Mutational Analysis of Carbamyl Phosphate Synthetase. Substitution of Glu841 Leads to Loss of Functional Coupling between the Two Catalytic Domains of the Synthetase Subunit†

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ABSTRACT: The synthetase subunit of *Escherichia coli* carbamyl phosphate synthetase has two catalytic nucleotide-binding domains, one involved in the activation of HCO_3^- and the second in phosphorylation of carbamate. Here we show that a Glu841 → Lys841 substitution in a putative ATP-binding domain located in the carboxyl half of the synthetase abolishes overall synthesis of carbamyl phosphate with either glutamine or NH_3 as the nitrogen source. Measurements of partial activities indicate that while HCO_3^--dependent ATP hydrolysis at saturating concentrations of substrate proceeds at higher than normal rates, ATP synthesis from ADP and carbamyl phosphate is nearly completely suppressed by the mutation. These results indicate Glu841 to be an essential residue for the phosphorylation of carbamate in the terminal step of the catalytic mechanism. The Lys841 substitution also affects the kinetic properties of the HCO_3^- activation site. Both k_{cat} and K_m for ATP increase 10-fold, while K_m for HCO_3^- is increased 100-fold. Significantly, NH_3 decreases rather than stimulates P_i release from ATP in the HCO_3^--dependent ATPase reaction. The increase in k_{cat} of the HCO_3^--dependent ATPase reaction, and an impaired ability of the Lys841 enzyme to catalyze the reaction of NH_3 with carboxy phosphate, strongly argues for interactions between the two catalytic ATP sites that couple the formation of enzyme-bound carbamate with its phosphorylation.

In *Escherichia coli*, carbamyl phosphate, an essential intermediate in the arginine and pyrimidine biosynthetic pathways, is synthesized by glutamine-dependent carbamyl phosphate synthetase (Pierard et al., 1965). Carbamyl phosphate is formed from glutamine (or NH_3) and HCO_3^- in a reaction requiring two molecules of ATP, only one of which donates its phosphoryl group to carbamyl phosphate (Anderson & Meister, 1965). Carbamyl phosphate synthetase is composed of two catalytic subunits (Matthews & Anderson, 1972; Trotta et al., 1971). The smaller glutaminase subunit hydrolyzes glutamine and transfers enzyme-bound NH_3 (eq 1 in Scheme I) to a site located on the synthetase subunit (Trotta et al., 1971, 1974b).

Scheme I

$$\text{E + glutamine + } H_2O \rightleftarrows \text{glutamate + E·NH}_3 \quad (1)$$

$$\text{E + ATP + } HCO_3^- \rightleftarrows \text{E·CO}_3PO_3^{3-} \text{ + ADP + } H^+ \quad (2)$$

$$\text{E·CO}_3PO_3^{3-} + NH_3 \rightleftarrows \text{E·CO}_2^-NH_2 + P_i \quad (3)$$

$$\text{E·CO}_2^-NH_2 + \text{ATP} \rightleftarrows \text{E + carbamyl-P + ADP} \quad (4)$$

The mechanism of carbamyl phosphate synthesis is known to proceed through carboxy phosphate (Anderson & Meister, 1965; Raushel & Villafranca, 1980; Wimmer et al., 1979). This intermediate is formed by an initial phosphorylation of bicarbonate resulting in an enzyme-bound carboxy phosphate (eq 2). Although the enzyme-bound carboxy phosphate is not easily detected because of its lability, the rate of its breakdown can be assayed by measuring HCO_3^--dependent ATPase (Anderson & Meister, 1966). In the second step, the bound carboxy phosphate undergoes a nucleophilic attack by NH_3, yielding enzyme-bound carbamate (eq 3). Carbamate is phosphorylated by the second molecule of ATP to form carbamyl phosphate in the terminal step (eq 4). The latter reaction, because its equilibrium favors ATP formation, can be assayed by measuring the synthesis of ATP from ADP and carbamyl phosphate (Anderson & Meister, 1966).

The above mechanism suggests the existence of at least three functionally distinct sites in the enzyme, each defined by its ability to bind and act on specific substrates and intermediates of the catalytic cycle. On the basis of primary sequence homology with other amidotransferases and in vitro mutagenesis studies, the region of the glutaminase with the active-site cysteine involved in glutamine hydrolysis has been identified (Nyunoya & Lusty, 1984; Rubino et al., 1986). The sequences of both glutamine- and ammonia-dependent carbamyl phosphate synthetases have also disclosed the existence of at least two nucleotide-binding sites in the synthetase component (Lusty et al., 1983). Amino acid substitutions in two of the proposed nucleotide-binding sites of *E. coli* carbamyl phosphate synthetase have confirmed that the initial activation of bicarbonate and the terminal phosphorylation of carbamate are functions of two separate domains, located in the amino- and carboxyl-terminal halves of the synthetase (Post et al., 1990). Even though some of the partial reactions have been successfully dissected by mutational (Post et al., 1990) as well chemical means, biochemical and, more recently, genetic data also point to the existence of reciprocal modulatory influences of reaction centers on the synthetase and the glutaminase components (Anderson & Carlson, 1975; Foley et al., 1971; Pinkus & Meister, 1972; Rubino et al., 1986, 1987). Such evidence implies either conformationally transmitted signals between active sites or, alternatively, that some of the active sites may physically overlap and share common residues.

To gain additional insights into the organization of the functional domains in carbamyl phosphate synthetase, we have undertaken a mutational analysis of the *E. coli* enzyme. The *carA* and *carB* genes of this bacterium code for the glutaminase and synthetase subunits, respectively (Mergeay et al.,

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Table I: *Escherichia coli* K12 Strains

strain	genotype	source or ref
K12	F^-	CSH collection[a]
P4X	Hfr, *met*B1, *rel*A1, *spo*T1	B. Bachmann[b]
C600	F^-, r_k^-, r_m^-, Δ*carB*-8	M. Crabeel[c]
RC50	F^-, *carA*-50, *thi*-1, *mal*A1, *xyl*-7, *rps*L135, λ^R, λ^-, *tsx*-273	Mergeay et al. (1974)
L623	as RC50, but *car*-A50 *zab-27*::*Tn10*	Guillou et al. (1989)
RR1	F^-, pro^-, *leu*B, *thi*-1, *lac*Y1, *hsd*R, *end*A, *rps*L20, *ara*-14, *gal*K2, *xyl*-5, *mtl*-1, *sup*E44, λ^-	Bolivar & Backman (1979)
L634	as RR1, but *car-50* *zab-27*::*Tn10*	$P1_{vir}$(L623) × RR1
L649	as RR1, but *carA-50*, Leu^+	$P1_{vir}$(RC50) × L634
L814	as L649, but *recA56*, *srlC-300*::*Tn10*	JC10240[d] × L649
L895	C600, but *carAB*$^+$ *zab*-21::*Tn*10	this laboratory
L683	as RR1, but *carB*-2117 *zab*-21::*Tn10*	this study
L705	as RR1, but *carB*-2117 *zab*-21::*Tn10*	this study
L829	C600(pLLK12, *carAB*$^+$)	this study
L871	L814(pLL43, *carB*-2117)	this study
L880	C600(pLL117, *carAB*-2117)	this study

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1974). In the present paper, we describe a mutation in *carB* resulting in a single amino acid substitution in the synthetase subunit that suppresses overall carbamyl phosphate synthesis by selectively blocking the terminal step of the catalytic mechanism (eq 4). Kinetic analysis of the mutant enzyme has uncovered a novel form of functional coupling between the sites involved in carboxy phosphate formation and in the conversion of carbamate to carbamyl phosphate. The identification of the residue responsible for the altered properties of the mutant synthetase subunit permits further refinements in the model of the carbamate phosphorylating domain in the carboxyl half of the protein.

Materials and Methods

Chemicals and Enzymes. Restriction endonucleases and other DNA-modifying enzymes were purchased from New England Biolabs. Deoxyoligonucleotides (17-mers) complementary to both strands of *carAB* were synthesized with an ABI Model 380A DNA synthesizer. [γ-^{32}P]ATP (3000 Ci/mmol), deoxyadenosine 5′-α-[^{35}S]thiotriphosphate (>1000 Ci/mmol), [U-^{14}C]ADP (550 mCi/mmol), and adenosine 5′-*O*-(3-[^{35}S]thiotriphosphate) (ATPγ^{35}S)[1] (650 Ci/mmol) were obtained from Amersham. ATP, ATPγS, ADP, NADP, and NAD were purchased from Pharmacia. Diammonium carbamyl phosphate (Sigma) was converted to the potassium salt, and its purity (98.5%) was determined in the ornithine transcarbamylase reaction. Other reagents were of the highest quality available commercially.

Bacterial Strains. The genotypes and sources of the mutant and wild-type *E. coli* strains used in this study are presented in Table I. The mutant bacteriophage $P1_{vir}$ was used in all transductional crosses, as described by Miller (1972). The *carB* mutants L683 and L705 were obtained by transducing a wild-type strain of *E. coli* to tetracycline resistance with phage $P1_{vir}$(L895) that had been mutagenized with hydroxylamine according to the procedure of Hong and Ames (1971). Tet^R transductants were screened for arginine and uracil auxotrophs on minimal medium.

Western Analysis and Sucrose Gradient Centrifugation. Western analysis and sucrose gradient centrifugation of carbamyl phosphate synthetase in extracts of *E. coli* cells carrying mutations in *carB* were performed as previously described (Guillou et al., 1989).

Recombinant DNA Procedures. Standard protocols were used for isolation of chromosomal and plasmid DNA, digestion of DNA by restriction endonucleases, isolation of DNA fragments, ligation, and transformation of *E. coli* (Maniatis et al., 1982).

Cloning of Mutant carAB Genes. The *carAB* genes from mutants L683 (*carB-43*) and L705 (*carB-117*) were cloned by screening recombinant plasmid libraries prepared from chromosomal DNA of each strain. The chromosomal DNAs were digested with *Bgl*II, and fragments ranging from 4.5 to 6.5 kb in size were isolated by preparative agarose gel electrophoresis and ligated to the *Bam*HI site of pUC18. *E. coli* transformants were screened for the presence of the mutant *carAB* genes by colony hybridization. The two libraries yielded plasmids pLL43 and pLL117 each with the complete mutant *carAB* operon on a 5.6-kb *Bgl*II fragment. A similar method was used to clone a 5.6-kb *Bgl*II fragment containing the wild-type *carAB* operon from an isogenic strain.

DNA Sequencing. Plasmid DNA was isolated by the method of Birnboim and Doly (1979) and was further purified by cesium chloride equilibrium centrifugation. The nucleotide sequences of the mutant and wild-type *carAB* genes were obtained by the chain termination method (Sanger et al., 1987) using double-stranded plasmid DNA templates (Zhang et al., 1988) and a series of synthetic 17-mer oligodeoxynucleotides as primers.

Purification of Carbamyl Phosphate Synthetase. Wild-type carbamyl phosphate synthetase (specific activity, 3.6 μmol min^{-1} mg^{-1}, at 37 °C) was purified from *E. coli* L829 essentially as described by Rubino (1987). Mutant carbamyl phosphate synthetases were purified by the same procedure, except that the proteins were chromatographed on MonoQ columns (Pharmacia). The mutant and wild-type enzymes fractionated identically on sieving and ion-exchange columns. Enzyme purity was estimated to be about 98% by Coomassie staining of the proteins after SDS–polyacrylamide gel electrophoresis (Laemmli, 1970). Both wild-type and mutant enzyme preparations were determined to be free of contaminating ATPases, and of biotin-dependent carboxylases. The purified enzymes were desalted either by centrifugation through columns of Sephadex G-50 equilibrated with 0.1 M Tris–acetate (pH 7.6)/0.2 M sodium acetate/1 mM EDTA or by dialysis against 0.12 M potassium phosphate, pH 7.6, containing 1 mM EDTA.

Protein Determination. Protein concentration was determined from the absorbance at 280 nm, by using an extinction coefficient of 0.686/mg of protein (Anderson, 1977; Rubino et al., 1986).

Enzyme Assays. Carbamyl phosphate synthesis was determined by measuring the amount of citrulline formed in a coupled assay with ornithine transcarbamylase. Reaction mixtures contained, in a final volume of 0.10 mL, 0.05 M Hepes/NaOH, pH 7.6, 100 mM KCl, 5 mM ATP, 20 mM magnesium chloride, 20 or 200 mM $NaHCO_3$, 20 mM glutamine, 5 mM ornithine, and 2 units of ornithine trans-

[1] Abbreviations: ATPγS, adenosine 5′-*O*-(3-thiotriphosphate); Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EDTA, ethylenediaminetetraacetic acid; CAD, multifunctional pyrimidine-specific enzyme comprising glutamine-dependent carbamyl phosphate synthetase, aspartate transcarbamylase, and dihydroorotase.

carbamylase. The reaction was initiated by addition of wild-type (1–2 μg) or mutant (10–50 μg) enzyme. After incubation for 10–30 min at 37 °C, the reaction was terminated by addition of 100 μL of 2 M perchloric acid; after standing at 0 °C for 10 min, the insoluble protein was removed by centrifugation. An aliquot (180 μL) of the supernatant solution was removed, and citrulline formed was quantitated as described by Rubino (1986). Two control tubes containing all of the components of the assay except for enzyme or ATP were included in each experiment.

HCO_3^--dependent ATPase activity was determined by measuring [^{32}P]P_i formed from [γ-^{32}P]ATP. Reaction mixtures contained, in a final volume of 0.20 mL, 0.05 M Hepes/NaOH, pH 7.6, 100 mM KCl, 5 mM [γ-^{32}P]ATP [(1–4) × 10^6 cpm/μmol], 20 mM magnesium chloride, and 20 or 200 mM $NaHCO_3$. The reaction was started by addition of 5–20 μg of desalted wild-type or mutant enzyme. After incubation for 10 min at 37 °C, the reaction was stopped by adding 1.0 mL of 0.1 N HCl containing acid-washed charcoal (50 mg/mL). After brief centrifugation, the radioactivity of an aliquot (0.70 mL) of the supernatant layer was determined by scintillation counting. Because [γ-^{32}P]ATP is hydrolyzed nonenzymatically during the incubation, the experimental values were corrected for phosphate released in duplicate reactions carried out without added enzyme.

ATP synthesis from carbamyl phosphate and ADP was measured at 25 °C by following the increase in absorbance at 340 nm of NADPH generated in a coupled assay with hexokinase and glucose-6-P dehydrogenase. Each cuvette contained, in a total volume of 0.5 mL, 50 mM Hepes/NaOH, pH 7.0, 100 mM KCl, 5 mM ADP, 20 mM magnesium chloride, 10 mM dipotassium carbamyl phosphate (pH 6.5), 50 mM glucose, 1 mM NADP, 20 units of hexokinase, 2 units of glucose-6-phosphate dehydrogenase, and 6 mM P_i (added with the enzyme). The reaction was initiated by addition of 20 μL of dialyzed wild-type (20 μg) or mutant (100 μg) enzyme.

Glutaminase activity was assayed in reaction mixtures containing 50 mM potassium phosphate, pH 7.6, and 20 mM glutamine, in a final volume of 0.20 mL. The reaction was started by addition of 25 μg of wild-type or mutant protein. A control without enzyme was included for each assay. After incubation for 60 min at 37 °C, the reaction was terminated by addition of 20 μL of 1 N HCl; after standing at 0 °C for 5 min, the solution was neutralized by addition of 25 μL of 1 M Tris. The amount of glutamate formed was measured from the fluorescence of NADH after reaction with glutamate dehydrogenase and NAD (Brent & Bergmeyer, 1974).

Kinetic Analysis. The steady-state kinetics of the HCO_3^--dependent ATPase reaction were measured at 25 °C by coupling of the reaction to pyruvate kinase and lactate dehydrogenase and monitoring the disappearance of NADH at 340 nm. Each cuvette contained, in a final volume of 0.5 mL, 50 mM Hepes/NaOH, pH 7.6, 100 mM KCl, 20 mM magnesium chloride, 0.2 mM NADH, 1 mM phosphoenolpyruvate, 20 μg each of pyruvate kinase and lactate dehydrogenase, and varying concentrations [in the range (0.5–5)K_m] of HCO_3^- at different fixed concentrations of MgATP. When the concentration of $NaHCO_3$ in the assay mixtures exceeded 20 mM, the pH of the buffers was adjusted so that the final pH of the reaction mixtures was pH 7.5. In the case of the wild-type enzyme where the K_m for HCO_3^- is low, the actual concentration of HCO_3^- in the assay was taken as the sum of the concentrations of the HCO_3^- added and the endogenous HCO_3^- (≤0.4 mM) present in the assay components.

When ADP was added as a product inhibitor, the rate of ATP hydrolysis was determined at 25 °C by measuring the production of [^{32}P]P_i from [^{32}P]ATP in duplicate assays with and without enzyme as described in the preceding section. Inhibition of ATP hydrolysis by ADP was determined with varying ATP and inhibitor concentrations at a fixed concentration (10 mM) of bicarbonate. Under these conditions, the kinetic data yielded a competitive pattern of inhibition, consistent with ADP being the last product released.

All of the kinetic data were analyzed according to the procedures described by Cleland (1979); constants and the standard error of their estimation were obtained by least-squares fit of initial rates to the appropriate equation, by using programs obtained from Dr. Ronald E. Viola (University of Akron, Akron, OH).

Binding Experiments. Binding of MgADP and MgATPγS by wild-type and mutant carbamyl phosphate synthetases was determined at 4 °C by equilibrium dialysis, using dialysis cells (100-μL volume) and the methods described by Anderson (1977). In measurements of MgADP binding, the protein compartment contained either wild-type (42.2 μM) or mutant (58 μM) carbamyl phosphate synthetase in a solution of 0.05 M Hepes/NaOH, 100 mM KCl, 10 mM potassium phosphate, 25 mM $MgCl_2$, and 0.2 mM EDTA, pH 7.4. The ligand compartment contained [^{14}C]ADP (~25 000 cpm) and varying concentrations (20–1200 μM) of ADP in the same buffer. In the MgATPγS-binding experiments, the protein compartment contained wild-type (78.7 μM) or Lys841 (75.9 μM) mutant enzyme in 0.05 M Hepes/NaOH, 100 mM KCl, 10 mM potassium phosphate, 50 mM $MgCl_2$, and 0.2 mM EDTA (pH 7.5). The ligand compartment contained ATPγ^{35}S (~40 000 cpm) and varying concentrations (0.05–2 mM) of ATPγS in the same buffer. The samples were dialyzed for 21 h at 4 °C. After equilibration, the radioactivity in the protein and ligand compartments was determined, and the amount of free and enzyme-bound ligand was calculated. All (>98%) of the radioactivity added initially was recovered at the end of the experiment. In control experiments, it was determined that ATP was not hydrolyzed to any significant extent during the course of the experiments. The binding constants n (number of sites) and K' (dissociation constant) were obtained after linear least-squares fitting of the binding data to the Scatchard equation.

Binding of UMP and ornithine was also determined in two different experiments by equilibrium dialysis. The wild-type or mutant enzyme (30 μM) in 50 mM Hepes/NaOH, 100 mM KCl, 10 mM potassium phosphate, and 0.2 mM EDTA, at pH 7.4, was dialyzed at 23 °C for 8 h against the same buffer containing [^{14}C]UMP (30 μM) or [^{14}C]ornithine (100 μM). The wild-type and Lys841 enzymes bound, respectively, 0.33 and 0.31 mol of [^{14}C]UMP/mol of monomer. Both enzymes bound the same amount of [^{14}C]ornithine, 0.18 ± 0.02 mol/mol of protein. When MgADP (10 mM) was included in the mixtures, binding increased to 0.34 ± 0.04 mol/mol of enzyme monomer. From these measurements at a single concentration of the allosteric ligand, the dissociation constants of UMP and of ornithine were calculated to be 22 and 154 μM, respectively. These values are comparable with the values of the dissociation constants previously reported by Anderson (1977), suggesting that the lysine substitution does not impair binding of these allosteric ligands.

Results

Phenotype of carB Mutants. To identify mutations in catalytically important domains of the synthetase subunit, *E. coli* mutants auxotrophic for arginine and uracil were screened

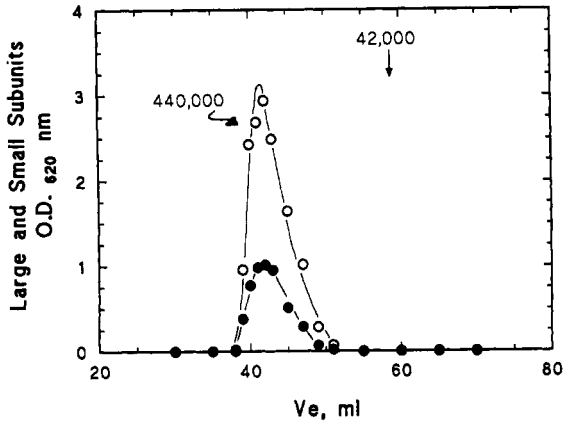

FIGURE 1: Elution profile of the α and β subunits of Lys841 carbamyl phosphate synthetase upon chromatography on Superose 12. The purified mutant enzyme (0.94 mg) in 0.5 mL of 50 mM Hepes/NaOH, pH 7.5, containing 0.1 M KCl, 10 mM potassium phosphate, and 0.5 mM EDTA was applied to a column (1.6 × 50 cm) of Superose 12 equilibrated with the same buffer. The column was developed at 4 °C at a flow rate of 1 mL/min, and 1-mL fractions were collected. Samples (15 μL) of each fraction were dissociated with SDS, and the subunits were separated in 9% polyacrylamide gels and stained with Coomassie Blue. The optical densities at 620 nm of the stained bands corresponding to the (●) glutaminase and (○) synthetase subunits were quantitated by densitometry and are expressed in arbitrary units. The protein concentration of the peak fraction was 0.103 mg/mL. The molecular weight of the carbamyl phosphate synthetase complex indicated in the figure was estimated from its elution volume (V_e) relative to standards of known molecular weight. The arrow indicates the elution volume corresponding to the molecular weight of the glutaminase subunit.

genetically and biochemically. Mutants with alleles mapping to the *carB* gene were analyzed as follows. Total cell extracts were first tested immunologically by the Western technique to exclude mutants with truncations or deletions in the synthetase subunit. Strains displaying normal concentrations of full-length synthetase and glutaminase subunits were further ascertained to have an $\alpha\beta$ carbamyl phosphate synthetase complex by sucrose gradient centrifugation.

Two independent isolates, L683 and L705, had carbamyl phosphate synthetase with physical properties indistinguishable from those of the wild-type enzyme. Since both strains were determined to have the identical mutation, only the *carB*-117 mutation of L705 will be discussed. The results of analytical gel chromatography of the enzyme in L705, illustrated in Figure 1, indicate that both subunits of carbamyl phosphate synthetase coelute in a single peak. A similar elution pattern was observed with the wild-type carbamyl phosphate synthetase (data not shown). An average molecular weight of 440 000 is estimated for both wild-type and mutant enzymes. The salt and buffer conditions used for the gel filtration cause the enzyme to self-associate, and the estimated molecular weight is probably an average of an equilibrium mixture of monomer–dimer–tetramer. The two enzymes also have identical sedimentation properties in sucrose gradients. Both subunits cosediment as a single protein peak with an apparent molecular weight of about 160 000. The salt conditions in this analysis were chosen to minimize self-association of the enzyme and explain the lower molecular weight, which corresponds to the monomer. Neither method gave any indication of subunit dissociation nor was there any suggestion of a protein species with an elution volume (V_e) corresponding to the glutaminase subunit whose expected position in the elution profile is shown by the arrow in Figure 1. The *carB*-117 mutation, therefore, does not affect the ability of the synthetase and glutaminase subunits to form a stable complex.

Table II: Specific Activities of Wild-Type and Mutant Carbamyl Phosphate Synthetases in the Overall and Partial Reactions[a]

	sp act. (μmol min^{-1} mg^{-1})	
enzymatic act.	wild-type enzyme	Lys841 mutant enzyme
glutamine-dependent carbamyl phosphate synthesis	2.60	0
NH_3-dependent carbamyl phosphate synthesis	1.81	0
glutaminase	0.025	0.020
HCO_3^--dependent ATPase		
20 mM HCO_3^-	0.11	0.25
200 mM HCO_3^-	0.12	0.80
ATP synthesis from ADP and carbamyl phosphate	0.62	0.01

[a] The assay conditions used to determine each of the enzyme activities are described under Materials and Methods.

The stability and normal physical behavior of the mutant enzyme coupled with its failure to catalyze any detectable synthesis of carbamyl phosphate with either glutamine or NH_3 as nitrogen donor are consistent with a mutation in a catalytic domain. In addition to the preliminary mapping data, several other lines of evidence also suggest a mutation in the synthetase component. L705 is unable to grow on minimal medium supplemented with 100 mM NH_4Cl. This phenotype is most easily explained by a defect in the synthetase. *E. coli* mutants with a complete deletion of *carA*, for example, can synthesize sufficient carbamyl phosphate to support growth when supplied with high concentrations of ammonium ion in the medium. More significantly, transformation of the mutant with a specific region of *carB* yielded wild-type recombinants (see section below). Two other observations are worthy of note. The fact that the *carB* deletion strain C600 transformed with the mutant gene on high-copy plasmids fails to grow on minimal medium containing high NH_4^+ indicates a stringent mutation. Secondly, L705 has a reversion rate (1×10^{-8}) consistent with a single point mutation.

Sequence Analysis of carB-43 and carB-117 Mutations. Complementation of the mutant with a set of fragments of wild-type *carAB* on multicopy plasmids allowed the *carB*-43 and *carB*-117 mutations to be mapped to a 664 bp region between the *Sph*I and *Sma*I sites of *carB*. The nucleotide sequences of this region of the mutant and the isogenic wild-type genes were obtained by the chain termination method (Sanger et al., 1987) with double-stranded DNA serving as templates for a set of oligodeoxynucleotide (17-mer) primers complementary to the sense strand of *carB*. The sequences indicate both mutants to have the identical G → A transition at nucleotide 2521 of the *carB* reading frame. This single base pair substitution (GC for AT) changes the normal GAA codon of Glu841 to an AAA codon for lysine.

Properties of Mutant Carbamyl Phosphate Synthetase. The rates of overall carbamyl phosphate synthesis and of the partial reactions reflecting different steps of the mechanism are summarized in Table II for the wild-type and Lys841 enzymes. The mutant enzyme synthesizes carbamyl phosphate with a specific activity corresponding to less than 0.01% of wild type. Even though the glutaminase activity of the mutant enzyme appears to be normal, a more detailed kinetic analysis indicates this reaction to be affected by the Lys841 substitution as well (C. J. Lusty and M. Liao, unpublished results).

The enzymatic defects of the mutant enzyme were further characterized by assays of bicarbonate-dependent ATPase and transfer of the phosphoryl group from carbamyl phosphate to ADP, a reversal of the last step of the mechanism. The replacement of Glu841 with Lys has no apparent effect on the

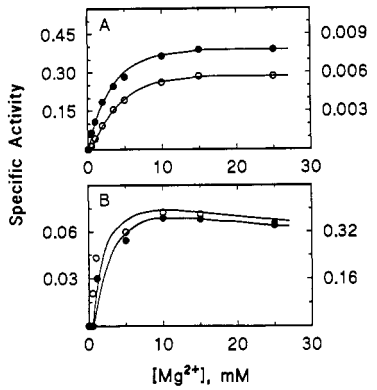

FIGURE 2: Dependence of ATP synthesis and HCO_3^--dependent ATPase on Mg^{2+} concentration. In panel A, formation of ATP from carbamyl phosphate and ADP was measured at 25 °C, at pH 7.0, in reaction mixtures (0.5 mL) containing 50 mM Hepes/NaOH, pH 7.0, 50 mM KCl, 0.1 mM ADP, 30 mM potassium carbamyl phosphate, $MgCl_2$ as indicated, and either 15 μg of (○) wild-type or 112 μg of (●) Lys841 enzyme. In panel B, ATPase activity was assayed as ADP formed at 25 °C at pH 7.5 in a coupled assay with pyruvate kinase and lactate dehydrogenase. Each cuvette contained, in a final volume of 0.5 mL, 50 mM Hepes/NaOH, pH 6.8, 100 mM KCl, 100 mM $NaHCO_3$, 0.1 mM ATP, $MgCl_2$ as indicated, and either 61.2 μg of (○) wild-type or 11 μg of (●) Lys841 enzyme. Left ordinate, specific activity of wild type; right ordinate, specific activity of Lys841 carbamyl phosphate synthetase.

Mg^{2+} concentration dependence in either partial reaction. Both wild-type and mutant enzymes are saturated at the same concentration of the cation as shown by the normalized specific activities of the two enzymes in Figure 2. The HCO_3^--dependent ATPase activity is 2.5 times higher in the mutant than the wild-type carbamyl phosphate synthetase (Table II). A further 3-fold enhancement of this rate is seen in the mutant but not the wild-type complex at higher concentrations of HCO_3^-. The Lys841 substitution has no effect on the pH dependence of the ATPase reaction which exhibits a broad optimum around pH 7.5.

In contrast to the nearly normal rate of HCO_3^--dependent ATPase in the mutant, synthesis of ATP from ADP and carbamyl phosphate occurs at only 1.6% of the wild-type rate. The differential loss of ATP synthesis but not ATPase activity suggests that the mutation affects predominantly the site involved in the phosphorylation of carbamate. The increased rate of ATP hydrolysis catalyzed by the Lys841 enzyme at the high concentrations of substrate, however, also indicates a second important effect of the mutation on the bicarbonate activation site in the amino-terminal half of the synthetase.

Binding Interactions of ADP and ATPγS. Binding of MgADP and of the analogue MgATPγS to carbamyl phosphate synthetase was determined from equilibrium dialysis experiments. The affinities of wild-type and Lys841 enzymes for each nucleotide were calculated from binding saturation curves obtained by measuring protein-bound ligand with different concentrations of ligand in the equilibration buffer. The apparent dissociation constants of the MgADP and MgATPγS complexes derived from the titrations are summarized in Table III. The dissociation constants for both MgADP and MgATPγS are increased only by a factor of about 1.5–2. Scatchard plots were linear over the range of ligand concentrations used, suggesting that the dissociation constants for each nucleotide are similar at the two binding sites in the complex. The dissociation constant of ADP at the bicarbonate activation site was also determined kinetically as K_i by assaying inhibition of the ATPase by ADP. The dissociation constants for ADP in the wild-type and mutant enzymes estimated by this means are almost identical to those determined by equilibrium dialysis (cf. Table V below).

Table III: Binding of MgADP and MgATPγS[a]

binary complex	no. of sites, n_{app}	dissociation constant, K_i, 4 °C (mM)	ΔG_b (kcal mol^{-1})
Glu841 enzyme·MgADP	1.8 ± 0.1	0.11 ± 0.01	
Lys841 enzyme·MgADP	1.9 ± 0.1	0.23 ± 0.02	0.4
Glu841 enzyme·MgATPγS	1.5 ± 0.1	0.42 ± 0.06	
Lys841 enzyme·MgATPγS	1.5 ± 0.1	0.64 ± 0.10	0.2

[a] The number of binding sites (n_{app}) and the dissociation constants (K_i) at 4 °C were determined as described under Materials and Methods. Changes in the binding energies (ΔG_b) resulting from the amino acid replacement were calculated from the relationship $\Delta G = RT \ln [K_{i(mutant)}/K_{i(wild\text{-}type)}]$ (Fersht, 1985).

Table IV: Kinetic Parameters of Wild-Type and Lys841 Carbamyl Phosphate Synthetases in the ATP Synthesis Reaction[a]

kinetic parameter	wild-type enzyme	Lys841 enzyme
k_{cat} (s^{-1})	1.60	0.05
K_m for ADP (μM)	30	32
k_{cat}/K_m for ADP (s^{-1} M^{-1})	5.3×10^4	1700
K_m for ADP (5 mM Orn) (μM)	6	25
K_m for carbamyl-P (mM)	0.90	10.8
k_{cat}/K_m for carbamyl-P (s^{-1} M^{-1})	1780	4.5

[a] Reaction conditions: 25 °C, pH 7.0, 50 mM Hepes/NaOH, 100 mM KCl, 15 mM free Mg^{2+}, 30 mM potassium carbamyl phosphate (when MgADP was the varied substrate) or 5 mM MgADP (when carbamyl-P was the varied substrate), and 25 μg of wild-type or 100 μg of Lys841 carbamyl phosphate synthetase. Kinetic constants were obtained after fitting of the initial velocity data to the Michaelis–Menten equation.

The Scatchard plot for the mutant complex extrapolated to about 1.5 mol of adenine nucleotide bound per mole of monomer. This value is the same as that seen in the wild-type complex. The difference in the binding constants corresponds to a decrease in the mutant enzyme of some 0.4 and 0.2 kcal mol^{-1} in the binding energies of MgADP and MgATPγS, respectively. The results of these experiments argue against a direct involvement of Glu841 in nucleotide binding. The marginal effect of the mutation on the dissociation constants and the total amount of nucleotide bound suggests that replacement of Glu841 with lysine is unlikely to cause a serious perturbation in the conformation of the two adenine nucleotide binding domains. Measurements of ornithine and UMP binding also fail to reveal any major effects of Lys841 on the interaction of these allosteric effectors with the enzyme.

Kinetic Analysis of ATP Synthesis. As reported in Table II, a survey of the three easily measurable partial activities of carbamyl phosphate synthetase indicates a differential inactivation of the terminal ATP-dependent transphosphorylation reaction as a result of the Lys841 substitution. This reaction has been proposed to be catalyzed at a nucleotide-binding site located in the carboxyl half of the synthetase subunit (Post et al., 1990). To better understand the mechanistic basis for the lesion, a more detailed analysis of the catalytic properties of the mutant enzyme was carried out.

Because of its low ATP synthesis activity, the effect on the initial velocity of varying the concentration of either ADP or carbamyl phosphate at fixed but nonsaturating levels of the second substrate could not be determined accurately for the Lys841 enzyme. It was possible, however, to determine the steady-state constants k_{cat} (turnover number) and K_m by varying each substrate individually at saturating concentrations of the other. These values for the mutant and normal complexes are compared in Table IV. The turnover number is at least 30 times lower in the mutant enzyme. K_m for ADP, on the other hand, is essentially unaffected. The 31-fold

reduction in the apparent second-order association constant k_{cat}/K_m for the binding of ADP is due almost entirely to the decrease in the rate constants for catalysis. These kinetic data indicate that the amino acid replacement does not strongly influence the binding interactions of ADP, a conclusion not inconsistent with the results of the equilibrium dialysis experiments which also exclude any compromising effect of the mutation on ADP binding.

A telling difference between the two enzymes with respect to the reverse reaction is the 12-fold higher K_m for carbamyl phosphate in the mutant. Combined with the 30 times lower k_{cat}, it reduces the overall catalytic efficiency by about 400-fold. This dramatic decrease in k_{cat}/K_m explains, in part, the virtually complete inhibition of carbamyl phosphate synthesis. The 30 times lower turnover even in the presence of saturating carbamyl phosphate indicates that the principal effect of the Lys841 substitution is on the catalytic conversion of the enzyme-bound intermediates to carbamate and ATP in the backward reaction and inferentially to carbamyl phosphate and ADP in the forward synthetic direction. The higher K_m and the decrease in k_{cat}/K_m also suggest a second effect of the amino acid replacement on binding of carbamyl phosphate. A consequence of a lower affinity for carbamyl phosphate might be a reduced affinity of the enzyme for carbamate as well, since the latter intermediate presumably binds to the same site.

Two other findings are of interest. Ornithine is a well-documented allosteric activator of *E. coli* carbamylphosphate synthetase that acts by decreasing the K_m for ADP (Trotta et al., 1974a). The K_m of the wild-type enzyme measured in the presence of ornithine is lowered 5-fold (Table IV). Although addition of ornithine causes a decrease in the K_m for ADP in the mutant, the change is small compared to the normal complex. Also significant is the absence of an effect on the reaction rate by the allosteric inhibitor UMP under conditions where the wild-type enzyme is inhibited by more than 95% (data not shown). These observations indicate that the changes in the active site induced by these allosteric molecules are no longer operative in the mutant enzyme.[2]

Coupling between HCO_3^- Activation and Carbamate Phosphorylation. The results of the ATPase assays, while confirming the principal effect of the Glu841 → Lys841 substitution to be on phosphorylation of carbamate, also hinted at some perturbation of the catalytic site involved in carboxy phosphate synthesis previously mapped to the amino-terminal half of the synthetase subunit. This is evidenced in the faster rate of HCO_3^--dependent ATP hydrolysis, particularly at high concentrations of the anion (Table II).

This partial reaction was examined in more detail to elucidate how the mutation in the carboxyl domain affects the first step in carbamyl phosphate synthesis catalyzed in the amino half of the synthetase. The rates of ATP hydrolysis, a measure of carboxy phosphate synthesis, were determined as a function of ATP and HCO_3^- concentrations in the assay. The initial velocities measured with the mutant complex in the presence of different concentrations of each substrate fit (with a value of σ equal to 0.016) the equation for the sequential mechanism:

$$v = VAB/K_AB + K_BA + AB + K_{i_A}K_B \qquad (5)$$

[2] Binding of either UMP or ornithine was measured at a single concentration of each effector by equilibrium dialysis. The calculated K_d values of 22 and 150 μM, respectively, for UMP and ornithine are similar to those reported previously for wild-type enzyme. This would suggest that the lack of effect on the kinetics of the enzyme is not likely to be due to loss of binding of either UMP or ornithine.

Table V: Kinetic Parameters in the HCO_3^--Dependent ATPase Reaction[a]

kinetic parameter	wild-type enzyme	Lys841 enzyme
k_{cat} (s^{-1})	0.23 ± 0.001	2.95 ± 0.02
K_m for ATP (μM)	10 ± 0.1	90 ± 3
k_{cat}/K_m for ATP (s^{-1} M^{-1})	2.3×10^4	3.3×10^4
K_{i_A} for ATP (μM)	97 ± 1	158 ± 6
K_m for HCO_3^- (mM)	0.64 ± 0.01	59 ± 1
k_{cat}/K_m for HCO_3^- (s^{-1} M^{-1})	360	50
K_{i_B} for HCO_3^- (mM)	5.9 ± 0.1	103 ± 6
K_i for ADP (mM^{-1})	0.12 ± 0.01	0.20 ± 0.03
E_a (kcal mol^{-1})	17.3 ± 0.1	14.2 ± 0.2

[a] Reaction conditions: 25 °C, pH 7.5, 0.05 M Hepes/NaOH, 100 mM KCl, 20 mM $MgCl_2$, varying concentrations [(0.5–5)K_m] of $NaHCO_3$, and different fixed concentrations [(0.5–4)K_m] of MgATP. The K_i for ADP was determined as described under Materials and Methods.

suggesting that the Lys841 enzyme catalyzes HCO_3^--dependent ATP hydrolysis according to the same kinetic mechanism as wild type. The values of the kinetic constants obtained for the two enzymes are summarized in Table V. The turnover number for the ATPase reaction is 13 times greater in the mutant enzyme. K_m for ATP is 10-fold higher. The ratio k_{cat}/K_m, equivalent to the apparent second-order rate constant (k_{1A}) for addition of this substrate, is increased by 1.5-fold. A similar increase in the substrate inhibition constant K_{i_A} agrees with the binding data obtained with the ATP analogue ATPγS (Table III). With K_{i_A} and k_{1A}, we can calculate dissociation rates of 2.1 and 5.2 s^{-1}. These values indicate that the mutation results in only a moderate increase in the rate constant for dissociation of ATP. In contrast, K_m for HCO_3^- is increased by 2 orders of magnitude. The resultant 7-fold lowering of k_{cat}/K_{mB} associated with the large increase in K_{i_B} indicates that the Lys841 substitution results in an enzyme that not only is less competent in binding carbamyl phosphate but also has an equally reduced affinity for HCO_3^-. The increased K_m for HCO_3^- implies that the lower binding is due to an increased dissociation of the enzyme–substrate complex E·ATP·HCO_3^- as well as the enzyme-bound carboxy phosphate·ADP intermediate.

A reasonable explanation for the observed increases in k_{cat} and K_m for HCO_3^- and, to a lesser extent, in K_m for ATP and in K_i for ADP is a destabilization of the Michaelis complexes E·ATP and E·ATP·HCO_3^- and of the ground-state complexes E·carboxy phosphate·ADP and E·ADP. Some degree of stabilization of the E·carboxy phosphate·ADP transition state may also contribute to the increase in k_{cat}. The changes in substrate binding energy due to the replacement of Glu841 can be estimated by comparing the relative changes in the dissociation constants K_{i_A} and K_{i_B} of the mutant and wild-type enzymes using the equation noted in Table III. The apparent decreases in the binding energies ΔG_b of ATP and of HCO_3^- are calculated as 0.2 and 1.7 kcal mol^{-1}, respectively. The lower binding energies of ATP and HCO_3^- in the enzyme–substrate complex, by contributing a decrease of about 1.9 kcal mol^{-1} in the activation energy for k_{cat}, could account to a large extent for the higher reaction rate at saturating concentrations of substrate. A decrease in the activation energy for k_{cat} was substantiated experimentally when the mutant and wild-type enzymes were compared by measuring their Arrhenius energies of activation (E_a). Over the temperature range 38–14 °C used in the experiments, Arrhenius plots of log V versus 1/K were linear with calculated slopes of −3798 ± 30 K^{-1} for wild-type enzyme and −3118 ± 54 K^{-1} for the mutant enzyme. From the derived values of E_a (Table V), we calculate the enthalpy of activation $\Delta H^{\ddagger}$(25 °C) for k_{cat} to be lower by about 3.1 kcal

Table VI: Effect of NH_3 on HCO_3^--Dependent P_i Release from ATP[a]

components added	[^{32}P]P_i released (μmol min^{-1} mg^{-1}) wild-type enzyme	Lys841 enzyme
$NaHCO_3$ (20 mM)	0.10	0.20
$NaHCO_3$ (20 mM), NH_4Cl (300 mM)	0.97	0.11
$NaHCO_3$ (200 mM)	0.12	0.72
$NaHCO_3$ (200 mM), NH_4Cl (300 mM)	1.02	0.42

[a] Reaction conditions: 37 °C, pH 7.6, 50 mM Hepes/NaOH, 100 mM KCl, 4 mM [γ-^{32}P]ATP (4×10^6 cpm/μmol), 20 mM $MgCl_2$, and $NaHCO_3$ and NH_4Cl as indicated.

mol^{-1} as a result of the amino acid substitution.

Another novel property of the mutant carbamyl phosphate synthetase is the lack of stimulation of HCO_3^--dependent ATPase by NH_4^+. Normally, this activity is 10 times higher in the presence of ammonium ion (Table VI). In contrast, addition of NH_4^+ to the Lys841 enzyme reduces the ATPase activity. Since P_i release from ATP in the presence of ammonia is a measure of carboxy phosphate conversion to carbamate (eq 3), the failure of the cation to stimulate this partial reaction suggests that the Glu841 → Lys substitution has a further effect on formation of carbamate.

Discussion

The synthesis of carbamyl phosphate proceeds consecutively through two different enzyme-bound intermediates, carboxy phosphate and carbamate (Anderson & Meister, 1966). The mechanism by which the intermediates and carbamyl phosphate are formed has been outlined, but the nature of the catalytic site or sites responsible for catalyzing the individual steps is not well understood. Recently, site-directed mutagenesis has been successfully used to selectively inactivate two different domains in the synthetase subunit, one involved in activation of HCO_3^- and the other in phosphorylation of carbamate to carbamyl phosphate (Post et al., 1990). While this functional dissection has helped to confirm earlier speculations about the locations of active-site domains in the amino and carboxyl halves of the synthetase component in the *E. coli* glutamine-dependent complex (Lusty et al., 1983), the topology of the two sites vis-à-vis each other is still unclear. Are the sites physically separated, necessitating a transfer of intermediates through a conduit similar to the indole channel spanning the α and β subunits of tryptophan synthase (Hyde et al., 1988), or are they part of a single complex structure?

Mutational analysis, particularly if the lesions in the enzyme are confined to specific steps of the overall reaction, not only can provide valid answers to questions of mechanism but also can clarify aspects of structure–function relationships. The *E. coli* mutant L705 reported in this paper is one of a large group of strains that were judged to have mutations in *carB*, based on their growth phenotypes and on genetic mapping data. L705(*carB* allele 2117) was selected for more detailed kinetic studies because it satisfied criteria generally recognized to indicate a mutation in an active site. Even though the mutation abolishes virtually all detectable carbamyl phosphate synthesis activity, it has no effect on the in vivo stability of the protein. Nor does it discernibly change the $\alpha\beta$ structure and physical properties of the complex. Only 10 other mutants out of more than 250 tested exhibit these properties.

The *carB*-2117 allele of L705 was confirmed by sequencing to be in the synthetase component of the complex. The single base change in the sense strand from G → A at nucleotide 2521 of the gene results in a substitution of a lysine for glutamic acid at residue 841 of the synthetase component. The amino acid change occurs within a highly conserved sequence context previously proposed to be an adenine-binding domain (Lusty et al., 1983) and more recently shown to function in phosphorylation of enzyme-bound carbamate (Post et al., 1990). These authors reported that substitution of Gly722 with isoleucine reduced phosphorylation of ADP by carbamyl phosphate but did not affect the ATPase activity of the enzyme. Glu841 located 120 amino acids away from Gly722 in the glycine-rich sequence of the adenine nucleotide binding fold is probably part of the same active-site domain. Glu841 is an invariant residue situated at the end of a cluster of hydrophobic amino acids (VYLIE) present in this domain in all carbamyl phosphate synthetases examined to date. The importance of the glutamic acid in catalysis is attested to by its presence in homologous domains of other enzymes such as pyruvate carboxylase, acetyl-CoA carboxylase, and carbamate kinase (Table VII). These enzymes can synthesize ATP from ADP and carbamyl phosphate (Attwood & Keech, 1984; Baur et al., 1989; Polakis et al., 1972) and in at least two cases (pyruvate and acetyl-CoA carboxylase) also catalyze an ATP-dependent activation of HCO_3^- to form enzyme-bound carboxy phosphate (Attwood & Keech, 1984; Polakis et al., 1972). Each of these proteins has an active-site domain ho-

Table VII: Sequence Context of Glu841 in Carbamyl Phosphate Synthetases and Homologous Carboxylases[a]

enzyme/organism		residues	amino acid sequence (Glu841 ▼)	ref
CPS	*E.coli*	827-860	N V Q F A V K N N E - V Y L I **E** V N P R A A R T V P F V S K A T G V P	*b*
CPS	*M.barkeri*		N I Q M A E K G G K - V Y V L **E** A N P R S S R T I P F V S K A V G I P	*c*
CPS-P	*B.subtilis*	818-851	N I Q F V L S Q G E - V Y Y V L **E** V N P R S S R T V P F L S K I T G I P	*d*
CPS-A	Yeast	846-880	N M Q I I K D G E H T L K V I **E** C N I R A S R S F P F V S K V L G V N	*e*
CPS-P	Yeast	1246-1279	N I Q F I A K D N E - I K V I **E** C N V R A S R S F P F I S K V V G V N	*f*
CPS	*D.melan.*	1219-1252	N M Q L I A K N N E - L K V I **E** C N V R V S R S F P F V S K T L D H D	*g*
CPSI	Rat	1241-1274	N V Q F L V K G N D - V L V I **E** C N L R A S R S F P F V S K T L G V D	*h*
CAD	Hamster	1045-1078	N L Q L I A K D D Q - L K V I **E** C N V R V S R S F P F V S K T L G V D	*i*
CK	*P.aeruginosa*	278-310	K D A V I G S L A D I V A I T **E** G K A G T R V S T R K A G I E Y R *	*j*
PC	Yeast	293-327	T A E F L V D N Q N R H Y F I **E** I N P R I Q V E H T I T E E I T G I D	*k*
ACAC	Chicken	422-456	T V E Y L Y S Q D G S F Y F L **E** L N P R L Q V E H P C T E M V A D V N	*l*

[a] The sequences are aligned to give maximal amino acid identities. *Gaps* represent postulated deletions/insertions. Invariant amino acid residues are *boxed*. Legend abbreviations: CPS, carbamyl phosphate synthetase; CPS-A, arginine-specific carbamyl phosphate synthetase; CPS-P, pyrimidine-specific carbamyl phosphate synthetase; CAD, carbamyl phosphate synthetase–aspartate transcarbamylase–dihydroorotase; PC, pyruvate carboxylase; ACAC, acetyl-CoA carboxylase; CK, carbamate kinase. [b] Nyunoya & Lusty (1983). [c] Morris & Reeve (1988). [d] C. Quinn and R. L. Switzer (personal communication). [e] Lusty et al. (1983). [f] Souciet et al. (1987). [g] Freund & Jarry (1987). [h] Nyunoya et al. (1985). [i] Simmer et al. (1990). [j] Baur et al. (1989). [k] Lim et al. (1988). [l] Takai et al. (1988).

mologous to the carbamate phosphorylation domain of carbamyl phosphate synthetase with a conserved glutamic acid proximal to a stretch of hydrophobic amino acids. Also significant is that 5′-[*p*-(fluorosulfonyl)benzoyl]adenosine reacts with *E. coli* (Boettcher & Meister, 1980) and rat liver (Powers et al., 1983) carbamyl phosphate synthetases, and the carbamyl phosphate synthetase component of hamster CAD (Kim et al., 1991), causing complete loss of activity in each case. In studies of CAD (Kim et al., 1991), two of the tryptic peptides labeled by the ATP analogue originate from the carboxyl half of the carbamyl phosphate synthetase component. One of the peptides is an eight amino acid long fragment with the sequence VIENCRX. The glutamic acid residue in this peptide corresponds to Glu841 in the homologous *E. coli* carbamyl phosphate synthetase (cf. Table VII).

The aforementioned evidence indicates Glu841 to be an essential residue in a catalytic domain of the enzyme. This conclusion is also supported by the catalytic properties of the mutant enzyme. The replacement Glu841 → Lys confers a 10000-fold lower rate of carbamyl phosphate synthesis. Since the assay is capable of detecting a single turnover of the enzyme, this corresponds to a virtually complete absence of activity. The dramatic loss of enzymatic competence in overall carbamyl phosphate synthesis can be rationalized in terms of distinct effects on both carbamate phosphorylation and HCO_3^- activation. Qualitatively and quantitatively, the more damaging effect of the mutation is seen in the new kinetic parameters describing the terminal part of the catalytic cycle. Phosphorylation of ADP by carbamyl phosphate, a partial reaction reflecting reversal of the last step of the mechanism, is reduced by more than 2 orders of magnitude. The lowered k_{cat} and higher K_m for carbamyl phosphate in this reaction suggest less efficient conversion of the transition-state intermediate E·ADP·carbamyl phosphate to E·ATP·carbamate.

Among the possible functions of Glu841 are (1) binding of ATP through Mg^{2+}, (2) formation of a phosphorylated enzyme intermediate, (3) extraction of a proton from carbamic acid in preparation for a nucleophilic attack on ATP, and (4) provision of an electronically favorable local environment for the removal of a highly negatively charged product. Of these, only phosphorylation of the glutamic acid residue can be discounted in view of the absence of any evidence for the participation of a phosphorylated intermediate in the catalytic mechanism (Meister, 1989). The involvement of glutamic acid in adenine nucleotide binding through Mg^{2+}, either directly or through a water molecule, seems unlikely in view of the ability of the mutant enzyme to bind ADP with an affinity similar to wild type. At present, we favor the notion that Glu841 may participate in catalysis by acting as a proton-withdrawing (forward reaction) or -donating group (reverse reaction). The large decrease in the k_{cat} of the mutant enzyme for overall carbamyl phosphate synthesis is consistent with a role of Glu841 as a general base or acid. A similar role of aspartic acid residues has been implicated in phosphoryl group transfer catalyzed by hexokinase (Viola & Cleland, 1978) and phosphofructokinase (Hellinga & Evans, 1987; Shirakihara & Evans, 1988). The hydrolysis of GTP promoted by *ras* p21 also occurs by a general base type of mechanism dependent on a glutamine residue (Pai et al., 1990).

A second factor contributing to the absence of overall carbamyl phosphate synthesis is the change in the kinetic parameters of HCO_3^- activation. At saturating concentrations of HCO_3^-, the K_m for ATP in this reaction is increased 10-fold; however, the magnitude of the increase in K_m corresponds almost entirely to the increase in k_{cat}, indicating that the lysine substitution has little effect on the rate of association of ATP, but increases one or more of the rate constants for ATP and bicarbonate conversion to ADP and carboxy phosphate. These results tend to argue against any important role of Glu841 in binding of ATP in the initial activation step or in any direct involvement of Glu841 in the formation of carboxy phosphate. Despite the 10 times higher HCO_3^--dependent ATPase activity assayed in the presence of saturating concentrations of both substrates, the overall catalytic efficiency of the bicarbonate activation site is 7-fold lower in the mutant enzyme due to the 100-fold increase in K_m for bicarbonate and an increased dissociation of enzyme-bound carboxy phosphate.

The altered kinetic parameters of the ATPase reaction indicate that the mutation also affects this catalytic step. The reaction of HCO_3^- with ATP has been demonstrated to be catalyzed by a domain located in the amino-terminal half of the synthetase (Post et al., 1990). The apparent functional coupling of two physically separated domains in the protein, one responsible for bicarbonate activation and the other for phosphorylation of carbamate, suggests that they may be part of a single structural unit with amino acid residues recruited from both domains. According to this interpretation, the Glu841 → Lys841 replacement could be visualized not only to lead to the loss of a critical catalytic residue but also to induce other more subtle distortions in a local configuration of residues necessary for stabilizing the enzyme-bound intermediates. The composition of such a complex site in fact might also include side chains originating in the glutaminase component. In this context, a Cys269 → Ser substitution in the glutaminase subunit that abolishes the hydrolysis but not the binding of glutamine also results in stimulation of the HCO_3^--dependent ATPase activity of the synthetase. This enhancement in ATPase has been shown to be a consequence of destabilization and increased dissociation of the carboxy phosphate intermediate (Mullins et al., 1991).

An important aspect of the mechanism and one thus far neglected in this discussion is the conversion of carboxy phosphate to carbamate. In the presence of NH_3, carbamate and P_i are formed in equimolar amounts (eq 3), and the HCO_3^--dependent P_i release from ATP has been generally assumed to be a measure of carbamate formation. NH_3 normally causes the rate of P_i formation to be stimulated by a factor of 10–15 (Table VI). In contrast to wild-type carbamyl phosphate synthetase, addition of NH_3 to the Lys841 enzyme fails to elicit any increase in P_i production. This observation suggests an impairment in the mutant of carbamate formation from carboxy phosphate and NH_3 as well (eq 3). The apparent inability of the mutant carbamyl phosphate synthetase to convert carboxy phosphate to carbamate further points to a possible role of Glu841 in this reaction as well.

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