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The Differentially Conserved Residues of Carbamoyl-Phosphate Synthetase*

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Carbamoyl-phosphate synthetase (CPS) from *Escherichia coli* is a heterodimeric protein. The larger of the two subunits (M_r ~118,000) contains a pair of homologous domains of approximately 400 residues each that are ~40% identical in amino acid sequence. The carboxy phosphate (residues 1–400) and carbamoyl phosphate domains (residues 553–933) also contain ~79 differentially conserved residues. These are residues that are conserved throughout the bacterial evolution of CPS in one of these homologous domains but not the other. The role of these differentially conserved residues in the structural and catalytic properties of CPS was addressed by swapping segments of these residues from one domain to the other. Nine of these chimeric mutant enzymes were constructed, expressed, purified, and characterized. A majority of the mutants were unable to synthesize any carbamoyl phosphate and the rest were severely crippled. True tandem repeat chimeric proteins were constructed by the complete substitution of one homologous domain sequence for the other. Neither of the two possible chimeric proteins was structurally stable. These results have been interpreted to demonstrate that the two homologous domains in the large subunit of CPS are functionally and structurally nonequivalent. This nonequivalence is a direct result of the specific functions each of these domains must perform during the overall synthesis of carbamoyl phosphate in the wild type enzyme and the specific structural alterations imposed by the differentially conserved residues.

Carbamoyl-phosphate synthetase (CPS)[1] is a complicated protein. This enzyme catalyzes the formation of carbamoyl phosphate via a reaction mechanism that requires two molecules of ATP, bicarbonate, and either NH_4^+ or glutamine as a source of ammonia. The full reaction is presented in equation 1.

$$2ATP + HCO_3^- + \text{glutamine} \rightarrow 2ADP + P_i + \text{glutamate} + \text{carbamoyl-P} \quad \text{(Eq. 1)}$$

The bacterial protein is found as a heterodimer (1–4). The smaller of the two subunits (M_r ~42,000) is required for the hydrolysis of glutamine and delivery of the ammonia intermediate to the large subunit (1, 5). The large subunit (M_r ~118,000) functions to activate bicarbonate for two phosphorylation events and amide bond formation (6–8). This subunit can catalyze the formation of carbamoyl-P in the absence of the small subunit only when ammonia is used as the nitrogen source (1).

The amino acid sequence of the large subunit has articulated the presence of four major domains. The domain boundaries for the wild type enzyme (***N-C***) are graphically illustrated in Fig. 1. Nyunoya and Lusty (4) have discovered that the amino acid sequence of the segment, which extends from residue 1 to 400, is approximately 40% identical to the domain, which ranges from residue 553 to 933. This finding has led to the suggestion that the evolutionary development of CPS progressed from the duplication and subsequent fusion of an ancestral gene that encoded for a protein whose inherent catalytic properties were critical for the activation of bicarbonate.

The functional properties of the various domains within the large subunit of CPS have been unveiled by an extensive set of site-directed mutants (9–11), protein modification (12–15), and x-ray crystallographic analyses (16–18). The chemical mechanism for the formation of carbamoyl phosphate has been elucidated by steady-state kinetics (19), partial reactions (6, 20), and positional isotope exchange investigations (8, 21–23). The chemical mechanism most consistent with all of the available biochemical data is presented below in Scheme 1. The domain at the N-terminal end of the large subunit (residues 1–400) is required for the initial phosphorylation of bicarbonate to carboxy phosphate and the subsequent displacement of this phosphate group by ammonia to form the carbamate intermediate (9, 24). The domain found in the C-terminal half of the large subunit of CPS (residues 553–933) is required for the ultimate phosphorylation of carbamate to form carbamoyl phosphate (19). These two segments of the large subunit have been designated as the carboxy phosphate and carbamoyl phosphate domains, respectively. The domain at the extreme C terminus (933–1073) of the large subunit is required for the binding of the two allosteric effectors, ornithine and UMP (25). The precise function of the remaining domain (residues 400–533) is unknown but it appears critical for the oligomerization of the heterodimer (16).

The recent x-ray crystallographic investigation of CPS from the laboratories of Holden and of Rayment (16–18) has provided structural support for the properties of the various domains of this enzyme. Most remarkably, the x-ray study has demonstrated that the three active sites within the heterodimer are separated from one another by ~45 Å. Moreover, the three individual active sites within the heterodimer are connected by two molecular tunnels, which presumably allow for the translocation of reactive intermediates from one domain to the next. A ribbon representation of the heterodimer is presented in Fig. 2.

Given the functional similarities required for the catalytic operation of the carboxy phosphate and the carbamoyl phosphate domains, it is not too surprising that the amino acid

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[1] The abbreviation used is: CPS, carbamoyl-phosphate synthetase.

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sequence and three-dimensional structure of these segments of the large subunit are similar to one another. However, the x-ray structure has clearly shown that whereas the structures of these two domains are similar, they are not identical. Moreover, the functional demands within the wild type protein on these two homologous domains are significantly different. For example, only the carboxy phosphate domain is required to have a binding site for association with the small subunit and to possess a conduit for the transfer of ammonia from the small to the large subunit. Moreover, only the carboxy phosphate domain must be able to catalyze amide bond formation. In contrast, the allosteric activation and inhibition of catalytic activity via ligand binding to the allosteric domain is limited to modulation of the carbamoyl phosphate domain.

In this investigation we have set out to explore the limits of the structural and functional flexibility of the carboxy phosphate and carbamoyl phosphate domains. We seek to address the specific regions of these domains that differentiate the properties of one domain from the other. It is known that ~40% of the amino acid sequence of these domains is identical and thus ~60% of the sequence is different. Here we probe the structural and functional properties imposed on each of these domains by the differentially conserved residues in CPS. These are amino acid residues in either of the two domains that are conserved throughout the bacterial evolution of CPS but where the specific amino acid is not conserved with an identical amino acid at the homologous position within the other domain. The amino acid sequence alignment between the carboxy phosphate and carbamoyl phosphate domains, based on the x-ray crystal structure, is presented in Fig. 3. The differentially conserved residues in both domains are highlighted by the black background. There are 51 residues in the carboxy phosphate domain and 28 residues in the carbamoyl phosphate domain that are differentially conserved.

To address the functional flexibility of the two homologous domains in the large subunit of CPS, we have assembled an extensive series of chimeric reconstructions of the associated domains. In the initial set of mutations, a true tandem repeat has been created by the duplication of one entire domain and substitution of this domain for its homologous counterpart within the large subunit of CPS. These two chimeric proteins have been abbreviated as either ***N-N*** or ***C-C*** mutants. These mutant proteins are crude examples of a devolved CPS. To address the functional significance of the differentially conserved residues within each of the two homologous domains in the large subunit of CPS, clusters of differentially conserved residues were systematically reinserted back into the appropriate positions of the two tandem repeat mutants. The object here was to gradually make either of the tandem repeat proteins more like the wild type protein and to determine the minimum number of changes that would regenerate catalytic activity comparable to the wild type protein. These modifica-

1 400 553 933 1073

N-C

N-N

C-C

N_C-C

N-C_N

N-N_C

C_N-C

FIG. 1. **Domain structure for the wild type CPS and the rearrangements of these domains for the chimeric mutants constructed in this investigation.**

FIG. 2. **Ribbon representation of the large and small subunits of CPS (16, 17).** The small subunit is depicted in *magenta*. The carboxy phosphate (residues 1–400), oligomerization (residues 400–553), carbamoyl phosphate (residues 553–933), and allosteric domains (residues 933–1073) are depicted in *green*, *yellow*, *blue,* and *red*, respectively. The drawing was constructed from the x-ray coordinates of CPS from Thoden *et al.* (17).

$$HOC(O)O^- \underset{MgADP}{\overset{MgATP}{\rightleftharpoons}} {}^-OC(O)OP(O)(O^-)O^- \underset{Pi}{\overset{NH_3}{\rightleftharpoons}} H_2NC(O)O^- \underset{MgADP}{\overset{MgATP}{\rightleftharpoons}} H_2NC(O)OP(O)(O^-)O^-$$

(Gln $\rightleftharpoons$ Glu with H_2O supplies NH_3)

SCHEME 1

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tions are abbreviated as either ***N-N***$_c$ or ***C***$_n$***-C*** mutants. Finally, to determine if these differentially conserved regions could be moved from one domain into the homologous domain within the wild type protein, a series of such substitutions were created in the series ***N***$_c$***-C*** and ***N-C***$_n$. Graphical representations of these reconstructions of the wild type and chimeric proteins are illustrated in Fig. 1.

MATERIALS AND METHODS

Chemicals and Enzymes—All chemicals and coupling enzymes used in the kinetic assays were purchased from Sigma. Bacto-tryptone and yeast extract were purchased from Difco. All restriction enzymes were supplied by Promega, Stratagene, New England Biolabs, or Amersham Pharmacia Biotech. The Vent^R polymerase was obtained from New England Biolabs, whereas the *pfu* DNA polymerase was provided by Stratagene. For the isolation of DNA fragments, the Wizard miniprep polymerase chain reaction DNA purification kit was used. All plasmid DNA was isolated using Wizard miniprep plasmid DNA kits. The DNA sequence analysis and the synthesis of all oligonucleotides were provided by the Gene Technologies Facility in the Biology Department of Texas A&M University.

Bacterial Strains and Plasmids—The *Escherichia coli* strains used for this study were RC50 (*car* A50, *thi*-1, *mal*A1, *xyl*-7, *rsp*L135, λ^r, λ^-, and *tsx*-237) and XL1-Blue (*rec*A1, *end*A1, *gyr*A96, *thi*-1, *hsd*R17($r_k^-m_k^+$), *sup*E44, *rel*A1, λ^-, (lac)). The RC50 strain used for protein expression was a generous gift from Dr. Carol J. Lusty (Public Health Research Institute of New York). All plasmids used in this project were derived from pMS03 (10). Site-directed mutagenesis was performed using the polymerase chain reaction and the overlap extension method of Ho *et al.* (26). The introduction and elimination of restriction sites within the plasmid pMS03 and the derivatives of this plasmid were done in every case by site-directed mutagenesis. All of the site-directed changes made to the wild type CPS were confirmed by DNA sequencing of the modified plasmids.

*Construction of the **N-N** and **C-C** Chimeric Tandem Repeat Mutants*—The initial goal of this investigation was to make two mutant proteins that contained an internal tandem repeat of either the carboxy phosphate or carbamoyl phosphate domain. A scheme for the construction of these mutants is shown in Fig. 4. It was necessary to create two plasmids from which the carboxy phosphate and carbamoyl phosphate domains could be excised by restriction digest at homologous positions. The plasmid pMS03 with the wild type sequence for CPS contains two *Acc*I restriction sites at codon positions 556 and 837 in the *car*B gene. The restriction site at position 837 was removed to make plasmid pFMR02, and then a new *Xho*I restriction site at codon position 935 was introduced to make pFMR03. The *Acc*I restriction site at codon position 556 in the plasmid pFMR02 was removed by site-directed mutagenesis to make plasmid pFMR04. An *Acc*I restriction site was added to pFMR04 at codon position 4, and then an *Xho*I site was introduced at codon position 402 to form pFMR07. The *Acc*I/*Xho*I restriction fragments from pFMR03 and pFMR07 were isolated after incubation of the parent plasmids with the restriction enzymes *Acc*I and *Xho*I. These fragments were swapped with one another and then religated to construct pNN01 and pCC01 according to the scheme that is presented in Fig. 4.

*Construction of the **N**$_c$**-C** and **N-C**$_n$ Mutants*—Nine clusters of differentially conserved residues were selected for mutagenesis. Four of these differentially conserved clusters were identified within the carbamoyl phosphate domain, whereas five of these clusters were found in the carboxy phosphate domain. The entire sequence of amino acids within each of these nine differentially conserved clusters was substituted for those residues found in the homologous domain of the wild type protein. The plasmids for the ***N***$_c$***-C*** series of mutants were constructed using pFMR07 as the template, whereas pFMR03 was used to construct the plasmids for the ***N-C***$_n$ series of mutants. The sequences of the nine differentially conserved clusters from the carboxy phosphate and carbamoyl phosphate domains are highlighted in Fig. 3. The exact sequence substitutions and the specific protein designations for the ***N***$_c$***-C*** and ***N-C***$_n$ series of mutants are listed in Table I.

*Construction of **N-N**$_c$ and **C**$_n$**-C** Mutants*—The ***N-N***$_c$ mutants were constructed by excision of the mutated **N**$_c$ domain from the plasmids containing the gene for the ***N***$_c$***-C*** mutants. This fragment was then substituted for the corresponding fragment in the plasmid pNN01. The plasmids for the ***C***$_n$***-C*** series of mutants were made in an analogous manner.

Expression and Purification of Mutant Proteins—Plasmids containing the genes for the mutants of CPS were transformed into the CPS-deficient *E. coli* strain RC50 for expression and subsequent purification of the mutant proteins. All *E. coli* cells were grown in a modified Luria-Bertani broth medium containing 24 g of yeast, 12 g of tryptone, 0.4% glycerol (per liter), and 0.1 M potassium phosphate (9). The transformed cells were grown in presence of 50 μg/ml ampicillin at 28 °C and harvested in the stationary phase.

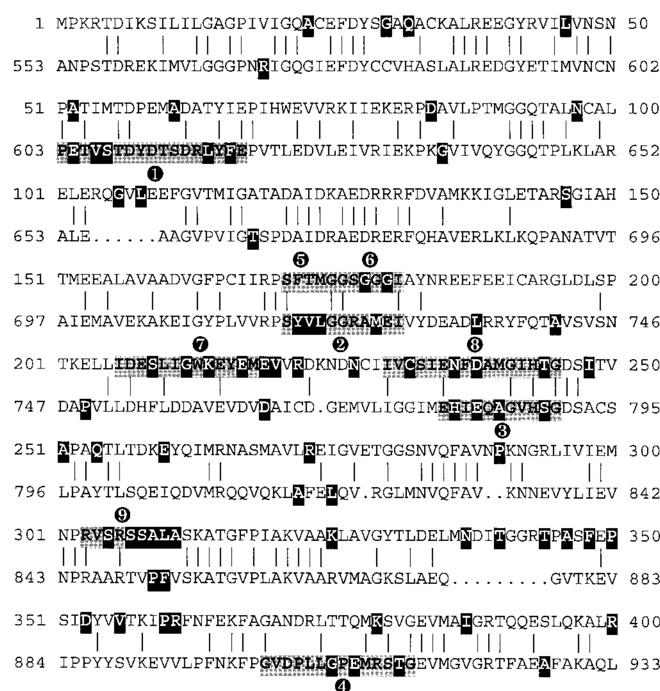

FIG. 3. **Sequence alignment of the carboxy phosphate and carbamoyl phosphate domains of CPS from *E. coli*.** The differentially conserved residues are depicted in a black background. The differentially conserved residues were identified from an alignment of 24 amino acid sequences for CPS from bacterial sources. The segments of differentially conserved residues used to construct the chimeric mutants are highlighted with a gray background. Each of these segments has been identified with a number that is carried forward to Table I.

The majority of the mutant proteins were purified by the standard protocol as described previously by Mareya and Raushel (27). In some cases DNase I was used in place of protamine sulfate to precipitate DNA, and additional inhibitors were added to inactivate cellular proteases. In a few cases the gel filtration step was eliminated, and the protein was applied directly to the ion exchange column after filtration through a 0.45-micron cellulose acetate filter. The protein-containing fractions were identified by visualization on a 12.5% denaturing Phastgel. The fractions were pooled, concentrated to approximately 2 mg/ml, and stored at −70 °C until needed.

Determination of Enzymatic Activity—The enzymatic activity for the forward and reverse reactions were conducted as described previously (27). The formation of carbamoyl phosphate was determined by measuring the amount of citrulline formed in a coupled assay with ornithine transcarbamoylase and ornithine (28).

Statistical Analysis of Kinetic Data—The kinetic parameters, V_m and K_m, were determined by fitting the data to Equation 2 with computer programs obtained from Savanna Shell Software. In this equation, v is the initial velocity, V_m is the maximal velocity, K_m is the Michaelis constant, and A is the substrate concentration. The estimated errors were less than 20% for each of the catalytic constants presented in Tables II-IV.

$$v = V_mA/(K_m + A) \quad \text{(Eq. 2)}$$

RESULTS

*Characterization of the **N-N** and **C-C** Chimeric Mutants*—The pNN01 and pCC01 plasmids were transformed into the RC50 cell line, and multiple attempts were made to express and purify the two tandem repeat mutant proteins, ***N-N*** and ***C-C***. The presence of both the large and small subunits of the ***N-N*** tandem repeat mutant of CPS in whole cells was observed with the aid of SDS-polyacrylamide gel electrophoresis. How-

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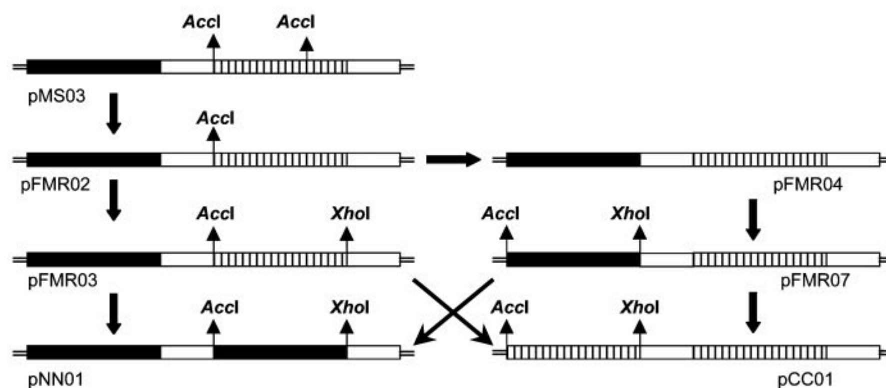

FIG. 4. **The flowchart for the introduction and removal of restriction sites needed to construct the chimeric mutants used in this investigation.**

ever, the large subunit was rapidly degraded to a molecular mass of approximately 102 kDa. The majority of the truncated large subunit was localized in the insoluble pellet, and attempts to solubilize the protein with urea failed. The truncated ***N-N*** mutant protein was partially purified (~70%) and found unable to catalyze the formation of carbamoyl phosphate (<0.007 units/mg). With this mutant we were unable to detect the catalytic hydrolysis of ATP that was dependent on the presence of bicarbonate nor were we able to measure the synthesis of ATP from ADP (partial back reaction) that was dependent on the presence of carbamoyl phosphate. Transformation of the pCC01 plasmid into RC50 failed to produce detectable quantities of the ***C-C*** chimeric protein, because only the small subunit was observed via SDS-polyacrylamide gel electrophoresis.

*Characterization of the **N-N**$_c$ and **C**$_n$-**C** Mutants*—Attempts were made to rescue the unstable ***N-N*** and ***C-C*** chimeric mutants by the incorporation of one or more segments of the differentially conserved residues back into the chimeric insert (see Fig. 1 and Table I). These new mutants, ***N-N***$_1$, ***N-N***$_2$, ***N-N***$_3$, ***N-N***$_4$, and ***N-N***$_{1,2}$ (containing segments 1 and 2), were transformed into RC50. In every case both subunits were expressed, but the large subunit was always cleaved to a smaller fragment of 102 kDa and was insoluble. These ***N-N***$_c$ mutants were not purified or further characterized. The series of mutants for the ***C-C***$_n$ chimeric protein, ***C-C***$_7$, ***C-C***$_8$, and ***C-C***$_9$, failed to show any expression of the large subunit.

*Characterization of the **N**$_c$-**C** Mutant Proteins*—The last two series of mutants were designed to test the effect of substitution of whole segments of differentially conserved residues for their homologous counterparts in the other domain. Four mutants in the series ***N***$_c$-***C*** were expressed in RC50 when grown at 28 °C. All four mutants were expressed in large quantities and the large subunit was the size expected for the full-length protein. A complete set of kinetic parameters for all four mutants is presented in Tables II–IV. In general, the incorporation of differentially conserved segments from the carbamoyl phosphate domain into the carboxy phosphate domain results in a crippling of the catalytic reactions that are known to occur within this domain. For example, neither ***N***$_1$-***C*** nor ***N***$_2$-***C*** was able to catalyze the formation of carbamoyl phosphate at a significant rate. The mutant ***N***$_4$-***C*** made carbamoyl phosphate in a coupled manner (forming one carbamoyl phosphate molecule for every two molecules of ATP hydrolyzed). In contrast, the mutant ***N***$_3$-***C*** required five molecules of ATP molecules to be hydrolyzed for every carbamoyl phosphate formed. The ***N***$_1$-***C*** and ***N***$_2$-***C*** mutants had no ATPase activity that was dependent on the addition of bicarbonate or glutamine. The ATPase activity of the ***N***$_3$-***C*** mutant was not enhanced by the presence of a nitrogen source and possessed a high K_m for bicarbonate. Overall, the effects on the catalytic activity by these mutations

TABLE I
Amino acid sequence of the native target regions and the corresponding replacement

Mutant	Amino acid sequence	
N$_1$–C	51 PATIMIDPEMADATYIE 67	wild type sequence
	603 PETVSTDYDTSDRLYFE 619	replacement sequence
N$_2$–C	171 SFTMGGSGGGI 181	wild type sequence
	717 SYVLGGRAMEI 727	replacement sequence
N$_3$–C	235 ENFDAMGIHTG 245	wild type sequence
	780 EHIEQAGVHSG 790	replacement sequence
N$_4$–C	369 GANDRLTTQMKSVG 382	wild type sequence
	902 GVDPLLGPEMRSTG 915	replacement sequence
N–C$_5$	717 SYVLG 721	wild type sequence
	171 SFTMG 175	replacement sequence
N–C$_6$	722 GRAMEI 727	wild type sequence
	176 GSGGGI 181	replacement sequence
N–C$_7$	752 LDHFLDDAVEVDVDA 766	wild type sequence
	206 IDESLIGWKEYEMEV 220	replacement sequence
N–C$_8$	775 IGGIMEHIEQAGVHSG 790	wild type sequence
	230 IVCSIENFDAMGIHTG 245	replacement sequence
N–C$_9$	845 RAARTVPF 852	wild type sequence
	303 RVSRSSAL 310	replacement sequence

were less severe on the partial back reaction. The k_{cat} values for all four mutants were diminished, but the K_m values for carbamoyl-P and ADP were generally lower than those exhibited by the wild type enzyme (Table III).

*Characterization of the **N-C**$_n$ Mutant Proteins*—The expression levels of the five ***N-C***$_n$ mutants constructed for this investigation were comparable with that of the wild type enzyme. The kinetic constants for the catalytic activity for each of the purified mutants are presented in Tables II-IV. Substitution of the differentially conserved regions from the carboxy phosphate domain into the homologous parts of the carbamoyl phosphate domain has virtually eliminated the ability of the ***N-C***$_7$, ***N-C***$_8$, and ***N-C***$_9$ mutants to catalyze the formation of carbamoyl phosphate using either glutamine or ammonia as a nitrogen donor. For the mutants ***N-C***$_5$ and ***N-C***$_6$, the rate of carbamoyl phosphate formation was significantly reduced relative to the wild type enzyme, and the hydrolysis of ATP is measurably uncoupled from the assembly of carbamoyl phosphate.

Perturbations were also noted in the rate of the bicarbonate-dependent ATP hydrolysis. For the ***N-C***$_8$ and ***N-C***$_9$ mutants, the rate of the ATP turnover was reduced by 5- and 4-fold, respectively. Conversely, the ***N-C***$_5$, ***N-C***$_6$, and ***N-C***$_7$ mutants

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Table II
Kinetic constants for the forward reaction catalyzed by chimeric CPS mutants
pH 7.6, 25°C, 50 mM HEPES, 100 mM KCl, 20 mM Mg^{2+}.

Enzyme	Effector[c]	Glutamine-dependent ATPase[a]			Carbamoyl Phosphate Synthesis[b]	
		K_{ATP}	k_{cat}	K_{HCO3-}	k_{cat} with Gln	k_{cat} with ammonia
		μM	*min*$^{-1}$	*mM*	*min*$^{-1}$	*min*$^{-1}$
Wild-type	None	440	610			
	Ornithine	52	540	1.6	290	94
	UMP	1100	610			
N_1–C	Ornithine		<1		<1	<1
N_2–C	Ornithine		<1		<1	<1
N_3–C	Ornithine	5	64	190	13	5
N_4–C	Ornithine	150	37	10	22	21
N–C_5	None	270	540			
	Ornithine	98	460	9	83	26
	UMP	120	140			
N–C_6	None	190	130			
	Ornithine	210	130	30	21	43
	UMP	230	150			
N–C_7	Ornithine	100	86	86	<1	<1
N–C_8	Ornithine	720	7.5	41	<1	<1
N–C_9	Ornithine	1700	7.5	120	<1	<1

[a] 5.0 mM ATP (when $KHCO_3$ was the variable substrate) or 50 mM $KHCO_3$ (when ATP was variable substrate).
[b] 5.0 mM ATP, 50 mM $KHCO_3$, and either 10 mM glutamine or 300 mM NH_4Cl.
[c] Effector concentrations: ornithine, 10 mM; UMP, 100 μM.

Table III
Kinetic constants for ATP synthesis reaction catalyzed by chimeric CPS mutants
pH 7.5, 25°C, 20 mM Mg^{2+}, 100 mM KCl, 20 mM carbarnoyl phosphate (when ADP was the variable substrate) or 5.0 mM ATP (when carbamoyl phosphate was variable substrate).

Enzyme	Effector[a]	K_{ADP}	k_{cat}	K_{C-P}
		μM	*min*$^{-1}$	*mM*
Wild type	None	170	67	
	Ornithine	22	64	2.0
	UMP	2200	35	
N_1–C	Ornithine	64	6	4.8
N_2–C	Ornithine	4.9	14	0.4
N_3–C	Ornithine	9.6	22	0.3
N_4–C	Ornithine	4.7	8	0.2
N–C_5	None	60	16	
	Ornithine	11	16	1.1
	UMP	1790	19	
N–C_6	None	930	61	
	Ornithine	900	66	60
	UMP	1040	13	
N–C_7	Ornithine	170	1.4	22
N–C_8	Ornithine	700	0.5	5
N–C_9	Ornithine	22	0.5	120

[a] When allosteric effector was present, 10 mM ornithine or 100 μM UMP was used.

Table IV
Kinetic constants for bicarbonate-dependent ATPase reaction
pH 7.5, 25°C, 20 mM Mg^{2+}, 100 mM KCl, 5 mM ATP (when bicarbonate was varied) and 50 mM bicarbonate (when ATP varied).

Enzyme	K_{ATP}	k_{cat}	K_{HCO3-}
	μM	*min*$^{-1}$	*mM*
Wild-type	7.0	16	1.7
N_1–C		<1	
N_2–C		<1	
N_3–C	3.7	64	>600
N_4–C	84	21	5.8
N–C_5	50	91	6.7
N–C_6	25	24	2.3
N–C_7	53	70	53
N–C_8	820	4.3	29
N–C_9	2000	3.2	114

had an enhanced rate of ATP hydrolysis. The Michaelis constants for MgATP were increased for all of the mutants. The rate of ATP synthesis during the partial back reaction was significantly reduced by every mutation with the exception of ***N-C_6***. The K_m for MgADP was similar to the wild type value for the ***N-C_9*** and ***N-C_5*** mutants but was increased significantly for the remaining mutants.

*Allosteric Effects Exhibited by Ornithine and UMP with Mutants **N-C_5** and **N-C_6***—The allosteric effects of ornithine and UMP on the Michaelis constants for the nucleotides were measured for the wild type and two mutant enzymes. For the ***N-C_6*** mutant there was no longer any significant effect on the Michaelis constant for either ATP or ADP in the presence or absence of either ornithine or UMP. However, for the ***N-C_5*** mutant the Michaelis constants decreased for ATP and ADP in the presence of ornithine. Whereas this mutant displayed an increase in the K_m for ADP in the partial back reaction in the presence of UMP, the K_m for ATP actually decreases in the presence of UMP.

DISCUSSION

The structural differences that lead to the functional specialization of the two homologous domains within the large subunit of carbamoyl-phosphate synthetase are not clearly understood. In the wild type enzyme the carboxy phosphate domain within the N-terminal half of the large subunit of CPS contains the binding site for the ATP that phosphorylates bicarbonate (9). This domain has been shown to interact with the small subunit and to provide the molecular passageway for the delivery of the ammonia to the carboxy phosphate intermediate (16). Conversely, the homologous domain located within the C-terminal half of the large subunit of CPS contains the binding site for the ATP that phosphorylates carbamate (9). Moreover, the ATP bound within the carbamoyl phosphate domain is the primary target for the allosteric effects exerted by ornithine and UMP (29, 30). However, both of these domains contain a common molecular tunnel that has been proposed to facilitate the diffusion of carbamate between the two phosphorylation sites. It is highly likely that these two homologous domains in CPS evolved from a common ancestor and then diverged to accommodate the more specialized functions that each of these domains must perform to catalyze the formation of carbamoyl phosphate.

The two tandem repeat chimeric proteins, ***N-N*** and ***C-C***, were designed to determine the extent to which each of the two homologous domains within the wild type protein could functionally substitute for one another. The results obtained here

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demonstrate that the architecture of the large subunit cannot structurally accept identical copies of these domains within this context. Only a truncated fragment of the ***N-N*** chimeric protein could be isolated. The amino acid sequence of this fragment was undetermined but the missing ~150 residues are likely due to the cleavage of the allosteric domain from the C-terminal end of the full-length protein, because the native enzyme is very susceptible to proteolytic cleavage of the C terminus (25). No catalytic activity could be detected with the truncated chimeric ***N-N*** protein. The ***C-C*** chimeric protein could not be expressed under any of the conditions attempted and thus this particular construction of the large subunit must impart a great deal of thermodynamic instability to the folded protein.

The differentially conserved residues within the homologous domains of the large subunit of CPS are likely candidates for the structural determinants that govern the more specialized functional properties of this enzyme. For this investigation, nine clusters of differentially conserved residues in the large subunit of CPS were targeted for mutagenesis. These clusters of differentially conserved residues were substituted for their counterparts found within the homologous domain. These conservative substitutions should have been structurally less divisive than swapping of an entire domain. Nevertheless, five out of the nine proteins prepared in the two series of mutants, $\boldsymbol{N_c}$-***C*** and ***N***-$\boldsymbol{C_n}$, were unable to catalyze the formation of any measurable amounts of carbamoyl phosphate. Of the four mutants that were able to synthesize carbamoyl phosphate, only one them, $\boldsymbol{N_4}$-***C***, had a fully coupled chemical mechanism where just two molecules of ATP are consumed for every carbamoyl phosphate formed. Clearly, the functional properties of CPS are easily disrupted, and the unique components of one domain cannot be moved to the homologous part of the other domain.

The three-dimensional structure of CPS was unknown when these nine clusters of differentially conserved residues were targeted for detailed investigation. The location of these nine segments are graphically presented by a ribbon drawing of CPS in Fig. 5, *A–C*. Some of these segments are very close to the nucleotide binding sites, whereas others are further away from the two active sites. The mutant $\boldsymbol{N_1}$-***C*** cannot hydrolyze ATP and is unable to make carbamoyl phosphate. The residues modified in the mutant $\boldsymbol{N_1}$-***C*** are located in the carboxy phosphate domain at an interface with the carbamoyl phosphate domain and the C terminus of the protein. Similar effects on the catalytic activity are noted for the $\boldsymbol{N_2}$-***C*** mutant. Here the altered residues are found within the glycine-rich region that is adjacent to the ATP binding site required to phosphorylate bicarbonate. This mutant can no longer hydrolyze ATP and it is unable to make carbamoyl phosphate. The segment of residues mutated in the $\boldsymbol{N_3}$-***C*** protein is part of the binding site for ATP and the essential monovalent cation. The most salient effect with this mutant is the elevated K_m for bicarbonate. The $\boldsymbol{N_4}$-***C*** mutant is the only protein to retain a stoichiometric coupling between the two active sites of the large subunit. The residues involved in the construction of this mutation are found away from the active site at the interface between the carboxy phosphate and carbamoyl phosphate domains, and they form part of the proposed carbamate tunnel.

The remaining five mutations were made by substitution of differentially conserved residues found in the carboxy phosphate domain for those residues found in the carbamoyl phosphate domain. The alterations made to the ***N***-$\boldsymbol{C_5}$ mutant are located within the ATP binding site. The effect on the rate of carbamoyl phosphate formation is substantial and there is a significant uncoupling of the reactions at the two active sites. The ***N***-$\boldsymbol{C_6}$ mutant is adjacent to the ***N***-$\boldsymbol{C_5}$ mutant within the binding pocket for the ATP that phosphorylates carbamate. The most interesting effects displayed by this mutant are the complete loss of allosteric control by either ornithine or UMP. It would appear that the replaced amino acids at this site are critical for the transmission of the allosteric signals. None of the remaining mutants within the carbamoyl phosphate domain are able to make carbamoyl phosphate. The residues involved in the ***N***-$\boldsymbol{C_7}$ mutant are involved in ATP binding, whereas the residues in the ***N***-$\boldsymbol{C_8}$ mutation form the loop that binds the monovalent cation. The residues modified in the ***N***-$\boldsymbol{C_9}$ mutant are located in the carbamoyl phosphate domain at or near the interface with the carboxy phosphate domain and the allosteric domain. An interesting facet of the ***N***-$\boldsymbol{C_7}$, ***N***-$\boldsymbol{C_8}$, and ***N***-$\boldsymbol{C_9}$ chimeric mutants is the progressive increase in the K_m for ATP. The closer these mutations are to the allosteric domain, the higher the observed K_m for ATP. Interestingly, the ***N***-$\boldsymbol{C_9}$ mutant has the highest K_m for ATP of these three mutants and structurally it is near the $\boldsymbol{N_1}$-***C*** mutant, which cannot hydrolyze ATP nor is it able to make carbamoyl phosphate. Both of these chimeric mutants are near the C terminus of the large subunit, which was previously shown to be important for activity (25). The significance of these observations is unclear, but these results demonstrate the highly interactive nature of the various domains.

Our overall conclusion from the catalytic properties of the mutants made for this investigation is that the two homologous domains found within the large subunit of CPS are functionally nonequivalent. Each of these domains has apparently evolved to accommodate the specialized requirements that must be imposed on this remarkable protein in order for the regulated synthesis of carbamoyl phosphate to be catalyzed. This view of the structural and catalytic properties of CPS is at variance with previous models suggested for this protein where it has been postulated that the two homologous domains of CPS are functionally equivalent (31–35). This proposal is based, in part, on the reported ability of certain truncated variants of the wild type protein to catalyze the synthesis of carbamoyl phosphate via the formation of homodimeric complexes (31, 33). However, all of the amino acid residues that form interdomain contacts in the native enzyme have been eliminated in some of the truncated variants (31), and thus some of the reported homodimeric complexes appear incompatible with the x-ray structure of CPS (16, 18, 36). We have attempted to replicate the construction of some of the truncated mutants but thus far have been unable to demonstrate significant catalytic activity.[2] We are continuing to explore the reasons for the experimental differences.

The degree of functional equivalence shared by the carboxy phosphate and carbamoyl phosphate domains of CPS can also be explored by structural examination of those areas of each domain that appear to be unique or more specialized. If these areas impart functional nonequivalent properties on these domains, then one might expect to find a preponderance of differentially conserved residues at these locations. For example, in all of the crystal structures of CPS determined to date, the small subunit makes molecular contact only with residues in the carboxy phosphate domain and the unknown domain (residues 400–553). How unique is this binding surface and what is the likelihood that the homologous face of the carbamoyl phosphate and allosteric domains could accommodate an association with the small subunit? Fig. 6*A* shows the contact patch made by the small subunit on the face of the large subunit. Approximately two-thirds of the molecular interactions are made to the carboxy phosphate domain, whereas the rest are

[2] M. A. Stapleton, unpublished experiments.

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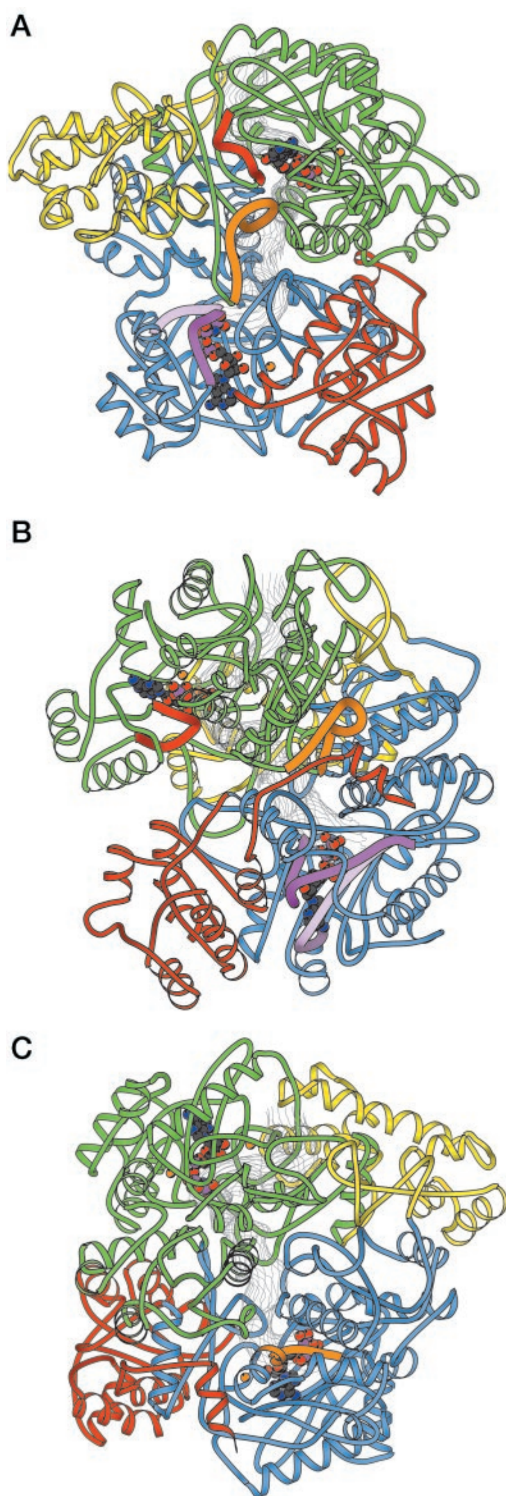

FIG. 5. **Ribbon drawings of CPS highlighting the locations where the nine differentially conserved segments were inserted.** The substituted segments are presented as colored tubes. The N_c-C and N-C_n mutants that retain the ability to catalyze the formation of carbamoyl phosphate are shown in *A*. The location of the mutants that can no longer catalyze the formation of carbamoyl phosphate is shown in *B* and *C*. *A,* N_3-C (*red tube*); N_4-C (*yellow tube*); N-C_5 (*green tube*); N-C_6 (*blue tube*). *B,* N_1-C (*red tube*); N_2-C (*yellow tube*); N-C_7 (*green tube*); N-C_8 (*blue tube*). *C,* N-C_9 (*yellow tube*).

made to the unknown domain. However, if one superimposes the homologous face from the C-terminal half of the large subunit onto this subunit-subunit interface, then the contact

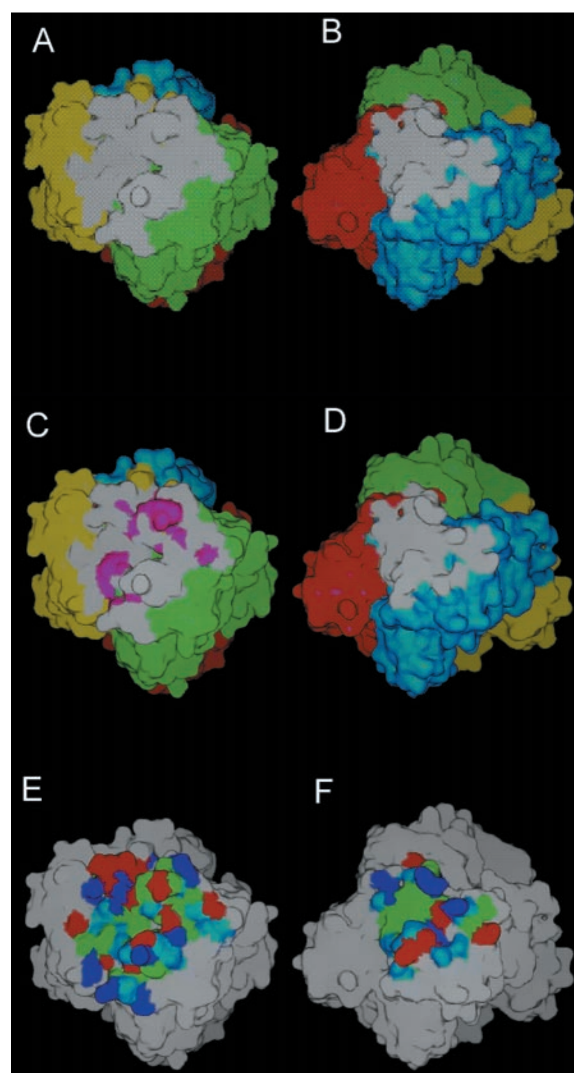

FIG. 6. **Molecular surface representations of the large subunit highlighting the observed and modeled locations in contact with the small subunit.** *A,* the contact patch (in *gray*) that is observed in the crystal structures of CPS (16, 17). *B,* the putative location of the hypothetical contact patch when the C-terminal half of CPS is superimposed on the actual subunit-subunit interface from the N-terminal half of CPS. The superposition was constructed with the Insight II program from BIOSYM. *C,* the *pink* surfaces indicate the location of the differentially conserved residues that participate in the native subunit-subunit interface between the large and small subunits of CPS. *D,* no differentially conserved residues are found in the hypothetical subunit-subunit interface modeled on the C-terminal half of CPS. *E,* the actual contact surface on the N-terminal half of CPS, colored according to surface character: acidic residues (*red*), basic residues (*dark blue*), hydrophobic residues (*green*), and all remaining residues (*light blue*). *F,* the contact surface on the hypothetical subunit-subunit interface located on the C-terminal half of CPS, colored according to surface character: acidic residues (*red*), basic residues (*dark blue*), hydrophobic residues (*green*), and all remaining residues (*light blue*). Additional details are given in the text.

patch shrinks considerably (Fig. 6*B*). Part of this diminution is due to the dramatically different structure of the allosteric domain relative to the unknown domain and subtle structural differences between the carboxy phosphate and carbamoyl phosphate domains.

At the surface of the subunit interface with the small subunit there are seven residues (Asn-227, Gln-254, Glu-260, Pro-290, Phe-348, Pro-350, and Asp-353) from the carboxy phosphate domain that are differentially conserved (Fig. 6*C*). There is not a single differentially conserved residue at an equivalent position in the carbamoyl phosphate domain (Fig. 6*D*). There are

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also 19 residues in the carboxy phosphate domain that make direct or indirect hydrogen bonds with the small subunit. However, in half of these residues the analogous residue in the carbamoyl phosphate domain is either of the opposite charge or is incapable of making a side chain hydrogen bond. This change in surface character is readily seen when the location of the acidic, basic, hydrophilic, and hydrophobic groups are mapped on the known small subunit binding interface of the carboxy phosphate domain and unknown domain as well the alternative small subunit binding interface of the carbamoyl phosphate domain and allosteric domain as shown in Fig. 6, *E* and *F*. Moreover, three of the four regions of the carbamoyl phosphate domain where there are deletions in the sequence relative to the sequence found for the carboxy phosphate domain would be part of the alternative binding site for the small subunit. It is thus highly unlikely that the small subunit will be able to make any type of productive association with the carbamoyl phosphate domain of CPS.

The other major structural feature, which distinguishes the carboxy phosphate domain from the carbamoyl phosphate domain, is the tunnel that leads from the small subunit to the nucleotide binding site. This tunnel serves as the conduit for the facilitated diffusion for ammonia from its site of production to its site of utilization. There is no structural or functional reason why the carbamoyl phosphate domain should possess a homologous passageway. Indeed, a pseudo-tunnel, which would occupy the homologous position within the carbamoyl phosphate domain, cannot be located using the same computer graphic techniques used to find the original NH_3 tunnel in the carboxy phosphate domain. There are 33 residues within the carboxy phosphate domain that have at least one atom 2.5 Å from the ammonia tunnel. Eleven of these residues (Glu-217, Glu-219, Cys-232, Ala-251, Ser-305, Ser-307, Ser-308, Leu-310, Ala-311, Pro-350, and Val-356) are differentially conserved. Thus, the percentage of differentially conserved residues that come together in the folded structure to mold the tunnel for ammonia is ~3-fold higher than the overall percentage of differentially conserved residues in the entire carboxy phosphate domain.

In summary, the two homologous domains within the large subunit of carbamoyl-phosphate synthetase appear to be functionally nonequivalent. Chimeric proteins containing identical copies of these domains are structurally unstable. Substitution of short segments of differentially conserved amino acid residues from one domain to the homologous domain is very disruptive of the catalytic properties of the mutant protein. The differentially conserved amino acids are positioned within the folded structures to define and articulate the specific properties that each of these homologous domains must possess to facilitate the synthesis of carbamoyl phosphate. Although each of these domains must be able to utilize ATP to phosphorylate a carboxylate functional group, only the carboxy phosphate domain must possess a docking site for the small subunit and a tunnel for the delivery of ammonia. Conversely, only the carbamoyl phosphate domain must have the structural apparatus to accept the regulatory signals from the allosteric domain.

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ENZYME CATALYSIS AND REGULATION:
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