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Role of the Four Conserved Histidine Residues in the Amidotransferase Domain of Carbamoyl Phosphate Synthetase†

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ABSTRACT: Carbamoyl phosphate synthetase from *Escherichia coli* catalyzes the formation of carbamoyl phosphate from ATP, bicarbonate, and glutamine. The amidotransferase activity of this enzyme is catalyzed by the smaller of the two subunits of the heterodimeric protein. The roles of four conserved histidine residues within this subunit were probed by site-directed mutagenesis to asparagine. The catalytic activities of the H272N and H341N mutants are not significantly different than that of the wild-type enzyme. The H353N mutant is unable to utilize glutamine as a nitrogen source in the synthetase reaction or the partial glutaminase reaction. However, binding to the glutamine active site is not impaired in the H353N enzyme since glutamine is found to activate the partial ATPase reaction by 40% with a K_d of 54 μM. The H312N mutant has a Michaelis constant for glutamine that is 2 orders of magnitude larger than the wild-type value, but the maximal rate of glutamine hydrolysis is unchanged. These results are consistent with His-353 functioning as a general acid/base catalyst for proton transfers while His-312 serves a critical role for the binding of glutamine to the active site.

Carbamoyl phosphate synthetase (CPS)[1] of *Escherichia coli* is a key enzyme in the biosynthetic pathways for arginine and pyrimidine nucleotides. This protein catalyzes the formation of carbamoyl phosphate according to the reaction

$$2\text{MgATP} + \text{HCO}_3^- + \text{H}_2\text{O} + \text{L-Gln} \rightarrow 2\text{MgADP} + \text{carbamoyl-P} + \text{P}_i + \text{L-Glu} \quad (1)$$

Although glutamine is the preferred nitrogen source, ammonia can be utilized as an alternate substrate at high concentrations.

The native enzyme is composed of two nonidentical subunits; a small one of molecular weight 41 270, and a large one of molecular weight 117 710 (Piette et al., 1984; Nyunoya & Lusty, 1983). The sole function of the small subunit is to hydrolyze glutamine for the production of NH_3, while the large subunit catalyzes the formation of carbamoyl phosphate from MgATP, HCO_3^-, and NH_3. The allosteric effectors (IMP, UMP, and ornithine) bind to the large subunit (Anderson & Meister, 1966) and control the activity of the enzyme by regulating the K_m of ATP.

The protein sequence of the heterodimeric enzyme of *E. coli* has been deduced from the nucleotide sequence of the *car*A (small subunit) and *car*B (large subunit) genes by Lusty and co-workers (Piette et al., 1984; Nyunoya & Lusty, 1983). Sequence comparisons of the small subunit have indicated that this protein belongs to the *trp*G-type glutamine amidotransferases (Piette et al., 1984; Nyunoya & Lusty, 1984; Werner et al., 1985). This class of enzymes also includes anthranilate synthetase (Nichols et al., 1980; Tso et al., 1980), *p*-aminobenzoate synthetase (Kaplan & Nichols, 1983; Kaplan et al., 1985), GMP synthetase (Zalkin et al., 1985), CTP synthetase (Wang et al., 1986), and formylglycinamide ribonucleotide synthetase (Schendel et al., 1989).

The *trp*G-type glutamine amidotransferases are all thought to involve an essential cysteine residue in the chemical mechanism for the hydrolysis of glutamine (Zalkin, 1985). The critical involvement of a cysteine residue in the amidotransferase activity of the *E. coli* CPS has been demonstrated by labeling experiments with a chloroketone analogue of glutamine (Khedouri et al., 1966; Pinkus & Meister, 1972) and cyanate (Anderson et al., 1973; Anderson & Carlson, 1975). All of the other *trp*G-type amidotransferase enzymes are inactivated by 6-diazo-5-oxo-L-norleucine (DON), an activated glutamine analogue (Levenberg et al., 1957; Patel et al., 1977), and it has been shown in most cases that a cysteine residue is labeled at the active site (Ohnoki et al.,

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[1] Abbreviations: CPS, carbamoyl phosphate synthetase; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; GMPS, GMP synthetase; AS II, anthranilate synthetase; PABS, *p*-aminobenzoate synthetase; CTPS, CTP synthetase.

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Table I: Sequence Comparisons for the Conserved Histidine Residues in the Amidotransferase Domain of CPS

Source	267	277	308	316	338	356	Reference
HAM	G I C L G H Q L L A L		T S Q N H G F A V		E G I V H D S L P F F S V Q F H P E H		*a*
CPS1	G I S T G N I I T G L		T A Q N H G Y A L		E G I M H E S K P F F A V Q F H P E V		*b*
DROS	G I C L Q H Q L L A K		T S Q N H G Y A V		E G I V H A S K P Y F S V Q F H P E H		*c*
DICT	G V C M G N Q L L G L		T S Q N H G F V I		E G I Y H E S K P W F S V Q F H P E A		*d*
URA2	G I C L G H Q L I A R		T S Q N H G F A V		E R F Y H S E L P Y F S V Q F H P E S		*e*
CPA1	G I C L G H Q L L A L		T S Q N H G Y A V		E G M I H L Q R P I F S T Q F H P E A		*f*
COLI	G I C L G H Q L L A L		T A Q N H G F A V		Q G I H R T D K P A F S F Q G H P E A		*g*
STY	G I C L G H Q L L A L		T A Q N H G F A V		Q G I H R T D K P A F S F Q G H P E A		*h*
	272		312		341	353	

[a] Simmer et al., (1990). [b] Lusty et al. (1983). [c] Freund and Jarry (1987). [d] Faure et al. (1988). [e] Souciet et al. (1987). [f] Werner et al. (1985). [g] Piette et al. (1984). [h] Kilstrup et al. (1988). The numbering is related to the *E. coli* sequence.

1977; Truitt et al., 1978; Tso et al., 1980). Covalent labeling with the glutamine analogues abolishes activity with glutamine as the nitrogen source but does not impair the activity when ammonia is used directly.

It has been postulated that the most likely mechanism for the hydrolysis of glutamine by this class of enzymes involves the formation of a thioester intermediate. The most direct evidence for such an intermediate has been provided with CTP synthetase where [^{14}C]glutamine has been bound to the enzyme and a covalent adduct isolated after gel filtration under denaturing conditions (Levitzki & Koshland, 1971). In the case of anthranilate synthetase, the replacement of Cys-84 with glycine by mutagenesis abolishes the glutamine-dependent activity without affecting the NH_3-dependent activity (Paluh et al., 1985). This conclusion was supported by the replacement of Cys-269 of the *E. coli* CPS with either serine or glycine (Rubino et al., 1986). The mutant proteins were unable to hydrolyze glutamine, but they were able to synthesize carbamoyl phosphate from NH_3, MgATP, and HCO_3^-.

The role of other amino acids at the active site of these amidotransferase enzymes is not well understood. However, site-directed mutagenesis of His-170 to a tyrosine in anthranilate synthetase (Amuro et al., 1985) has suggested that this residue participates in the glutaminase activity and perhaps acts as a general base to promote the reactivity of the active site Cys-84. Recent protein sequence alignment of the amidotransferase domains for carbamoyl phosphate synthetase from eight different species has indicated that only four histidines are conserved (Simmer et al., 1990). Portions of this alignment, highlighting the conserved histidine residues, are illustrated in Table I. It is interesting to note that these histidine residues are all clustered at the carboxyl-terminal end of the small subunit of the *E. coli* enzyme.

In order to critically examine the functional role, if any, for these histidines, we have mutated all of these conserved residues to asparagine. The mutant enzymes, H272N, H312N, H341N, and H353N, have been purified to homogeneity and their kinetic constants compared with those of the wild-type enzyme.

Materials and Methods

Chemicals and Enzymes. The chemicals and coupling enzymes used for activity measurements were purchased from Sigma, Aldrich, or Boehringer. The Ultrogel AcA-34 was obtained from IBF Biotechnics. The DNA sequencing kit was obtained from Bio-Rad. Bactotryptone and Bacto-yeast were obtained from Difco.

Bacterial Strains and Plasmids. The *E. coli* strains used for this study were RC50 (*car*A50, *thi*-1, *mal*A1, *xyl*-7, *rsp*L135, λ^r λ^-, *tsx*-273); XL1-Blue (*rec*A1, *end*A1, *gyr*A96, *thi*-1, *hsd*R17 (r_k^-, m_k^+), *sup*E44, *rel*A1, λ (lac) [F′, *pro*AB, *lac*I^qZΔM15, Tn 10 (*tet*^{r})]); and CJ236 (*dut*1, *ung*1, *thi*-1, *rel*A1/pCJ105[F′(cm^r)]) (Bullock, 1987; Kunkel et al., 1987; Joyce & Grindley, 1984). The strain RC50 (Glansdorff et al., 1976) was a gift from Dr. Carol J. Lusty (Public Health Research Institute of New York). The *car*AB operon obtained from Dr. Carol J. Lusty was subcloned into the *Hin*dIII site of the pBS$^+$ plasmid (Stratagene, La Jolla, CA) and used under the name of pDP412 for the mutagenesis experiments as previously described (Post et al., 1990).

Growth Conditions. The cells were grown in Luria-Bertani (LB) broth (Maniatis et al., 1982) or modified LB media (Post et al., 1990). The transformed cells were grown in the presence of 50 μg/mL ampicillin and harvested in stationary phase.

Site-Directed Mutagenesis. Site-directed mutations in the amidotransferase subunit of the *E. coli* carbamoyl phosphate synthetase were constructed according to the general method described by Kunkel et al. (1987). The oligonucleotides used to change the four histidine residues at positions 272, 312, 341, and 353 to asparagine were $^{5'}$CAG CAG CTG ATT* ACC GAG$^{3'}$, $^{5'}$CGC AAA ACC GTT* GTT CTG$^{3'}$, $^{5'}$GGT GCG ATT* AAT GCC CTG$^{3'}$, and $^{5'}$GGC TTC AGG GTT* CCC GTG$^{3'}$, respectively. These primers, obtained from the DNA Sequencing Laboratory, Biology Department of Texas A&M University, introduce a single base change (G into T) into the wild-type sequence. *E. coli* strain CJ236 (dut, ung, F′) was transformed with the plasmid pDP412, and the uracil-containing single-stranded DNA was prepared by use of helper phage M13K07 (Vieira & Messing, 1987). Site-directed mutations were produced according to the method of Kunkel et al. (1987), with the mutagenesis kit from Bio-Rad on the plasmid carrying the entire *car*AB insert. Transformant colonies were screened for the correct base changes by dideoxy sequencing of the denatured plasmids (Kraft et al., 1988) in a 200-base-pair region surrounding the mutation point. The mutated plasmids were transformed into the *car*AB$^-$ strain RC50 for the expression and purification of the mutant enzymes.

Purification of Wild-Type and Mutant Enzymes. The purification protocol involved lysis of the cells by a French press, protamine sulfate and ammonium sulfate precipitations, gel filtration chromatography, and anion-exchange chromatography, as described by Post et al. (1990). The purity of all enzyme preparations was >95% as determined by polyacrylamide gel electrophoresis.

Determination of Enzyme Activity. The rate of carbamoyl phosphate synthesis was determined by use of a coupled enzyme assay with ornithine transcarbamoylase (Snodgrass &

Parry, 1969). This assay measures the rate of appearance of citrulline formed from carbamoyl phosphate and ornithine. The assay mixture contained 50 mM HEPES, pH 7.6, 100 mM KCl, 10 mM ATP, 20 mM $MgCl_2$, 20 mM $NaHCO_3$, 10 mM ornithine, 10 mM glutamine or 600 mM NH_4Cl, 0.8 unit of ornithine transcarbamoylase, and 2–5 μg of CPS in a final volume of 500 μL. After incubation at 25 °C for 10 min, the reaction was stopped by the addition of an acid reagent and diacetyl monoxime (Rubino et al., 1986). The citrulline concentration was determined from the absorbance at 464 nm with an extinction coefficient of 37 800 M^{-1} cm^{-1} (Snodgrass & Parry, 1969).

The rate of formation of ADP in the presence and absence of a nitrogen source (NH_3 or glutamine) was determined with use of a pyruvate kinase/lactate dehydrogenase coupled assay, which measures the disappearance of NADH at 340 nm. The assay mixtures contained 25 mM HEPES, pH 7.6, 1.0 mM phosphoenolpyruvate, 0.2 mM NADH, 100 mM KCl, 20 mM $MgCl_2$, 10 mM ATP, 10 mM glutamine or 450 mM NH_4Cl, 40 mM $NaHCO_3$, 20 units of pyruvate kinase, 30 units of lactate dehydrogenase, and 10–100 μg of CPS in a final volume of 2.5 mL. The K_m for ATP was determined with and without 10 mM ornithine in order to determine the effect of the allosteric activator. The MgATP concentration was varied from 3 to 2000 μM. The K_m for NH_4Cl was obtained by varying the concentration from 0 to 400 mM while the K_m for glutamine was determined from concentrations ranging from 0.1 to 40 mM.

The rate of MgATP formation from MgADP and carbamoyl phosphate was measured with use of the hexokinase/glucose-6-phosphate dehydrogenase coupled assay. The assay mixtures contained 25 mM HEPES, pH 7.6, 125 mM KCl, 0.75 mM NAD^+, 10 mM ADP, 10 mM carbamoyl phosphate, 1.0 mM glucose, 15 mM magnesium acetate, 20 units of hexokinase, 10 units of glucose-6-phosphate dehydrogenase, and 20–100 μg of CPS in a final volume of 2.0 mL. The formation of NADH was followed at 340 nm. The K_m for ADP was obtained by varying the concentration of MgADP from 3 to 1400 μM.

The glutaminase activity of CPS was assayed by coupling the formation of L-glutamate to the production of α-ketoglutarate with L-glutamate dehydrogenase in a stepwise manner. The enzyme (20 μg) was incubated with 0.1 M buffer and 10 mM L-glutamine at 25 °C in a volume of 2.0 mL. The reaction was stopped by the addition of 50 μL of 1 M HCl followed by 15 min on ice. The pH was neutralized by the addition of 50 μL of 1.0 M Tris, pH 10, and then 700 μL of an assay mixture composed of 0.15 M Tris, pH 8.0, 0.8 mM acetylpyridine adenine nucleotide, and 5 units of L-glutamate dehydrogenase were added. The absorbance at 363 nm of the reduced acetylpyridine adenine nucleotide was measured after incubation at 25 °C for 1 h. The concentration of L-glutamate was calculated from the standard curve established with known amounts of L-glutamate.

Data Analysis. The kinetic parameters (V_{max} and V/K_m) from double-reciprocal plots were determined by fitting the experimental velocity data to eq 2 with the Fortran program

$$v = VA/(K + A) \quad (2)$$

$$\log (V/K) = \log [C/(1 + H/K_1 + K_2/H)] \quad (3)$$

$$\log (V/K) = \log [C(1 + H/K_2)/(1 + H/K_1)(1 + K_3/H)] \quad (4)$$

of Cleland (1979). The pH profiles that decreased with a slope of +1 at low pH and a slope of −1 at high pH were fit to eq 3. The pH profile for V/K of the wild-type enzyme was fit to eq 4 by use of the Marquart–Levenberg algorithm. In eqs 2–4, A and H are the reactant and hydrogen ion concentrations, respectively. K is the Michaelis constant for A, v is the initial velocity, and V is the maximum velocity. The constants K_1, K_2, and K_3 represent dissociation constants for ionizable groups on the enzyme or substrate, and C is the pH-independent value of V/K.

The plots for the enhancement or inhibition of ATP turnover by glutamine with the H353N mutant were fit to (Cleland, 1970)

$$v = V_0(\alpha I + K_i)/(I + K_i) \quad (5)$$

where V_0 is the initial velocity in the absence of the effector I, and K_i is the dissociation constant of the EI complex. The constant α is the ratio of the velocities at saturating and zero effector concentration.

Results

The four conserved histidine residues of the amidotransferase domain of carbamoyl phosphate synthetase were mutagenized to asparagine by site-directed alteration of the *car*A gene of *E. coli*. The mutant proteins were expressed and purified to homogeneity by the same methods previously used to isolate the wild-type protein. No significant differences in the stability of these proteins were observed, and the two subunits remained fully associated during gel filtration chromatography. Alterations in the kinetic constants for these mutant proteins were determined by measurement of the full biosynthetic reaction and the various partial reactions catalyzed by carbamoyl phosphate synthetase. These kinetic parameters are summarized in Tables II and III.

Kinetic Properties of H272N. The kinetic parameters for the mutant enzyme, H272N, are virtually identical with those of the wild-type enzyme. The rate of synthesis of carbamoyl phosphate using either glutamine or ammonia as the nitrogen source is reduced approximately 50% when compared to that of the wild-type enzyme. The hydrolysis of glutamine alone is reduced about 2-fold with a 3-fold increase in the Michaelis constant. There is no change in the K_m for ADP or the V_{max} for the partial back-reaction (Table II). The kinetic constants for ATP in the full biosynthetic reaction and the bicarbonate-dependent ATPase reaction are altered only slightly, although the maximal velocity for the ATPase reaction is reduced about 2-fold.

Kinetic Properties of H312N. The substitution of an asparagine for a histidine at position 312 has resulted in a protein with significantly increased Michaelis constants for glutamine. The rate of carbamoyl phosphate synthesis is reduced about 2-fold when either glutamine or ammonia is used as a nitrogen source. However, the maximal rate with glutamine is achieved only at significantly elevated levels of this substrate. The K_m for glutamine in the full biosynthetic reaction has been increased from 105 μM to 20 mM with this mutation. A similar increase in the K_m for glutamine in the glutaminase reaction is also noted, but again there is no change in the V_{max} for this partial reaction. The kinetic parameters for the partial back-reaction are unchanged. For the bicarbonate-dependent ATPase reaction, the Michaelis constants for the nucleotide are unaltered but the maximal rate of this reaction is reduced about 4-fold.

The pH rate profiles for the glutaminase reaction of the wild type and H312N mutant are shown in Figure 1. The wild-type enzyme shows two ionizations. A group with a pK_a of 9.9 must be protonated for activity, and another group with a pK_a of 9.9 must be unprotonated for maximal activity. However, protonation of this second group reduces the binding

Table II: Kinetic Parameters for Reactions Catalyzed by Carbamoyl-P Synthetase and Mutants

		reaction monitored					
		carbamoyl-P synthesis[a] nitrogen source		glutaminase[b]		ATP synthesis[c]	
enzyme	ornithine (mM)	glutamine (μmol/min·mg)	NH_4^+ (μmol/min·mg)	V_{max} (μmol/h·mg)	K_{gln} (mM)	V_{max} (μmol/min·mg)	K_{ADP} (μM)
wild type	0			1.1 ± 0.2	0.3 ± 0.1	0.39 ± 0.05	90 ± 5
	10	1.8	1.0			0.39 ± 0.05	5 ± 1
H272N	0			0.5 ± 0.1	1.0 ± 0.4	0.34 ± 0.01	57 ± 2
	10	0.8	0.6			0.32 ± 0.01	12 ± 1
H312N	0			1.2 ± 0.1	14 ± 3	0.33 ± 0.01	61 ± 6
	10	0.7[d]	0.5			0.29 ± 0.01	9 ± 1
H341N	0			0.5 ± 01	1.4 ± 0.4	0.35 ± 0.08	65 ± 4
	10	1.0	0.6			0.30 ± 0.01	14 ± 1
H353N	0			≤0.006		0.31 ± 0.01	92 ± 8
	10	≤0.002	1.6			0.26 ± 0.01	16 ± 1

[a] pH 7.6, 100 mM KCl, 20 mM $NaHCO_3$, 20 mM $MgCl_2$, 10 mM ATP, and either 10 mM glutamine or 600 mM NH_4Cl. [b] pH 7.6. [c] pH 7.6, 125 mM KCl, 15 mM Mg^{2+}, and 10 mM carbamoyl phosphate. [d] 38 mM glutamine.

Table III: Kinetic Parameters for the Formation of ADP from ATP

		reaction monitored							
		ATP hydrolysis[a]		glutamine-dependent ATP hydrolysis[b]			NH_4-dependent ATP hydrolysis[c]		
enzyme	ornithine (mM)	V_{max} (μmol/min·mg)	K_{ATP} (μM)	V_{max} (μmol/min·mg)	K_{ATP} (μM)	K_{GLN} (μM)	V_{max} (μmol/mg·min)	K_{ATP} (μM)	$K_{NH_4^+}$ (mM)
wild type	0	0.43 ± 0.04	20 ± 2	3.6 ± 0.4	350 ± 40		1.9 ± 0.1	130 ± 7	
	10	0.40 ± .05	7.0 ± 2	3.2 ± 0.1	34 ± 5	105 ± 6	2.0 ± 0.4	51 ± 3	150 ± 14
H272N	0	0.18 ± 0.077	18 ± 2	2.2 ± 0.1	440 ± 40		2.2 ± 0.1	180 ± 9	
	10	0.15 ± 0.01	5.0 ± 0.5	2.1 ± 0.3	32 ± 2	88 ± 3	2.5 ± 0.6	70 ± 4	240 ± 26
H312N	0	0.10 ± 0.01	9 ± 0.5	2.3 ± 0.2	350 ± 24	12900 ± 3000	1.2 ± 0.1	57 ± 10	
	10	0.10 ± 0.01	4.3 ± 0.6	2.4 ± 0.6	32 ± 2	20100 ± 5000	1.3 ± 0.2	36 ± 5	130 ± 20
H341N	0	0.21 ± 0.01	23 ± 3	3.1 ± 0.1	710 ± 90		2.3 ± 0.1	130 ± 7	
	10	0.18 ± 0.01	5.5 ± 0.7	2.6 ± 0.3	100 ± 20	68 ± 3	2.1 ± 0.1	51 ± 5	111 ± 19
H353N	0	0.45 ± 0.01	13 ± 2	0.61 ± 0.1	20 ± 2	(37 ± 9)	3.6 ± 0.1	160 ± 20	
	10	0.46 ± 0.01	12 ± 2	0.63 ± 0.1	13 ± 1	(54 ± 9)	3.7 ± 0.6	120 ± 6	79 ± 7

[a] pH 7.6, 100 mM KCl, 20 mM $NaHCO_3$, 20 mM $MgCl_2$, and a variable amount of ATP. [b] Same as in *a* except that variable amounts of glutamine were also added. [c] Same as in *a* except that variable amounts of ammonia were also included.

of glutamine by only 1 order of magnitude. The pH rate profile for the mutant enzyme shows a single ionization at pH 9.8 that must be protonated for activity, but the group that is preferred to be unprotonated for binding of glutamine with the wild-type enzyme does not appear in this profile.

Kinetic Properties of H341N. The kinetic constants for the mutant enzyme H341N are nearly identical with that of the wild-type enzyme. The rate of carbamoyl phosphate synthesis with either glutamine or ammonia as a nitrogen source is reduced about 2-fold. The rate of hydrolysis of glutamine is reduced about 2-fold, and the Michaelis constant is increased about 4-fold, although this increase in the K_m for glutamine is not apparent when glutamine is used as a nitrogen source for the full biosynthetic reaction. The kinetic constants of this mutant for the partial back-reaction are unchanged relative to that of the wild-type enzyme as are the constants for the bicarbonate-dependent ATPase reaction and the full biosynthetic reaction when the rate of formation of ADP is monitored.

Kinetic Properties of H353N. The replacement of the histidine with an asparagine at residue 353 of the small subunit of carbamoyl phosphate synthetase results in a protein that is unable to utilize glutamine as a substrate. The mutant H353N can use ammonia as a nitrogen source for the synthesis of carbamoyl phosphate, but the activity with glutamine has been reduced by at least 3 orders of magnitude relative to that of the wild-type enzyme. The lack of reactivity with this compound is confirmed by the significant decrease in the glutaminase partial reaction catalyzed by this enzyme (Table II). There are no significant changes in either the partial back-reaction (Table II) or the bicarbonate-dependent ATPase

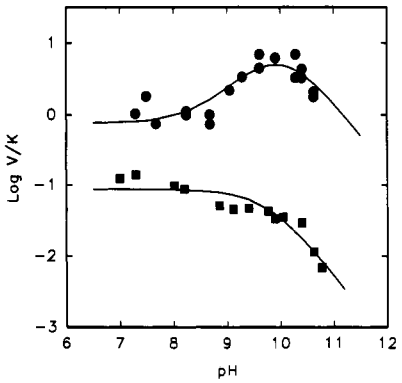


FIGURE 1: pH rate profile for the hydrolysis of glutamine. The data for the wild-type CPS (●) were fit to eq 4 while the data for the mutant H312N (■) were fit to eq 3. The vertical scale is in arbitrary units. Additional details are given in the text.

reaction (Table III). However, when saturating amounts of glutamine are added to the assay mixture for the ATPase reaction, the rate of ATP hydrolysis is increased by 40% (Table III). Shown in Figure 2 is a plot of the increase in the ATPase rate as a function of the concentration of added glutamine (no glutamine is actually hydrolyzed). A fit of the data to eq 5 gives a K_i for glutamine of 54 μM and an activation constant, α, of 1.4. When saturating glutamine is added to an assay mixture containing ATP, bicarbonate, and ammonia, the rate of carbamoyl phosphate and ADP formation is reduced by 36%. Shown in Figure 2 is the reduction in ATP turnover at various levels of glutamine. A fit of the data to eq 5 gives an apparent K_i of 140 μM at 300 mM NH_4^+ and an inhibition

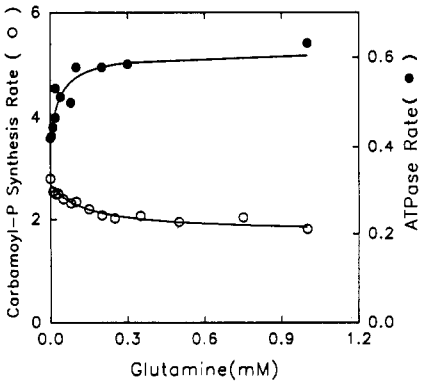


FIGURE 2: The activation and inhibition of the ATPase (●) and ammonia-dependent carbamoyl phosphate synthesis reaction (O) by various amounts of glutamine as catalyzed by the mutant H353N. The rates are in units of micromoles per minute per milligram of protein. The data in both plots were fit to eq 5. Additional details are given in the text.

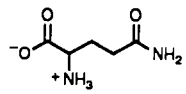
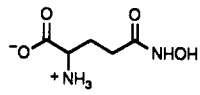
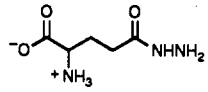
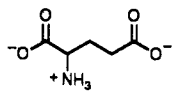
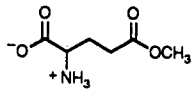
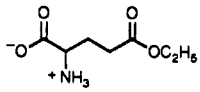
constant, α, of 0.64. The apparent dissociation constant for glutamine was found to increase with increases in the NH_4^+ concentration. A fit of the data (not shown) to eq 6 (Cleland,

$$K_i^{app} = K_i([NH_4^+]/K_d + 1) \quad (6)$$

1970) gave a dissociation constant of 70 mM for NH_4^+ at the glutamine binding site.

The enhancement by glutamine on the rate of the bicarbonate-dependent ATPase reaction was used to probe the binding of other glutamine analogues. Shown in Table IV are the structures, dissociations constants, and activation constants for a series of glutamine analogues interacting with the small subunit of mutant H353N. These compounds have previously been shown to inhibit the glutaminase activity of the wild-type enzyme (Wellner et al., 1973).

Table IV: Effects of Glutamine Analogues on the ATPase Reaction Catalyzed by H353N[a]

compound	K_i[b]	α[b]
	54 μM	1.4
	9 μM	1.7
	340 μM	1.5
	28 mM	1.4
	25 mM	2.1
	no effect	

[a] pH 7.5, 10 mM ornithine, 20 mM $NaHCO_3$, 1 mM ATP, and 10 mM $MgCl_2$. [b] From fits of the data to eq 5.

Discussion

The objective of this study was to investigate the role of the conserved histidine residues in the amidotransferase domain of carbamoyl phosphate synthetase by site-directed mutagenesis. Histidine was chosen as an initial target because this amino acid can participate both in the chemical reaction via general acid/base catalysis and in the binding of the substrate via hydrogen-bonding and/or electrostatic interactions. Of the 15 histidines in the small subunit of CPS from *E. coli*, only four of these residues are conserved in the eight sequences available to us from various sources (see Table I). In this study the histidine residues at positions 272, 312, 341, and 353 of the amidotransferase subunit of CPS from *E. coli* were mutated to asparagine. This replacement is sterically conservative, and some hydrogen-bonding schemes can be maintained. However, the asparagine side chain cannot replace an imidazole in general acid/base mechanisms or participate in electrostatic interactions.

The kinetic properties for two of the mutants constructed in this study, H272N and H341N, were altered only slightly when compared to those of the wild-type enzyme. These mutants utilized glutamine in the full biosynthetic reaction and in the glutaminase reaction about as well as the native protein. Moreover, there were only minor differences in the kinetic constants for the adenine nucleotides in either the full biosynthetic reaction or any of the partial reactions catalyzed by CPS. Therefore, it appears that neither of these histidines contributes in any critical way to either the binding or hydrolysis of glutamine.

The replacement of His-353 with an asparagine residue has resulted in a protein that is unable to utilize glutamine as a substrate. The glutaminase activity is lost as well as the ability to use glutamine as a nitrogen source during the synthesis of carbamoyl phosphate. The effect is localized to the small subunit since none of the other reactions catalyzed by this enzyme are affected to any significant extent. However, the mutant H353N is still able to bind glutamine since the addition of glutamine causes an enhancement of the bicarbonate-dependent ATPase reaction and a diminution in the rate of carbamoyl phosphate synthesis when ammonia is used as the nitrogen source. The affinity of glutamine for the amidotransferase domain has not been diminished by this modification since the activation constant for glutamine in the ATPase reaction (54 μM) is smaller than the Michaelis constant for glutamine (K_m = 105 μM) with the wild-type enzyme. Therefore, it appears that His-353 is involved primarily in catalysis rather than in the binding of the substrate.

The enhancement in the rate of the ATPase reaction by glutamine indicates that the binding of ligands to the small subunit causes conformational changes that are transmitted to the large subunit. These effects have been previously observed in a mutant in which the essential Cys-269 was changed to either serine or glycine (Rubino et al., 1986; Mullins et al., 1991). With these mutants all glutaminase activity was lost, but the binding of glutamine to these proteins resulted in an order of magnitude increase in the ATPase reaction. It therefore appears that neither Cys-269 nor His-353 contributes much to the binding of glutamine to the amidotransferase domain of CPS.

The binding of glutamine to the small subunit of CPS is, however, severely affected by the replacement of His-312 with an asparagine. The Michaelis constants for glutamine in the glutaminase and carbamoyl phosphate synthesis reactions are increased by 2 orders of magnitude. There are no effects on V_{max}, and thus this alteration is restricted primarily to the binding of glutamine. The pH rate profile of V/K for the glutaminase reaction with the wild-type enzyme shows two ionizations that affect the kinetic parameters. There is a group with a pK_a of about 9.9 that must be protonated for activity.

Table V: Sequence Comparisons among CPS from *E. coli* and Other Aminotransferases

Source	267 … 277	309 … 316	338 … 356	Ref
CPS	G I C L G H Q L L A L	A Q N H G F A V	Q G I H R T D K P A F S F Q G H P E A	*a*
GMPS	G V C Y G M Q T M A M	W M S H G D K V	A I M A N E E K R F Y G V Q F H P E V	*b*
AS II	G I C L G H Q A I V E	A R Y H S - L V	M A V R H D A D R V C G F Q F H P E S	*c*
PABS	G V C L G H Q A M A Q	T R Y H S L Y V	M G I R H R Q W D L E G V Q F H P E S	*d*
CTPS	G I C L G M Q - V A L	R H R H R Y E V	I I E V P N H P W F V A C Q F H P E F	*e*

[a] Piette et al. (1984). [b] Zalkin et al. (1985). [c] Nichols et al. (1980). [d] Kaplan et al. (1985). [e] Weng et al. (1986). The numbering relates to the carbamoyl phosphate synthetase from *E. coli*.

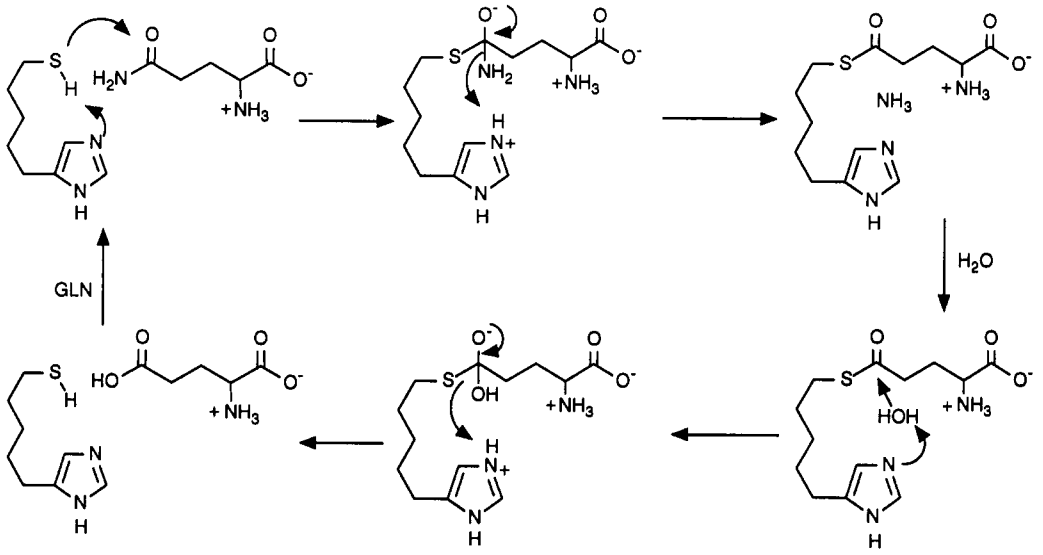


FIGURE 3: Proposed mechanism for the hydrolysis of glutamine by the amidotransferase domain of carbamoyl phosphate synthetase from *E. coli*. The thioester is formed from Cys-269, and the thiol group is activated via general base catalysis by His-353.

This group also appears in the pH rate profile of the mutant H312N. In addition, there is an ionization of a group that appears in the pH rate profile of the wild-type enzyme that is absent in the mutant.[2] When this group with a pK_a of 9.9 is protonated, there is an order of magnitude reduction in the V/K for glutamine with the wild-type enzyme. This ionization may be due directly to His-312 since this kinetic effect is not observed when this amino acid is replaced with the nonionizing asparagine. However, further studies will be required before a more definitive description can be presented.

The two histidines that are unaffected by mutagenesis are not universally conserved (see Table I). The other two histidines in the amidotransferase domain of carbamoyl phosphate synthetase showed significant changes in the kinetic parameters after mutagenesis to asparagine. His-353 is critical for bond cleavage since the H353N mutant enzyme has lost all catalytic activity but will still bind glutamine with high affinity. In contrast, His-312 is critical for binding since the Michaelis constant of glutamine with the H312N mutant increased by 2 orders of magnitude but the maximal velocity at saturating glutamine remained unaltered when compared to the wild-type values. These results are also consistent with the sequence alignments of the other *trp*G-type amidotransferases. Shown in Table V are the amino acid sequence alignments for four other amidotransferase enzymes in the regions of the histidines modified in this study. For the histidines that appear at positions 272 and 341 of the *E. coli* carbamoyl phosphate synthetase, there is no consensus with the other four sequences at equivalent positions. His-353 is totally conserved, and Amuro et al. (1985) have mutated this histidine to tyrosine with the loss of all catalytic activity in anthranilate synthetase. In previous published alignments of the *trp*G-type amidotransferases, the histidine at position 312 of CPS is not conserved (Amuro et al., 1985). Although this histidine is located within a region of some insertions and/or deletions, the equivalent histidine for all five proteins can be made to align with little difficulty (see Table V).

A consistent chemical mechanism for the hydrolysis of glutamine by the amidotransferase domain of CPS is illustrated in Figure 3. In this mechanism Cys-269 is activated by His-353 prior to formation of the thioester intermediate. In subsequent steps this histidine functions as a general acid/base catalyst for proton donation and abstraction. This mechanism is similar to the one proposed by Amuro et al. (1985) for anthranilate synthetase and by Mei and Zalkan (1989) for glutamine phosphoribosylpyrophosphate amidotransferase.

[2] The glutaminase activity of the wild-type carbamoyl phosphate synthetase exhibits another pH optimum below pH 7 (Trotta et al., 1973).

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