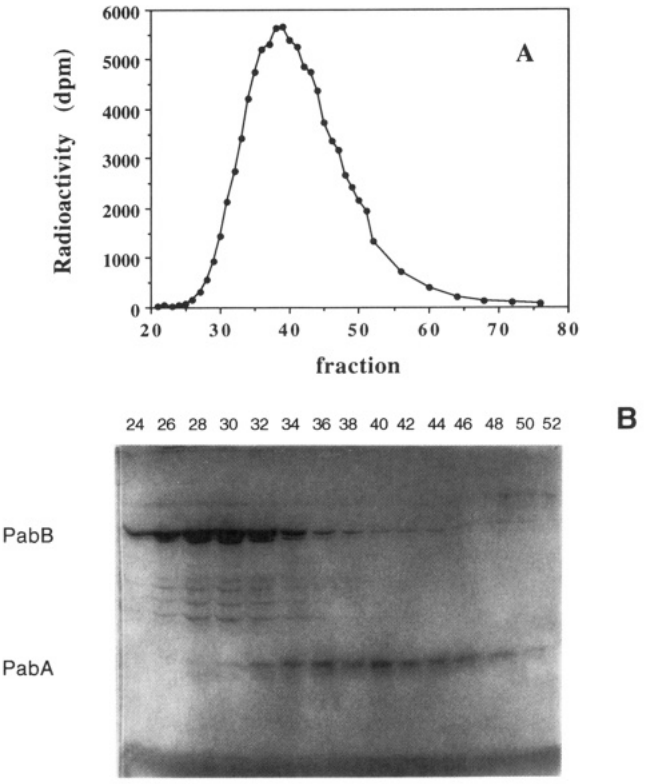

FIGURE 7: Gel filtration of the intermediate between PabA/PabB and [^{14}C]glutamine. The acylenzyme between a 1:1 PabA/PabB complex (10 nmol of each) and [^{14}C]glutamine was formed and trapped as described under Materials and Methods. The assay mixture was chromatographed on a 1.6 × 36-cm Sephadex G-100-120 column equilibrated with 100 mM sodium acetate (pH 4.0) containing 1% SDS. The first 17 mL (void volume) was collected in one pool and then the fraction size was adjusted to 280 μL. (A) ^{14}C radioactivity in dpm of 170 μL of fractions from the gel filtration. (B) SDS–PAGE (14% resolving gel) on 50 μL of corresponding fractions.

DISCUSSION

The overproduction system described in this paper allows rapid purification of PabA to homogeneity in substantial quantity and permits its enzymatic characterization. The behavior of PabA as a straightforward or a conditional PabB-activated glutaminase was a question to be addressed due to the variety of behaviors reported for *trpG*-encoded amidotransferase subunits or domains: *Pseudomonas putida* anthranilate synthase component II, exhibiting a full glutaminase activity (Goto et al., 1976); carbamyl phosphate synthetase light subunit alone with a higher K_m for glutamine (Trotta et al., 1974); and *Salmonella typhimurium* anthranilate synthase II, having no glutamine hydrolase activity by itself (Bauerle et al., 1987). Several preparations of PabA appeared to be devoid of glutaminase activity and only acquired glutaminase hydrolyzing capacity in the presence of PabB as predicted by Nichols (Green & Nichols, 1991).

We have characterized several aspects of the conditional glutaminase activity of PabA. By itself, it has a glutaminase k_{cat} of less than 0.0025 min^{-1}, reflecting less than one hydrolytic event per 400 min. Pure *E. coli* PabB accelerates k_{cat} at least 6700-fold to a value of 17 min^{-1}. The effect of PabB appears to be as a stoichiometric positive allosteric effector on the PabA subunit since the gain of glutaminase activity on titration plateaus at exactly 1:1 PabA/PabB ratios. The addition of chorismate, the PabB-specific substrate, produces

another 2-fold increases in PabA glutaminase activity to 40 min^{-1}, reflecting another subtle conformational effect. PabB is not catalytically active as a chorismate aminase under these conditions until Mg^{2+} is added, and that has only minor additional effect, so it is unlikely that removal of nascent NH_3 is at issue in the allosteric activation of PabA by PabB.

While a 1:1 stoichiometry is indicated by gain of activity titrations and dilution analysis indicates a K_d of $<10^{-8}$ M, the PabA and PabB subunits do not form a long-lived stable complex detectable by gel filtration analysis, in contrast to the anthranilate synthase subunits (Ito & Yanofsky, 1969; Pabst et al., 1973). It may be that addition of purified PabC in stoichiometric amounts (not available in these studies) may produce a stable trimeric PabA, -B, -C PABA synthase complex. To assess the specificity of *E. coli* PabB as a positive activator of *E. coli* PabA glutaminase activity, we purified and tested two homologous anthranilate synthase chorismate aminating subunits. The structure of AS I/AS II anthranilate complexes is very diverse depending on the species: in enteric bacteria such as *E. coli* or *S. typhimurium*, the *trpG*-encoded glutaminase subunit is fused to the *trpD*-encoded anthranilate phosphoribosyl transferase leading to a multifunctional protein, whereas in *S. marcescens* or *P. putida* AS II is unfused. Instead of the *E. coli* protein, we utilized the closely related *S. typhimurium* AS I, easily prepared from an overproduction construct (Caligiuri & Bauerle, 1991). We also purified the *trpE* gene product from a *S. marcescens* overproducer (Zalkin, 1985). Neither of these chorismate-aminating enzymes could replace *E. coli* PabB as an allosteric activator. The *S. typhimurium* AS I fragment is 90% identical to the *E. coli trpE*-encoded species (Yanofsky & VanCleemput, 1982) and should be a reasonable test for specificity. Thus it would appear that the protein–protein interaction between *E. coli* PabA and PabB is highly specific even though it has been reported that in bacteria such as *Bacillus* and *Acinetobacter* a single glutaminase subunit is shared by both PabB and AS I subunits (Kane & O'Brien, 1975; Sawula & Crawford, 1973).

To assess further the intrinsic ability of PabA separately to recognize glutamine, two additional probes were conducted: the use of diazooxonorleucine (DON) as an affinity label and the ability to produce the anticipated γ-glutamyl-*S*–enzyme intermediate. DON is a classic affinity label for this family of glutaminase subunits, binding to the glutamine site, undergoing enzyme-assisted protonation of the diazo ketone group to unmask the reactive alkyldiazonium species that then releases N_2 and covalently alkylates and inactivates the enzyme. DON is indeed an inactivator of the PabA/PabB complex's glutaminase activity with K_i of 210 μM and k_{inact} of 1.25 min^{-1}. The protection of PabA/PabB by glutamine against inactivation by DON may suggest that DON acts at the active site of the glutaminase. In the absence of PabB, DON covalently modifies PabA at least 100-fold more slowly, and that low rate may be adventitious, nonspecific reactivity. Thus it is clear that PabA by itself shows drastically reduced susceptibility to DON binding and alkylation, as was observed with *Bacillus subtilis* anthranilate synthase II (Kane et al., 1973).

The anticipated glutaminase mechanism of PabA, in complex with PabB, is hydrolysis via a γ-glutamyl–*S*-enzyme, and Cys 79 is the highly conserved cysteine residue analogous to that identified as the catalytic nucleophile in AS II (Paluh et al., 1985). By use of [^{14}C]glutamine and acid quench experiments, it was indeed possible to detect covalent glutamyl–enzyme formation and hydrolytic decay. Again PabA alone had no detectable ability to undergo ^{14}C glutamylation. Coupled to its inability to catalyze net glutamine hydrolysis, it would appear that the active site of PabA is not in an active conformation able to initiate nucleophilic attack on glutamine until PabB has induced a conformational change. PabA clearly is a conditional glutaminase. In the PabA/PabB complex the [^{14}C]glutamyl–enzyme builds up in steady-state turnover to 0.56 equiv, allowing an estimation that deacylation is slightly rate determining in turnover. Subsequent separation of PabA from PabB by gel filtration allowed demonstration that the [^{14}C]glutamyl group was attached to PabA. Current studies are directed at determination of the role of specific residues in PabA involved in glutaminase catalysis and on what features of PabB produce the specific association and activation of PabA.

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