Regulatory Changes in the Control of Carbamoyl Phosphate Synthetase Induced by Truncation and Mutagenesis of the Allosteric Binding Domain†

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ABSTRACT: Carbamoyl phosphate synthetase from *Escherichia coli* catalyzes the synthesis of carbamoyl phosphate from bicarbonate, ammonia, and two molecules of MgATP. The enzyme is composed of two nonidentical subunits. The small subunit catalyzes the hydrolysis of glutamine to glutamate and ammonia. The large subunit catalyzes the formation of carbamoyl phosphate and has the binding sites for bicarbonate, ammonia, MgATP, and the allosteric ligands IMP, UMP, and ornithine. The allosteric ligands are believed to bind to the extreme C-terminal portion of the large subunit. Truncation mutants were constructed to investigate the allosteric binding domain. Stop codons were introduced at various locations along the *car*B gene in order to delete amino acids from the carboxy-terminal end of the large subunit. Removal of 14−119 amino acids from the carboxy-terminal end of the large subunit resulted in significant decreases in all of the enzymatic activities catalyzed by the enzyme. A 40-fold decrease in the glutamine-dependent ATPase activity was observed for the Δ14 truncation. Similar losses in activity were also observed for the Δ50, Δ65, Δ91, and Δ119 mutant proteins. However, formation of carbamoyl phosphate was detected even after the deletion of 119 amino acids from the carboxy-terminal end of the large subunit. No allosteric effects were observed for UMP with either the Δ91 or Δ119 truncation mutants, but alterations in the catalytic activity were observed in the presence of ornithine even after the removal of the last 119 amino acids from the large subunit of CPS. Six conserved amino acids within the allosteric domain were mutated. These sites included two glycine residues at positions 921 and 968, a threonine at position 977, an asparagine at position 1015, and two arginines at positions 1030 and 1031. The glycine residues were mutated to alanine, valine, and isoleucine. The other amino acids were changed to alanine residues. The allosteric effects exhibited by both ornithine and UMP were gradually diminished as the glycine residue at position 968 was changed to alanine, valine, and finally isoleucine. This effect was observed in both the glutamine-dependent ATP hydrolysis and the ATP synthesis reactions. The G921A mutant showed no alteration in any of the allosteric properties. The mutant proteins G921V and G921I were unstable and were found to be defective for the synthesis of carbamoyl phosphate. The T977A mutant was not regulated by UMP, but the full allosteric effects were observed with ornithine. The R1030A and R1031A mutants exhibited wild-type properties whereas the N1015A mutant could not be purified. These results demonstrate that the allosteric effects exhibited by UMP and ornithine can be functionally separated. The mutation of a single conserved threonine residue switches off the allosteric effects exhibited by UMP while not altering the ability of this protein to be activated by ornithine. In contrast, the allosteric effects exhibited by both UMP and ornithine can be gradually diminished by the substitution of a single glycine residue at position 968 with larger hydrophobic side chains.

Carbamoyl phosphate synthetase (CPS)[1] from *Escherichia coli* catalyzes the synthesis of carbamoyl phosphate according to the reaction depicted in eq 1.

$$2\text{ATP} + \text{bicarbonate} + \text{glutamine} \rightarrow 2\text{ADP} + \text{P}_i + \text{glutamate} + \text{carbamoyl phosphate} \quad (1)$$

The product of this reaction is the key intermediate in the biosynthesis of arginine and pyrimidine nucleotides. The production and enzymatic activity of CPS are therefore under strict metabolic control. The enzyme isolated from *E. coli* is a heterodimer. The small subunit, encoded by the *car*A gene, hydrolyzes glutamine and is responsible for the transfer of NH_3 to the large subunit for carbamoyl phosphate synthesis. The large subunit, encoded by the *car*B gene, contains the binding sites for the other substrates and the allosteric effects ornithine, IMP, and UMP. The enzyme activity is subject to feedback inhibition by UMP, and is activated by ornithine and IMP (Anderson & Meister, 1966; Pierard, 1966; Anderson & Marvin, 1968; Braxton et al., 1992).

The binding sites for the allosteric effectors are located on the large subunit (Trotta et al., 1971). Previous work has demonstrated that the allosteric effectors act by altering the apparent affinity of the enzyme for MgATP (Anderson & Meister, 1966; Anderson & Marvin, 1968). The presence of UMP increases the concentration of ATP required to give half-maximal velocity, whereas the presence of ornithine or IMP has the opposite effect. A quantitative assessment of the allosteric ligands on the various partial reactions catalyzed by the *E. coli* CPS has been examined in detail by Braxton et al. (1992). Their results indicate that IMP has a rather

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[1] Abbreviations: CPS, carbamoyl phosphate synthetase; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; OTCase, ornithine transcarbamoylase; PCR, polymerase chain reaction.

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small effect on the overall reaction and either of the two partial reactions involving ADP or ATP. UMP and ornithine were found to have a pronounced effect on the apparent K_m for MgATP in the overall reaction and for MgADP in the partial ATP synthesis reaction. The saturation profiles for ATP and the allosteric effectors are hyperbolic (Braxton et al., 1992).

The precise locations of the binding sites and the functional domains within the large subunit of CPS are not known. However, a 20-kDa domain located at the extreme carboxy-terminal end of the mammalian carbamoyl phosphate synthetase I has been shown to bind the positive effector *N*-acetylglutamate (Rodriguez-Aparicio et al., 1989). Even though *N*-acetyl-glutamate is not an allosteric effector of the *E. coli* enzyme, it has been shown that the homologous region of the bacterial enzyme may have a similar function in regulation. It has been proposed that UMP and IMP bind to the same general site on the large subunit of the *E. coli* enzyme (Boettcher & Meister, 1982). Studies involving a variety of UMP and IMP analogues have shown that dUMP can diminish the apparent inhibition induced by UMP and activation by IMP, but has no effect on the level of enhancement exhibited by ornithine. These results have been interpreted to be in accord with the view that UMP and IMP bind to the same or overlapping allosteric sites of CPS which are apparently distinct from the binding site for ornithine (Boettcher & Meister, 1982).

It has been demonstrated that UMP binds to the 15-kDa carboxy-terminal region of the large subunit of the *E. coli* CPS (Rubio et al., 1991). The large subunit labeled with [^{14}C]UMP has been found to be resistant to proteolytic cleavage. Removal of a 15-kDa polypeptide from the carboxy-terminal end of CPS by prior treatment of the enzyme with trypsin prevents subsequent labeling with [^{14}C]-UMP. The protection of CPS from proteolysis by ornithine has been interpreted by Rubio et al. (1991) to suggest that ornithine binds to the same domain as UMP. It has also been shown that cleavage of this domain from the C-terminal end of CPS results in complete inactivation of enzymatic activity (Rubio et al., 1991). However, limited proteolysis of the *E. coli* enzyme with endoproteinase Asp-N results in the cleavage of shorter peptides from the C-terminal end of CPS. These truncated versions of CPS are still catalytically active but have greatly diminished capacity for allosteric regulation (Mareya & Raushel, 1995).

In this investigation, attempts were made to further disrupt and perturb the structure of the putative allosteric region using three complementary approaches. First, truncation mutants were constructed by introducing termination codons at specific locations along the *car*B gene. The effect of these truncations on the binding of allosteric effectors has been analyzed by measuring the change in the apparent affinity for MgATP and MgADP in the various partial reactions catalyzed by CPS. Second, conserved glycine residues at positions 921 and 968 were increased in size by substitution to alanine, valine, and isoleucine. Third, the side chains of four other conserved amino acids (Thr-977, Asn-1015, Arg-1030, and Arg-1031) were excised by mutating them to alanine residues. We have found that with a single point mutation we can completely abolish the inhibitory effect exerted by UMP but can retain the full catalytic ability to synthesize carbamoyl phosphate and be regulated by ornithine. We have also demonstrated that mutants can be created that are variable in their ability to be maximally regulated by these effectors.

Table 1: Mutagenic Primers Used in the Construction of Truncated Proteins[a]

plasmid	DNA sequence	truncation
pSM14	5′GCG ACT TAA TAA GTA ATT TCG3′ 3′CGC TGA ATT ATT CAT TAA AGC5′	Δ14
pSM39	5′GT GCG CTG TAA TAA AAA GTG C3′ 3′CA CGC GAC ATT ATT TTT CAC G5′	Δ39
pSM50	5′GT GCG ATT TAA TAA TCC CGC G3′ 3′CA CGC TAA ATT ATT AGG GCG C5′	Δ50
pSM65	5′G AATGGC TAA TAA ACC TAC AT3′ 3′C TTA CCG ATT ATT TGG ATG TA5′	Δ65
pSM91	5′GTG CTG GGC TAA TGA GGT ATC3′ 3′CAC GAC CCG ATT ACT CCA TAG5′	Δ91
pSM119	5′GC GAT AAA TAA TGA GTG GTG G3′ 3′CG CTA TTT ATT ACT CAC CAC C5′	Δ119
pSM144	5′CG TTT GCC TAA TAG CAG CTG G3′ 3′GC AAA CGG ATT ATC GTC GAC C5′	Δ144

[a] The flanking primers used to isolate the truncation mutants were 5′TGG CTG GCA AAT CGC TGG C and 5′AAA TAT CGA TTC CCC AAA. The respective positions of these primers are 4245 and 4978 within the *car*B gene.

MATERIALS AND METHODS

Chemicals and Enzymes. All chemicals and coupling enzymes used in assays were purchased from Sigma Chemical Co., Aldrich Chemical Co., Fisher Scientific, or United States Biochemical Co. Bacto-tryptone and yeast extract were obtained from Difco. The Ultrogel AcA-34 was purchased from IBF Biotechnics. All enzymes used for the manipulation of recombinant DNA were purchased from Progema, Statagene, New England Biolabs, or United States Biochemical. Sequenase version 2.0 DNA sequencing kits were obtained from United States Biochemical. Gene Clean DNA purification kits were purchased from Bio 101. Magic Minipreps DNA purification kits and Magic PCR Preps DNA purification kits were purchased from Promega. All oligonucleotides used in this work were synthesized by the Gene Technology Laboratory of the Biology Department of Texas A&M University.

Bacterial Strains and Plasmids. The *E. coli* strains used for this study were RC50 (obtained as a generous gift of Carol J. Lusty) and XL1-Blue (Joyce & Grindley, 1984; Bullock et al., 1987). The plasmid pDP412 carrying the *car*AB genes in the *Hin*dIII site of the vector pBS+ was constructed as previously described (Post et al., 1990).

Growth Conditions. All cells were grown at 37 °C in Luria-Bertani (LB) broth (Maniatis et al., 1982) or a modified LB media which contained 24 g of yeast extract, 12 g of tryptone, 0.4% glycerol (per liter), and 0.1 M potassium phosphate. Transformed cells were grown in the presence of 50 μg/mL ampicillin and harvested in the stationary phase.

Construction of Truncation Mutants. Site-directed mutagenesis by overlap extension with the polymerase chain reaction (Ho et al., 1989) allowed stop codons to be introduced at various positions of the *car*B gene within the pDP412 plasmid. The pairs of internal complementary oligonucleotides encoding the stop codons and the flanking primers for the truncation mutants are listed in Table 1. The PCR-amplified mutagenic fragments of pDP412 were purified and digested with either *Bpu*1102I and *Bst*XI or *Bpu*1102I and *Bam*HI to facilitate insertion into the plasmid.

Table 2: Primers Used for Site-Directed Mutagenesis[a]

plasmid	DNA sequence	mutation
pBC412−921A	5′AAGGTGCGGGCCACGCCCAT3′ 3′TTCCACGCCCGGTGCGGGTA5′	G621A
pBC412−921V	5′AAGGTGCGGACCACGCCCAT3′ 3′TTCCACGCCTGGTGCGGGTA5′	G921V
pBC412−921I	5′AAGGTGCGGATCACGCCCAT3′ 3′TTCCACGCCTAGTGCGGGTA5′	G921I
pBC412−968A	5′AGCTCGAAGGCCTGTTTCAG3′ 3′TCGAGCTTCCGGACAAAGTC5′	G968A
pBC412−9668V	5′AGCTCGAAGACCTGTTTCAG3′ 3′TCGAGCTTCTGGACAAAGTC5′	G968V
pBC412−968I	5′AGCTCGAAGATCTGTTTCAG3′ 3′TCGAGCTTCTAGACAAAGTC5′	G968I
pBC412−977	5′ACAATCGCCGCGCCGTGGGT3′ 3′TGTTAGCGGCGCGGCACCCA5′	T977A
pBC412−1015	5′TGAGGTGGTGGCGATGATGT3′ 3′ACTCCACCACCGCTACTACA5′	N1015A
pBC412−1030	5′GCACTGCGAGCAATCACGCG3′ 3′CGTGACGCTCGTTAGTGCGC5′	R1030A
pBC412−1031	5′AGCGCACTGGCACGAATCAC3′ 3′TCGCGTGACCGTGCTTAGTG5′	R1031A

[a] The flanking primers used to isolate the mutants were 5′CGT GCG GCG GCT ACC GTT and 5′AAC GGT CCT CAT CATG AGA. These respective positions of these primers are 4172 and 4919 within the *car*B gene.

The resulting expression vectors were transformed into the *E. coli* RC50 cell line (*car*A50, *thi*-1, *mal*A1, *xyl*-7, *rsp*L135, λ^r, λ^-, *tsx*-273).

Site-Directed Mutagenesis. Two glycine residues at positions 921 and 968 were individually changed to alanine, valine, and isoleucine. In addition, Thr-977, Asn-1015, and Arg-1030 and Arg-1031 were individually changed to alanines using the method of overlap extension PCR (Ho et al., 1989). The 18- to 20-base oligonucleotide primers used to make these mutants are listed in Table 2. The amplified mutagenic DNA fragment was purified from an agarose gel, cut with *Bpu*1102I then *Bst*XI, concentrated with pDP412, digested with *Bpu*1102I/*Bst*XI, ligated with T4 DNA ligase, and finally transformed into XL1-Blue cells. Colonies were screened as described below for those plasmids containing the correct mutation. The plasmids for the mutants were subsequently transformed into RC50 cells.

Sequencing of Mutants. The insert for all of the mutants was completely sequenced using the dideoxynucleotide chain termination method (Sanger et al., 1977) to ensure that only the desired base changes were present. Double-stranded plasmid DNA (dsDNA) was isolated using the Magic Miniprep DNA purification system and cut 3′ to the mutation site with *Hin*dIII. The linear dsDNA was then incubated with the T7 5′−3′ exonuclease to make single-stranded DNA (ssDNA), which was then sequenced using the Sequenase version 2.0 DNA sequencing kit.

Purification of Wild-Type and Mutant Enzymes. Wild-type CPS was purified from *E. coli* XL1-Blue carrying the plasmid pDP412. Carbamoyl phosphate synthetase, with site-directed mutations in positions G921A, G921V, G921I, G968A, G968V, G968I, T977A, N1015A, R1030A, and R1031A, was purified from *E. coli* RC50 carrying the appropriate plasmids. Purification was performed as previously described by Mareya and Raushel (1994). However, in most purifications of the truncated enzymes, the protein from the protamine sulfate precipitation step was filtered and directly applied to the Waters Protein-Pack column. The protein concentration was determined using an absorbance coefficient of A_{280} = 0.69/mg (Rubino et al., 1986).

Determination of Enzyme Activity. For the determination of the glutamine-dependent ATPase reaction, the formation of MgADP was followed using the pyruvate kinase/lactate dehydrogenase (PK/LDH) coupling system as previously described (Mareya & Raushel, 1994). For determination of the bicarbonate-dependent ATPase reaction, the formation of MgADP from MgATP and HCO_3^- was determined using the pyruvate kinase/lactate dehydrogenase coupled assay with the omission of glutamine from the reaction mixture. For the determination of MgATP synthesis from MgADP and carbamoyl phosphate, the reaction was followed using a hexokinase/glucose-6-phosphate dehydrogenase coupled assay as previously described (Mareya & Raushel, 1994). The reaction was measured spectrophotometrically at 340 nm. The reaction mixture was assayed at 25 °C upon addition of carbamoyl phosphate synthetase (20−150 μg). Glutamine-dependent carbamoyl phosphate synthesis was determined by measuring the rate of citrulline formation in a coupled assay containing ornithine transcarbamoylase and ornithine (Mareya & Raushel, 1994).

Kinetic data were fit to eq 2 using the computer programs supplied by Savanna Shell Software. In these equations, v is the initial velocity, V_{max} is the maximal velocity, and K_m is the Michaelis constant.

$$v = \frac{V_{max}[S]}{K_m + [S]} \quad (2)$$

Variable concentrations of ATP and ADP were used in order to determine the effect of allosteric ligands on the binding of the nucleotides. Saturating concentrations of ornithine (15 mM), UMP (100 μM), and IMP (1 mM) were added to assay mixtures to determine the effect on the Michaelis constants of the nucleotides. The ATP concentrations used for most of the site-directed mutants ranged from 0.02 to 8 mM for the glutamine-dependent ATPase activity. For the bicarbonate-dependent ATPase activity, concentrations were varied from 0.02 to 0.50 mM ATP. For the ATP synthesis activity the ADP concentration was varied from 0.010 to 2.0 mM ADP.

RESULTS

Construction of Truncated Mutant Proteins. Seven truncation mutants were synthesized as outlined in the previous section. Multiple stop codons were introduced at various positions near the 3′ end of the *car*B gene in order to delete amino acid residues from the carboxy-terminal end of the large subunit. Truncation mutants with 14, 39, 50, 65, 91, 119, and 144 amino acids missing from the C-terminal end of the large subunit were constructed and labeled as Δ14, Δ39, Δ50, Δ65, Δ91, Δ119, and Δ144, respectively. These mutants correspond to truncation directly after residues 1059, 1034, 1023, 1008, 982, 954, and 929, respectively. The expression of these mutant proteins varied, and in each case the production of the mutant enzymes was lower than that of the wild-type enzyme.

Purification of Wild-Type and Mutant Enzymes. The wild-type carbamoyl phosphate synthetase and five mutant enzymes were purified in order to assess the catalytic properties and consequences of truncating the C-terminal end

Table 3: Kinetic Parameters for the Glutamine-Dependent ATPase Reaction Catalyzed by the Truncation Mutants[a]

	none		10 mM ornithine[b]		100 μM UMP[b]		1 mM IMP[b]	
enzyme	V_{max} (μmol/min·mg)	K_{ATP} (mM)	V_{max} (μmol/min·mg)	K_{ATP} (mM)	V_{max} (μmol/min·mg)	K_{ATP} (mM)	V_{max} (μmol/min·mg)	K_{ATP} (mM)
WT	3.9	0.5	3.5	0.05	1.9	1.2	3.3	0.4
Δ14	0.09	4.1	0.11	3.9	0.08	3.8	0.08	3.2
Δ50	0.05	2.2	0.07	3.5	0.05	2.3	0.04	2.4
Δ65	0.07	3.2	0.09	3.3	0.07	3.0	0.09	3.1
Δ91	0.02	2.8	0.03	5.1	0.03	3.2	0.03	3.5
Δ119	0.04	8.4	0.04	8.2	0.03	8.2	0.03	9.3

[a] Reactions were performed in the presence of 10 mM glutamine, variable ATP, 40 mM bicarbonate, and 20−40 mM Mg^{2+} at pH 7.6 and 25 °C. [b] Allosteric effector added.

Table 4: Kinetic Parameters for the Bicarbonate-Dependent ATPase Reaction Catalyzed by the Truncation Mutants[a]

	none		10 mM ornithine[b]		100 μM UMP[b]		1 mM IMP[b]	
enzyme	V_{max} (μmol/min·mg)	K_{ATP} mM	V_{max} (μmol/min·mg)	K_{ATP} (mM)	V_{max} (μmol/min·mg)	K_{ATP} (mM)	V_{max} (μmol/min·mg)	K_{ATP} (mM)
WT	0.11	0.016	0.10	0.007	0.11	0.025	0.13	0.021
Δ14	0.03	1.7	0.04	1.7	0.03	1.1	0.03	1.1
Δ50	0.01	0.32	0.02	0.46	0.01	0.22	0.01	0.34
Δ65	0.03	1.5	0.03	1.3	0.02	0.80	0.03	0.78
Δ91	0.01	1.5	0.01	1.9	0.02	1.6	0.01	1.5
Δ119	0.01	2.5	0.01	3.2	0.01	2.0	0.02	3.5

[a] Reactions were performed at pH 7.6, 25 °C, in the presence of 40 mM bicarbonate, variable ATP, and 20 mM Mg^{2+}. [b] Allosteric effector added.

Table 5: Kinetic Parameters for the ATP Synthesis Reaction Catalyzed by the Truncation Mutants[a]

	none		10 mM ornithine[b]		100 μM UMP[b]		1 mM IMP[b]	
enzyme	V_{max} (μmol/min·mg)	K_{ADP} (mM)	V_{max} (μmol/min·mg)	K_{ADP} (mM)	V_{max} (μmol/min·mg)	K_{ADP} (mM)	V_{max} (μmol/min·mg)	K_{ADP} (mM)
WT	0.42	0.17	0.35	0.02	0.15	1.9	0.27	0.10
Δ14	0.02	1.6	0.02	0.68	0.01	0.62	0.02	0.52
Δ50	0.43	0.73	0.37	0.66	0.43	0.69	0.42	1.3
Δ65	0.03	0.63	0.04	1.7	0.03	0.55	0.04	0.55
Δ91	0.17	0.49	0.16	0.95	0.17	0.53	0.17	1.0
Δ119	0.02	1.7	0.02	3.1	0.02	1.2	0.01	1.1

[a] Reactions were performed at pH 7.6, 25 °C, in the presence of 10 mM carbamoyl phosphate, 20 mM Mg^{2+}, and variable ADP. [b] Allosteric effector added.

of this protein. The wild-type enzyme was purified to greater than 95% homogeneity, whereas the five mutants were purified to at least 85% homogeneity. The purification of the Δ39 and Δ144 mutant proteins was unsuccessful. Irreversible precipitation of the Δ39 protein during the protamine sulfate step prevented the successful isolation of this protein, while the expression of the Δ144 mutant enzyme was followed by the rapid degradation of the large subunit.

Kinetic Properties of Carbamoyl Phosphate Synthetase and the Truncation Mutants. The kinetic parameters K_m and V_{max} for the truncated mutants and wild-type enzyme are presented in Tables 3, 4, and 5. The kinetic constants have been obtained for the glutamine-dependent ATPase, the bicarbonate-dependent ATPase, and ATP synthesis reactions in the absence of the allosteric effectors, and in the presence of each of the three effectors ornithine, IMP, and UMP.

Δ14 Truncation Mutant. The catalytic properties of this mutant demonstrate the rather severe effect on the enzyme when relatively few amino acids are deleted from the carboxy-terminal end of the large subunit. The V_{max} values for all reactions monitored were significantly lower than the wild-type values. Two- to three-fold reductions in the K_m for ADP were obtained with each allosteric effector in the ATP synthesis reaction (Table 5). A detectable decrease in the V_{max} was observed for the ATP synthesis reaction in the presence of UMP. The three allosteric effectors did not produce any significant changes in the kinetic parameters of the other partial reactions monitored.

Δ50 Truncation Mutant. The deletion of 50 amino acids from the carboxy-terminal end of the large subunit resulted in a significant decrease in the overall biosynthetic reaction, but the ATP synthesis activity was comparable to the wild-type protein. A two-fold increase in the K_m value for ADP was observed when the ATP synthesis reaction was carried out in the presence of IMP. Ornithine increased the K_m for ATP nearly 2 fold in the glutamine-dependent ATPase reaction.

Δ65 Truncation Mutant. The addition of UMP or IMP produced 2-fold decreases in the K_m for ATP when the bicarbonate-dependent ATPase reaction was measured for this mutant (Table 4). The ATP synthesis reaction exhibited a 2- to 3-fold increase in the K_m for ADP in the presence of ornithine. The binding of all three allosteric effectors to the truncated Δ65 protein was therefore detected.

Δ91 Truncation Mutant. The ATP synthesis activity of this mutant was found to be higher than most of the other mutants with the exception of Δ50 (Table 5). The K_m for ADP in the presence of either ornithine or IMP increased

Table 6: Kinetic Parameters for the Glutamine-Dependent ATPase Reaction Catalyzed by the Site-Directed Mutants[a]

	none		15 mM ornithine[b]		100 μM UMP[b]		1 mM IMP[b]	
enzyme	V_{max} (μmol/min·mg)	K_{ADP} (μM)	V_{max} (μmol/min·mg)	K_{ADP} (μM)	V_{max} (μmol/min·mg)	K_{ADP} (μM)	V_{max} (μmol/min·mg)	K_{ADP} (μM)
WT	3.9	560	3.5	50	1.9	1200	3.3	390
G921A	2.3	370	2.1	57	0.6	520	1.3	81
G921V	0.1	73	0.08	55	0.1	73	0.1	76
G921I	0.1	140	0.09	180	0.1	140	0.08	260
G968A	1.6	920	1.2	100	0.7	760	0.7	170
G968V	1.7	570	1.6	150	1.5	450	1.6	290
G968I	0.9	580	1.0	390	1.1	630	0.8	530
T977A	1.1	610	1.0	75	1.0	600	0.9	440
R1030A	1.6	550	2.5	80	1.5	1400	1.1	370
R1031A	2.0	340	2.6	67	1.3	950	2.0	200

[a] Reactions were performed in the presence of 10 mM glutamine, variable ATP, 50 mM bicarbonate, and 20−40 mM Mg^{2+} at pH 7.6 and 25 °C. [b] Allosteric effector added.

Table 7: Kinetic Parameters for the Bicarbonate-Dependent ATPase Reaction Catalyzed by the Site-Directed Mutants[a]

	none		15 mM ornithine[b]		100 μM UMP[b]		1 mM IMP[b]	
Enzyme	V_{max} (μmol/min·mg)	K_{ADP} (μM)	V_{max} (μmol/min·mg)	K_{ADP} (μM)	V_{max} (μmol/min·mg)	K_{ADP} (μM)	V_{max} (μmol/min·mg)	K_{ADP} (μM)
WT	0.11	16	0.10	7	0.11	25	0.13	21
G921A	0.26	59	0.16	23	0.18	31	0.20	45
G921V	0.06	5	0.07	5	0.06	5	0.07	4
G921I	0.03	15	0.07	34	0.05	45	0.05	12
G968A	0.13	110	0.07	10	0.14	100	0.12	54
G968V	0.26	40	0.21	22	0.24	37	0.27	33
G968I	0.24	70	0.32	80	0.24	80	0.19	50
T977A	0.16	31	0.13	10	0.16	31	0.20	43
R1030A	0.15	20	0.15	7	0.13	18	0.05	17
R1031A	0.32	21	0.26	10	0.21	29	0.34	32

[a] Reactions were performed in the presence of variable ATP, 50 mM bicarbonate, and 20−40 mM Mg^{2+} at pH 7.5 and 25 °C. [b] Allosteric effector added.

2-fold for this reaction. A 2-fold increase in the K_m for ATP for the glutamine-dependent ATPase reaction was observed in the presence of ornithine. Insignificant changes in the kinetic parameters were obtained for the bicarbonate-dependent ATPase reaction.

Δ119 Truncation Mutant. After the deletion of 119 residues from the carboxy-terminal end of the large subunit, measurable activity was still obtained for the partial reactions. However, ornithine was the only allosteric effectors that produced any changes in the apparent binding of the nucleotide substrates. A 2-fold increase in the K_m for ADP was obtained for the ATP synthesis reaction in the presence of ornithine.

Construction and Purification of Mutant Enzymes at Glycine Residues 921 and 968. The two conserved glycine residues at positions 921 and 968 within the large subunit of CPS were mutated to alanine, valine, and isoleucine. In general the levels of expression of these mutant proteins were less than those of the wild-type enzyme under similar growth conditions. All of the mutant proteins were purified to greater than 90% homogeneity using the same isolation procedures developed originally for the wild-type enzyme.

Kinetic Properties of the Glycine-Modified Mutants. The kinetic properties of the mutant enzymes that were made at the two conserved glycine residues were determined. The catalytic constants V_{max} and K_m were determined for the bicarbonate-dependent ATPase activity, the glutamine-dependent ATPase activity, the ATP synthesis activity, and the overall synthesis of carbamoyl phosphate. The results are presented in Tables 6, 7, and 8. These measurements were made in the presence and absence of saturating concentrations of the allosteric effectors ornithine, UMP, and IMP.

Bicarbonate-Dependent ATP Hydrolysis. The kinetic constants for the bicarbonate-dependent hydrolysis of ATP in the absence of glutamine for the wild-type and glycine mutants are listed in Table 7. The observed rate of ATP hydrolysis activity for this partial reaction of CPS is not dramatically affected by the substitution of larger hydrophobic residues for the original glycine residues. However, there is a minor decrease in the maximal rate of the ATPase reaction for the G921I and G921V mutants. The Michaelis constants for ATP of these mutants were altered by a factor of 0.5 to 4 relative to the wild-type value except for G921V and G968I. The K_m values for ATP with the G921V mutant are significantly less than the wild-type value, whereas the K_m for ATP of the G968I mutant is an order of magnitude higher in the presence of ornithine.

Glutamine-Dependent ATP Hydrolysis. The kinetic constants for the utilization of ATP measured in the presence of glutamine are listed in Table 6. With respect to the wild-type enzyme there are significant decreases in the V_{max} for ATP turnover with glutamine as a nitrogen source for the G921V and G921I mutants. However, there is relatively little effect upon substitution of an alanine for the original glycine at this position. The K_m for ATP increases in the presence of ornithine as the amino acid at position 968 is changed from glycine to isoleucine. The inhibitory effect of UMP is diminished for the G968A mutant and is completely lost for the G968V and G968I substitutions.

ATP Synthesis Reaction. The rate of ATP production from ADP and carbamoyl phosphate was monitored for the mutants and wild-type enzyme, and the kinetic constants are listed in Table 8. A large decrease in the maximal catalytic

Table 8: Kinetic Parameters for the ATP Synthesis Reaction Catalyzed by the Site-Directed Mutants[a]

	none		15 mM ornithine[b]		100 μM UMP[b]		1 mM IMP[b]	
Enzyme	V_{max} (μmol/min·mg)	K_{ADP} (μM)	V_{max} (μmol/min·mg)	K_{ADP} (μM)	V_{max} (μmol/min·mg)	K_{ADP} (μM)	V_{max} (μmol/min·mg)	K_{ADP} (μM)
WT	0.42	170	0.35	22	0.15	1900	0.27	95
G921A	0.32	110	0.27	14	0.42	880	0.22	110
G921V	<0.003	ND	<0.002	ND	<0.002	ND	<0.002	ND
G921I	0.07	430	0.04	120	0.07	380	0.07	260
G968A	0.08	130	0.08	14	0.17	360	0.05	40
G968V	0.27	370	0.28	130	0.29	340	0.38	260
G968I	0.20	150	0.19	100	0.18	150	0.21	160
G977A	0.21	180	0.18	36	0.24	160	0.21	180
R1030A	0.18	600	0.26	26	0.12	780	0.19	490
R1031A	0.43	100	0.40	22	0.16	1200	0.44	270

[a] Reactions were performed at pH 7.6, 25 °C, in the presence of 10 mM carbamoyl phosphate, 15 mM Mg^{2+}, and variable ADP. [b] Allosteric effector added.

activity is observed for the G921V mutation. This decrease in V_{max} is greater than 2 orders of magnitude relative to the wild-type enzyme. The allosteric effects of ornithine are diminished for the G968V and G968I mutations. Moreover, the allosteric effect of UMP is reduced for the G968A mutant and lost for the G968V and G968I proteins.

Carbamoyl Phosphate Synthesis by Glycine Mutants. All of the glycine mutants, as well as the wild-type enzyme, were assayed to determine if these mutants were able to synthesize carbamoyl phosphate. All of these mutants were able to synthesize carbamoyl phosphate at a rate approximately one-half that observed for the rate of ATP turnover (data not shown). However, no detectable activity (<0.004 μmol/min mg) was observed for the G921V and G921I mutants.

Construction, Purification, and Characterization of Mutant Enzymes at Nonglycine Conserved Residues. The four nonglycine conserved amino acid residues within the allosteric binding domain of the large subunit of CPS, (Thr-977, Asn-1015, Arg-1030, and Arg-1031) were mutated to alanine residues. The mutant proteins, with the exception of N1015A, were purified to homogeneity and their kinetic constants determined. The N1015A mutant was rapidly degraded during attempts at isolation. The catalytic constants for the remaining three mutants were determined for the partial reactions as well as for the full biosynthetic reaction using glutamine as a nitrogen source in the presence and absence of the allosteric effectors. The values for K_m and V_{max} are listed in Tables 6, 7, and 8.

Bicarbonate-Dependent ATP Hydrolysis. The kinetic constants with this set of mutants for the hydrolysis of ATP in the absence of glutamine are listed in Table 7. The observed rates for the ATP hydrolysis activity are not significantly different from the wild-type values. The net effects exhibited by the allosteric ligands are quite small.

Glutamine-Dependent ATP Hydrolysis. The kinetic constants for the hydrolysis of ATP in the presence of glutamine are listed in Table 6. The observed rate of ATP hydrolysis for the T977A mutant is decreased by a factor of about 3 relative to the wild-type enzyme. The observed rate of hydrolysis of ATP for the R1030A and R1031A mutants is decreased by less than 50% relative to the wild-type enzyme. The apparent K_m for MgATP in the presence of ornithine is not altered in the T977A, R1030A, or R1031A mutants relative to the wild-type enzyme. However, the apparent K_m for MgATP in the presence of UMP for the T977A mutant has been altered relative to the wild-type enzyme. The T977A mutant has lost the ability of UMP to inhibit the enzyme by increasing the K_m for ATP. In contrast, the R1030A and R1031A mutants are still inhibited in the presence of UMP. All three mutants synthesize carbamoyl phosphate at a rate that is approximately one-half the rate of ATP turnover in the presence of glutamine (data not shown).

ATP Synthesis. The rate of ATP production was measured for the mutant and wild-type enzymes, and the catalytic constants are listed in Table 8. The values for V_{max} with the T977A, R1030A, and R1031A mutants are essentially the same as found for the wild-type enzyme. The apparent K_m values for MgADP in the presence of ornithine did not vary from the wild-type values. In the presence of UMP, the K_m for MgADP is not changed with the T977A mutant, indicating that the ability of UMP to inhibit the enzyme has been lost. For the R1030A mutant the effect on the K_m for MgADP in the presence of UMP was diminished slightly from the wild-type value, while the R1031A mutant was inhibited by UMP very much like the wild-type enzyme.

DISCUSSION

Carbamoyl phosphate synthetase from *E. coli* is a complex enzyme. The heterodimeric enzyme has one small subunit for the amidotransferase activity and another larger subunit for the direct synthesis of carbamoyl phosphate. In addition, the amino and carboxyl terminal halves of the large subunit are homologous. The amino acid sequence from residues 1 to 400 is 40% identical to the sequence from residues 553 to 933 (Nyunoya & Lusty, 1983). These two regions are currently thought to contain the binding sites for each of the two molecules of ATP that are required for the synthesis of carbamoyl phosphate (Post et al., 1990). The N-terminal half is utilized for the phosphorylation of bicarbonate whereas the C-terminal half is utilized for the phosphorylation of the intermediate, carbamate. The allosteric effectors ornithine, UMP, and IMP all bind to the large subunit and affect catalytic activity by modulating the Michaelis constant for the ATP used in the phosphorylation of the carbamate intermediate. Previous experiments by Rubio et al. (1991) have strongly suggested that the binding site for UMP is located at the extreme C-terminal end of the large subunit (between residues 933 and 1073). The location of the ornithine site has not been determined but has often been assumed to be located within the same C-terminal domain as the binding site for UMP.

This investigation was prompted by two reports. First, we have recently demonstrated that limited proteolysis of the large subunit of CPS from *E. coli* with endoproteinase Asp-N produced a series of nested fragments of CPS after relatively short peptides (10−100 residues) were cleaved from the C-terminal end of large subunit. These truncated fragments of the large subunit were catalytically active but appeared to have lost all allosteric control (Mareya & Raushel, 1995). Second, chemical labeling experiments with the rat liver enzyme suggested that portions of the ATP binding site(s) were composed of side chains that were no farther than 50 residues from the extreme C-terminal end of the large subunit (Potter & Powers-Lee, 1992). The first report suggests that the catalytic activity of CPS could be physically separated from the allosteric controls, whereas the chemical labeling experiments imply that the catalytic and regulatory domains are spacially quite close.

Our initial aims for the experiments reported here were to probe the mechanism, pathway, and specificity for allosteric control of the large subunit by ornithine and UMP. We were especially interested in determining whether the binding sites for ornithine and UMP could be differentiated and if the allosteric signal from the site of binding to the site of action on ATP proceeded via a common or distinct pathway for these two allosteric effectors with opposite functions. However, the three-dimensional structure of CPS is not known from any source. Therefore, in order to disrupt the site of allosteric ligand binding and/or linkage to the catalytic site, we had to rely on non−structural-based methods for selection of specific residues for mutation. The approach taken here has been to mutate conserved residues within the putative allosteric binding domain and to shorten the allosteric domain as a way of locally disrupting the structure of the wild-type enzyme.

In order to make fragments of the large subunit of defined length, we elected to put termination codons selectively at various locations along the *car*B gene. These truncated proteins were then utilized to probe the makeup of the allosteric domain of CPS and to determine further if the UMP and ornithine control sites could be experimentally distinguished. Removal of 14 to 119 amino acids from the carboxy-terminal end of CPS results in the significant loss of catalytic activity. This loss in catalytic activity is expressed in the full biosynthetic reaction in addition to the two partial reactions exhibited by this enzyme. At least a 40-fold decrease in activity was observed for the glutamine-dependent ATPase reaction, and approximately 10-fold decreases in activity were observed for the bicarbonate-dependent ATPase reaction. The partial ATP synthesis activities of the mutants Δ50 and Δ91 were higher than those of the other mutants. ATP synthesis rates comparable to the wild-type enzyme were obtained with the Δ50 mutant and 2- to 3-fold decreases in the ATP synthesis activity were exhibited by the Δ91 protein.

Although these mutants are catalytically sluggish, enzymatic activity was observed for these mutants even after the deletion of 119 amino acids from the carboxy-terminal end of the large subunit. Guillou et al. (1989) have previously reported the complete loss of enzymatic activity when 171, 350, and 495 amino acids were removed from the carboxy-terminal end of the large subunit, and the results obtained in this investigation are consistent with those results. However, the fact that catalytic activity is still retained by the mutants prepared for this investigation strongly suggests that the binding sites for ATP do not extend into this region of the large subunit. Potter and Powers-Lee (1992) have proposed that the binding site for the ATP that phosphorylates carbamate includes portions of the extreme C-terminus of the rat liver enzyme. This proposal was based on the isolation of radiolabeled peptides after incubation of the enzyme with the reactive ATP analogue FSBA. In particular, they found a labeled peptide that corresponds to residues 1445−1454 of the rat liver CPS (these residues are homologous to residues 1022−1031 of the *E. coli* enzyme). This putative site is therefore only 43−52 amino acid residues away from the extreme carboxy-terminal end of the large subunit. If this portion of the large subunit constituted a significant part of the nucleotide binding pocket, it is unlikely that catalytic activity would remain after deletion of this whole domain.

The truncation mutants were also tested for their ability to be regulated by the addition of the allosteric effectors ornithine, IMP, and UMP. Changes in the Michaelis constants for ATP and/or ADP were observed for some of these mutants with these compounds. However, the overall effects were not always the same as those observed in the wild-type enzyme. For example, ornithine showed a tendency to decrease the affinity of the nucleotides with these mutants, whereas with wild-type enzyme this molecule exclusively activates by reducing the Michaelis constant for either ATP or ADP. Nevertheless, a measurable effect for ornithine was still observed after 119 amino acids were removed from the C-terminal end of the large subunit. This observation suggests that the ornithine binding site is still partially intact but has been perturbed by the deletion. In contrast the allosteric effects exhibited by UMP are, in general, no longer observed after more than 65 amino acids have been excised from the C-terminal end of the large subunit. These results support the proposal that places the allosteric binding site for UMP within the extreme C-terminal domain. The location of the binding site for ornithine is still unresolved, but the allosteric effects by ornithine are still observed after the removal of 119 amino acids from the carboxy-terminal end of CPS. These results suggest that the binding site for ornithine may be farther away from the carboxy-terminal end of CPS than UMP.

The detrimental character of deleting amino acids from the carboxy-terminal end was further demonstrated by the construction of Δ39 and Δ144. The purification of Δ39 was hindered because of problems with precipitation of the protein during cell lysis. Proteolytic degradation of the large subunit during purification also prevented the isolation of Δ144. The degradation and instability of the truncation mutants demonstrate the requirement of the carboxy-terminal end of CPS for the stabilization of the overall enzyme conformation. The lower enzymatic activities observed with the truncation mutants may also be due to folding problems associated with their construction. Alterations in the global or local structural conformation of the large subunit may also be required to explain the reversal in the allosteric effects by ornithine.

Two additional sets of site-directed mutants were constructed in order to complement the truncation experiments. The first set concentrated on two conserved glycine residues. In order to restrict the conformational flexibility of the protein in the region of these glycine residues, the side chain on the

α-carbon was elaborated by successive mutation to alanine, valine, and isoleucine. These bulkier hydrophobic additions are larger in size, and thus any potential disruptions to the structure and/or function may be modulated by the specific mutation at this location within the protein. The alternative approach has been to take other conserved residues within the allosteric binding domain and eliminate the side chain by mutation to an alanine residue. We have constructed and kinetically characterized mutations at six conserved residues within the allosteric binding domain.

The mutations constructed at Gly-968 are well within the putative allosteric binding domain. Changes in this residue had relatively little effect on the k_{cat} for the hydrolysis of ATP or formation of carbamoyl phosphate. Therefore, alteration at this site has not perturbed, in any significant way, the catalytic machinery for carbamoyl phosphate synthetase. However, mutations at this site have significantly perturbed the ability of both ornithine to activate and UMP to inhibit the protein by altering the K_m for MgATP. With the wild-type enzyme the ratio of the Michaelis constants for MgATP in the presence and absence of the effector is approximately 10 when ornithine activates the protein. As this residue is changed from glycine to other amino acids with larger side chains, the effectiveness of ornithine to activate the protein is gradually diminished until almost no difference in the K_m for MgATP is observed with the G968I mutant. The very same trend is observed when UMP is used to inhibit the catalytic activity of the enzyme. For the wild-type enzyme, the K_m for MgADP is increased by an order of magnitude in the presence of UMP. With these mutants the inhibitory effect by UMP is drastically diminished upon the simple addition of an extra methyl group (G968A) and lost altogether with the valine (G968V) and isoleucine (G968I) substitutions. We have thus constructed a series of mutants whereby the allosteric signals from the allosteric binding site for each effector can be attenuated simply by the size of the side chain that is substituted for the original glycine at residue 968.

The series of mutations at residue Gly-921 have more seriously affected the catalytic function of the domain that is required for the phosphorylation of carbamate. The G921V and G921I proteins are apparently unable to synthesize carbamoyl phosphate, and there is essentially no enhancement in MgATP hydrolysis in the presence of either glutamine or ammonia. In addition, the G921V protein is unable to catalyze the partial back reaction where carbamoyl phosphate is used to phosphorylate ADP. This latter result provides further confirmation that the domain encompassing residues 553−933 is primarily involved in the phosphorylation of carbamate rather than the phosphorylation of bicarbonate (Post et al., 1990).

Conserved residue were also mutated at positions Thr-977, Arg-1030, and Arg-1031. The N1015A mutant must be relatively unstable since no carbamoyl phosphate synthetase could be purified from cells expressing this enzyme. The three remaining enzymes were easily purified and displayed catalytic properties that were essentially unchanged relative to those observed for the wild-type enzyme in the absence of allosteric effectors. Therefore, the side chains for these three residues do not appear critical for the normal functions of CPS. This result is also consistent with the extreme C-terminal domain functioning exclusively as the site of binding and transduction of signals for the control of catalysis through the binding of allosteric ligands.

This last series of mutants also exhibited quite interesting allosteric properties. When these three residues were mutated to alanine, there was essentially no alteration in the allosteric properties exhibited by ornithine. There is, however, a significant difference for the allosteric effects exhibited by UMP. Although mutation of the side chains for either of the arginine residues results in the production of protein with wild-type activity, the T977A mutant has totally lost its ability to be regulated by UMP. However, it is unknown at this time whether this is a result of a disruption in the binding of the allosteric ligand UMP, or the formation of a short circuit in the transmission of the allosteric signal from the site of binding to the site of action. If the latter is true then the signal pathways for ornithine and UMP are apparently distinct. Nevertheless, the net result is the construction of a molecular switch for gating off the allosteric signal from UMP while retaining the effectiveness of activation by ornithine. This latter mutant (T977A) may therefore find some utility in metabolic studies for an investigation of the consequences of a CPS that is no longer controlled by pyrimidines.

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