Differential Roles for Three Conserved Histidine Residues within the Large Subunit of Carbamoyl Phosphate Synthetase†

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ABSTRACT: Three conserved histidine residues, His-243, His-781, and His-788, located within the large subunit of carbamoyl phosphate synthetase from *Escherichia coli* were identified by sequence identity comparisons. These three histidine residues were individually mutated to asparagine residues. The H243N mutant enzyme was found to be critical for carbamoyl phosphate synthesis as the mutant protein was unable to synthesize carbamoyl phosphate at a significant rate (<1/1500). By analysis of the effects of this mutation on the partial reactions catalyzed by CPS, it was determined that this mutation blocked the formation of the carbamate intermediate from carboxyphosphate and ammonia. The H781N mutant enzyme had an order of magnitude reduction for both the rate of carbamoyl phosphate formation and ATP synthesis which is consistent with the proposal that the carboxyl-terminal half of the large subunit is primarily involved in the phosphorylation of the putative carbamate intermediate. This mutation also reduced the effects of the allosteric activator ornithine on the K_m parameters for ATP in the overall biosynthetic reaction and ADP in the ATP synthesis reaction. The H788N mutant enzyme is a functional protein which maintains the ability to synthesize carbamoyl phosphate at a rate comparable to that of the wild-type enzyme. The effects of this mutation are 10-fold reductions of the ATP synthetase and the bicarbonate-dependent ATPase activities with substantial increases in the K_m values for ATP in the full biosynthetic reaction and for ADP in the ATP synthesis reaction. The reaction of the wild-type and mutant enzymes with diethylpyrocarbonate (DEPC) was also investigated. All of the activities of the enzyme are inactivated by treatment with high concentrations of DEPC. For the bicarbonate-dependent ATPase activity of the wild-type H781N and H788N enzymes, treatment with DEPC results in an initial increase in ATPase activity followed by a slower inactivation. This biphasic behavior was not observed with the H243N mutant enzyme.

Carbamoyl phosphate synthetase (CPS)[1] of *Escherichia coli* catalyzes the synthesis of carbamoyl phosphate according to eq 1. The enzyme can also utilize ammonia as a nitrogen

$$\text{Gln} + HCO_3^- + 2\text{MgATP} \rightarrow \text{Glu} + 2\text{MgADP} + P_i + \text{carbamoyl phosphate} \quad (1)$$

source. The enzyme is composed of two nonidentical subunits, a small subunit of molecular weight 41 270 encoded by the *car*A gene, which contains the amidotransferase activity, and a large subunit of molecular weight 117 710 encoded by the *car*B gene, which catalyzes the formation of carbamoyl phosphate from bicarbonate, ammonia, and ATP (Piette et al., 1984; Nyunoya & Lusty, 1983). The large subunit also contains the binding sites for the allosteric effectors IMP, UMP, and ornithine (Anderson & Meister, 1966; Trotta et al., 1971).

The enzymatic mechanism for the synthesis of carbamoyl phosphate involves the activation of bicarbonate through the formation of carboxyphosphate, which then undergoes nucleophilic attack by ammonia to form carbamate.[2] The carbamate intermediate is then phosphorylated by the second ATP to give the final product carbamoyl phosphate (Meister, 1989; Raushel et al. 1978). The overall mechanism is illustrated in Scheme I. In addition to the carbamoyl phosphate synthesis reaction, the enzyme catalyzes three partial reactions: a bicarbonate dependent hydrolysis of ATP shown in eq 2, the synthesis of ATP from carbamoyl phosphate and ADP shown in eq 3, and the hydrolysis of glutamine to glutamate and ammonia shown in eq 4.

$$\text{MgATP} + H_2O \xrightarrow{HCO_3^-} \text{MgADP} + P_i \quad (2)$$

$$\text{MgADP} + \text{carbamoyl phosphate} \rightarrow \text{MgATP} + CO_2 + NH_3 \quad (3)$$

$$\text{glutamine} + H_2O \rightarrow \text{glutamate} + NH_3 \quad (4)$$

There is a considerable amount of evidence to indicate that there are two physically distinct ATP binding sites within the large subunit (Powers et al., 1977; Powers & Meister, 1978; Raushel et al., 1978; Raushel & Villafranca, 1979; Boettcher & Meister, 1980; Lusty et al., 1983; Post et al., 1990). The large subunit has a high degree of internal homology between the amino-terminal and carboxyl-terminal halves which has been suggested to have arisen as the result of a gene duplication

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[1] Abbreviations: CPS, carbamoyl phosphate synthetase; DEPC, diethylpyrocarbonate; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; EDTA, ethylenediaminetetraacetic acid; Tris, tris-(hydroxymethyl)aminomethane; PCR, polymerase chain reaction.

[2] Alternatively, carbon dioxide may be the active carboxylating agent. The role of the carboxyphosphate intermediate would then be to dehydrate bicarbonate providing a high, localized concentration of electrophilic CO_2 to react with ammonia and form the carbamate intermediate (Sauers et al., 1975).

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Scheme I

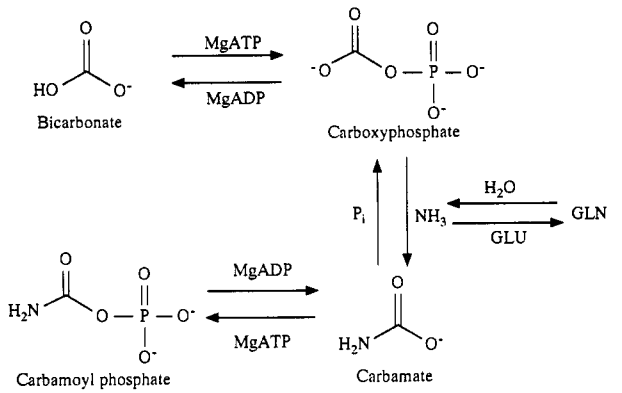

Table I: Sequence Comparisons for the Conserved Histidine Residues in the Large Subunit of CPS[a]

source	range		ref
E. coli carB	232–246	C S I E N F D A M G I H T G D	*b*
B. subtilis pyrAB	232–246	C N M E N I D P V G I H T G D	*c*
yeast ura2	659–673	C N M E N F D P L G I H T G D	*d*
yeast cpa2	252–266	C N M E N F D P L G V H T G D	*e*
D. melanogaster CAD	632–646	C N M E N F D P L G I H T G E	*f*
hamster CAD	462–476	C N M E N L D P L G I H T G E	*g*
rat cps1	649–663	C N M E N V D A M G V H T G D	*h*
E. coli carB	777–791	G I M E H I E Q A G V H S G D	*b*
B. subtilis pyrAB	777–791	G I M E H I E R A G V H S G D	*c*
yeast ura2	1177–1191	V V S E H V E N A G V H S G D	*d*
yeast cpa2	797–811	A I S E H V E N A G V H S G D	*e*
D. melanogaster CAD	1170–1184	L F P E H V E N A G V H S G D	*f*
hamster CAD	996–1010	A I S E H V E N A G V H S G D	*g*
rat cps1	1192–1206	A I S E H V E D A G V H S G D	*h*

[a] The boxed regions identify the conserved histidine residues that were mutated in this study. [b] Nyunoya and Lusty (1983); [c] Quinn et al. (1991); [d] Souciet et al. (1989); [e] Lusty et al. (1983); [f] Freund and Jarry (1987); [g] Simmer et al. (1990); [h] Nyunoya et al. (1985).

and fusion of an ancestral protein during the evolution of this enzyme (Nyunoya & Lusty, 1983). The homology in the primary structure between these two halves of the large subunit suggests that each domain may contain a separate binding site for one of the two molecules of ATP required for the overall synthesis of carbamoyl phosphate. Site-directed mutations of conserved glycine residues within the putative nucleotide binding site on the amino-terminal half (G176I and G180I) indicate that this domain of the *E. coli* enzyme is involved in the phosphorylation of bicarbonate, while the corresponding mutation in the carboxyl-terminal half (G722I) indicates that this region is involved in the phosphorylation of carbamate (Post et al., 1990).

The determination of the amino acid residues essential for substrate binding and catalytic activity is critical for the elucidation of the molecular events that occur along the enzyme-catalyzed reaction pathway. In this study, the role of conserved histidine residues of the large subunit in the overall carbamoyl phosphate synthesis and partial reactions has been investigated. Identity comparisons of CPS from *E. coli* with those enzymes isolated from several other sources demonstrate that there are only three strictly conserved histidine residues in the large subunit (Simmer et al., 1990; Quinn et al., 1991). A portion of these sequence alignments that highlight the conserved histidine residues is illustrated in Table I. Two of these conserved histidine residues (H788 and H781) are located in the carboxyl-terminal half while the other histidine is located in the amino-terminal half (H243). These three conserved histidine residues were chosen as targets for site-directed mutagenesis and individually substituted with asparagine. These studies have been complemented by inactivation of the catalytic functions with the histidine selective reagent diethylpyrocarbonate (Lunbald & Noyes, 1984; Miles, 1977).

MATERIALS AND METHODS

Chemicals and Enzymes. All chemicals and coupling enzymes used in assays were purchased from either Sigma or Aldrich. Restriction enzymes were purchased from Bethesda Research Laboratories. The Sequenase kit was obtained through U.S. Biochemical. The Ultrogel AcA-34 and AcA-36 gel filtration media were purchased from IBF Biotechnics. Site-directed mutagenesis was performed using the T7-Gen in vitro mutagenesis kit from United States Biochemical Corp. or Mutagene obtained from Bio-Rad. The Green A resin was obtained from Amicon. [^{14}C]Diethylpyrocarbonate was purchased from Sigma (9.4 mCi/mmol), and radioactivity measurements were made using a Beckman 6000 SE scintillation counter.

Bacterial Strains and Plasmids. The *E. coli* strains used in this study were RC50 (*car*A50, *thi*-1, *mal*A1, *xyl*-7, *rsp*L135, λ^r, λ^-, *tsx*-273), RR1 (*pro*A, *leu*B, *thi*-1, *lac*Y, *hsd*R, *end*A, *rsp*L20, *ara*-14, *gal*K2, *mtl*-1, *sup*E44), 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^{q}ZΔM15, Tn10(*tet*^{r})]), and CJ236 (*dut*1, *ung*1, *thi*-1, *rel*A1/pCJ105[F′(*cm*^{r})]) (Bullock et al., 1987; Kunkel et al., 1987; Joyce & Grindley, 1984). The strains RR1, RC50, and the plasmids pMC41 and pSR21 were obtained from Dr. Carol J. Lusty (Public Health Research Institute of New York). The plasmids pDP4115 and pDP4117 were derived from the plasmid pMC41 containing the *car*AB operon as described below.

Construction of the H788N and H243N Mutant Enzymes. The *car*AB operon (Piette et al., 1984) was subcloned into the *Hin*dIII site of the phagemid pBS(+) (Stratagene, La Jolla, CA) creating a new plasmid pDP412. Uracil containing single-stranded DNA was generated in the *E. coli* strain CJ236 (*dut*$^-$, *ung*$^-$, F′) using helper phage M13K07 (Viera & Messing, 1987). Site-directed mutations were produced as described by Kunkel et al. (1987), and the resulting plasmids were transformed into *E. coli* strain XL1-Blue (*dut*$^+$, *ung*$^+$, F′). Helper phage M13K07 was used to generate single-stranded DNA from the resulting colonies, which were screened for correct sequence changes by dideoxy sequencing (Sanger et al., 1977) of a 300 base pair region surrounding the site-directed target sequence. Plasmids isolated from colonies with altered sequences were transformed into the *car*AB$^-$ strain RC50 for enzyme production and subsequent purification. The plasmid containing the site-specific change for His-243 to an asparagine was named pDP4115, and the plasmid encoding the His-788 to asparagine change was named pDP4117. The oligonucleotides used to mutate the two histidine residues at amino acid positions 243 and 788 of the large subunit to asparagines were 5′ACC GGT GTT* GAT GCC CAT3′ and 5′ACC GGA GTT* CAC GCC CGC3′, respectively. These primers, obtained from the DNA sequencing laboratory, Biology Department, Texas A&M University, introduced a single base change of G to T into the wild-type sequence. Revertants to the wild-type enzyme were also produced for both mutant plasmids by repeating the mutagenesis protocol with oligonucleotides of the wild-type sequence.

Construction of the Mutant Enzyme H781N. The mutant allele of *car*AB encoding the single amino acid substitution of an asparagine for histidine at position 781 was constructed by site-directed mutagenesis of the pDP412 plasmid using the

polymerase chain reaction (PCR) and the overlap extension protocol of Ho et al. (1989). The pair of internal complementary oligonucleotides encoding a base change of C to A into the wild-type sequence to create the desired amino substitution were 5′C ATG GAG* AAT ATT GAG CAG G3′ and 5′C CTG CTC AAT ATT* CTC CAT G3′. The flanking oligonucleotide primers used to isolate the mutant allele were 5′GCG AAG CGA ATC CGT CTA CC3′ and 5′CTT CAA TCA GGT AGA CTT CG3′. The PCR-amplified mutagenic fragment of pDP412 was purified and digested with *Acc*I to facilitate insertion into the *car*AB gene. In the corresponding wild-type plasmid (pDP412), the *Acc*I site in the multiple cloning site of pBS(+) was removed by digestion with the restriction enzymes *Bam*HI and *Pst*I. This fragment was replaced with a *Bam*HI–*Pst*I adaptor obtained from Stratagene (La Jolla, CA), and the resulting plasmid was named pSM10. This new plasmid was digested with *Acc*I, and the restriction fragment corresponding to the one isolated from the PCR-amplified mutagenic fragment was removed. The resulting vector was then ligated with the purified PCR fragment and transformed into RC50. The entire *Acc*I fragment generated by PCR was sequenced to confirm the site-specific mutation. The plasmid encoding the His-781 to an asparagine residue was named pSM111.

Purification of Wild-Type and Mutant Enzymes. Wild-type carbamoyl phosphate synthetase was purified from *E. coli* RR1 carrying the pMC41 plasmid. All cells were grown in Luria-Bertani (LB) media (Maniatis et al., 1982) in the presence of 50 μg/mL ampicillin and harvested in stationary phase. The histidine mutant enzymes, H243N, H781N, and H788N, were purified from *E. coli* RC50 carrying plasmids pDP4115, pSM111, and pDP4117, respectively. The cells (30–50 g/preparation) were lysed by passage through a French press (12 000 psi) and then treated with 1 mM EDTA and 0.2 mM phenylmethanesulfonyl fluoride. Protamine sulfate was added drop wise to the lysate until a final concentration of 0.5% was reached. The precipitate was then removed by centrifugation at 18000*g* for 30 min. The supernatant solution was fractionated with 0–65% saturated ammonium sulfate. The resulting precipitate was resuspended in 70 mL of 0.1 M potassium phosphate buffer, pH 7.6, and 1.0 mM EDTA and subsequently applied to an Ultrogel AcA-34 column (5.0 × 200 cm) and eluted with the same buffer. The fractions containing CPS were determined by assaying enzymatic activity, SDS–PAGE, and Western blots. The fractions containing CPS were pooled and applied to a Pharmacia FPLC Mono-Q 10/10 column. The CPS was eluted with a 100-mL gradient of 0.1 M potassium phosphate buffer, pH 7.6, containing 0–1.0 M KCl. The wild-type and mutant proteins eluted at a concentration of approximately 0.15 M KCl. The purified enzymes were stored at −70 °C until required.

Purification of the Small Subunit of CPS. The small subunit was isolated from *E. coli* strain RC50 containing the plasmid pSR21 (Guillou et al., 1989) following steps similar to those used for the purification of wild-type and mutant CPS except for an additional chromatography step using the Green A dye matrix material from Amicon. The pooled fractions from the Ultrogel AcA-36 chromatographic step were loaded onto a column containing the Green A material which had been equilibrated with 50 mM HEPES buffer at pH 7.6. The column was washed with one or two bed volumes (150–300 mL) of the HEPES buffer, and then a linear gradient of the same buffer with 0–1 M KCl was applied to elute the protein. The small subunit was identified by SDS–PAGE.

Determination of Enzyme Activity. The rate of carbamoyl phosphate synthesis was assayed by measuring the rate of citrulline formation in a coupled assay containing ornithine transcarbamoylase and ornithine (Rubino et al., 1986). The assay mixtures contained 50 mM HEPES buffer (pH 7.6), 100 mM KCl, 10 mM ATP, 20 mM $MgCl_2$, 20 mM $NaHCO_3^-$, 10 mM ornithine, 0.9 units/mL ornithine transcarbamoylase, and either 20 mM glutamine or 300 mM ammonia. In a typical measurement, 10–40 μg of CPS was added to 0.5 mL of the assay mixture and incubated at 25 °C for a specified time. The reaction was quenched by the addition of 1 mL of an acid reagent which consisted of 3.7 g of antipyrine, 2.5 g of ferric ammonium sulfate, 250 mL of H_2SO_4, 250 mL of 85% H_3PO_4, and water to give a final volume of 1 L. To the quenched solution, 0.5 mL of 0.4% diacetyl monoxime was added, and the solution was vortexed and boiled for 15 min. The citrulline concentration was measured from the absorbance at 464 nm using an extinction coefficient of 37 800 M^{-1} cm^{-1} (Snodgrass & Parry, 1969).

The rate of production of ADP was assayed using a pyruvate kinase/lactate dehydrogenase coupling system by measuring the rate of loss of NADH spectrophotometrically at 340 nm. A 3-mL assay mixture contained 25 mM HEPES (pH 7.6), 1.0 mM PEP, 0.2 mM NADH, 100 mM KCl, 20 mM $MgCl_2$, 10 mM ATP, 40 mM $NaHCO_3^-$, 10 mM ornithine, 20 units of pyruvate kinase, 30 units of lactate dehydrogenase, and either 10 mM glutamine or 300 mM NH_3. To determine the bicarbonate dependent ATPase activity, the nitrogen source was omitted. All assays were conducted at 25 °C.

The rate of ATP synthesis was assayed using a hexokinase/glucose-6-phosphate dehydrogenase coupling system by measuring the rate of production of NADH at 340 nm. Each assay mixture consisted of 25 mM glycylglycine buffer (pH 7.6), 125 mM KCl, 0.5 mM NAD^+, 10 mM ADP, 15 mM magnesium acetate, 10 mM carbamoyl phosphate, 1.0 mM glucose, 10 mM ornithine, 20 units of hexokinase, and 10 units of glucose-6-phosphate dehydrogenase.

The glutaminase activity of CPS was assayed by coupling the formation of L-glutamate to the production of α-ketoglutarate using L-glutamate dehydrogenase (Buchanan et al., 1978). The enzyme (20 μg) was incubated with 0.1 M CAPS buffer (pH 10.0) and 10 mM L-glutamine at 25 °C in a volume of 200 μL. The isolated small subunit was assayed by incubating 20 μg of the small subunit in 0.1 M phosphate buffer (pH 6.8) with 150 mM L-glutamine. The reaction was stopped at various times by the addition of 50 μL of 1 N HCl. The solutions were neutralized with the addition of 50 μL of 1 M Tris (pH 10.5), and then 700 μL of an assay mixture consisting of 0.15 M Tris (pH 8.0), 0.8 mM acetylpyridine adenine nucleotide (APAD), and 5 units of glutamate dehydrogenase was added to make a final volume of 1 mL. The samples were incubated at 25 °C for 1 h, and the increase in the APADH concentration was measured at 363 nm using an extinction coefficient of 8.3 mM^{-1} cm^{-1}.

Enzyme Modification with Diethylpyrocarbonate. Diethylpyrocarbonate was used to modify histidine residues. A stock solution of diethylpyrocarbonate (DEPC) was made with anhydrous ethanol immediately before use. The final DEPC concentration was determined by adding an aliquot of the stock solution to 10 mM imidazole (pH 6.9) and measuring the concentration of *N*-carbethoxyimidazole from the absorbance at 230 nm ($\epsilon = 3.0 \times 10^3$ M^{-1} cm^{-1}; Miles, 1977). The hydrolysis rate of the DEPC in the 0.1 M phosphate buffer (pH 6.9) used in the inactivation studies was determined by incubation of DEPC in the buffer and removing aliquots at

Table II: Kinetic Parameters of the Wild-Type and Mutant Enzyme

		reaction monitored						
		ATP synthetase[a]		bicarbonate-dependent ATP hydrolysis[b]		glutamine-dependent ATP hydrolysis[c]		carbamoyl phosphate synthetase[c]
enzyme	ornithine (mM)	K_m (μM)	V_{max} (μmol/min·mg)	K_m (μM)	V_{max} (μmol/min·mg)	K_m (μM)	V_{max} (μmol/min·mg)	V_{max} (μmol/min·mg)
wild-type	0	90 ± 2	0.40 ± 0.03	20 ± 2	0.43 ± 0.06	350 ± 60	3.6 ± 0.4	
	10	5.0 ± 0.2	0.40 ± 0.02	7 ± 2	0.40 ± 0.01	34 ± 4	3.3 ± 0.2	1.8 ± 0.1
H243N	0	55 ± 16	0.23 ± 0.01	55 ± 8	0.35 ± 0.01	60 ± 7	0.33 ± 0.01	
	10	7 ± 2	0.23 ± 0.01	50 ± 3	0.35 ± 0.01	30 ± 8	0.35 ± 0.01	0.0006 ± 0.0001
H781N	0	180 ± 30	0.025 ± 0.001	72 ± 1	0.16 ± 0.01	410 ± 40	3.7 ± 0.1	
	10	100 ± 10	0.026 ± 0.001	38 ± 2	0.15 ± 0.01	225 ± 30	3.2 ± 0.1	0.15 ± 0.01
H788N	0	820 ± 14	0.041 ± 0.002	18 ± 3	0.053 ± 0.002	780 ± 120	1.9 ± 0.7	
	10	81 ± 1	0.035 ± 0.003	10 ± 1	0.051 ± 0.003	240 ± 30	1.9 ± 0.7	1.1 ± 0.2

[a] pH 7.5, 25 °C, variable ADP, 10 mM carbamoyl phosphate, 20 mM Mg^{2+}. [b] pH 7.5, 25 °C, variable ATP, 40 mM bicarbonate, 20 mM Mg^{2+}. [c] pH 7.5, 25 °C, varible ATP, 40 mM bicarbonate, 20 mM Mg^{2+}, 10 mM glutamine.

various time intervals and determining the residual DEPC concentration by reaction with 10 mM imidazole as described above. The inactivation of CPS by DEPC was determined by incubation of 0.2–0.5 mg of the protein in 0.1 M potassium phosphate buffer (pH 6.9) at 25 °C. The inactivation was initiated by the addition of DEPC, aliquots were removed at regular time intervals, and the remaining enzyme activity was assayed. The reversibility of the inactivation was investigated by incubating the inactivated enzyme with hydroxylamine (Lunbald & Noyes, 1984; Miles, 1977). An equal volume of 100 mM hydroxylamine and 10 mM imidazole (pH 6.9) was added to solutions of inactivated enzyme. The number of histidines modified by diethylpyrocarbonate was monitored by the increase in absorbance in the 230–245-nm region using an extinction coefficient of $\epsilon = 3000\ M^{-1}\ cm^{-1}$ (Miles, 1977).

Carbamoyl phosphate synthetase was also modified with [^{14}C]DEPC to determine the relative extent of modification to the large and small subunits. In a typical [^{14}C]DEPC modification experiment, 2 mg of CPS was incubated in 90 mM phosphate buffer (pH 6.8) at 25 °C in a final volume of 1.1 mL. The modification was initiated with the addition of sufficient radiolabeled DEPC to give a final concentration of 5 mM. At various times, a 50-μL aliquot was removed and added to 20 μL of 10 mM imidazole to quench the modification reaction. The unreacted reagent was removed from the enzyme solution by centrifugation of each sample through a column of Sephadex G-50 equilibrated with 50 mM phosphate buffer (pH 7.6) (Penefsky, 1977). SDS–PAGE was then conducted at neutral pH using a Bis–Tris and TES buffer system (Moos et al., 1988) at 20 mA for 1.5 h at room temperature. A control gel containing nonradiolabeled DEPC-treated enzyme was stained with Coomassie blue to locate the bands corresponding to the large and small subunits of CPS. Each lane containing the ^{14}C-labeled enzyme was sliced into 4–5-mm sections and sonicated, and the radioactivity was measured.

RESULTS

Construction of Site-Directed Carbamoyl Phosphate Synthetase Mutations. Site-directed mutants of carbamoyl phosphate synthetase were constructed and purified by the procedures described above. Histidine residues at positions 243, 781, and 788 of the large subunit were individually changed to asparagine residues.

Kinetic Properties of Carbamoyl Phosphate Synthetase and the Site-Directed Mutants. The kinetic parameters, V_{max} and K_m, for the wild-type and histidine mutant enzymes are presented in Table II. The kinetic parameters were determined for the bicarbonate-dependent ATPase activity, ATP synthetase activity, and carbamoyl phosphate synthetase activity in both the presence and absence of the allosteric activator ornithine. The replacement of His-243 with an asparagine residue results in an enzyme unable to synthesize carbamoyl phosphate at a significant rate. Remarkably, the mutant protein still has ATP synthetase and bicarbonate-dependent ATPase activities with V_{max} and K_m values comparable to those of the wild-type enzyme (Table II). However, there is no significant difference in the rate of ATP hydrolysis in the presence and absence of glutamine or ammonia, and essentially no carbamoyl phosphate is produced.

The substitution of an asparagine for H781 results in a 10-fold reduction in the rates of carbamoyl phosphate and ATP synthesis. The rate of the glutamine-dependent ATPase reaction is essentially identical to that of the wild-type protein. The bicarbonate-dependent ATPase velocity is similar to that of the wild-type protein. However, the ATP hydrolysis and carbamoyl phosphate formation have been uncoupled. The K_m values for ATP and ADP have been significantly increased relative to those of the wild-type protein, while the allosteric effects of ornithine decreased.

The kinetic properties for the H788N mutant protein constructed in this study were only slightly different than those of the wild-type enzyme (Table II). The modified enzyme was able to synthesize carbamoyl phosphate at a rate approximately 60% that of the wild-type enzyme, thus illustrating that this residue is not critical for catalytic activity. However, the K_m for ATP in the overall biosynthetic reaction was substantially increased in both the presence and absence of ornithine. Interestingly, a similar effect was observed for the K_m of ADP in the ATP synthetase reaction. This mutation also caused a 10-fold reduction in V_{max} for both the bicarbonate-dependent ATPase and the ATP synthetase activities.

Diethylpyrocarbonate Modification of Wild-Type Carbamoyl Phosphate Synthetase. The addition of diethylpyrocarbonate to CPS inactivated the carbamoyl phosphate synthesis, glutaminase, and ATP synthesis activities. For the carbamoyl phosphate synthesis and ATP synthesis catalyzed reactions, the inactivation process followed pseudo-first-order kinetics. The remaining activity, corrected for the hydrolysis of DEPC as a function of time, is described by eq 5, where

$$\ln (A/A_o) = -(k/k')I_o(1 - e^{-k't}) \quad (5)$$

A/A_o is the fraction of activity remaining at time t, I_o is the initial concentration of DEPC, k is the apparent second-order rate constant for the loss of enzyme activity, and k' is the

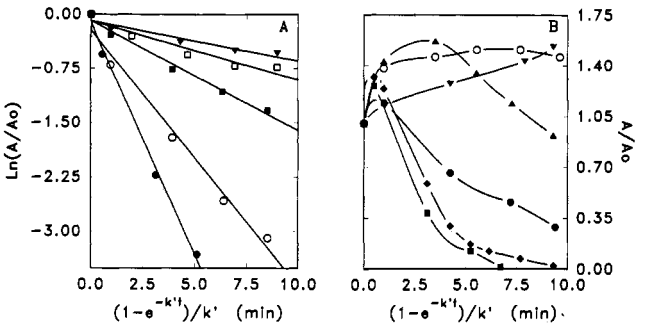

FIGURE 1: Diethylpyrocarbonate modification of wild-type CPS. (A) The effect of DEPC on the carbamoyl phosphate synthesis reaction using glutamine as the nitrogen source. The enzyme (1.5 μM) was incubated with 0.5 (▼), 1.0 (□), 2.5 (■), 5.0 (○), and 10 (●) mM DEPC. (B) The effect of DEPC modification of the bicarbonate-dependent ATPase activity. The enzyme (1.5 μM) was incubated with 0.5 (▼), 1.0 (○), 2.5 (▲), 5.0 (●), 10 (◆), and 20 (■) mM DEPC. Additional details are presented in the text.

Table III: Effects of Diethylpyrocarbonate on the Various Activities of the Wild-Type and Mutant Proteins[a]

	reaction inactivated by diethylprocarbonate treatment			
enzyme	ATP synthetase (M^{-1} min^{-1})	ammonia-dependent ATP hydrolysis (M^{-1} min^{-1})	glutamine-dependent ATP hydrolysis (M^{-1} min^{-1})	bicarbonate-dependent ATP hydrolysis (M^{-1} min^{-1})
wild type	57 ± 6	47 ● 1	61 ± 5	biphasic
H243N	49 ± 5	96 ± 8	98 ± 6	93 ± 6
H781N	15 ± 5	23 ± 3	16 ± 4	biphasic
H788N	29 ± 7	23 ± 2	49 ● 5	biphasic

[a] Second-order rate constants determined at pH 6.9, 25 °C.

first-order rate constant for the buffer-catalyzed hydrolysis of the DEPC (Gomi & Fujioka, 1983).

A representative plot (Figure 1A) shows the loss of carbamoyl phosphate synthetase activity using glutamine as the nitrogen source. Similar plots (data not shown) were obtained for the carbamoyl phosphate synthetase activity using ammonia as the nitrogen source and for the ATP synthesis reaction. A plot of the pseudo-first-order rate constants for inactivation, k_{obs}, versus the DEPC concentrations yielded linear plots with slopes equal to the apparent second-order rate constants of inactivation, k. The second-order rate constants for inactivation of the various catalytic activities of carbamoyl phosphate synthetase are listed in Table III.

Substrate protection studies were performed to determine if the DEPC inactivation was due to modification of histidine residues at or near the active site of the enzyme. As illustrated in Figure 2A, combinations of substrates and allosteric ligands were found to protect the enzyme against inactivation. A mixture of ATP, $MgCl_2$, and ornithine provided the best protection.

The effect of DEPC on the bicarbonate-dependent ATPase activity of CPS was found to be biphasic with an initial increase in the ATPase activity followed by inactivation, as shown in Figure 1B. Low concentrations of DEPC (<1 mM) resulted in a net increase in the ATPase activity. The addition of substrates and allosteric ligands protected the bicarbonate-dependent ATPase activity against DEPC inactivation, but they did not hinder the activation as shown in Figure 2B.

The effects of DEPC on the glutaminase activity were also examined (data not shown). For the wild-type CPS, the time-dependent loss of glutaminase activity was pseudo-first-order, and the second-order rate constant was found to be 9 ± 2 M^{-1} min^{-1}. For the isolated small subunit, the DEPC inactivation

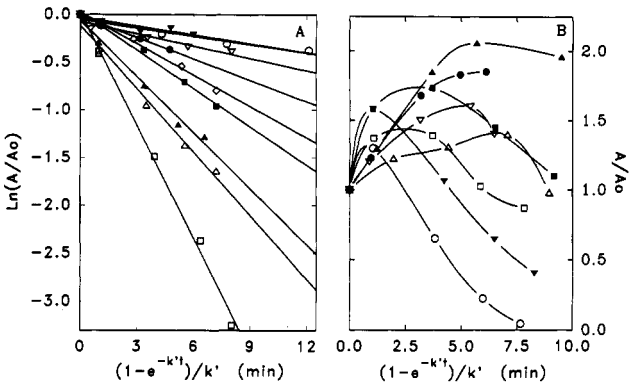

FIGURE 2: Substrate protection against diethylpyrocarbonate inactivation of wild-type CPS. The enzyme (1.5 μM) was preincubated with various substrates followed by the addition of DEPC to give a final DEPC concentration of 5.0 mM. (A) Substrate protection against DEPC inactivation of the carbamoyl phosphate synthesis reaction using glutamine as a nitrogen source. (□) No substrates added, (△) 1 mM glutamine, (▲) 5 mM ADP, (■) 5 mM carbamoyl phosphate, (◇) 5 mM ATP and 5 mM $MgCl_2$, (●) 10 mM ATP and 10 mM $MgCl_2$, (▽) 1 mM ornithine, (▼) 5 mM ATP and 10 mM $MgCl_2$, and (○) 1 mM ornithine, 5 mM ATP, and 10 mM $MgCl_2$. (B) Substrate protection of the bicarbonate-dependent ATPase activity. (○) No substrates added, (●) 1 mM ornithine, (▽) 5 mM ADP and 10 mM $MgCl_2$, (▼) 5 mM ADP, (□) 5 mM ATP, (■) 5 mM ATP and 5 mM $MgCl_2$, (△) 5 mM ATP and 10 mM $MgCl_2$, and (▲) 1 mM ornithine, 5 mM ATP, and 10 mM $MgCl_2$.

was also pseudo-first-order, and the second-order rate constant was determined to be 200 ± 30 M^{-1} min^{-1}.

There are 30 histidine residues in the carbamoyl phosphate synthetase from *E. coli*: 15 in the large subunit and 15 in the small subunit (Piette et al., 1984; Nyunoya & Lusty, 1983). The number of histidines that can be chemically modified with DEPC was determined spectrophotometrically (Miles, 1977) and through the use of radiolabeling using [^{14}C]DEPC (Burstein et al., 1974). The use of radiolabeled DEPC permitted the determination of the number of histidine residues modified on each subunit, as shown in Table IV. The protection by ornithine against enzyme modification by DEPC is shown both by the number of histidines modified and the percent activity remaining. From Table IV, it appears that incubation of the enzyme with ornithine protects three to four histidine residues from modification with DEPC.

Diethylpyrocarbonate has been shown to modify lysine, cysteine, tyrosine, and N-terminal nitrogens (Lunbald & Noyes, 1984; Miles, 1977, Horiike et al., 1979; Cousineau & Meighen, 1976). DEPC modification of tyrosine residues can be determined by spectral changes at 278 nm (Miles, 1977). The reaction between 2.5 μM enzyme and 10 mM DEPC was monitored at 278 nm, and no change in the absorbance was observed. Evidence that the loss of activity during DEPC modification is not the result of lysine modification can be obtained by reversing the modification reaction with hydroxylamine. DEPC modification of lysines is not reversible with hydroxylamine, while DEPC modification of histidines, cysteines, and tyrosines is reversible (Miles, 1977). The reversibility of DEPC modification was tested by treating the wild-type enzyme with 2.9 mM DEPC. There were 14 histidine residues modified, leaving the enzyme with 28% of the initial ammonia-dependent ATPase activity and 22% of the initial carbamoyl phosphate synthetase ability. Hydroxylamine was then added to give a final hydroxylamine concentration of 33 mM. After 2 h, 7.4 histidines were still modified, liberating 7 histidine residues. The ammonia-dependent ATPase activity increased to 75% of the initial

Table IV: Quantitation of the Number of Histidine Residues Modified by Diethylpyrocarbonate[a]

DEPC conc (mM)	substrates added	no. histidines modified determined photometrically	no. of ^{14}C labels incorporated into whole enzyme	no. of ^{14}C labels incorporated into small subunit	no. of ^{14}C labels incorporated into large subunit	% HCO_3^- dependent ATPase act. remaining	% Gln dependent ATPase act. remaining
1		10	ND[b]	ND	ND	83%	43%
2.9		14	ND	ND	ND	53%	13%
2.9	1 mM ornithine	10	ND	ND	ND	112%	52%
5		18	18	9	9	17%	2%
5	1 mM ornithine	15	10	5	5	76%	44%
10		25	ND	ND	ND	0%	0%

[a] pH 6.9, 25 °C, 60-min incubation period. [b] ND, not determined.

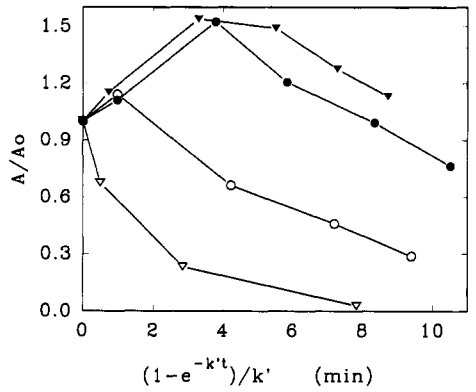

FIGURE 3: Effect of diethylpryrocarbonate modification on the bicarbonate-dependent ATPase activity of the wild-type (○) and mutant enzymes H243N (▽), H781N (▼), and H788N (●). The enzymes were incubated with 5.0 mM DEPC, and the effect on the bicarbonate-dependent ATPase activity was measured.

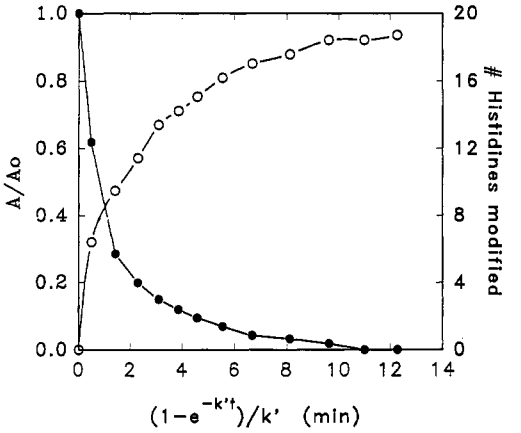

FIGURE 4: Correlation between the DEPC modification of histidine residues and the loss of carbamoyl phosphate synthesis ability. The wild-type CPS enzyme was incubated with 5 mM DEPC, and the number of histidine residues modified was determined (○) as well as the fraction of remaining carbamoyl phosphate synthesis activity (●).

activity, but there was no increase in carbamoyl phosphate synthetase activity.

Diethylpyrocarbonate Modification of the Mutant Enzymes. The addition of DEPC to the H243N enzyme resulted in the pseudo-first-order loss of activity for all of the reactions catalyzed by CPS. The second-order rate constants for DEPC inactivation of the carbamoyl phosphate synthetase activities are tabulated in Table III. The addition of DEPC to the H788N and H781N enzymes resulted in the pseudo-first-order loss of activity for the glutamine- and ammonia-dependent ATP hydrolysis reactions and the ATP synthetase activity. The effect of DEPC on the bicarbonate-dependent ATPase activity of the H788N and H781N mutants was biphasic with an initial increase in the ATPase activity followed by inactivation. Figure 3 illustrates the effect of 5 mM DEPC on the bicarbonate-dependent ATPase activity of the wild-type, H243N, H781N, and H788N enzymes. Substrates and the allosteric activator ornithine protected these histidine mutant enzymes against inactivation in the same manner as observed with the wild-type enzyme (data not shown).

DISCUSSION

Histidine residues have been found to be essential for the catalytic activity of many enzymes. This observation is due directly to the pK_a of the imidazole ring of histidine, which is close to neutrality, allowing histidine residues to function in enzyme catalysis as a general acid and/or as a general base. Histidine residues can also participate in substrate and metal ion binding through hydrogen-bonding and elctrostatic interactions. Two histidine residues located within the small subunit of CPS have previously been identified as critical for the glutaminase activity of carbamoyl phosphate synthetase of *E. coli* (Miran et al., 1991). In this study, the role of histidine residues within the large subunit of CPS were investigated.

Evidence that at least one histidine residue is critical for enzyme catalysis can be obtained from chemical modification studies with the histidine-specific reagent diethylpyrocarbonate. The role of specific histidine residues can be determined by site-directed mutagenesis. Treatment of CPS with diethylpyrocarbonate inactivates carbamoyl phosphate synthesis. There is a direct correlation between the rate of histidine modification and inactivation of catalytic activity, suggesting that at least one histidine residue is essential for enzyme catalysis (Figure 4). In an attempt to identify the specific histidine residues essential for enzyme activity, the three strictly conserved histidine residues (H243, H781, and H788) were individually mutated to asparagines. The choice of substitution with asparagine residues was made with the intention of removing the ability of the selected histidine residue to act as a general acid/base in the catalytic mechanism or as a potential ligand to an enzyme-bound metal ion without perturbing the structural integrity of the enzyme by making a sterically conservative replacement in which some of the hydrogen bonds may be maintained.

The addition of diethylpyrocarbonate to the wild-type CPS enzyme modifies histidine residues with equal labeling of the large and small subunits. Treatment of carbamoyl phosphate synthetase with high concentrations of DEPC ultimately inactivates all of the functions of the enzyme. The addition of substrates and/or the allosteric effector ornithine protects the enzyme from loss of catalytic activity, suggesting that the inactivation process is due to modification of a residue at or near the active site. The kinetics of inactivation were pseudo-first-order for the full forward reaction using either glutamine

or ammonia, the glutamine hydrolysis reaction of the small subunit, and the ATP synthesis reaction.

The bicarbonate-dependent ATPase activity of CPS is a measure of the hydrolysis of the putative carboxyphosphate intermediate in the absence of a nitrogen source. For the wild-type CPS enzyme, the rate of bicarbonate dependent ATP hydrolysis is an order of magnitude lower than the overall rate of carbamoyl phosphate synthesis. The chemical rate of formation of the carboxyphosphate intermediate is rapid (Raushel & Villafranca, 1979). Thus, the low net rate of hydrolysis must be due to the enzyme protection of the intermediate from the bulk H_2O or slow carboxyphosphate release from the active site. The rate of bicarbonate-dependent ATPase activity is modulated by allosteric interactions between the glutamine binding site on the small subunit and the site responsible for the bicarbonate activation on the large subunit (Khedouri et al., 1966). The addition of DEPC to CPS produces an initial increase in the bicarbonate-dependent ATPase activity followed by inactivation (Figure 1B), suggesting that there are at least two histidine residues involved. Similar increases in the bicarbonate-dependent ATPase activity have been observed with inhibitors that selectively inhibit the glutaminase activity of CPS by modification of the reactive Cys-269 residue located in the small subunit (Khedouri et al., 1966; Pinkus & Meister, 1972; Anderson et al., 1973; Trotta et al., 1974; Wellner & Meister, 1975; Anderson & Carlson, 1975; Kaseman, 1980). If the increase in the bicarbonate-dependent ATPase activity was due to the modification of the critical sulfhydryl residue within the small subunit of CPS, then the rate of increase in the ATPase activity would be concomitant with the loss of glutaminase activity. The second-order rate constant for DEPC activation of the bicarbonate-dependent ATPase activity is greater than 100 M^{-1} min^{-1}, while the rate constant for DEPC inactivation of the glutaminase activity is 7 M^{-1} min^{-1}, which is relatively slow. The order of magnitude difference between these two rate constants rules out the possibility that the net increase in activity is due to the modification of the critical Cys-269 residue of the small subunit and indicates that a histidine residue maybe participating in the transduction of allosteric effects between the two subunits or helps protect the enzyme-bound carboxyphosphate intermediate from attack by H_2O. An alternative explanation is that there is a relaxation of the tertiary structure of the enzyme upon modification by CPS which makes the enzyme-bound carboxyphosphate intermediate more susceptible to hydrolysis. The inactivation step would then be the inhibition of carboxyphosphate formation by modification of an essential residue. This explanation is supported by the substrate protection study shown in Figure 2B. The addition of substrates and/or ornithine protects the bicarbonate-dependent ATPase activity from inactivation, implying that the inactivation is due to modification of a residue at or near the active site, but the addition of substrates and/or effectors does not inhibit the activation of the ATPase activity.

Two putative domains have been proposed for the large subunit of CPS on the basis of the extensive similarity of the carboxyl and amino terminal halves of this protein (Lusty et al., 1983; Post et al., 1991). The amino-terminal half has been proposed to be primarily involved in the phosphorylation of bicarbonate, while the carboxyl-terminal half catalyzes the phosphorylation of the carbamate intermediate to form the ultimate product, carbamoyl phosphate. If these two putative domains are physically distinct, then the phosphorylation of bicarbonate would occur in the amino-terminal half, which can be analyzed by evaluation of the kinetic parameters for the bicarbonate-dependent ATPase activity, and the phosphorylation of carbamate would be located in the carboxyl-terminal half and could be measured by analysis of the ATP synthetase activity. The replacement of His-788 to an asparagine residue results in a protein that still maintains the ability to synthesize carbamoyl phosphate at a reasonable rate (Table II). Thus it is not essential for catalytic activity. However, the mutant enzyme has 10-fold reductions in both the bicarbonate-dependent ATPase and the ATP synthetase activities. This effect on both of the partial activities suggests that the two domains do not function independent of each other. Attempts to isolate catalytically active carboxyl-terminal or amino-terminal domains through genetic manipulation failed to produce truncated mutants with partial catalytic activities, indicating that these two domains cannot function independently of each other (Raushel et al., 1992). There is little effect on the K_m for ATP in the bicarbonate-dependent ATPase reaction, but there is a remarkable increase in the K_m for the ATP used in the synthesis of carbamoyl phosphate and a comparable increase in the K_m for ADP in the partial reverse reaction. This mutation thus affects the ATP binding site in the carboxyl-terminal half but not the ATP site in the amino-terminal half. The mutant enzyme has a V_{max}/K_m value for the bicarbonate-dependent ATPase activity one-tenth that of the wild-type protein and a V_{max}/K_m value for the ATP synthetase activity reduced by a factor of 100 compared with the wild-type enzyme, consistent with the proposition that this region is primarily involved in the phosphorylation of carbamate to form carbamoyl phosphate (Post et al., 1990).

The site-specific substitution of an asparagine residue for His-781 has resulted in a protein where the glutamine- and ammonia-dependent ATPase activities are uncoupled from the production of carbamoyl phosphate (Table II). The wild-type enzyme efficiently uses two molecules of ATP to produce one molecule of carbamoyl phosphate as illustrated by the 2:1 ratio of the nitrogen-dependent ATP hydrolysis rate to the rate of carbamoyl phosphate synthesis. However, with the H781N mutant enzyme, the ratio of glutamine-dependent ATP hydrolysis to carbamoyl phosphate formation is 20:1. The glutamine-dependent ATP hydrolysis activity is the same as with the wild-type enzyme, but the net rate of carbamoyl phosphate formation has been reduced by a factor of 10. The rate of ATP synthesis has also been diminished by a factor of 10. Since the bicarbonate-dependent ATPase and the glutamine-dependent ATPase activities of this mutant are comparable to those activities of the wild-type enzyme but both the rates of ATP and carbamoyl phosphate synthesis decreased by a factor of 10, it appears that this mutation primarily affects the efficiency of phosphorylation of carbamate. The mutation also diminished the allosteric effects of ornithine, which activates the enzyme by decreasing the K_m for ATP. In the absence of ornithine, the K_m values for ADP and ATP were found to be only slightly higher than the corresponding kinetic constants for the wild-type enzyme. The addition of 10 mM ornithine to the H781N mutant resulted in only a 2-fold reduction of the K_m values for ATP. A 10-fold reduction of the K_m parameters is observed for the wild-type enzyme. This histidine is located within the proposed carbamate phosphorylation domain. The order of magnitude decrease in both the ATP and carbamoyl phosphate synthesis reactions is consistent with this hypothesis. A similar 10-fold decrease in the rate of ATP and carbamoyl phosphate formation was observed in the G722I mutation (Post et al., 1990).

The replacement of His-243 with an asparagine residue resulted in a protein that was unable to utilize ammonia or glutamine to synthesize carbamoyl phosphate at a significant rate (<1/1500). The mutation produced essentially no effect on the bicarbonate-dependent ATPase, the ATP synthetase, and the glutaminase activities of CPS. There is no effect on either of the two partial reactions involving ATP, but this mutant protein cannot synthesize carbamoyl phosphate; therefore, this mutation results in a protein that is unable to form carbamate from carboxyphosphate and ammonia as illustrated in Scheme I.

In light of the identification of His-243 as a residue critical for enzyme catalysis of carbamoyl phosphate synthesis, several possible functions can be proposed for the involvement of this histidine in the mechanism of CPS. Analysis of the effects of the His-243 to asparagine mutation on the partial reactions suggests that this histidine is involved in the formation of the carbamate intermediate from ammonia and carboxyphosphate. There is no obvious requirement for general base catalysis in either of the phosphorylation reactions involving ATP. The function of the critical His-243 may be to act as a general base removing a proton from ammonia to facilitate its nucleophilic attack on the putative carboxyphosphate intermediate. Alternatively, this histidine residue may function as a ligand to a divalent metal such as magnesium. The divalent metal can function as an electrophilic catalyst by polarizing the carbonyl oxygen bond and stabilizing any negative charges formed during the formation of enzyme-bound intermediates.

The determination of the effects of diethylpyrocarbonate on the various activities of wild-type carbamoyl phosphate synthetase produced a set of parameters that could be used to further characterize the three histidine mutants. The effects of DEPC modification of the H788N and H781N mutant enzymes were very similar to the effects of DEPC on the wild-type enzyme (Table III). The inactivation of the ammonia-dependent and glutamine-dependent ATPase activities together with the inactivation of the ATP synthesis reaction were pseudo-first-order, while DEPC modification caused an initial increase in the bicarbonate-dependent ATPase activity followed by a slower inactivation process (Figure 3). The rates of inactivation were lower than the values observed for the wild-type enzyme. The effects of DEPC modification with the H243N mutant were all pseudo-first-order. The initial increase in the bicarbonate-dependent ATPase activity is no longer observed (Figure 3). It therefore seems plausible to suggest that His-243 is the residue that causes the increase in the rate of hydrolysis of ATP upon DEPC modification.

With the identification of His-243 as a residue critical for the formation of carbamate from carboxyphosphate, every step in the proposed enzyme-catalyzed pathway (Scheme I) has now been blocked by a site-specific mutation. The glutamine hydrolysis reaction catalyzed by the small subunit has been destroyed by mutations of Cys-269 to serine or glycine (Rubino et al., 1986) and the mutation of His-353 to asparagine (Miran et al., 1991). The formation of carboxyphosphate has been diminished by the mutations of Gly-176 and Gly-180 to isoleucine residues. The phosphorylation of carbamate to carbamoyl phosphate has been significantly reduced by the Gly-722 to isoleucine mutation (Post et al., 1990) and His-781 to asparagine.

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