Comparison of the Functional Differences for the Homologous Residues within the Carboxy Phosphate and Carbamate Domains of Carbamoyl Phosphate Synthetase†

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ABSTRACT: Carbamoyl phosphate synthetase (CPS) from *Escherichia coli* catalyzes the formation of carbamoyl phosphate from two molecules of MgATP, bicarbonate, and glutamine. It has been previously shown that the amino- and carboxy-terminal halves of the large subunit of this protein are homologous. A working model for the active site structure of the carboxy-terminal domain of the large subunit of CPS was constructed based upon amino acid sequence alignments and the previously determined three-dimensional structures of two mechanistically related proteins, biotin carboxylase and D-alanine:D-alanine ligase. The model was tested by mutation of ten amino acid residues predicted to be important for binding and/or catalysis. The mutated residues were as follows: R571, R675, R715, D753, E761, N827, Q829, E841, N843, and R845. The mutant proteins were expressed, purified to homogeneity and the catalytic properties determined for a variety of assay formats. The mutants E761A, E841Q, N843D, and R845Q were diminished in their ability to synthesize carbamoyl phosphate. The R715A, Q829A, and R675A mutants displayed elevated Michaelis constants for MgADP in the partial back reaction. The mutants E761A, N827A, E841Q, N843D, and R845Q showed significant increases in the Michaelis constants for either bicarbonate or carbamoyl phosphate. No significant alterations were noted upon mutation of either R571 or D753 to an alanine residue and thus these amino acids do not appear essential for structure or catalytic activity. These results have been utilized to further support the proposal that the C-terminal half of the large subunit of CPS is primarily responsible for the phosphorylation of the carbamate intermediate during the final formation of carbamoyl phosphate. The measured effects on the catalyic activities displayed by these mutations were found to be comparable to the previously determined effects after mutation of the homologous residues located on the N-terminal half of CPS and also for those residues mutated within D-alanine:D-alanine ligase [Shi, Y., & Walsh, C. T. (1995) *Biochemistry 34*, 2768−2776].

It is currently believed that the large subunit of carbamoyl phosphate synthetase (CPS)[1] arose from a gene duplication and fusion of a smaller ancestral protein (Nyunoya & Lusty, 1983). This proposal is supported by the high degree of amino acid sequence identity between the amino- and carboxy-terminal halves of the large subunit of CPS (Nyunoya & Lusty, 1983) and differential mutagenesis experiments for residues that are at or near the binding sites for each of the two nucleotides that are required for the overall synthesis of carbamoyl phosphate (Post *et al.*, 1990). Working models of the active sites of the carboxy phosphate and carbamate domains were constructed based upon primary sequence alignments of the two homologous halves of the large subunit of CPS and two mechanistically related enzymes, biotin carboxylase and DD-ligase. The working models were further refined by comparison to the X-ray structures of biotin carboxylase (Waldrop *et al.*, 1994) and DD-ligase (Fan *et al.*, 1994). In the preceding paper (Stapleton *et al.*, 1996) we successfully identified a number of residues within the carboxy phosphate domain of CPS that are important for the binding of bicarbonate and the MgATP that is utilized for the synthesis of the carboxy phosphate intermediate.

The experiments reported in this paper are directed toward the identification of those residues that are responsible for comparable functions within the segment of the active site in the carbamate domain of CPS where the second ATP phosphorylates the carbamate intermediate for the ultimate synthesis of carbamoyl phosphate. Ten residues within the carbamate domain of the large subunit of CPS were selected as targets for mutagenesis. These residues consist of R571, R675, R715, D753, E761, N827, Q829, E841, N843, and R845. In our preliminary working model, residues R675, R714, D753, and E761 are proposed to interact directly with the nucleotide while residues Q829, E841, and N843 are proposed to bind directly to one of the two required metal ions used in the overall reaction. Residues R571, N827, and R845 are proposed to bind to either carbamoyl phosphate or carbamate. These sites have now been mutated and the corresponding proteins have been expressed, purified, and characterized by analysis of the kinetic constants for the full biosynthetic reaction and the two partial reactions catalyzed by CPS. Functional roles for these residues in the carbamate domain have now been assigned based upon the observed catalytic properties of these mutants in comparison with those

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[1] Abbreviations: CPS, carbamoyl phosphate synthetase; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; SDS, sodium dodecyl sulfate; PCR, polymerase chain reaction; BC, biotin carboxylase; DD-ligase, D-alanine:D-alanine ligase.

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Table 1: Primers Used in the Site-Directed Mutagenesis of the Carbamate Domain of CPS[a]

plasmid	DNA sequence	enzyme
pMS06	5′-GTT AAC CCG CAA GCG GCG-3′ 3′ CAA TTG GGC GTT CGC CGC 5′	E845Q
pMS08	5′-CTG ATT CAA GTT AAC CCG-3′ 3′-GAC TAA GTT CAA TTG GGC-5′	E841Q
pMS09	5′-ATT GAA GTT GAC CCG CGT-3′ 3′-TAA CTT CAA CTG GGC GCA-5′	N843D
pMS17	5′-CGT GCA GAA GAC GCT GAA CG-3′ 3′-GCA CGT CTT CTG CGA CTT GC-5′	R675A
pMS19	5′-GGT GGT AGC TCC GTC TTA CG-3′ 3′-CCA CCA TCG AGG CAG AAT GC-5′	R715A
pMS20	5′-GTT GCT GGC GCA CTT CCT CG-3′ 3′-CAA CGA CCG CGT GAA GGA GC-5′	D753A
pMS21	5′-CGC GGT AGC AGT TGA CG-3′ 3′-GCG CCA TCG TCA ACT GC-5′	E761A
pMS24	5′-CCT GAT GGC CGT GCA GTT TGC 3′ 3′-GGA CTA CCG GCA CGT CAA ACG 5′	N827A
pMS27	5′-CCC GAA CGC TAT CGG TCA GGG 3′ 3′-GGG CTT GCG ATA GCC AGT CCC 5′	R571A
pMS29	5′-GAT GAA CGT GGC GTT TGC GG-3′ 3′-CTA CTT GCA CCG CAA ACG CC-5′	Q829A

[a] These mutagenic primers introduced base changes at the underlined positions.

mutants constructed at the homologous sites within the carboxy phosphate domain of the large subunit.

MATERIALS AND METHODS

General Materials. The restriction enzymes, reagents, and coupling enzymes were obtained from the suppliers identified in the preceding paper (Stapleton *et al.*, 1996). The bacterial strains and associated plasmids used in this study were also the same as those described previously.

Construction of the R571A, R675A, R715A, D753A, E761A, N827A, R829A, E841Q, N843D, and R845Q Mutants. Site-directed mutagenesis was performed on the plasmid pMS03 using the polymerase chain reaction (PCR) and the overlap extension method of Ho *et al.* (1989). The pairs of internal oligonucleotides which were used to initiate these mutations are listed in Table 1. A flanking primer with the sequence 5′-CATGGAGAATATTGAGCAGG-3′ at position 3291 of the *car*AB gene was used to make all of the mutants except for R571A. For the R571A mutation, flanking primers with the sequences 5′-CGTGCATTAT-TGCCCCATC-3′ at position 2133 and 5′-CGTTCAGCGTCT-TCTGCACG-3′ at position 3650 were used. The 3′-flanking primer used in the creation of the R675A mutant was at position 3785 with the sequence 5′-CGTAAGACGGAGC-TACCACC-3′. The 3′-flanking primer used to make the R715A, D753A, and E761A mutants was located at position 4111 of the *car*AB gene, and the sequence of this primer was 5′-GCAAACTGCACGGCCATCAGG-3′. In order to make the mutants N827A and Q829A, the 3′-flanking primer at postion 4392 with the sequence 5′-CCCATGACT-TAATCGGTAGAGCAC-3′ was used. The final PCR product was digested with *Eco*RI and *Sac*II during the construction of the R571A mutant. The PCR fragment used to construct the R675A mutant was digested with *Eco*RI and *Sph*I. To make the R715A, D753A, and E761 mutants, the PCR fragments were digested with *Sph*I and *Afl*II. The PCR fragments used to make the N827A, Q285A, E841Q, N843D, and R845Q mutants were digested with *Afl*II and *Bpu*1102I. The digested fragments were inserted into the pMS03 plasmid at the appropriate locations and then fully sequenced using the Sequenase 2.0 sequencing kit from USB/Amersham. The mutant plasmids were transformed into the RC50 cell line for expression and subsequent purification of the mutant proteins.

Purification of Wild-Type and Mutant Proteins. The wild-type and site directed mutants of CPS were purified according to the protocols provided by Mareya *et al*. (1994) with the exception of the N843D mutant. The mutant N843D was purified using the truncated procedure as described previously for the N301D mutant of CPS (Stapleton *et al.*, 1996).

Kinetic Measurements. The specific details for the measurement of the kinetic parameters were the same as those described previously by Stapleton *et al.* (1996). Carbamoyl phosphate synthesis was determined by measuring the rate of citrulline formation in a coupled assay with ornithine transcarbamoylase and ornithine (Snodgrass *et al.*, 1969). The rate of ATP formation from MgADP and carbamoyl phosphate was determined spectrophotometrically using a hexokinase/glucose-6-phosphate dehydrogenase coupling system. The rate of ATP hydrolysis was monitored at 340 nm in the presence or absence of a nitrogen source (NH_3 or glutamine) by coupling the production of ADP to the oxidation of NADH with the enzymes pyruvate kinase and lactate dehydrogenase.

Statistical Analysis of Kinetic Data. The kinetic parameters, V_m and V/K_m, were determined by fitting the data to eq 1 with computer programs obtained from Savanna Shell Software. In this equation v is the initial velocity, K_m is the Michaelis constant, and A is the substrate concentration. Nonlinear double-reciprocal plots were fit to eq 2. In eq 2, A represents the substrate concentration, K_1 and K_2 are the Michaelis constants at high and low substrate concentration while V_1 and V_2 are the maximal velocities at high and low substrate concentration. The data for the enhancement of ATP hydrolysis in the presence of a nitrogen source with all mutants were fit to eq 3. In this equation, A is the concentration of the nitrogen source (either ammonia or glutamine), V_o is the initial enzyme velocity in the absence of a nitrogen source, and K_a is the activation constant for the nitrogen source. The value of α defines the ratio of the observed velocities at saturating and zero levels of either glutamine or ammonia.

$$v = V_m A/(K_m + A) \quad (1)$$

$$v = [V_1 A/(K_1 + A)] + [V_2 A/(K_2 + A)] \quad (2)$$

$$v = V_o(\alpha A + K_a)/(A + K_a) \quad (3)$$

Three-Dimensional Model of the Carbamate Domain of CPS. The three-dimensional model of the carbamate domain of CPS was constructed in the manner described in the preceding paper for the model of the carboxy phosphate domain. The only difference being in the assignments of the structurally conserved regions relative to those of DD-ligase (DdlB). The assigned structurally conserved regions for the model of the carbamate domain were: (1) DdlB 2−16:CPS-553 558−572, (2) DdlB 30−41:CPS-553 590−600, (3) DdlB 50−63:CPS-619--632, (4) DdlB 69−73:CPS-553 638−642, (5) DdlB 79−105:CPS-553 651−677, (6) DdlB 110−112:CPS-553 682−684, (7) DdlB 137−146:CPS-553 709−718, (8) DdlB 154−157:CPS-553 726−729, (9)

Table 2: Kinetic Parameters for Reactions Catalyzed by Carbamoyl Phosphate Synthetase Mutants

		glutamine-dependent ATPase reaction[a]			carbamoyl phosphate synthesis reaction[b]	
enzyme	effector[c]	K_{ATP} (μM)	V_m(μmol/min·mg)	K_{HCO_3} (mM)	V_m with Gln (μmol/min·mg)	V_m with ammonia (μmol/min·mg)
wild-type	none	440	3.8			
	ornithine	52	3.4	1.6	1.8	0.59
	UMP	1100	3.8			
R571A	none	320	4.8			
	ornithine	70	5.1	0.8	2.3	2.0
	UMP	120	1.5			
R675A	none	760	1.5			
	ornithine	240	1.8	1.3	0.31	0.35
	UMP	95	3.2			
R715A	none	220	3.0			
	ornithine	210	4.7	1.8	0.93	0.68
	UMP	140	0.7			
D753A	none	220	4.7			
	ornithine	55	4.4	3.2	0.64	0.70
	UMP	260	3.1			
E761A	none	60	2.5			
	ornithine	68	2.5	72	0.02	0.04
	UMP	52	2.5			
N827A	none	73	1.5			
	ornithine	72	1.7	51	0.20	0.10
	UMP	46	1.2			
R829A	none	920	2.0			
	ornithine	340	2.5	0.7	1.0	0.33
	UMP	950	0.6			
E841Q	none	130	2.5			
	ornithine	100	2.0	56	0.06	0.02
	UMP 2100	0.7				
N843D	none	470	1.3			
	ornithine	220	1.5	17	<0.003	<0.005
	UMP 9300	0.7				
R845Q	none	550	2.2			
	ornithine	200	1.1	26	<0.005	<0.005
	UMP	120	0.5			

[a] pH 7.6, 25 °C, 50 mM HEPES, 100 mM KCl, 20 mM Mg^{2+}, 50 mM $KHCO_3$, 5 mM ATP (when $KHCO_3$ was the variable substrate) or 50 mM $KHCO_3$ (when ATP was variable substrate), variable amounts of glutamine or ammonia. [b] pH 7.6, 25 °C, 50 mM HEPES, 100 mM KCl, 5 mM ATP, 10 mM Orn, 20 mM Mg^{2+}, 50 mM $KHCO_3$, either 10 mM of glutamine or 300 mM of NH_4Cl. [c] Effector concentrations: ornithine, 10 mM; UMP, 100 μM.

DdlB 176−186:CPS-553 750−760, (10) DdlB 202−209: CPS-553 773−780, (11) DdlB 218−223:CPS-553 790−795, (12) DdlB 231−260:CPS-553 804−833, (13) DdlB 267−273:CPS-553 839−845, (14) DdlB 283−303:CPS-553 851−871.

RESULTS

Ten conserved amino acids, located within the carbamate domain of CPS, were mutated to other residues in order to more fully explore the active site(s) of this enzyme and to compare the functional differences and similarities of these specific residues with their homologous counterparts from the carboxy phosphate domain. The residues R571, R675, R715, D753, E761, N827, and Q829 were changed to alanine while E841, N843, and R845 were mutated to glutamine, aspartate, and glutamine, respectively, using the method of overlap extension PCR. The choice of residues selected for mutation was based on conservation of these specific sites within the CPS primary sequence from various sources and sequence alignments with the mechanistically similar enzymes, biotin carboxylase and DD-ligase. The mutant proteins were expressed and purified to homogeneity as judged by SDS-polyacrylamide gel electrophoresis. The effects of these modifications on the kinetic constants were determined for each mutant by measuring the full biosynthetic reaction and both of the two partial reactions catalyzed by CPS in the presence and absence of the allosteric effectors, ornithine and UMP. The kinetic parameters, K_m and V_{max}, obtained for the wild-type and the mutant enzymes are summarized in Tables 2−5. A model for the three-dimensional fold of the carbamate domain of CPS was constructed with the Homology program from Biosym, based on the X-ray coordinates for DD-ligase (Fan *et al.*, 1994) and biotin carboxylase (Waldrop *et al.*, 1994) and is illustrated in Figure 1. The approximate spacial locations of the ten residues mutated in this investigation are indicated by spheres within the α-carbon backbone of the model. Of the ten residues of CPS that were mutated in this investigation there are eight that have fully analogous residues in BC, DD-ligase, and the carboxy phosphate domain of CPS (N845 of CPS does not have an analogous residue in DD-ligase, and R571 is not conserved in the carboxy phosphate domain of CPS). A superimposition of the α-carbon atoms of these eight homologous residues within the structure of DD-ligase and the model for the carboxy phosphate domain of CPS gives an RMS deviation of 3.3 Å. However, the primary sequence alignment of the carbamate domain of CPS with DD-ligase was slightly different from the one used to create the model for the carboxy phosphate domain of CPS. In this altered alignment, R675 of CPS was not aligned with K97 of DD-ligase. Removal of R675 from the superimposition gave an RMS deviation of only 1.11 Å for the seven remaining residues. The area of lowest RMS deviation between the BC and DD-ligase structures was found in the C-terminal

Table 3: Kinetic Parameters for the Wild-Type and Mutant Enzymes for the ATP Synthesis Reaction

		ATP Synthesis Reaction[a]		
enzyme	effector[b]	K_{ADP} (μM)	V_{max} (μmol/min·mg)	K_{CP} (mM)
wild-type	none	170	0.42	
	ornithine	22	0.40	2.0
	UMP	2200	0.22	
R571A	none	170	0.68	
	ornithine	17	0.66	3.5
	UMP	3200	0.47	
R675A	none	440	0.04	
	ornithine	79	0.03	8.8
	UMP	nd	nd	
R715A	none	410	0.10	
	ornithine	76	0.11	8.9
	UMP	1400	0.03	
D753A	none	21	0.12	
	ornithine	4	0.11	0.7
	UMP	180	0.10	
E761A[c]	none	2400, 32	0.06, 0.02	
	ornithine	2900, 25	0.06, 0.02	31
	UMP	180	0.025	
N827A	none	180	0.015	
	ornithine	570	0.043	54
	UMP	110	0.005	
R829A	none	1800	0.22	
	ornithine	220	0.31	13
	UMP	1300	0.04	
E841Q	none	340	0.011	
	ornithine	31	0.007	5
	UMP	420	0.006	
N843D	none	68	0.007	
	ornithine	94	0.006	16
	UMP	25	0.005	
R845Q	none	120	0.005	
	ornithine	102	0.004	24
	UMP	160	0.004	

[a] pH 7.5, 25 °C, 20 mM Mg^{2+}, 100 mM KCl, 20 mM carbamoyl phosphate (when ADP was the variable substrate) or 5 mM ADP (when carbamoyl phosphate was variable substrate). [b] When allosteric effector was present, 10 mM ornithine or 100 μM UMP was used. [c] The kinetic data for the E761A mutant were fit to eq 3 as described under Materials and Methods.

Table 4: Kinetic Parameters for Bicarbonate-Dependent ATPase Reaction[a]

enzyme	K_{ATP} (μM)	K_{HCO_3} (mM)	V_{max} (μmol/min·mg)
wild-type	7.0	1.7	0.10
R571A	14	1.1	0.95
R675A	47	1.0	0.09
R715A	41	2.3	0.16
D753A	16	3.8	0.62
E761A	25	70	1.6
N827A	21	20	0.64
R829A	67	1.3	0.27
E841Q	100	70	1.5
N843D	82	27	0.53
R845Q	58	26	0.85

[a] pH 7.5, 25 °C, 20 mM Mg^{2+}, 100 mM KCl, 5 mM ATP (when bicarbonate varied), 50 mM bicarbonate (when ATP varied).

domains of these two proteins. Superimposition of the five analogous resides located in these sub-domains gave RMS deviations of 0.63 Å between BC and DD-ligase, 1.09 Å between DD-ligase and the carbamate domain of the CPS model and 1.11 Å between BC and the carbamate domain of the CPS model. The dissimilarities in the structurally conserved regions and the primary sequence alignments of DD-ligase with the carboxy phosphate and carbamate domains of CPS was the direct cause for the slightly different

Table 5: Effect of Nitrogen Source on the Glutamine-Dependent ATP Hydrolysis Reaction[a]

enzyme	α for Gln	K_i for Gln (μM)	α for NH_4^+	K_i for NH_4^+ (mM)
wild-type	28	100	7.7	440
R571A	8.2	90	3.0	190
R675A	8.0	160	5.0	200
R715A	47	130	6.3	150
D753A	6.8	200	4.3	130
E761A	2.3	20	ND	ND
N827A	2.5	70	ND	ND
R829A	17	110	5.9	57
E841Q	1.7	70	ND	ND
N843D	5.1	260	4.2	170
R845Q	4.1	100	2.0	69

[a] pH 7.6, 25 °C, variable glutamine or ammonium chloride, 5 mM ATP, 50 mM HEPES, 100 mM KCl, 10 mM ornithine, 20 mM Mg^{2+}, 50 mM $KHCO_3$, and 5 mM ATP.

appearance of the model structure for the carbamate domain of CPS in comparison to the structure for the model of the carboxy phosphate domain. Again, it must be emphasized that this model of the carbamate domain of CPS should not be taken as a undistorted representation of the three-dimensional structure of CPS since it was based only on a portion of the entire primary sequence. The main purpose of the model was as an aid in the design of mutational studies.

Glutamine- and Ammonia-Dependent Carbamoyl Phosphate Synthesis. The mutant enzymes, R571A, R675A, R715A, D753A, E761A, N827A, Q829A, E841Q, N843D, and R845Q were examined for their ability to synthesize carbamoyl phosphate using either glutamine or ammonia as a nitrogen source. With the exception of R571A, the rates of carbamoyl phosphate synthesis with either glutamine or ammonia as a nitrogen source have been reduced with all of these mutants. Two- to six-fold reductions in the rate of carbamoyl phosphate synthesis were observed for the R675A, R715A, D753 and R829A mutants. For the mutants E761A, N827A, and E841Q the rate of carbamoyl phosphate synthesis has been reduced between 10- and 100-fold while no detectable carbamoyl phosphate synthesis was found for the N843D and R845Q mutants. These results are summarized in Table 2.

ATP Synthesis Reaction. The rate of the ATP synthesis reaction was supressed by most of the mutations constructed within the carbamate domain of carbamoyl phosphate synthetase. The most severe losses in the catalytic activity of the partial back reaction were observed with the N827A, E841Q, N843D, and R845Q mutants while smaller reductions in rate were observed for the R675, E761A and N827A mutants. The apparent K_m for MgADP in the absence of any allosteric effector was measurably increased relative to the wild-type value for the R675A, R715A, R829A, and E841Q mutations. Moreover, the E761A mutant showed a nonlinear double-reciprocal plot when the ADP concentration was varied. The largest increases in the K_m for carbamoyl phosphate were measured for the E761A, N827A, and R845Q mutants. The kinetic constants are summarized in Table 3.

Bicarbonate-Dependent ATPase Activity. For the bicarbonate-dependent ATPase reaction, the effects on the catalytic turnover were in general rather small. At one extreme the apparent maximal velocity for the R675A mutant was

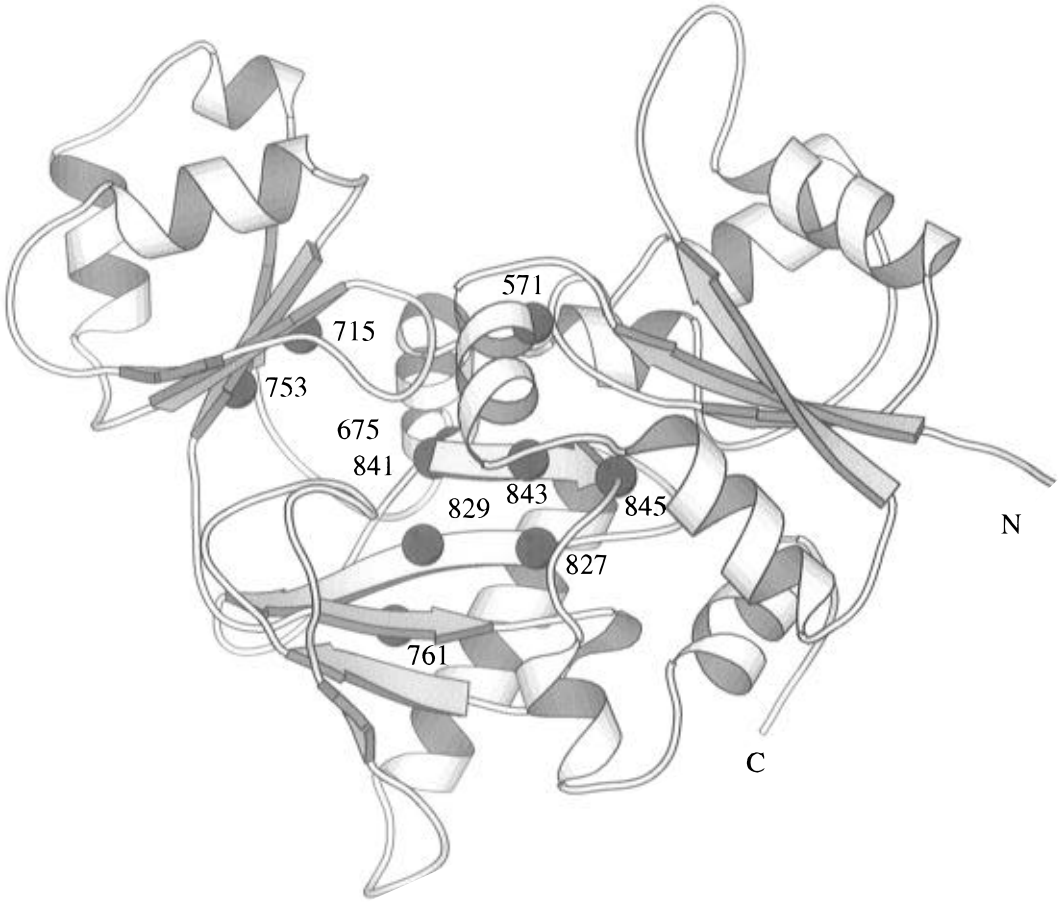

FIGURE 1: Model for the three-dimensional fold of the carbamate domain of CPS from *E. coli*. This structure was created by mapping the CPS amino acid sequence onto the coodinates for the crystal structure of DD-ligase and biotin carboxylase from *E. coli* using the Homology program from Biosym, Inc. Those residues mutated in this investigation are indicated by spheres. The figure was made with Molscript (Kraulis, 1991).

reduced slightly while for the E761A and E841Q mutants the rate of ATP turnover was increased about 15-fold. However, there were some significant increases in the apparent K_m for bicarbonate. For the E761A, N827A, E841Q, N843D, and R845Q mutants, the K_m for bicarbonate increased by at least an order of magnitude relative to the wild-type value. Surprisingly, there were also increases in the Michaelis constant for MgATP for some of the mutants. The biggest effect was noted with the E841Q mutant where the value increased to 100 μM from 7 μM for the wild-type enzyme. All of the kinetic constants are summarized in Table 4.

Glutamine-Dependent ATP Turnover. For the wild-type enzyme, the rate of turnover for ATP increases by at least an order of magnitude in the presence of glutamine. For all of the mutants examined in this investigation, the rate of ADP formation increased in the presence of glutamine. The largest enhancement was observed with the R715A mutant while the smallest effect was obtained for the E841Q mutant (Table 5). A small elevation was observed in the Michaelis constant for MgATP with the R675A and Q829A mutants in the absence of allosteric effectors. Significantly, many of the mutants have a higher affinity for the nucleotide in the presence of UMP. This is in sharp contrast with the effect of this allosteric effector on the wild-type enzyme. These results are summarized in Tables 2 and 5.

DISCUSSION

Ten conserved amino acids located within the carbamate domain of the large subunit of CPS were mutated in an attempt to identify the roles of these residues during the biosynthesis of carbamoyl phosphate. These particular amino acids were targeted for mutagenesis on the basis of multiple sequence alignments with the homologous carboxy phosphate domain of CPS and the mechanistically related proteins, DD-ligase and biotin carboxylase. Further insights into the potential functions for these ten residues were supplied by the X-ray crystal structures for DD-ligase (Fan *et al.*, 1994) and biotin carboxylase (Waldrop *et al.*, 1994). The mutant proteins, R571A, R675A, R715A, D753A, E761A, N827A, Q829A, E841Q, N843A, and R845Q, were expressed and purified, and the kinetic constants for the full biosynthetic reaction and the two partial reactions were quantified using a variety of assay conditions. In the following analysis of the observed effects on the kinetic constants exhibited by these mutatations, we made the explicit assumption that the perturbations on the bicarbonate-dependent ATPase reaction were a direct reflection on that portion of the active site involved in the phosphorylation of bicarbonate. It then follows that the partial back reaction (ATP synthetase reaction) would serve as a reporter for modulations on the other nucleotide site that is primarily responsible for the phosphorylation of the carbamate intermediate for the ultimate formation of carbamoyl phosphate. On the basis

of our previous investigations, we anticipated that the major effects exhibited by these mutations within the carbamate domain of CPS would primarily, but not exclusively, be found on the partial back reaction rather than on the bicarbonate-dependent ATPase reaction (Post *et al.*, 1990). This prediction has been confirmed by analysis of the experimental kinetic properties of these specific mutants.

Residues Involved in the Binding of Nucleotide. Four of the ten residues investigated in this study were originally postulated to be associated with the binding of the nucleotide used in the phosphorylation of the carbamate intermediate. One of these residues, D753, was proposed to interact with the amino group at C-6 of the adenine ring. However, examination of the kinetic constants that appear in Tables 2−5 indicates that there are no significant changes when this particular aspartate is mutated to an alanine residue. Therefore, it appears unlikely that the carboxylate side chain of D753 is interacting directly with the adenine ring as indicated in the original model. Two arginine residues, R675 and R715, were proposed to directly coordinate with the nucleotide. The side chain for R675 was postulated to ion pair with the β-phosphoryl group of ADP while R715 was proposed to make contacts with the α-phosphoryl group and the nitrogen at N7 of the adenine ring. With either of the mutations made at these positions, there were measureable increases in the K_m for MgADP in the absence of any allosteric effectors relative to the value observed for the wild-type enzyme. The remaining member of this group, E761, was proposed to hydrogen bond with the *cis*-diol of the ribose sugar of the nucleotide. The mutant constructed at this position catalyzed a barely detectable rate of carbamoyl phosphate synthesis. The bicarbonate-dependent ATPase reaction was enhanced relative to the wild-type value while the partial back reaction was characterized by nonlinear double-reciprocal plots with very slow turnovers. The limiting value for the K_m of MgADP in the absence of an allosteric effector (2.4 mM) was greatly increased relative to the wild-type value. However, it should be noted that the K_m values for both carbamoyl phosphate and bicarbonate were each increased by an order of magnitude relative to the values obtained for the wild-type enzyme. It therefore appears that three of the four residues postulated to interact directly with the nucleotide responsible for the phosphorylation of carbamate exhibit catalytic constants after mutagenesis consistent with the original proposal.

Residues Involved in the Binding of Divalent Cations. Three of the original ten residues (Q829, E841, and N843) mutated for this investigation were postulated to be involved in the binding of one of the two divalent cations needed for this reaction. One of these metal ions coordinates the tripolyphosphate moiety of ATP while the other metal serves some other less well-defined function. Thermodynamic binding and associated kinetic studies with the CPS from *E. coli* have demonstrated that this enzyme binds one divalent cation (Mg^{2+} or Mn^{2+}) prior to the binding of the first MgATP to the enzyme (Raushel *et al.*, 1979). However, it is unclear whether this divalent cation is required for each of the three distinct reactions catalyzed by the large subunit of CPS. Nevertheless, E841, N843 and Q829 were postulated to interact directly with one of the two divalent cations utilized in the carbamate domain of the large subunit of CPS in analogy with the model based on the structures of DD-ligase and biotin carboxylase. The Q829A mutant was able to synthesize carbamoyl phosphate at a respectable rate with a moderate increase in the K_m for the MgATP and no significant change in the K_m for bicarbonate. However, in the partial back reaction there was a significant increase in the K_m for MgADP that substantiates the predicted effect on the binding of the metal nucleotide. The other two residues, E841 and N843, are very sensitive to changes in the side chain structure. Neither of the mutants made at these two positions were able to make carbamoyl phosphate at a significant rate and the partial back reaction was equally diminished. The Michaelis constants for bicarbonate and carbamoyl phosphate were all elevated above the wild-type values. Similar result have been previously observed by Guillou and Lusty (1992) for an E841K mutant. Overall, these results are consistent with a model for the binding of either of the two metal ions with the phosphoryl groups of both ADP and carbamoyl-P.

Residues that Interact with Carbamoyl Phosphate. The remaining three residues (R571, N827, and R845) were originally postulated to constitute a portion of the binding site for carbamate or carbamoyl phosphate. The replacement of the arginine at position 845 with a glutamine resulted in a mutant that was unable to synthesize carbamoyl phosphate and unable to catalyze the partial back reaction. However, the bicarbonate-dependent ATPase reaction was largely unaffected although the K_m values for bicarbonate and ATP were elevated. Similar effects on the catalytic constants were observed upon mutation of N827. The N827A mutant had a significant drop in the ability to synthesize carbamoyl phosphate. The bicarbonate-dependent ATPase reaction was elevated but the ATP synthesis reaction was greatly diminished relative to the wild-type enzyme. This mutant enzyme had the highest K_m for carbamoyl phosphate of any of the mutants constructed for this investigation. The remaining mutant, R571A, displayed properties that were nearly identical with the wild-type enzyme and thus it appears that this arginine is not important for either the maintenance of protein structure or catalytic activity of carbamoyl phosphate synthetase.

Working Model for the Carbamate Domain of CPS. In the previous paper, directed at the identification of essential residues within the carboxy phosphate domain of CPS (Stapleton *et al.*, 1996) we constructed a working model for the active site that was based upon the known three-dimensional structure for DD-ligase (Fan *et al.*, 1994) and the associated mutagenesis experiments of Shi and Walsh (1995). Shown in Figure 2 is an analogous model for the carbamate domain of CPS refined to incorporate the results obtained in this investigation. Not included in this model are R571 and D753 since the kinetic parameters for the alanine mutants were largely unaltered relative to the wild-type enzyme. The remaining eight residues are inserted into the working model based upon the sequence alignments with DD-ligase and biotin carboxylase in association with the known three-dimensional X-ray structures. The measured catalytic properties of the mutants created at each of these positions are fully consistent with the postulated working model. While investigations of this type can be effectively utilized to identify critical residues within the separate domains it does not appear possible to make further deductions with regard to how these putative functional domains interact with one another for the concerted synthesis of carbamoyl phosphate. However, this particular feature of

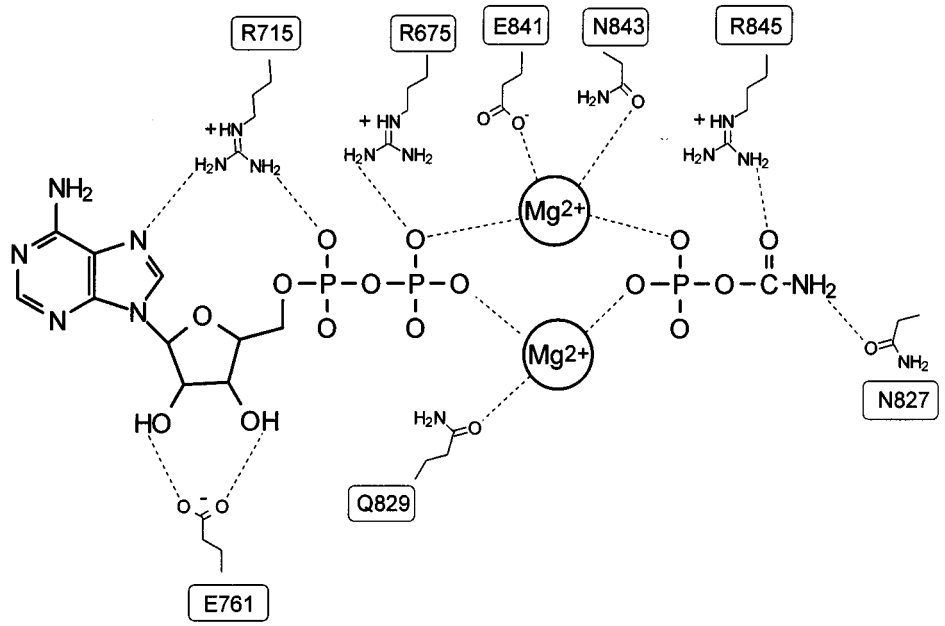

FIGURE 2: Working model for the interaction of the active site residues of the carbamate domain of CPS with MgADP and carbamoyl phosphate. This model was generated by sequence alignment studies with DD-ligase and biotin carboxylase in conjunction with the X-ray crystal structures and the catalytic properties of the mutants of CPS constructed in this investigation.

Table 6: Comparison of Homologous Mutations within DD-Ligase and the Carboxy Phosphate and Carbamate Domains of CPS

carboxy phosphate domain of CPS[a]		carbamate domain of CPS[b]		DD-ligase[c]	
mutant	modification	mutant	modification	mutant	modification
R82A	no significant effect on kinetic parameters	K634	not mutated	H63Q	elevated K_m for D-Ala$_1$
R129A	elevated K_m for ATP in bicarbonate-dependent ATPase reaction	R675A	elevated K_m for ADP in partial back reaction	K97	not mutated
R169A	elevated K_m for ATP in bicarbonate-dependent ATPase reaction	R715A	elevated K_m for ADP in partial back reaction	K144A	K_m for ATP increased
D207A	no significant effects on kinetic parameters	D753A	no significant effects on kinetic parameters	E180	not mutated
E215A	elevated K_m for bicarbonate suppressed V_{max} for carbamoyl-P formation	E761A	elevated K_m for carbamoyl-P and bicarbonate suppressed V_{max} for carbamoyl-P formation	E187	not mutated
N283A	elevated K_m for bicarbonate no carbamoyl-P formation	N827A	elevated K_m for carbamoyl-P and bicarbonate very low carbamoyl-P formation	R255A	no detectable activity
Q285A	elevated K_m for ATP in bicarbonate-dependent ATPase reaction	Q829A	elevated K_m for ADP in partial back reaction	D257N	no detectable activity
E299Q	elevated K_m for bicarbonate low carbamoyl phosphate formation	E841Q	elevated K_m for bicarbonate suppressed V_{max} for carbamoyl-P formation	E270Q	no detectable activity
N301D	elevated K_m for ATP and bicarbonate low carbamoyl-P formation	N843D	elevated K_m for bicarbonate and carbamoyl-P no carbamoyl-P formation	N272	not mutated
R303Q	elevated K_m for bicarbonate suppressed carbamoyl-P formation	R845Q	elevated K_m for bicarbonate and carbamoyl-P no carbamoyl-P formation		

[a] Stapleton *et al.* (1996). [b] This study. [c] Shi and Walsh (1995).

the carbamoyl phosphate synthetase reaction is what makes this enzyme so fascinating. Structural clues for how this enzyme coodinates the biosynthesis of carbamoyl phosphate must await completion of the three-dimensional X-ray stucture (Thoden *et al.*, 1995; Marina *et al.*, 1995).

Comparison of Homologous Mutations in DD-ligase with Those for the Carboxy Phosphate and Carbamate Domains of CPS. Most of the mutants constructed and characterized in this investigation of the carbamate domain of CPS have comparable sites within the carboxy phosphate domain of CPS and in the active site of DD-ligase [for example see Figure 1 of Stapleton *et al.* (1996)]. Shown in Table 6 is a direct comparison of the most salient effects after mutagensis of these critical residues. As expected, the mutations constructed within the carboxy phosphate domain of CPS show major perturbations in the ability of the modified proteins to phosphorylate bicarbonate while homologous mutations in the carbamate domain have, in general, specifically perturbed the ability of the mutants to phosphorylate carbamate. Moreover, this table also indicates that comparable modulations in the kinetic properties are observed for those residues that have been mutated in DD-ligase. It will be quite interesting to determine if mutagenesis of homologous sites in biotin carboxylase show similar properties. It should also be noted that many of the active site residues identified in this investigation have also been conserved in the active site of glutathione synthetase (Fan *et al.*, 1995).

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