p-Aminobenzoate Synthesis in *Escherichia coli*: Mutational Analysis of Three Conserved Amino Acid Residues of the Amidotransferase PabA†

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ABSTRACT: *p*-Aminobenzoate synthesis in *Escherichia coli* requires three enzymes, PabA, PabB, and PabC, acting respectively as glutaminase, chorismate aminase, and 4-amino-4-deoxychorismate aromatase. PabA requires stoichiometric amounts of PabB to display glutaminase activity. PabA has conserved cysteine (C79), histidine (H168), and glutamate (E170) residues that have been suggested in the analogous anthranilate synthase to form a type of catalytic triad in an acylenzyme mechanism. Mutations at each of these residues of PabA lead to the following observations. C79S PabA has 40-fold lower k_{cat} and 10^4 lower k_{cat}/K_m with no detectable acylenzyme accumulation in steady-state turnover (vs wild-type PabA at 0.56 mol fraction of γ-glutamyl-enzyme). H168Q has no catalytic activity and does not compete with wild-type PabA for PabB (this may indicate a folding defect). Four E170 mutants give three outcomes. E170D and E170A yield active PabA species, down 4-fold and 150-fold, respectively, in k_{cat}/K_m ratios from wild-type PabA. E170Q has no detectable glutaminase activity but does bind to PabB in competition with wild-type PabA while E170K has neither detectable catalytic activity nor the ability to be recognized by PabB.

In microorganisms and plants, the dihydroaromatic compound chorismate serves as a branchpoint 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 (Figure 1). As previously shown (Roux & Walsh, 1992), PabA, a 21-kDa subunit, is a conditional glutaminase activated by 1:1 complexation with PabB. The nascent ammonia released during the hydrolysis of glutamine is subsequently used by PabB, a 51-kDa subunit, to transform chorismate to the dihydroaromatic intermediate 4-amino-4-deoxychorismate (Anderson et al., 1991). Although PabA requires the presence of PabB to be catalytically active, PabB can directly use NH_3 to aminate chorismate (Ye et al., 1990). The third subunit, PabC, is still poorly characterized, but it has been shown to be required for the elimination of the pyruvate moiety from 4-amino-4-deoxychorismate and its aromatization to yield PABA (Nichols et al., 1989; Ye et al., 1990).

Amidotransferases can be separated into two classes according to their amino acid sequence. The *purF*-type subfamily includes amidophosphoribosyltransferase (Tso et al., 1982), asparagine synthetase (Andrulis et al., 1987), glucosamine-6-P synthase (Walker et al., 1984), and *Rhizobium leguminosarum* NodM (Surin & Downie, 1988). The other subfamily, the *trpG* type, includes anthranilate synthase (Nichols et al., 1980; Tso et al., 1980), *p*-aminobenzoate synthase (Kaplan & Nichols, 1983; Kaplan et al., 1985), carbamoyl-phosphate synthetase (Piette et al., 1984; Werner et al., 1985), CTP synthetase (Weng et al., 1986), formylglycinamide ribonucleotide synthetase (Ebbole & Zalkin, 1987; Schendel et al., 1989), GMP synthetase (Zalkin et al., 1985; Teideman et al., 1985), and imidazoleglycerol-phosphate synthetase (Carlomagno et al., 1988). The sequences of the *trpG*-type amidotransferases are characterized by three highly conserved regions which are thought to be important for the function and the structure of the protein (Weng et al., 1987). Affinity labeling studies of anthranilate synthase by 6-diazo-5-oxo-L-norleucine (DON), an activated analogue of glutamine, have shown that a cysteine (Cys-84) was involved at the active site (Tso et al., 1980). The replacement by mutagenesis of this cysteine residue with a glycine abolished the glutamine-dependent activity of *Serratia marcescens* anthranilate synthase without affecting the NH_3-dependent activity (Paluh et al., 1985). In the same way, after replacement of Cys-269 in *Escherichia coli* carbamoyl-P synthetase by serine or glycine, the enzyme was not able to hydrolyze glutamine (Rubino et al., 1986). The role of other amino acids at the active site is still not well understood. However, in *S. marcescens* anthranilate synthase, the site-directed mutagenesis of a totally conserved histidine (His-270) to tyrosine with loss of activity had led to the suggestion that this residue could act as a general base to promote the reactivity of active-site Cys-84 (Amuro et al., 1985). In *E. coli* carbamoyl-P synthetase, the mutation of the corresponding His-353 to asparagine led also to complete

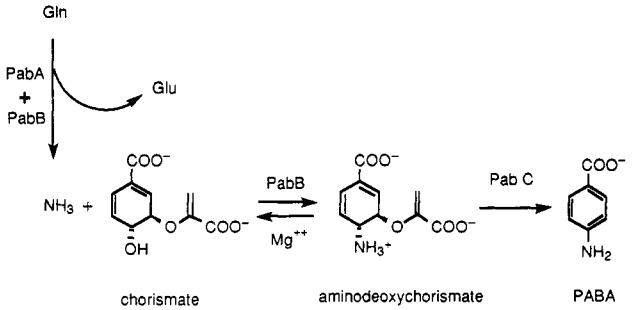

FIGURE 1: Formation of PABA from chorismate and glutamine by the action of the three enzymes PabA, PabB, and PabC.

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[1] Abbreviations: APAD, 3-acetylpyridine adenine dinucleotide; AS, anthranilate synthase; DON, 6-diazo-5-oxo-L-norleucine; GlDH, L-glutamate dehydrogenase; PabA, *p*-aminobenzoate synthase component II (amidotransferase); PabB, *p*-aminobenzoate synthase component I (chorismate aminase); PabC, *p*-aminobenzoate synthase component III (4-amino-4-deoxychorismate aromatase); PABA, *p*-aminobenzoate; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate; TCA, trichloroacetic acid.

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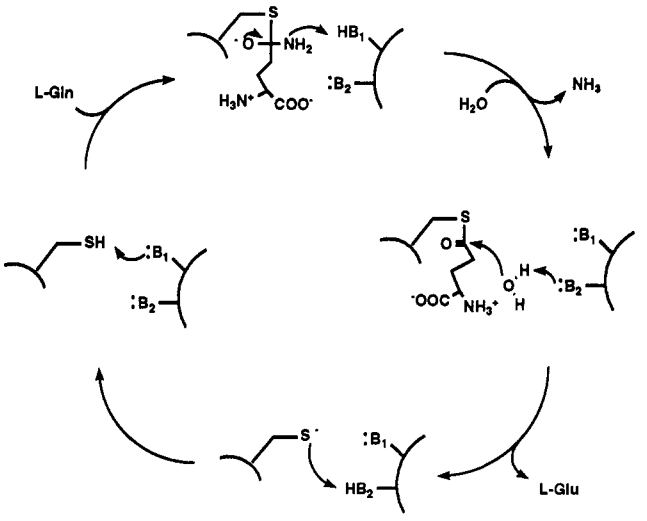

FIGURE 2: Hypothetical mechanism for the hydrolysis of glutamine by *trpG*-type glutaminases.

loss of glutaminase activity without affecting the binding of glutamine (Gaillard Miran et al., 1991). In this work, the other histidine residues, which are conserved in CPS synthetases but not in all *trpG*-type amidotransferases, were mutated. The replacement of His-272 and His-341 had no substantive effect on kinetics. However, the replacement of His-312 severely affected the binding of glutamine without decreasing k_{cat}. In this case, it has been proposed that, in addition to increasing the nucleophilicity of the active-site cysteine, His-353 could act as a general acid/base catalyst during the hydrolysis of the thioester intermediate. A totally conserved glutamic acid residue in *trpG*-type amidotransferases has also been inferred without direct experimentation to participate in catalysis (Mei & Zalkin, 1989). This acidic residue could help accelerate the hydrolysis of the thioester intermediate (Figure 2).

This paper reports the replacement by site-directed mutagenesis of the conserved Cys-79, His-168, and Glu-170 in *Escherichia coli* PabA. The results of this study support the role of Cys-79 as a catalytically active residue in PabA, as expected from the study of other *trpG*-type amidotransferases (Paluh et al., 1985; Rubino et al., 1986). However, the permissive replacement of Glu-170 by various amino acid residues rules out the hypothesis of this residue being directly essential in catalysis.

MATERIALS AND METHODS

Chemicals and Enzymes. Chorismate as barium salt, 3-acetylpyridine adenine dinucleotide (APAD), and L-glutamate dehydrogenase [EC 1.4.1.3 (GlDH)] were purchased from Sigma. [U-^{14}C]-L-Glutamine (285 mCi/mmol) was purchased from Amersham. The DNA sequencing kit was obtained from United States Biochemical Corp., and the plasmid purification kit was purchased from Quiagen. Enzymes used for in vitro transformation were obtained from New England Biolabs.

Strains, Plasmid, and Phage. The *E. coli* strains used for this study were XA90 (F′ Lac I^{Q} Z^{+} Y^{+} pro AB^{+} Δlac pro XIII ara^{-}, *nalA argE* am thi^{-} rif^{r}); XL1-Blue (recA1, endA1, gyrA96, thi-1, hsdR17 (r_{k^-}, m_{k^+}), supE44, relA1, λ (lac) [F′, proAB, lacIqZΔM15, Tn 10 (tet^{r})]; and CJ236 (dt1, ung1, thi-1, relA1/pCJ105 [F′(cm^{r})]) (Kunkel et al., 1987). Phagemid pNPA is a pHN1+ derivative that contains *E. coli* PabA gene (Ye et al., 1990). The helper phage R408 was purchased from Stratagene.

Site-Directed Mutagenesis. Site-directed mutagenesis of PabA cDNA was accomplished by the general method described by Kunkel (1987). Primers used for mutagenesis and sequencing were synthesized by Alex Nusbaum (Harvard Medical School). The mutagenic primers used were as follows: C79S, 5′-CTG ATG ACC GAG TGA GAC GCC AAG AAT-3′; H168Q, 5′-AAT ACT TTC TGG CTG GAA CTG CAC ACC-3′; E170D, 5′-GCT AAG AAT ACT ATC TGG ATG GAA CTG-3′; E170A, 5′-GCT AAG AAT ACT TGC TGG ATG GAA CTG-3′; E170Q, 5′-GCT AAG AAT ACT CTG TGG ATG GAA CTG-3′; E170K, 5′-GCT AAG AAT ACT TTT TGG ATG GAA CTG-3′. Bases encoding a mutated amino acid are underlined. *E. coli* strain CJ236 (dut^{-}, ung^{-}, F′) was transformed with the phagemid pNPA, and the uracil-containing single-stranded DNA (coding strand) was prepared by use of the helper phage R408. Site-directed mutations were produced according to the method of Kunkel et al. (1987). The hybrid cDNA constructs were screened and then sequenced in full by dideoxy sequencing of the denatured plasmids (Kraft et al., 1988). The mutated plasmids were transformed into *E. coli* XA90 for the expression of mutant enzymes.

Purification of Wild-Type and Mutant PabA. The purification protocol involved lysis of the cells (from a 1-L culture) by French press, ammonium sulfate precipitation, and gel filtration chromatography as described previously (Roux & Walsh, 1992). The proteins were >95% pure at this stage as determined by polyacrylamide gel electrophoresis. The enzymes were kept at −20 °C in 20 mM Tris·HCl, pH 7.4, containing 50% glycerol.

Enzyme Assays. The amidotransferase activity of PabA mutants was measured in the presence of 1.5 equiv of PabB, as described by Roux and Walsh (1992). The formation of L-glutamate was detected with an L-glutamate dehydrogenase-coupled assay by following the reduction of APAD, an analogue of NAD^{+}, at 364 nm (Nagano et al., 1970).

Isolation of a Covalent Intermediate between the PabA Mutant/PabB Complex and [^{14}C]Glutamine. As previously described (Roux & Walsh, 1992), the PabA acylenzyme intermediate was formed by incubating at room temperature a given PabA mutant (0.22–1.25 nmol) and 1.5 equiv of PabB with 7.5–10 nmol of [U-^{14}C]glutamine (specific activity 5.85 × 10^{5} dpm/nmol) and the desired concentration of L-glutamine. After TCA precipitation, the proteins were collected by filtration, and the radioactivity was measured.

RESULTS

The sequence identity between *E. coli* PabA and the mechanistically related anthranilate synthase component II, another *trpG*-type amidotransferase, is 44%. When several other members of the subfamily are considered, the global homology decreases, but three highly conserved regions remain. Two of these regions are represented in Figure 3 (Amuro et al., 1985). The first region contains the unique, totally conserved cysteine of this glutaminase subfamily (Cys-79 in PabA). It has been demonstrated that this residue acts as the active-site nucleophile in the case of *Serratia marcescens* anthranilate synthase component II (Tso et al., 1980). The second region contains two totally conserved residues, His-168 and Glu-170, which might act as general acid/base catalysts. These three potentially important residues, proposed to form a catalytic triad as shown in Figure 2 (Mei & Zalkin, 1989), have now each been replaced in *E. coli* PabA by site-directed mutagenesis of the *pabA* gene. The mutant proteins were expressed and purified to homogeneity by the same

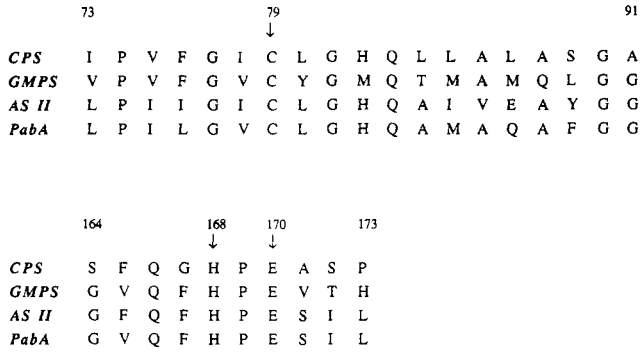

FIGURE 3: Amino acid alignment of two highly conserved regions of the amidotransferase domain in carbamoyl-P synthetase (CPS), GMP synthetase (GMPS), anthranilate synthase component II (AS II), and *p*-aminobenzoate synthase component II (PabA). The arrows indicate the mutated amino acids. The numbering scheme refers to the position of amino acids in *E. coli* PabA.

methods and in the same time frame (2 days between the cell breakage and the activity assays on pure enzyme) used previously to isolate the wild-type protein (Roux & Walsh, 1992).

Cys-79 Mutation. Cysteine-79 has been replaced by a less basic and less nucleophilic serine in order to test the role of this residue in the formation of the γ-glutamyl-acylenzyme intermediate between PabA and glutamine (Roux & Walsh, 1992). Expression of the C79S PabA mutant from freshly transformed *E. coli* XA90/pBRC79S was identical to the wild-type PabA overproduction. A 1-L culture gave 150 mg of purified mutant protein (≈20% of the total soluble proteins). In the obligate presence of PabB, C79S PabA exhibited glutaminase activity, when the formation of L-glutamate was followed with an L-glutamate dehydrogenase (GlDH)-coupled assay. C79S PabA reached a plateau of specific activity when 1 equiv of PabB was added to the assay, showing that the glutaminase activity was not due to contamination by nonspecific amidotransferases. This activity, although very low compared to the wild-type enzyme, was significantly reproducible and above background. The use of 7 nmol of C79S PabA in the assay (7-fold increase compared to assays with wild-type PabA) allowed us to measure activities representing at least 10-fold signal over experimental noise ratios. It should be noted that the lag time of ≈1 min observed with wild-type PabA (Roux & Walsh, 1992) increased to about 3 min with C79S PabA mutant. As a consequence, the initial velocity was determined between 5 and 7 min after mixing, when the activity was stabilized. The steady-state kinetic parameters were determined in the presence of 1.5 equiv of PabB. As shown in Table I, the K_m for glutamine has been increased ca. 250-fold from 0.33 to 83 mM, and the k_{cat} has been decreased about 40-fold from 20 to 0.49 min^{-1}, leading to a 10^4-fold decrease of the catalytic efficiency ratio k_{cat}/K_m compared to wild-type PabA.

A competition assay between wild-type and mutant PabA was used to assess the ability of PabA mutant to form a complex with PabB. As glutaminase activity of PabA is only detected when PabA is complexed with PabB (Roux & Walsh, 1992), the addition of a conformationally-conserved PabA mutant should decrease the activity of a wild-type PabA/PabB complex. More extensive direct measurements would be necessary to determine if a decreased PabA/PabB interaction is linked to a change in mutant folding or in the PabA/PabB interface. In addition, the kinetics of PabA/PabB complexation should be investigated. This assay was carried out with C79S PabA in the presence of 3 mM L-glutamine. This concentration was 10-fold higher than the K_m of wild-type PabA but 25-fold smaller than the K_m of C79S PabA. In these conditions, the role of C79S PabA in the global glutaminase activity could be neglected. A decrease of the PabA activity was detected upon C79S PabA addition, as shown in Figure 4. However, this decrease is smaller than expected from a simple competition between two similar proteins, plateauing at about 40% inhibition. This may indicate that C79S PabA, although catalytically active, does not interaction with PabB as strongly as wild-type PabA. Such a perturbation in mutant enzyme conformation could explain the 250-fold increase in glutamine K_m between wild-type and C79S PabA.

An acylenzyme intermediate derived from glutamine accumulates to 0.56 mol/mol of PabA with wild-type PabA (Roux & Walsh, 1992). The formation of such an intermediate between glutamine and the less nucleophilic active-site serine of C79S PabA mutant was studied as previously described (Chaparian & Evans, 1991; Roux & Walsh, 1992). After incubation of the PabA/PabB complex with [^{14}C]-L-glutamine, the intermediate was trapped by trichloroacetic acid precipitation, and the radioactivity associated with the denatured proteins was measured. With C79S PabA, the assay has been rendered difficult by the higher value of K_m for glutamine (83 mM) (the dilution of the 188 μM [^{14}C]-glutamine stock solution with nonlabeled glutamine led to very low specific radioactivity, and a lower limit of 0.2 mol fraction of acylenzyme could have been observed). No significant radioactivity was detected in precipitated proteins with glutamine concentrations ranging from 0.1 to 10 mM. These results seemed to indicate that the acylenzyme intermediate between C79S PabA and glutamine did not accumulate in a detectable amount.

His-168 Mutation. The conserved histidine at residue 168 was replaced by a sterically conservative glutamine, H168Q. Unfortunately, this mutation appears to prevent stable folding of the protein. As in the case of wild-type PabA, about 150 mg of H168Q PabA mutant was obtained from a 1-L culture, but nearly 90% of H168Q PabA was found in the cell pellet after French press lysis. Furthermore, during the gel filtration purification, the enzyme that was soluble eluted from the column in two distinct peaks corresponding to ≈45- and ≈20-kDa molecular mass. No significant PabB-dependent glutaminase activity was detected with the soluble monomeric H168Q PabA mutant. A competition assay with wild-type PabA was carried out in a PabB-dependent glutaminase assay, and as shown in Figure 4, no decrease of the glutaminase activity of wild-type PabA/PabB complex was detected. This indicated that even the soluble fraction of H168Q PabA was not interacting with PabB. It should also be noted that contrary to wild-type PabA whose overproduction was greatly enhanced when using freshly transformed cells, H168Q PabA gave the same result with 1-month- or 2-day-old single colonies.

Glu-170 Mutation. The conserved glutamic acid residue 170 has been mutated to a shorter acidic residue (aspartic acid), a small hydrophilic residue (alanine), a sterically conservative residue (glutamine), and a bulkier, positively-charged residue (lysine), with three distinct types of outcomes.

(*A*) **E170D PabA.* This conservative mutation which could move the side-chain carboxylate of E170 by ca. 1 Å turned out to have only a minimal effect on steady-state parameters (Table I): a 3-fold lessened affinity in the glutamine K_m and only a 25% decrease in k_{cat} (from 20 to 15 min^{-1} in E170D). Analysis of the steady-state level of the γ-glutamyl-enzyme intermediate gave a slightly increased accumulation at 0.77

Table I: Steady-State Kinetic Parameters for PabA Mutants[a]

enzyme	K_m (mM)		k_{cat} (min^{-1})		k_{cat}/K_m	
	-chorismate	+chorismate[b]	-chorismate	+chorismate[b]	-chorismate	+chorismate[b]
wild type	0.33 ± 0.03	0.96 ± 0.06	20 ± 1	39 ± 2	60 ± 8	41 ± 4
C79S[c]	83 ± 15	ND[e]	0.49 ± 0.05	ND	(6.1 ± 1.7) × 10^{-3}	ND
H168Q[d]			<0.1			
E170D	1.2 ± 0.15	1.8 ± 0.2	15 ± 1	16 ± 1	13 ± 2	9 ± 1
E170A	12.0 ± 1.5	10 ± 1	5.0 ± 0.2	3.0 ± 0.1	0.42 ± 0.07	0.30 ± 0.04
E170Q[d]			<0.1			
E170K[c]			<0.1			

[a] The activity of 1 nmol of PabA mutant with a saturating amount of PabB (1.5 equiv) was measured at room temperature using the GlDH-coupled assay in 100 mM triethanolamine, pH 9.0. The results are obtained by nonlinear regression. [b] Determined with a saturating concentration of chorismate (800 μM). [c] Determined with 7 nmol of PabA mutant. [d] Activity of 2 nmol of soluble PabA mutant (see text). [e] Not determined.

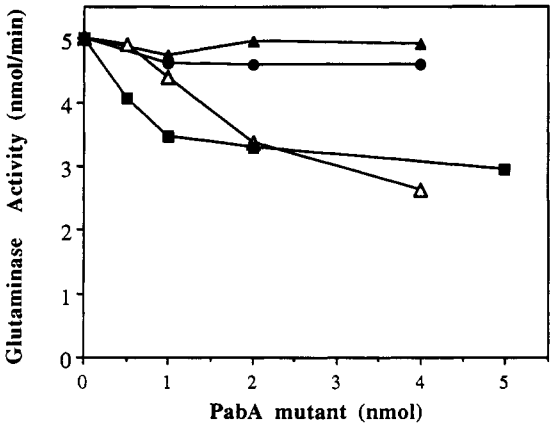

FIGURE 4: Probe of enzyme conformation by competition between wild-type and mutant PabA. The glutaminase activity of the wild-type PabA/PabB complex was determined in the presence of increasing amounts of the PabA mutants: E170K (▲); H168Q (●); E170Q (△); C79S (■). The L-glutamate dehydrogenase-coupled assay contained 1 nmol of wild-type PabA and 1.2 nmol of PabB in 1 mL of 100 mM triethanolamine, pH 9.0, 50 mM L-glutamine (except for C79S, 3 mM), 5 mM $MgCl_2$, 4 mM APAD, 20 units of GlDH, and PabA mutant.

mol/mol of enzyme vs 0.56 mol/mol for wild-type PabA in the PabA/PabB heterodimeric active complex.

*(B) *E170A PabA.* In E170A, the loss of the side-chain carboxylate altogether had a more substantial effect in catalytic impairment than E170D. The K_m for glutamine at 12 mM was up 35-fold from wild type, and the V_{max} was down 4-fold at 5 min^{-1} for a total decrement in catalytic efficiency, k_{cat}/K_m, of about 150-fold. Analysis of acylenzyme levels in the steady state detected less than 6% γ-glutamyl-enzyme, down from the 77% level in E170D.

From these two mutations, one would conclude E170 was not crucial to catalysis. The E170Q and E170K mutants noted below led, in contrast to inactive enzyme forms of PabA.

*(C) *E170Q PabA.* As in the case of H168Q PabA, this mutation has apparently perturbed the folding of the protein; 60% of the mutant was found aggregated in the cell pellet. From a 1-L culture, only 50 mg of soluble protein was obtained. No PabB-dependent glutaminase activity was detected with 4 nmol of E170Q PabA at glutamine concentrations ranging from 5 to 100 mM. Considering the background noise of the GlDH-coupled assay, k_{cat} was estimated to be lower than 0.1 min^{-1}.

The interaction of the soluble E170Q PabA with PabB was assessed with the same competition assay used for C79S and H168Q PabA mutants. The glutamine concentration was 10 mM, allowing wild-type PabA to be fully active. A decrease of glutaminase activity was detected when increasing amounts of E170Q PabA were added to an assay containing wild-type PabA/PabB complex (Figure 4). A replot of 1/activity vs mol of E170Q PabA gave a straight line, indicating that 2.4 equiv of E170Q PabA was necessary to decrease by half the wild-type PabA activity.

Table II: Accumulation of Acylenzyme in PabA Mutants[a]

enzyme	acylenzyme[b]	k_3/k_2[c]
wild type	0.56	0.8
C79S[d]	<0.01	
H168Q	ND[g]	ND
E170D	0.77	0.3
E170A[e]	<0.06	>15
E170Q[f]	<2 × 10^{-3}	
E170K	ND	ND

[a] Moles of acylenzyme for mole of PabA mutant. The acylenzyme between [^{14}C]glutamine and PabA mutant (complexed with 1.5 equiv of PabB) was formed as described under Materials and Methods. [b] Steady-state concentration of intermediate at saturating concentration of glutamine. [c] Deacylation rate constant (k_3) over acylation rate constant (k_2). [d] No radioactivity detected with 1.25 nmol of PabA mutant and [Gln] ranging from 0.1 to 10 mM. [e] Assay carried out with 0.44 nmol of PabA mutant and [Gln] from 2 to 15 mM. [f] No radioactivity detected with 0.5 nmol of PabA mutant and [Gln] ranging from 0.1 to 1 mM. [g] Not determined.

As the E170Q mutant seemed able to form a complex with PabB, the total loss of activity could have been caused by perturbation of the catalytic process. The accumulation of an acylenzyme intermediate was studied in order to determine if the first step of glutamine hydrolysis, the formation of a thioester, was kinetically perturbed. This time the assay was carried out in a different way. As the enzyme was not catalytically active, the usual study with increasing concentrations of labeled glutamine was not useful. A low concentration of [^{14}C]glutamine (75 μM, 585 000 dpm/nmol) was used, and the accumulation of acylenzyme was measured over a period of 30 min. No time-dependent increase of radioactivity was detected in denatured protein. The small radioactivity background in this assay (≈300 dpm) allowed estimation that less than 0.2% of E170Q PabA was labeled by [^{14}C]glutamine (Table II).

*(D) *E170K PabA.* The overproduction and purification of this mutant were equivalent to the wild-type PabA. Nearly 150 mg of purified protein was obtained from a 1-L culture. However, no PabB-dependent glutaminase activity was detected. In addition, no competition was observed between E170K mutant and wild-type PabA (Figure 4), indicating that E170K PabA was apparently not interacting with PabB. Therefore, it appeared that the complete loss of activity of the E170K mutant may be due to PabA/PabB complex disruption rather than perturbation of the catalytic process.

DISCUSSION

The results reported here describe construction and properties of one mutant each at C79 and H168 and four mutants

at E170, three fully conserved residues in the *trpG*-type family of glutamine amidotransferases, and permit some conclusions about PabA mechanism and structure (see Figure 2 for acylenzyme scheme).

The cysteine at residue 79 is analogous to the active-site cysteine in the homologous glutaminase subunit of anthranilate synthase, identified as the active-site nucleophile for γ-glutamyl-*S*-enzyme formation by DON inactivation (Tso et al., 1980) and by Cys to Gly mutagenesis (Paluh et al., 1985), yielding inactive enzyme. The C79S mutation was chosen to replace the nucleophilic cysteine thiolate by an alternate nucleophile, the serine hydroxyl. The serine alkoxide vs cysteine thiolate will be disfavored as catalytic nucleophile by two features: 7 pK_a units (pK_a 7 vs pK_a 14) and a markedly lower nucleophilicity of oxygen nucleophiles compared to thiol nucleophiles of comparable basicity.

In fact, the C79S PabA is still a respectable glutaminase when complexed with PabB. The k_{cat} decline of only 40-fold suggests chemical steps may not have been limiting catalysis in wild-type PabA. The loss of 250-fold in the glutamine K_m leads further to a net decline in the k_{cat}/K_m of some 10 000-fold relative to wild-type, validating a dramatic drop in the overall catalytic efficiency of C79S. The decreased catalytic efficiency could be due to lowered nucleophilicity and/or lessened concentration of the alkoxide species of Ser-79 in the C79S active site or to a less thermodynamically activated and hydrolytically more stable acyl-*O*-Ser-79 oxoester vs acyl-*S*-Cys-79 thiol ester intermediate in wild-type PabA. Direct analysis of acylenzyme levels in turnover revealed that as compared to 0.56 mol of [^{14}C]-γ-glutamyl-*S*-enzyme for wild-type PabA, no detectable [^{14}C]-γ-glutamyl-*O*-Ser enzyme was found in the C79S steady state. While there is the formal possibility of a change in mechanism from the two-step acylenzyme in wild-type PabA to a one-step direct H_2O addition in C79S, these data more likely suggest that γ-glutamyl-*O*-Ser enzyme formation is now substantially rate-limiting in catalysis. We have previously documented such a change in a C89S mutant of β-ketoacyl thiolase (Thompson et al., 1989) where the energetics of the acyl-*S*-enzyme were altered by conversion to an acyl-*O*-enzyme mechanism. Finally, as previously shown, the replacement of Cys-79 with Ser has a greater effect on K_m than k_{cat}. This may indicate that catalytic Cys-79 is directly involved in glutamine binding or that even a conservative mutation affects the conformation of the active site of the PabA/PabB complex. It should be noted that in carbamoyl-phosphate synthetase, the corresponding Cys-269 is not critical for glutamine binding (Rubino et al., 1986).

The proposed role of H168 as a general base in C79SH conversion to C79S$^-$ (Figure 2), by analogy to the postulate for anthranilate synthase's glutaminase activity (Amuro et al., 1985) and carbamoyl-phosphate synthetase's activity (Gaillard-Miran et al., 1991), was approached by the construction of a sterically conservative H168Q mutation. While this mutant was in fact without activity, the mechanistic significance may be moot because the enzyme was substantially insoluble and aggregated. The fraction of H168Q that was soluble and monomeric failed to displace wild-type PabA from the PabA/PabB active heterodimer as assessed by lack of effect in a competition assay. It is clear that even wild-type PabA by itself is only a conditional glutaminase (Roux & Walsh, 1992), requiring PabB to be present before PabA converts to an active conformer. Thus, PabA is probably a flexible polypeptide interconvertible among conformational isomers and rather rapidly loses its ability to be activated by PabB once purified. The H168Q form of PabA appears sufficiently destabilized relative to wild-type PabA that it has lost ability to be chaperoned into a native conformer by PabB. It remains to be seen if other H168 PabA mutants fold to metastable conformers such that a catalytic function for H168 can be assessed separate from the folding instability.

The conserved glutamate-170 was converted into four mutant forms, D170, A170, Q170, and K170, and each of the four mutants gave some distinct information. The E170D PabA mutant is only affected 4-fold in k_{cat}/K_m, arguing against some essential role for this invariant Glu side chain. The E170A mutant's retention of activity confirms that conclusion, but 170A is substantially more impaired than 170D, including a major redistribution in the concentration of γ-glutamyl-enzyme during turnover. The E170Q mutant is anomalous in this series in lacking any detectable glutaminase activity. Yet it appears to fold properly as indirectly assayed by competition with wild-type PabA for the activating PabB component. It may be that the E170 and D170 side chains do participate in some aspect of acylenzyme formation or hydrolysis and that the small A170 side chain may allow access of a water molecule instead that is excluded by the larger, neutral Q170 side chain. The replacement of the E170 anionic side chain by the cationic K170 is fully disruptive; not only is E170K inactive but also it will not detectably interact with PabB, suggesting an inactive conformer or perhaps a defective folding pathway. While these four mutants of E170 do not reveal its role in catalysis or structure, it does appear to be a sensitive region in this enzyme.

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