

Role of Conserved Residues within the Carboxy Phosphate Domain of Carbamoyl Phosphate Synthetase[†]

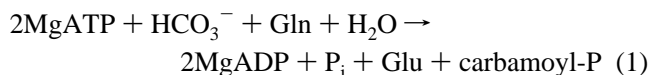
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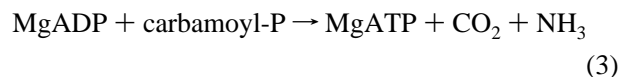
ABSTRACT: Carbamoyl phosphate synthetase (CPS) catalyzes the formation of carbamoyl phosphate from glutamine, bicarbonate, and 2 mol of MgATP. The heterodimeric protein is composed of a small amidotransferase subunit and a larger synthetase subunit. The synthetase subunit contains a large tandem repeat for each of the nucleotides used in the overall synthesis of carbamoyl phosphate. A working model for the three-dimensional fold of the carboxy phosphate domain of CPS was constructed on the basis of amino acid sequence alignments and the X-ray crystal structure coordinates for biotin carboxylase and D-alanine:D-alanine ligase. This model was used to select ten residues within the carboxy phosphate domain of CPS for modification and subsequent characterization of the kinetic constants for the mutant proteins. Residues R82, R129, R169, D207, E215, N283, and Q285 were changed to alanine residues; residues E299 and R303 to glutamine; and residue N301 to aspartate. No significant changes in the catalytic constants were observed upon mutation of either R82 or D207, and thus these residues appear to be nonessential for binding and/or catalytic activity. The Michaelis constant for ATP was most affected by modification of residues R129, R169, Q285, and N301. The binding of bicarbonate was most affected by the mutagenesis of residues E215, E299, N301, and R303. The mutation of residues E215, N283, E299, N301, and R303 resulted in proteins which were unable to synthesize carbamoyl phosphate at a significant rate. All of the mutations, with the exception of the N301D mutant, primarily affected the enzyme by altering the step for the phosphorylation of bicarbonate. However, mutation of N301 to aspartic acid also disrupted the catalytic step involved in the phosphorylation of carbamate. These results are consistent with a role for the N-terminal half of the synthetase subunit of CPS that is primarily directed at the initial phosphorylation of bicarbonate by the first ATP utilized in the overall synthesis of carbamoyl phosphate. The active site structure appears to be very similar to the ones previously determined for D-alanine:D-alanine ligase and biotin carboxylase.

Carbamoyl phosphate synthetase (CPS)¹ from *Escherichia coli* catalyzes the formation of carbamoyl phosphate, an intermediate in the biosynthesis of arginine and the pyrimidine nucleotides, by the following reaction:



The proposed catalytic mechanism involves four steps:² (1) the activation of bicarbonate via the formation of carboxy phosphate; (2) formation of ammonia by the hydrolysis of glutamine; (3) nucleophilic attack by ammonia on carboxy phosphate to generate carbamate; and (4) phosphorylation

of carbamate by the second molecule of ATP to form carbamoyl phosphate. This mechanism is illustrated in Scheme 1 (Meister, 1989; Raushel et al., 1978). In addition to the full forward reaction, the large subunit of CPS is able to catalyze two partial reactions; the bicarbonate-dependent ATPase reaction (eq 2) and the ATP synthesis reaction (eq 3).



The CPS protein is a heterodimer composed of a small subunit (42 kDa) and a large subunit of 118 kDa (Nyunoya et al., 1983). The small subunit is responsible for the hydrolysis of glutamine to glutamate and ammonia, while the large subunit contains the binding sites for bicarbonate, both molecules of MgATP, and several allosteric effectors (Anderson, 1966). The N- and C-terminal halves of the large subunit are homologous. The amino-terminal domain (residues 1–400) has 39% identical residues and 25% conservative replacements when compared to the corresponding carboxy-terminal domain extending from residues 553–933 (Nyunoya et al., 1983). It has been demonstrated by steady state kinetic analysis (Raushel et al., 1978), site-directed

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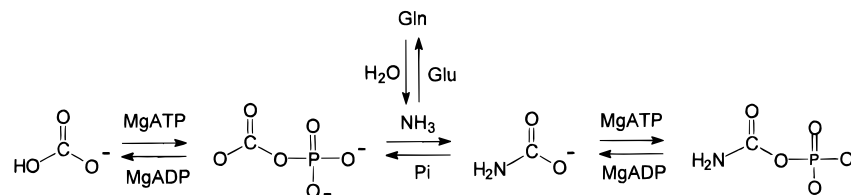
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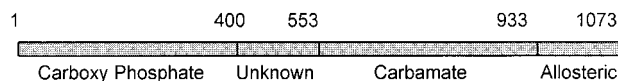
¹ Abbreviations: CPS, carbamoyl phosphate synthetase; HEPES, N-(2-hydroxyethyl)piperazine-N'-2-ethanesulfonic acid; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)aminomethane; PCR, polymerase chain reaction; BC, biotin carboxylase; DD-ligase, D-alanine:D-alanine ligase.

² It has been proposed by Sauers et al. (1975) that carboxy phosphate is cleaved to carbon dioxide and phosphate prior to the nucleophilic attack by ammonia.

Scheme 1



Scheme 2



mutagenesis (Post et al., 1990), and inactivation studies with 5'-*p*-fluorosulfonylbenzoyl adenosine (FSBA) (Boettcher & Meister, 1980) that there are at least two separate binding sites for MgATP on the large subunit of CPS.

The amino-terminal half (carboxy phosphate domain) of the large subunit is primarily responsible for the phosphorylation of bicarbonate and the formation of the reactive intermediate, carboxy phosphate. The carboxyl-terminal half (carbamate domain) of the large subunit is responsible for the phosphorylation of carbamate and the ultimate formation of carbamoyl phosphate (Post et al., 1990; Miles et al., 1993). The region of the large subunit, defined by residues 400–553, has, as yet, no known function. The domain from residues 933 to 1073 is responsible for the binding and allosteric regulation by UMP and IMP (Rubio et al., 1991). These very approximate domain boundaries are summarized in Scheme 2. However, this scheme is incomplete since no provision has been made for the transfer of ammonia from the small subunit of CPS to the site of carbamate formation.

The three-dimensional structure has not been solved for CPS from any source, but X-ray quality crystals have been obtained (Thoden et al., 1995; Marina et al., 1995). Nevertheless, sequence alignment analyses with related proteins have demonstrated that the CPS from *E. coli* exhibits a well-defined homology with many biotin-dependent enzymes such as the biotin carboxylase (BC) subunit of *E. coli* acetyl CoA carboxylase (Kondo et al., 1991), chicken and rat propionyl-CoA carboxylase (Browner, et al., 1989), and yeast pyruvate carboxylase (Lim et al., 1988). The amino acid sequences of BC and CPS from *E. coli* are 23% identical and 48% similar over the span of residues from 153 to 328 of BC (Kondo et al., 1991). One particular region conserved in all of these biotin containing proteins, and the N- and C-terminal halves of CPS, is located at amino acid residues 288–292 in BC, with the sequence ExNxR (Kondo et al., 1991; Best & Knauf, 1993).

In *E. coli*, acetyl CoA carboxylase is composed of three subunits that have distinct functions, and each of these subunits can be isolated separately (Guchhait et al., 1974). The biotin carboxylase subunit catalyzes the ATP-dependent carboxylation of the 1'-nitrogen of biotin. In addition to being homologous to one another in primary sequence, the reactions catalyzed by biotin carboxylase and CPS have mechanistic similarities. It has been proposed that biotin carboxylase utilizes carboxy phosphate as a reactive intermediate during the carboxylation of biotin (Ogita & Knowles, 1988).

An extensive sequence alignment can also be constructed between *E. coli* CPS and D-alanine:D-alanine ligase (DD-

ligase). This latter enzyme catalyzes the formation of the dipeptide D-ala-D-ala. A high degree of sequence identity is observed between the two halves of the CPS large subunit and the protein DD-ligase as illustrated in Figure 1. The N-terminal half of the large subunit of CPS contains 24% identical and 48% similar residues to that of D-ala:D-ala ligase. The proposed chemical mechanism for the reaction catalyzed by DD-ligase is initiated by the phosphorylation of the first D-alanine molecule to yield an acylphosphate intermediate (Mullins et al., 1990). The acylphosphate intermediate is subsequently attacked by the amino group of the second D-alanine substrate, and then phosphate is eliminated to produce the D-ala-D-ala dipeptide (Fan et al., 1994). The chemical mechanism of DD-ligase is therefore quite similar to the proposed mechanism for the formation of carbamoyl phosphate by CPS. Both reaction mechanisms involve the formation of an acyl phosphate intermediate followed by an attack with a nitrogen nucleophile and formation of an amide functional group.

In the study reported here, the coordinates from the crystal structures of the *E. coli* biotin carboxylase subunit of acetyl-CoA carboxylase (Waldrop et al., 1994) and the *E. coli* D-ala:D-ala ligase (Fan et al., 1994) were used to construct a three-dimensional model for the N-terminal half of the large subunit of *E. coli* carbamoyl phosphate synthetase. This three-dimensional model, in conjunction with the extensive sequence alignments, was subsequently used to identify potentially critical residues in the carboxy phosphate and in the carbamate domains of CPS as initial targets for site-directed mutagenesis. These mutants have been constructed, expressed, and purified, and the catalytic properties have been determined for a variety of assay formats. We have used these results to construct a working model for the coordinated synthesis of carbamoyl phosphate.

MATERIALS AND METHODS

Chemicals and Enzymes. All restriction enzymes were obtained from Promega, Stratagene, New England Biolabs, or USB/Amersham. The chemicals and enzymes used in the polymerase chain reaction (PCR) were purchased from Perkin-Elmer. The Sequenase 2.0 sequencing kit was supplied by USB/Amersham. All oligonucleotides were synthesized by the Gene Technologies Laboratory, Biology Department, Texas A&M University. Bacto-tryptone and yeast extract were purchased from Difco and Ultrogel AcA-34 was purchased from IBF Biotechnics. All chemicals and coupling enzymes used in assays were supplied by either Sigma or Aldrich.

Bacterial Strains and Plasmids. The *E. coli* strains used for this study were RC50 (*carA50*, *thi-1*, *malA1*, *xyl-7*, *rspL135*, λ^+ , λ^- , *tsx-273*) and XL1-Blue (*recA1*, *endA1*, *gyrA96*, *thi-1*, *hsdR17*($r_k^-m_k^+$), *supE44*, *relA1*, λ^- , (*lac*)). The RC50 strain was a generous gift from Dr. Carol J. Lusty (Public Health Research Institute of New York). All of the

BC	...MLDKIVI	ANRGEIALRI	LRACKELGIK	TVAVHSSADR	DLKHVLLADE	47
Dd1BTDKIIV	LLGGTSAER	EVSLNSGAAVLAGLR	31
CPS-1	MPKRTDIKSI	LILGAGPIVI	GQACEFDYSG	AQACKALREE	GYRVILVNSN	50
CPS-553	ANPSTDREKI	MVLGGGPNRI	GQGIFFDYCC	VHASLALRED	GYETIMVNCN	602
•						
BC	TVCIGPAPSV	KSYLNIPATI	SAAEITGAVA	IHPGYGFLSE	NANF.....	91
Dd1B	EGGIDAYPVD	PKEVDVTQLK	SMGFQKVFIA	LH...GRGGE	DGTL.....	72
CPS-1	PATIMTDPPEM	ADATYIEPTH	WEVVRKIIEK	ERPDVAVLPTM	GGQTALNCAL	100
CPS-553	PETVSTDYDT	SDRLYFEPVT	LEDVLEIVRI	EKPKGVIVQY	GGQTPLKLAR	652
•						
BC	...AEQVER	SGFIFIGPKA	ETTRIMGDKV	SAIAAMKKAG	VPC...VPGS	134
Dd1B	...QGMLEL	MGLPYTGSGV	MASALSMDKL	RSKLLWQAG	LPVAPWVALT	118
CPS-1	ELERQGVLEE	FGVTMIGATA	DAIDKAEDRR	REDVAMKIG	LETA.....	144
CPS-553	ALEAA... .	GVPVIGTSP	DAIDRAEDRE	RFQHAVERLK	LKQP.....	690
•						
BC	DGPLGDDM.D	KNRAIAKRIG	YPVIIKASGG	GGGRGMRVVR	GDAELAQSIG	183
Dd1B	RAEFEKGLSD	KQLAEISALG	LPVIVKPSRE	GSSVGMKVV	AENALQDALR	168
CPS-1	RSGIAHTM.E	EALAVAADVG	FPCIIRPSFT	MGGSGGGIAY	NREEFEE... .	190
CPS-553	ANATVTAI.E	MAVEKAKEIG	YPLVVRPSYV	LGGRAMEIVY	DEADLRR... .	736
•						
BC	MTRAEAKAAF	SNDMVYMEKY	LENPRHVEIQ	VLADGQGNAI	YLAELDCSMQ	233
Dd1B	LAFQH.....	.DEEVLEIKW	LSGP.EFTVA	ILGEEILPSI	RIQPSGTFYD	211
CPS-1	.ICARGLDLS	PTKELLIDES	LIGWKEYEME	VVRDKNDNCI	IVCSIEN.FD	238
CPS-553	.YFQTAVSVS	NDAPVLLDHF	LDDAVEVDVD	AICD.GEMVL	IGGIMEH.IE	783
•						
BC	RRHQKVVEEA	P...APGITP	ELRRYIGERC	AKACVDIGYR	.GAGTFEELF	279
Dd1B	YEAKYLSDET	QYFCPAGLEA	SQEANLQALV	LKAWTTLGCK	.GWGRIDVML	260
CPS-1	AMGIHTGDSI	TVAPAQTLTD	KEYQIMRNAS	MAVLRIGVE	TGGSNVQFAV	288
CPS-553	QAGVHSGDSA	CSLPAYTLSQ	EIQDVMRQQV	QKLAFELQVR	.GLMNVQFAV	832
•						
BC	E..NGEFYFI	EMNTRIQVEH	EVTEMITGVD	LIKEQLRIAA	GQPLSIKQEE	327
Dd1B	DS.DGQFYLL	EANT.....	..SPGMTSHS	LVPMAARQAG	..MSFSQLV	298
CPS-1	NPKNGRLIVI	EMNPRVSRSS	ALASKATGFP	IAKVAAKLAV	GYTLD.....	333
CPS-553	..KNNEVYLI	EVNPRARTV	PEVSKATGVP	LAKVAARVMA	GKSLA.....	875
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FIGURE 1: Sequence alignment of the large subunit of CPS (Nyunoya & Lusty, 1983) with biotin carboxylase (Kondo et al., 1991) and DD-ligase (Robinson et al., 1986). In this figure BC is the abbreviation for the biotin carboxylase subunit of acetyl Co-A carboxylase from *E. coli*. Dd1B is the abbreviation for DD-ligase from *E. coli*. CPS-1 designates the sequence of the carboxy phosphate domain and CPS-553 designates the sequence of the carbamate domain. Darker shading is made when any two of the four residues in the alignment are identical. Residues that are marked by dots are those which have been mutated in this study.

other plasmids used in this project were derived from the plasmid pDP412 (Miles et al., 1993).

Reconstruction of the Expression Plasmid. In order to facilitate the construction of the desired mutants, the polyclonal region of the plasmid pDP412 was excised. The plasmid pDP412 was first digested with *Hind*III to remove the *carAB* gene. This plasmid was then further digested with *Eco*RI and a portion of the polyclonal region was replaced by an oligonucleotide linker with the sequence 5'-AATTGGCGGTGCGA-3' hybridized to 3'-CCGCCACGCTTCGA-5'. The reconstructed plasmid (pMS02) contained the restored *Hind*III site but did not contain the restriction sites for *Eco*RI, *Sac*II, *Kpn*I, *Sma*I, *Bam*HI, *Xba*I, *Acc*I, *Pst*I, or *Sph*I. The *carAB* gene was reinserted into pMS02 at the *Hind*III site to form pMS03.

Site-Directed Mutagenesis. 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 encode for the mutations are listed in Table 1. The flanking primers used to produce the PCR fragments for the R82A, R129A, and R169A mutants were 5'-CGAGCGGTGCGAA-GACTCTC-3' at position 1306 and 5'-CCGATCAGC-GACTCAGCAATCAGC-3' at position 2251 of the *carAB* gene. The flanking primers used to make the D207A, E215A, N283A, and Q285A mutants were at positions 2133 and 2650 of the *carAB* gene. The sequences of these primers were 5'-CGTGCATTATTGCCCCATC-3' and 5'-CGTTCAGCGTCTTCTGCACG-3', respectively. In order to make the E299Q, N301D, and R303Q mutations, flanking primers at positions 2190 and 3028 were used with the sequences of 5'-ACCGTGAAGAGTTTGAAG-3' and 5'-CAGGAACCAGCCGTCAAT-3', respectively. For the construction of the E299Q, N301D, and R303Q mutants, the PCR fragments were digested with *Sac*II and *Eco*RI. The PCR fragments used to make the D207A, E215A, N283, and

Table 1: Primers Used for Synthesis of Site-Directed Mutants of CPS^a

plasmid	DNA sequence	enzyme
pMS11	5'-TCGAAATGGACCCACGCG-3' 3'-AGCTTTACCTGGGAGCGC-5'	N301D
pMS12	5'-GAACCCACAAGTGTCCCG-3' 5'-CTTGGGTGTTACACAGGGC-5'	R303Q
pMS13	5'-ATTGTTATCCAAATGAAC-3' 3'-TAACAATAAGGTTTACTTG-5'	E299Q
pMS14	5'-GAAAAAGAGGCCCGGACGC-3' 3'-CTTTTTCTCCGGGGCCTGCG-5'	R82A
pMS15	5'-CAGAAGACGCCCGTCGTTTC-3' 3'-GACTTCTGCGGACGCAAAG-5'	R129A
pMS16	5'-CGTGCATTATTGCCCATC-3' 3'-GCACGTAATAACGGGGTAG-5'	R169A
pMS23	5'-GCTGATTGCTGAGTCGCTGATCGG-3' 3'-CGACTAACGACTCAGCGACTAGCC-5'	D207A
pMS25	5'-GGCTGGAAAGCGTACGAG-3' 3'-CCGACCTTTCGTCATGCTC-5'	E215A
pMS26	5'-GGTTCCGCGGTTTCAGTTTGGCG-3' 3'-CCAAGGCGCAAGTCAAACGCC-5'	N283A
pMS28	5'-GGTTCCAACGTTTGGCGG-3' 3'-CCAAGTTGCAACGCAAACGCC-5'	Q285A

^a These primers introduce base changes at the positions indicated by the underlined bases.

Q285A mutants were digested with *Sac*II and *Eco*RI. For the formation of the R82A, R129A, and R169A mutants, the PCR products were digested with *Apa*I and *Sty*I. The digested fragments were inserted into the pMS03 plasmid and fully sequenced. The mutant plasmids were transformed into the RC50 cell line for expression and purification of the mutant proteins.

Growth Conditions. All *E. coli* cells were grown in a modified Luria–Bertani (LB) broth (Maniatis et al., 1982). The broth contained 24 g of yeast extract, 12 g of tryptone, and 0.4% glycerol (per liter), and 0.1 M potassium phosphate. The transformed cells were grown in the presence of 50 mg of ampicillin/mL and harvested in stationary phase.

Purification of Wild-Type and Mutant Proteins. The wild-type and mutant proteins were purified following the procedure of Mareya et al. (1994) with the exception of the N301D protein. The N301D protein was purified using a modified protocol in which the supernatant fluid from the protamine sulfate step was filtered and then applied directly to a Waters Protein-Pak column. The wild-type, R82A, R169A, D207A, E215A, N283A, Q285A, E299Q, and R303Q proteins were purified to greater than 95% homogeneity, as judged by SDS–polyacrylamide gel electrophoresis. The R129A and N301D proteins were judged to be at least 90% pure.

Kinetic Measurements. The formation of carbamoyl phosphate was determined by measuring the amount of citrulline formed in a coupled assay with ornithine transcarbamoylase and ornithine (Snodgrass et al., 1969). Each reaction mixture contained 50 mM HEPES (pH 7.6), 5 mM ATP, 100 mM KCl, 20 mM MgCl₂, 20 mM KHCO₃, 20 mM glutamine, 10 mM ornithine, and 2 units of ornithine transcarbamoylase in a final volume of 0.5 mL. Each reaction was initiated by addition of carbamoyl phosphate synthetase (50–100 μg) and incubated at 25 °C. At various time points, 0.5 mL aliquots were removed and the reaction was quenched with the addition of an acid reagent and diacetyl monoxime (Rubio et al., 1986). The absorbance at 464 nm of the resulting solution was measured, and the citrulline concentration was determined using an extinction

coefficient of 37 800 M⁻¹ cm⁻¹ (Snodgrass et al., 1969). Control experiments contained all of the assay components except for the CPS enzyme.

The rate of ATP formation from MgADP and carbamoyl phosphate was determined by using a hexokinase/glucose-6-phosphate dehydrogenase coupling system. Each reaction mixture contained 50 mM HEPES (pH 7.6), 100 mM KCl, 0.75 mM NAD⁺, variable concentrations of ADP and carbamoyl phosphate, 1 mM glucose, 15 mM magnesium acetate, 20 units of hexokinase, and 10 units of glucose-6-phosphate dehydrogenase in a final volume of 2.0 mL. Assays were conducted at 25 °C upon addition of the mutant CPS enzyme (50–250 μg). The increase in absorbance was monitored at 340 nm.

The rate of ATP hydrolysis was monitored at 340 nm in the absence or presence of a nitrogen source (NH₃ or glutamine) by coupling the production of ADP to the oxidation of NADH with the coupling enzymes pyruvate kinase and lactate dehydrogenase. Each assay mixture contained 50 mM HEPES (pH 7.6), 1 mM phosphoenolpyruvate, 10 mM glutamine, 50 mM bicarbonate, 20 units of pyruvate kinase, and 20 units of lactate dehydrogenase. Each reaction was performed at 25 °C and CPS was added to initiate the reaction. In the case of the mutant enzymes where the *K_m* for NH₄Cl is high, the ionic strength was held constant with KCl.

Statistical Analysis of Kinetic Data. The kinetic parameters, *V_m* and *K_m*, were determined by fitting the data to eq 4 with the computer programs obtained from Sahara Shell Software, where *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 5. In eq 5, *K₁* and *K₂* are the two Michaelis constants while *V₁* and *V₂* are the two maximal velocities. The data for the enhancement of ATP hydrolysis in the presence of a nitrogen source were fit to eq 6 according to the approach outlined by Cleland (1970). In this equation, *A* is the concentration of the nitrogen source (ammonia or glutamine), *V_o* is the initial enzyme velocity in the absence of a nitrogen source, and *K_a* is the apparent activation constant. The constant, *α*, defines the ratio of the velocities at saturating and zero concentration of the nitrogen source. The standard errors for all constants were less than 20%.

$$v = V_m A / (K_m + A) \quad (4)$$

$$v = V_1 A / (K_1 + A) + V_2 A / (K_2 + A) \quad (5)$$

$$v = V_o (\alpha A + K_a) / (A + K_a) \quad (6)$$

Three-Dimensional Model of the Carboxy Phosphate Domain of CPS. A primary sequence alignment was generated between BC, DD-ligase, and the carboxy phosphate domain of CPS in which the regions of secondary structure found in BC and DD-ligase were identified. On the basis of this primary sequence alignment and the presence of secondary structural elements, fourteen structurally conserved regions were assigned between DD-ligase and the carboxy phosphate domain of CPS. The assigned structurally conserved regions were as follows: (1) DdIB 2–7:CPS-1 5–10, (2) DdIB 22–32:CPS-1 29–39, (3) DdIB 46–52:CPS-1 62–68, (4) DdIB 73–80:CPS-1 105–112, (5) DdIB 88–105:CPS-1 120–137, (6) DdIB 128–144:CPS-1 153–169, (7)

DdIB 154–158:CPS-1 181–185, (8) DdIB 163–172:CPS-1 190–199, (9) DdIB 187–191:CPS-1 215–219, (10) DdIB 221–228:CPS-1 227–234, (11) DdIB 250–266:CPS-1 258–274, (12) DdIB 252–260:CPS-1 280–288, (13) DdIB 266–273:CPS-1 295–302, (14) DdIB 282–288:CPS-1 319–325. The coordinates for these regions in DD-ligase were transferred to the CPS amino acid sequence. The areas between the structurally conserved regions were assigned coordinates utilizing the loop searching algorithm in the program Homology from Biosym which finds best fit loops from Protein Data Bank coordinates. The model structure was minimized with Discover from Biosym using standard parameters until no abnormal bond distances or angles were detected and the total energy was equivalent or less than the total energy of the DD-ligase structure.

RESULTS

Identification of Target Residues for Site-Directed Mutagenesis. On the basis of the sequence alignment with the biotin carboxylase subunit of acetyl-CoA carboxylase from *E. coli*, three residues in the carboxy phosphate domain and three residues in the carbamate domain of CPS were chosen as initial targets for site-directed mutagenesis. These residues, E299, N301, and R303 of the carboxy phosphate domain of CPS and E841, N843, and R845 from the carbamate domain, were chosen because they are located in a highly conserved cluster in both domains and because the crystal structure of biotin carboxylase indicates that the homologous residues in BC are within the active site of the enzyme (Waldrop et al., 1994). Moreover, in a previous study by Lusty (Guillou et al., 1991), the E841 residue of the carbamate domain of CPS was mutated to a lysine residue. This non-conservative mutation resulted in an enzyme which was unable to catalyze the formation of carbamoyl phosphate with either ammonia or glutamine as the nitrogen source (Guillou et al., 1991). In this investigation more conservative replacements were chosen in which E299 and E841 were changed to glutamine, N301 and N843 were changed to aspartate, and R303 and R845 were changed to glutamine. The original glutamic acid and asparagine residues are also fully conserved in the amino acid sequence of DD-ligase from *E. coli*.

The crystal structure of DD-ligase, bound with ADP and a phosphorylated phosphinate analog of the proposed tetrahedral complex, has been solved to a resolution of 2.3 Å (Fan et al., 1994). A schematic diagram for the active site of DD-ligase is shown in Figure 2A. On the basis of this active site model and the sequence alignment shown in Figure 1, seven more residues in the carboxy phosphate domain of CPS and seven residues in the carbamate domain of CPS were chosen as additional targets for site-directed mutagenesis. The residues R82, R129, R169, D207, E215, N283, Q285, R571, R675, R715, D753, E761, N827, and Q829 were all mutated to alanine residues. Each of these residues is either strictly conserved or is a conservative replacement for those residues found in either biotin carboxylase, DD-ligase and in at least one of the two phosphorylation domains of the large subunit of CPS. A model for the active site of the carboxy phosphate domain of CPS was created by replacing those residues within the active site of DD-ligase with the homologous residues found in CPS. This working model for the active site is illustrated in Figure 2B. The ten mutants created within the carboxy phosphate domain were

purified and characterized by kinetic methods. The relative location of these ten residues is indicated in the sequence alignment presented in Figure 1. The characterization of the analogous mutations made within the carbamate domain are discussed in the following paper.

Glutamine-Dependent Carbamoyl Phosphate Synthesis. The rate of formation of carbamoyl phosphate was determined for the wild-type enzyme and all ten mutant proteins (Table 2). The E215A, N283A, E299Q, N301D, and R303Q mutants produced carbamoyl phosphate at a rate less than 1% of the observed rate for the wild-type enzyme. The R129A and the R169A mutants were able to make carbamoyl phosphate at rates of 1% and 2%, respectively, of the wild-type value. The R82A, D207A, and Q285A mutants retained 40%, 10%, and 10%, respectively, of the wild-type activity. All of the mutant proteins showed a reduction in the rate of formation of carbamoyl phosphate when ammonia was used as the nitrogen source rather than glutamine.

ATP Synthesis Reaction. All of the mutants, except for N301D and R303Q, were able to synthesize ATP at a velocity comparable to that of the native enzyme (Table 3). The R303Q enzyme had a very low rate of ATP synthesis, and the N301D mutant could only catalyze this reaction in the presence of high concentrations of ADP. The mutations made at positions 82, 129, 169, and 207 displayed very small deviations in the K_m for ADP compared with the wild-type enzyme. The N283A mutant showed a lowered value for the K_m of ATP that was not further reduced in the presence of ornithine. The double-reciprocal plots for the N301D mutant were nonlinear in the presence or absence of the allosteric activator, ornithine.

Bicarbonate-Dependent ATPase Activity. The maximal rate of the bicarbonate-dependent ATPase reaction was reduced by 10-fold for E299Q and R303Q and 5-fold for the E215A and the N301D mutant (Table 4). The N283A mutant showed a 5-fold increase in V_{max} compared to the wild-type value. All of the other mutants displayed rates of ATP hydrolysis which were similar to that of the wild-type enzyme. The R129A, R169A, N283A, Q285A, N301D, and R303Q mutants displayed increases in K_m for ATP between 4- and 32-fold, with R169A, Q285A, and N301D showing increases in K_m greater than 19 times that of the wild-type enzyme. Several mutations, E215A, N283A, E299Q, N301D, and R303Q, showed decreased affinities for the binding of bicarbonate. The R303Q mutant showed an 80-fold increase in the K_m for bicarbonate compared to the wild-type enzyme.

Glutamine-Dependent ATPase Activity. The rate of ADP formation is enhanced 10–40-fold in the presence of a nitrogen source for the native enzyme. The R129A, R169A, E215A, N283A, N301D, and R303Q mutants all showed less than a two-fold enhancement in the ATP hydrolysis rate in the presence of either glutamine or ammonia (Tables 2, 4, and 5). The rate of ADP formation for the R82A, D207A, and Q285A mutants was enhanced by the presence of ammonia in a similar fashion to that of the wild-type enzyme. For R82A, D207A, Q285A, and E299Q, the addition of glutamine produced enhancements in the rate of ATP hydrolysis from 2- to 10-fold compared to the rate of hydrolysis in the absence of a nitrogen source. The effects on the K_m for ATP for the glutamine-dependent ATPase reaction are different from the effects on the K_m for ATP seen on the bicarbonate-dependent reaction. The R82A, D207A, E215A, and Q285A mutants have K_m values for

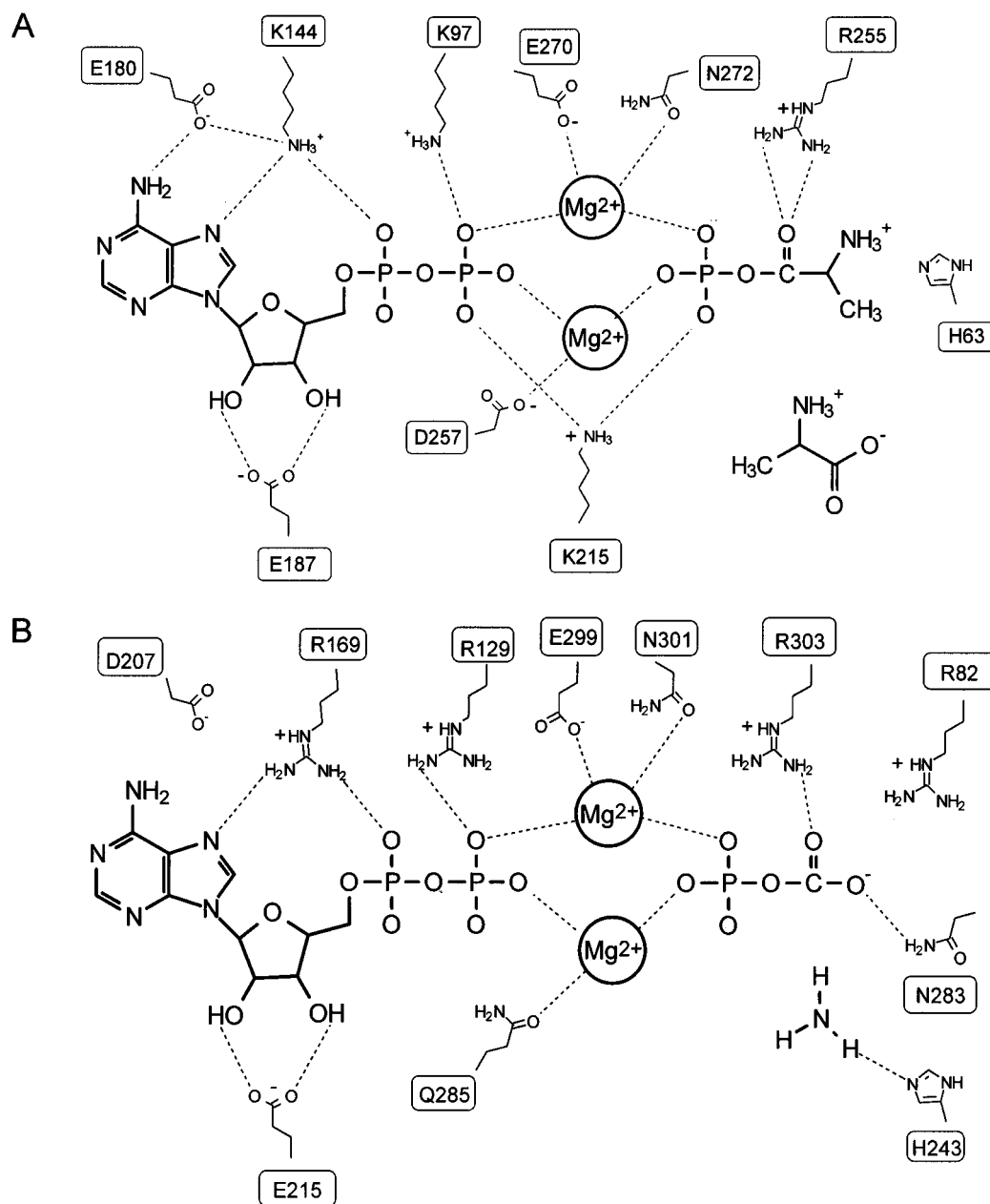


FIGURE 2: The active site residues for DD-ligase and the proposed working model for the active site of the carboxy phosphate domain of CPS. (A) Environment around the active site of DD-ligase. This model is adapted from the X-ray structure reported by Fan et al. (1994) and the mutagenesis experiments of Shi and Walsh (1995). (B) Working model for the active site of the carboxy phosphate domain of CPS. This model was generated by sequence alignment of CPS with DD-ligase and biotin carboxylase in conjunction with the reported X-ray crystal structures. Also included in this model is H243 interacting with ammonia. Miles et al. (1993) have demonstrated that mutation of this residue to an asparagine results in a protein that is unable to form carbamate from carboxy phosphate and ammonia. In the crystal structure of DD-ligase the homologous residue is Y216 and the phenol side chain appears to be interacting with the amino group of the substrate analog (Fan et al., 1994). It should be noted that only minor alterations in the catalytic activities of the R82A and D207A mutants of CPS were observed.

ATP which are similar to the wild-type values. The R129A, N283A, E299Q, N301D, and R303Q mutants showed decreases in the K_m for ATP in the absence of any allosteric effector. This observation is most likely due to the very low rates of carbamoyl phosphate formation by these same mutants. The E299Q mutant was more severely inhibited by UMP than the wild-type enzyme while the R303Q mutant was not affected by this allosteric inhibitor.

DISCUSSION

A crystal structure for carbamoyl phosphate synthetase is presently unavailable, and thus it was critical for the initial screening of potential active site residues to identify proteins

which are similar to CPS in both structure and catalytic reaction mechanism. Amino acid sequence alignments have revealed a high degree of sequence identity among the two phosphorylation domains of CPS, the biotin carboxylase subunit of acetyl-CoA carboxylase, and DD-ligase. Comparison of the X-ray crystal structures published for BC and DD-ligase demonstrates that this sequence identity occurs predominantly within the well-defined secondary structural elements contained in these two proteins. Using the commercially available software package BIOSYM/Insight II, the three dimensional crystal structures of these two enzymes were superimposed, and the two structures were found to be quite similar (Figure 3). Each protein has three subdo-

Table 2: Kinetic Constants for the Hydrolysis of ATP and the Formation of Carbamoyl Phosphate

enzyme	effector ^c	glutamine-dependent ATPase reaction ^a			carbamoyl phosphate synthesis reaction ^b	
		K_{ATP} (μ M)	V_{max} (μ mol/min·mg)	$K_{HCO_3^-}$ (mM)	V_{max} with Gln (μ mol/min·mg)	V_{max} with NH_4Cl (μ mol/min·mg)
wild-type	no effector	440	3.8			
	ornithine	52	3.4	1.6	1.8	0.59
	UMP	1100	3.8			
R82A	no effector	780	1.4			
	ornithine	60	1.2	0.82	0.71	ND
R129A	UMP	1100	0.67			
	no effector	81	0.34			
R169A	ornithine	42	0.29	9.0	0.02	0.006
	UMP	260	0.19			
D207A	no effector	400	0.32			
	ornithine	150	0.14	7.4	0.03	0.002
E215A	UMP	1500	0.48			
	no effector	670	1.2			
N283A	ornithine	57	0.84	2.9	0.22	0.04
	UMP	1500	0.41			
Q285A	no effector	620	0.05			
	ornithine	71	0.02	39	0.002	<0.001
E299Q	UMP	2000	0.06			
	no effector	23	0.48			
N301D	ornithine	86	0.64	24	<0.001	<0.001
	UMP	43	0.31			
R303Q	no effector	450	1.3			
	ornithine	180	1.4	3.8	0.17	0.04
R303Q	UMP	1700	0.82			
	no effector	32	0.008			
R303Q	ornithine	22	0.008	7.0	0.008	0.006
	UMP	430	0.007			
R303Q	no effector	200	0.013			
	ornithine	220	0.012	23	0.003	0.004
R303Q	UMP	500	0.014			
	no effector	87	0.010			
R303Q	ornithine	32	0.007	120	0.005	0.005
	UMP	110	0.014			

^a Rate of formation of ADP monitored. Reaction conditions: pH 7.5, 25 °C, variable ATP (50 mM bicarbonate), 20 mM Mg^{2+} or variable bicarbonate (5 mM ATP). ^b Rate of formation of carbamoyl phosphate monitored. Reaction conditions: pH 7.5, 25 °C, 5 mM ATP, 50 mM bicarbonate, 20 mM Mg^{2+} , and either 10 mM glutamine or 300 mM NH_4Cl . ^c Effector concentrations: ornithine, 10 mM; UMP, 100 μ M.

mains. In BC these domains have been labeled as A, B, and C (Waldrop et al., 1994) while in the DD-ligase structure they have been identified as the N-terminal, central, and C-terminal domains (Fan et al., 1994).

Two of the homologous domains within these two structures showed significant differences in the overall three-dimensional fold. There exist within the A-domain of biotin carboxylase an additional helix and strand that are located on the outer surface of this domain. The second distinct difference in the three-dimensional structures of BC and DD-ligase is most likely due to the fact that the DD-ligase structure includes $MgADP$ and an analog of the tetrahedral adduct complexed to the active site. In the DD-ligase structure, the central domain is folded over the active site to contain these substrates within a pocket. In BC, the homologous B-domain is extended out into the solvent and away from the center of the protein, leaving the putative active site exposed to solvent. Where the BC structure shows a long coil extending away from the A-domain, the DD-ligase structure contains a helix and a strand which allows the central domain to fold back over the center of the protein. The C-domain of BC and the C-terminal domain of DD-ligase contain the proposed active site located in a region of β -sheet. The tertiary structure in this area of the two proteins superimposes quite well.

A three-dimensional model of the carboxy phosphate domain of CPS was created using the Homology program from Biosym based on the BC and DD-ligase structures.

Structurally conserved regions were assigned based on primary sequence alignments and the presence of secondary structural elements found in the structures of both BC and DD-ligase. The coordinates for these regions in the DD-ligase structure were transferred to the CPS amino acid sequence. The areas between these structurally conserved regions were assigned coordinates based on best fit loops found in the Protein Data Bank. The CPS model was then minimized using the Biosym program Discover. The three-dimensional model of the carboxy phosphate domain of CPS derived using this procedure is illustrated in Figure 4. It is structurally more similar to DD-ligase in the areas in which DD-ligase differs from the BC structure.

Examination of this model reveals that nine of the ten residues within the carboxy phosphate domain of CPS that were chosen as the initial targets for site-directed mutagenesis are found at or near the proposed active site. The lone exception, residue R82, is located in the area analogous to the N-domain of DD-ligase and it appears to be quite removed from the putative active site.³ This observation is consistent with the overall behavior of the R82A mutant. The measured kinetic constants for the full and partial chemical reactions catalyzed by this mutant are all very similar to those obtained for the wild-type protein. Of the remaining nine residues,

³ His-63 of DD-ligase has been proposed to be in proximity to the amino and methyl groups of the D-alanine substrate (Shi & Walsh, 1995).

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

enzyme	effector ^b	K_{ADP} (μ M)	V_{max} (μ mol/min·mg)	K_{CP} (mM)
wild-type	no effector	170	0.42	
	ornithine	22	0.40	2.0
	UMP	2200	0.22	
R82A	no effector	79	0.22	
	ornithine	14	0.17	4.3
	UMP	960	0.15	
R129A	no effector	300	0.30	
	ornithine	18	0.21	0.51
	UMP	2200	0.19	
R169A	no effector	200	0.35	
	ornithine	16	0.24	2.1
	UMP	1700	0.29	
D207A	no effector	700	0.38	
	ornithine	18	0.30	0.78
	UMP	1500	0.25	
E215A	no effector	190	0.36	
	ornithine	12	0.19	0.75
	UMP	1500	0.37	
N283A	no effector	66	0.15	
	ornithine	44	0.28	2.3
	UMP	350	0.13	
Q285A	no effector	290	0.44	
	ornithine	58	0.27	2.6
	UMP	700	0.29	
E299Q	no effector	190	0.38	
	ornithine	8.0	0.31	2.0
	UMP	790	0.25	
N301D	no effector	8.0 ^c	7500	0.01
	ornithine	4.5	420	0.01
	UMP	143		0.02
R303Q	no effector	9.0		0.09
	ornithine	2.0		0.07
	UMP	77		0.06
				0.12

^a Reaction conditions: pH 7.5, 25 °C, 20 mM Mg²⁺, variable ADP (10 mM carbamoyl phosphate) or variable carbamoyl phosphate (5 mM ADP). ^b Effector concentrations: ornithine, 10 mM; UMP, 100 mM. ^c From fits of the data to eq 5.

Table 4: Kinetic Constants for the Bicarbonate-Dependent ATPase Reaction^a

enzyme	K_{ATP} (μ M)	V_{max} (μ mol/min·mg)	$K_{HCO_3^-}$ (mM)
wild-type	7.0	0.10	1.7
R82A	9.0	0.12	1.2
R129A	35	0.21	7.4
R169A	220	0.16	7.5
D207A	3.0	0.09	0.65
E215A	16	0.02	25
N283A	29	0.47	15
Q285A	130	0.17	5.4
E299Q	5.0	0.01	32
N301D	200	0.02	56
R303Q	33	0.01	130

^a Rate of formation of ADP monitored. Reaction conditions: pH 7.5, 25 °C, 10 mM ornithine, 20 mM Mg²⁺, variable ATP (50 mM bicarbonate) or variable bicarbonate (5 mM ATP).

eight have analogous residues in both BC and DD-ligase (R303 of CPS does not have an analogous residue in DD-ligase). A superimposition of the α -carbon atoms of these eight residues between DD-ligase and the carboxy phosphate domain model gives an RMS deviation of 4.36 Å. An inspection of the model reveals that D207 is located in an assigned loop not in a structurally conserved region, accounting for the large RMS deviation. Removal of D207 from the comparison gives an RMS deviation of only 1.08 Å for the seven remaining residues. The area of lowest RMS deviation

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

enzyme	α for Gln	K_a for Gln (μ M)	α for NH ₄ ⁺	K_a for NH ₄ ⁺ (mM)
wild-type	28	100	7.7	440
R82A	10	85	7.3	120
R129A	1.4	41	1.6	120
R169A	<1.04	ND	<1.04	ND
D207A	4.1	50	7.6	290
E215A	1.3	9.5	1.6	7.9
N283A	1.4	69	1.1	ND
Q285A	6.9	79	2.4	280
E299Q	2.1	84	4.0	2100
N301D	<1.04	ND	<1.04	ND
R303Q	1.1	ND	<1.04	ND

^a pH 7.5, 25 °C, variable glutamine or ammonium chloride, 5 mM ATP, 50 mM bicarbonate, 10 mM ornithine, 20 mM Mg²⁺. ND, not determined when the enhancement is less than 10%.

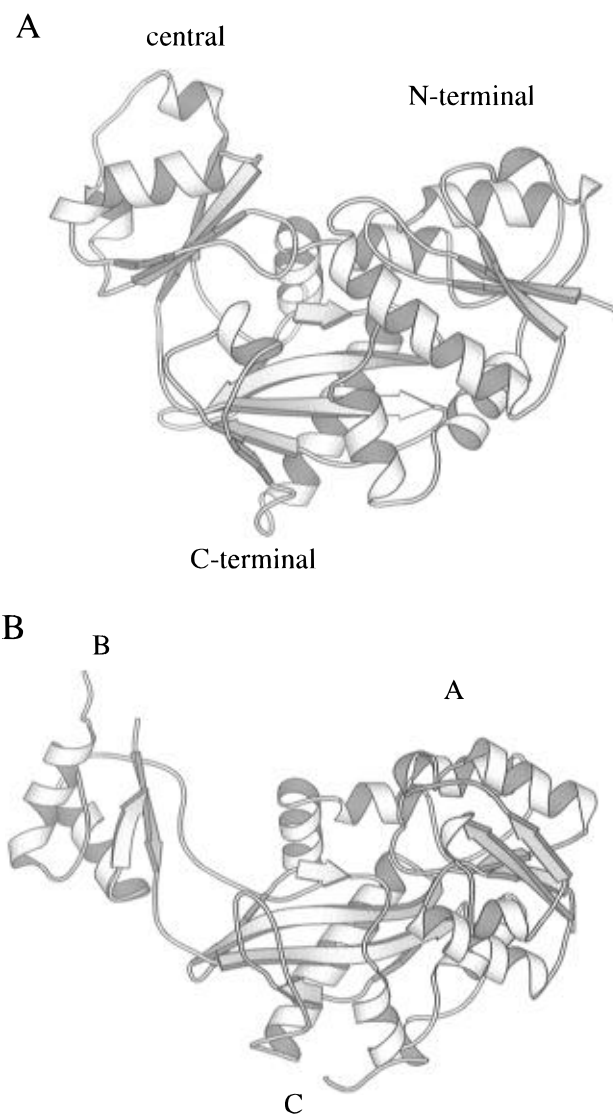


FIGURE 3: Comparison of the X-ray crystal structures of biotin carboxylase and DD-ligase. (A) DD-ligase (Fan et al., 1994). (B) Biotin carboxylase (Waldrop et al., 1994). The structures were drawn using Molscrip (Kraulis, 1991).

between the BC and DD-ligase structures is found in the C-terminal domains of these two proteins. Superimposition of the five analogous residues located in these domains give RMS deviations of 0.63 Å between BC and DD-ligase, 0.97 Å between DD-ligase and the CPS model, and 1.12 Å between

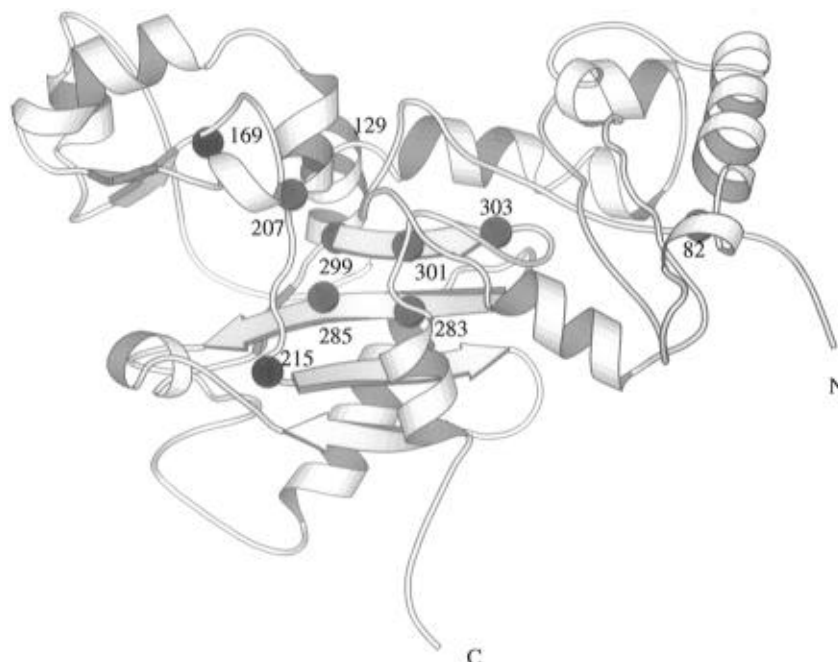


FIGURE 4: Computed model for the three-dimensional fold of the carboxy phosphate domain of CPS from *E. coli*. This structure was created by mapping the coordinates for the crystal structures of DD-ligase (Fan et al., 1994) and biotin carboxylase (Waldrop et al., 1994) onto the amino acid sequence of CPS using the Homology program from Biosym. Additional details are given in the text. Those residues mutated in this investigation are indicated by spheres. The structure was drawn using Molscrip (Kraulis, 1991).

BC and the CPS model. From these comparisons, it is evident that the model of the carboxy phosphate domain of CPS, based on the structures of BC and DD-ligase, is of sufficient quality and resolution to aid in the selection of mutants to be constructed within the two phosphorylation domains of CPS. It must be emphasized that this model should not be interpreted as an undistorted representation of the three-dimensional structure of CPS since it is based only on a portion of the entire primary sequence. The sole purpose of the model is as an aid in the design of mutational studies.

It was originally surmised that the active site of CPS could be subdivided into two general, but overlapping areas: the MgATP binding site composed of residues R129, R169, D207, E215, Q285, E299, and N301, and the bicarbonate binding site, consisting of residues R82, N283, and R303. The results of the kinetic studies with several of the mutant proteins support the predictions made about the roles of certain residues within the proposed active site. The R129A and R169A mutations caused 5- and 31-fold increases, respectively, in the K_m for ATP in the bicarbonate-dependent ATPase reaction. These results are consistent with the proposed electrostatic interaction of R129 with the β -phosphoryl group of ATP and for R169 to be interacting with the α -phosphoryl group. The effect of the R169A mutation is more pronounced; perhaps due to an additional interaction with the adenine ring. These two mutations also cause a 4-fold increase in the K_m for bicarbonate. The effect on the K_m of bicarbonate can most likely be attributed to the requirement for the binding of ATP prior to the binding of bicarbonate (Raushel et al., 1978) rather than to a direct interaction of either R129 or R169 with the bicarbonate. Both of these mutants were able to hydrolyze and synthesize ATP at a rate comparable to the wild-type enzyme. However, they were able to synthesize carbamoyl phosphate at less than 2% of the wild-type rate and the addition of a nitrogen source did not significantly increase the overall rate of ATP

hydrolysis. Thus, it appears that the inability of these mutants to synthesize carbamoyl phosphate is due either to a failure to bind ammonia and/or an inability to stabilize the carbamate or carbon dioxide intermediate long enough for the subsequent phosphorylation step to occur.

The other residues initially believed to be involved in ATP binding are D207 and E215. The D207A mutant had slightly lower affinities for ATP and bicarbonate but showed wild-type rates of hydrolysis. On the basis of these data, the D207 residue appears to be not critical for the catalytic activity of CPS. The E215A mutant is unable to hydrolyze ATP at a significant rate in either the presence or absence of a nitrogen source. However, the rate of synthesis of ATP from ADP and carbamoyl phosphate is similar to wild-type levels of activity. The rate of carbamoyl phosphate synthesis is reduced by over 2 orders of magnitude. Since the apparent K_m values for ATP are similar to the wild-type enzyme, it would appear that the E215 residue is not directly involved in the binding of ATP. However, the K_m for bicarbonate has been increased about 15-fold. These results are potentially in conflict with the original model as presented in Figure 2B. The E215 residue is maybe essential for efficient binding of bicarbonate and also for the catalytic competency of the enzyme. Alternatively, the very sluggish bicarbonate-dependent hydrolysis of ATP that is observed may originate solely from the binding of ATP and bicarbonate to the intact C-terminal domain.

Examination of the three-dimensional model of CPS, also led to the proposal that N283 and R303 would be directly involved in the binding of bicarbonate. The N283A mutation resulted in a 9-fold increase in the K_m for bicarbonate and a 4-fold increase in the K_m for ATP in the bicarbonate-dependent ATPase reaction. The rate of ATP hydrolysis in the absence of a nitrogen source was higher than the wild-type rate, but in the presence of a nitrogen source there was no further increase in the rate of ATP utilization and thus no carbamoyl phosphate can be synthesized with this mutant

protein. The asparagine at position 283 seems to affect the binding of both substrates to a small extent and abolishes the overall biosynthetic reaction.

The R303 residue also appears to facilitate the binding of bicarbonate. The K_m for bicarbonate in the bicarbonate-dependent ATPase reaction is increased by nearly 2 orders of magnitude relative to the wild-type enzyme. The rate of ATP hydrolysis in the presence or absence of a nitrogen source is at least an order of magnitude less than the wild-type protein. Moreover, the mutation at position 303 has also resulted in a 6-fold drop in the rate of ATP synthesis. It has previously been postulated that the ATP synthesis reaction occurs primarily within the C-terminal carbamate domain of CPS. However, the R303Q mutation affects the reactions at both of the phosphorylation sites. This observation would be consistent with the location of this residue near the interface between the carboxy phosphate and carbamate domains in the tertiary fold of the large subunit.

The three-dimensional model suggests that Q285, E299, and N301 may interact with the metal of bound MgATP. The mutations at these three positions did affect the kinetic constants for the binding of MgATP. The Q285A mutant showed elevations in the K_m for ATP. The K_m for ATP was increased by a factor of 19 for the bicarbonate-dependent ATPase reaction. The rates of ATP hydrolysis and ATP synthesis were similar to the wild-type enzyme, but the rate of carbamoyl phosphate synthesis was only 10% of the wild-type value. The E299Q mutation caused a significant decrease in the rates of ATP hydrolysis and carbamoyl phosphate synthesis. The only significant change in K_m is the 19-fold increase in Michaelis constant for bicarbonate. This may indicate that one of the two metals needed for catalytic activity may also be involved in the orientation and polarization of the bound bicarbonate and/or carboxy phosphate. It appears that the mutation at position 299 (and perhaps 285) inhibits the ammonia from interacting with the carboxy phosphate and/or allows the carbamate to be hydrolyzed or released before it can be phosphorylated.

One of the more interesting modifications is found in the N301D mutant. This mutation caused elevated K_m values for both ATP and bicarbonate and also decreased rates for all reactions. Most notably, the N301D mutation resulted in the appearance of nonlinear double-reciprocal plots. The double-reciprocal plot of $1/v$ vs $1/[MgADP]$ at saturating concentrations of carbamoyl phosphate and the plot of $1/v$ vs $1/[carbamoyl\ phosphate]$ were concave downward. The origin of this apparent "substrate activation" is uncertain but it is tempting to speculate that this mutant is able to catalyze the partial back reaction by both of the phosphorylation domains simultaneously but independently.

Several of the residues mutated in this study are homologous to residues in DD-ligase that have previously been mutated and characterized. Shi et al. (1994) have reported the results of mutagenesis studies on H63, K144, R255, D257, and E270 of DD-ligase, which are apparently homologous to R82, R129, N283, Q285, and E299 from CPS. The K144A mutant is an active ligase, but there is a 50-fold increase in the K_m for ATP. The E270 mutant is only about 1/2000 as catalytically efficient as the wild-type DD-ligase. The R255 and D257 mutants have no detectable catalytic activity. When comparing the results of Shi et al. (1995) with our own results, we see that the residue homologous to Q285 in DD-ligase is an essential residue, whereas a mutation

at this site in the carboxy phosphate domain of CPS shows an order of magnitude reduction in the overall synthesis of carbamoyl phosphate. However, the effects incurred by the mutation of R129, N283, and E299 mutations in CPS are similar to the homologous mutations in DD-ligase. In the following paper we extend these studies to include an analysis of similar mutations on the carbamate domain of CPS from *E. coli*.

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