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Dissection of the Functional Domains of *Escherichia coli* Carbamoyl Phosphate Synthetase by Site-directed Mutagenesis*

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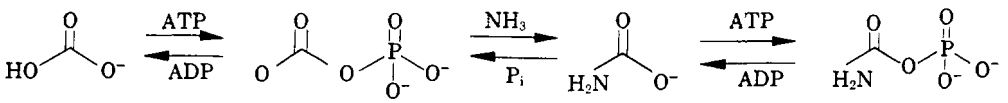
The catalytic functions of the amino-terminal and carboxyl-terminal halves of the large subunit of carbamoyl phosphate synthetase from *Escherichia coli* have been identified using site-directed mutagenesis. Glycine residues at positions 176, 180, and 722 within the putative mononucleotide-binding site were replaced with isoleucine residues. Each of these mutations resulted in at least a 1 order of magnitude reduction in the V_{max} for carbamoyl phosphate synthesis. The mutations on the amino-terminal half, G176I and G180I, caused slight reduction in the rate of synthesis of ATP from ADP and carbamoyl phosphate (the partial ATP synthesis reaction) but the bicarbonate-dependent ATPase reaction velocity was reduced to less than 10% of the wild-type rate. The mutant G722I, which is on the carboxy-terminal half, caused the partial ATP synthesis reaction to be reduced by 1 order of magnitude but the bicarbonate-dependent ATPase reaction was reduced only slightly. All three mutations are within regions which show homology to the putative glycine-rich loops of many ATP-binding proteins. These results have been interpreted to suggest that the two homologous halves of the large subunit of carbamoyl phosphate synthetase each contain a binding site for ATP. The NH_2-terminal domain contains the portion of the large subunit that is primarily involved with the phosphorylation of bicarbonate to carboxy phosphate while the COOH-terminal domain contains the region of the enzyme that catalyzes the phosphorylation of carbamate to carbamoyl phosphate.

Carbamoyl phosphate is a key intermediate in the biosynthesis of arginine and pyrimidine nucleotides. Carbamoyl phosphate synthetase from *Escherichia coli* catalyzes the formation of carbamoyl phosphate as shown in Equation 1, using glutamine as the preferred nitrogen source.

$$2\ ATP + bicarbonate + glutamine \rightarrow 2\ ADP + P_i + glutamate + carbamoyl\ phosphate \quad (1)$$

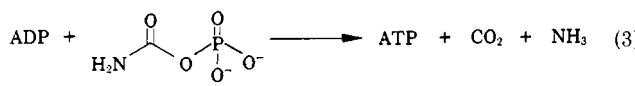
The chemical mechanism for the synthesis of carbamoyl phosphate has been shown to occur via the formation of two reactive intermediates, carboxy phosphate and carbamate, as illustrated in Scheme I (Anderson and Meister, 1965; Powers and Meister, 1976; Raushel *et al.*, 1978).



SCHEME I

This proposal is supported in part by two partial reactions catalyzed by this enzyme; a bicarbonate-dependent hydrolysis of ATP (Equation 2) and the synthesis of ATP from ADP and carbamoyl phosphate (Equation 3) (Anderson and Meister, 1965).

$$ATP \xrightarrow{HCO_3^-} ADP + P_i \quad (2)$$



(3)

The catalytically active protein is heterodimeric and is composed of a heavy subunit (M_r 130,000) and a light subunit (M_r 42,000) (Trotta *et al.*, 1971). The small subunit has been shown to bind glutamine while the large subunit binds the substrates MgATP, HCO_3^-, and NH_3, in addition to the allosteric effectors, IMP, UMP, and ornithine (Anderson and Meister, 1966). The isolated small subunit catalyzes the hydrolysis of glutamine, whereas the large subunit catalyzes the formation of carbamoyl phosphate using NH_3 as a nitrogen source (Trotta *et al.*, 1971). A recent review by Meister (1989) has summarized most of the catalytic and structural information available for the *E. coli* enzyme.

The *E. coli* operon for carbamoyl phosphate synthetase has been cloned and the genes for both subunits sequenced (Piette *et al.*, 1984; Nyunoya and Lusty, 1983). The amino acid sequence of the large subunit, as deduced from the DNA sequence of the *car*B gene, is of significant interest in that there appears to be considerable homology between the amino-terminal and carboxyl-terminal halves of the protein

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(Nyunoya and Lusty, 1983). For example, 39% of the residues between amino acid residues 1–400 and 553–933 are identical upon optimal alignment of the two halves and an additional 20% are conservative replacements. This observation suggests that a duplication and fusion of a gene for an ancestral protein occurred during the evolution of this enzyme. Moreover, the substantial sequence similarity strongly indicates that these portions of the protein are folded in an analogous manner. However, the functional role for each of these homologous domains is unknown at the present time. Both molecules of ATP may bind to a single catalytic domain while the other domain is required for regulation. Alternatively, each of the two putative domains may contain the binding site for one of the two molecules of ATP required for the complete synthesis of carbamoyl phosphate.

Several studies have shown that there are two physically distinct sites on carbamoyl phosphate synthetase which bind one of the two molecules of ATP utilized in the overall reaction (Raushel *et al.*, 1978; Meister, 1983; Powers *et al.*, 1977; Lusty *et al.*, 1983). One of these nucleotide sites is responsible for the phosphorylation of bicarbonate in the synthesis of carboxy phosphate while the other site is responsible for the phosphorylation of carbamate to form carbamoyl phosphate. Therefore, it should be possible to physically identify the approximate location for each adenine nucleotide-binding site by using the two partial reactions as diagnostic probes. Several regions within the large subunit have been proposed as possible components of the adenine nucleotide-binding sites (Powers-Lee and Corina 1987; Lusty *et al.*, 1983). One of these sites is a glycine-rich region located within the NH_2-terminal domain of the large subunit. This site has considerable homology with some of the proposed glycine-rich regions of other mononucleotide-binding proteins (Walker *et al.*, 1982; Fry *et al.*, 1985; Dreusicke and Schulz, 1986; Jurnak, 1985; la Cour *et al.*, 1985). The crystal structures of elongation factor-Tu and porcine adenylate kinase show this region to be within a flexible loop which is predicted to close around the phosphate moieties of the adenine nucleotides (Jurnak, 1985; Sachsenheimer and Schulz, 1977; Pai *et al.*, 1977). X-ray structures of the p21 protein bound with a nonhydrolyzable GTP analog (Pai *et al.*, 1989) and the porcine adenylate kinase bound with P^1P^5-di(adenosine 5′)-pentaphosphate (Ap_5A)[1] (Egner *et al.*, 1987) clearly indicate that the glycine-rich loop comprises a portion of the nucleotide-binding site. Site-directed mutagenesis studies on the glycine residues within the glycine-rich region of the F_1-ATPase β subunit from *E. coli* have shown considerable reduction in the F_1-ATPase activity when these residues were replaced with other hydrophobic residues (Parsonage *et al.*, 1987).

In order to examine the possibility of the proposed glycine-rich loop participating in the binding of at least one molecule of ATP on the large subunit of carbamoyl phosphate synthetase, 4 amino acid residues within the NH_2-terminal and COOH-terminal domains have been replaced with other amino acids. Two glycine residues, Gly-176 and Gly-180, within the glycine-rich region of the NH_2-terminal half have been mutated to isoleucines. The isoleucine substitutions are bulky, hydrophobic residues that are expected to reduce the flexibility of the proposed loop. On the COOH-terminal half, Gly-722 and Asp-733, have been mutated to isoleucine and alanine, respectively. Gly-722 and Asp-733 were chosen for replacement to determine if the homologous region within the COOH-terminal half is involved in the second ATP-binding site.

MATERIALS AND METHODS

Chemicals and Enzymes—All chemicals and coupling enzymes used in assays were purchased from Sigma or Aldrich. Bacto-tryptone and Bacto-yeast were obtained from Difco. The Ultrogel AcA-34 was purchased from IBF Biotechnics. Restriction enzymes were purchased from Bethesda Research Laboratories and the Sequenase kit was obtained from Bio-Rad.

Bacterial Strains and Plasmids—The *E. coli* strains used for this study were: RC50 (*car*A50, *thi*-1, *mal*A1, *xyl*-7, *rsp*L135, λ^r, λ^-, *tsx*-273); RR1 (*pro*A, *leu*B, *thi*-1, *lac*Y, *hsd*R, *end*A, *rsp*L20, *ara*-14, *gal*K2, *xyl*-5, *mtl*-1, *sup*E44); XL1-Blue (*rec*A1, *end*A1, *gyr*A96, *thi*-1, *hsd*R17(r_k^-, m_k^+), *sup*E44, *rel*A1, λ^-, (lac), [F′, *pro*AB, *lac*I^{q}ZΔM15, Tn10(tet^r)]), and CJ236 (*dut*1, *ung*1, *thi*-1, *rel*A1/pCJ105[F′(cm^r)]) (Bullock, 1987; Kunkel *et al.*, 1987; Joyce and Grindley, 1984). The strains RR1, RC50, and the plasmid pMC41 carrying the *car*AB genes in the *Hin*dIII site of the vector pUC19 (Norrander, 1983; Glansdorff *et al.*, 1976; Piette *et al.*, 1984) were obtained from Dr. Carol J. Lusty (Public Health Research Institute of New York). The plasmids pDP414, pDP415, pDP417, and pDP418 were derived from pMC41 as described under "Site-directed Mutagenesis."

Growth Conditions—All cells were grown in Luria-Bertani (LB) broth (Maniatis *et al.*, 1982) or a modified LB media which contained: 24 g of yeast, 12 g of tryptone, 0.4% glycerol (per liter), and 0.1 M potassium phosphate. Transformed cells were grown in the presence of 50 μg/ml ampicillin and harvested in stationary phase. For growth studies with mutant enzymes and revertants to the wild-type enzyme, cells were grown in minimal media 132 (Glansdorff, 1965).

Site-directed Mutagenesis—The *car*AB operon (Piette *et al.*, 1984) was subcloned into the *Hin*dIII site of the phagemid, pBS(+) (Stratagene, La Jolla, CA). Uracil-containing single-stranded DNA was generated in the *E. coli* strain CJ236 (*dut*, *ung*, F′) using helper phage M13K07 (Vieira and Messing, 1987). Site-directed mutations were produced as described by Kunkel *et al.* (1987) and the resulting plasmids were transformed into *E. coli* strain XL1-Blue (*dut*$^+$, *ung*$^+$,F′). Helper phage M13K07 was used to generate single-stranded DNA from the resulting colonies, which were screened for correct sequence changes by dideoxy sequencing (Sanger *et al.*, 1977) of a 300-base pair region surrounding the site-directed target sequence. Plasmids were isolated from colonies with altered sequences and transformed into the *car*AB$^-$ strain RC50 for enzyme production and purification. Table I describes the mutations produced and the resulting amino acid changes. Oligonucleotides complimentary to the sequences shown in Table I were obtained from the DNA Sequencing Laboratory, Biology Department, Texas A & M University. Following enzyme purification and characterization, revertants to wild-type were produced for each site-directed mutant by repeating the site-directed mutation protocol with wild-type oligonucleotides and then confirming the sequence alteration by dideoxy sequencing.

Expression of Revertants in E. coli—In order to determine that the mutant plasmids, pDP414, pDP415, pDP417, and pDP418, each contained only the single desired mutation, revertants of each of the plasmids were constructed as described under "Site-directed Mutagenesis." Upon confirmation of the reverted base change by DNA sequencing, each revertant plasmid was transformed into *E. coli* RC50. The transformants were grown in LB broth and harvested at stationary phase. Upon lysis of the cell pellets by French press as described below, the soluble fractions were tested for carbamoyl phosphate synthetase activity by measuring the formation of citrulline in a coupled assay with ornithine transcarbamoylase and ornithine as described below. Specific activities of the revertants were comparable to values obtained from numerous wild-type purifications.

Purification of Wild-type and Mutant Enzymes—Wild-type carbamoyl phosphate synthetase was purified from *E. coli* RR1 carrying the plasmid pMC41. Carbamoyl phosphate synthetase, with site-directed mutations in positions G176I, G180I, G723I, and D733A, was purified from *E. coli* RC50 carrying plasmids pDP415, pDP414, pDP417, and pDP418, respectively. Cells (30–50 g/preparation) were disrupted by two passages through a French press (12,000 psi). Following the addition of 1.0 mM EDTA and 0.2 mM phenylmethylsulfonyl fluoride, the lysate was treated with 2% protamine sulfate. The precipitate was removed by centrifugation at 15,000 rpm for 30 min and the supernatant solution was fractionated with ammonium sulfate. The protein fraction precipitating between 0 and 65% saturation was resuspended in approximately 70 ml of 0.1 M potassium

[1] The abbreviations used are: Ap_5A, P^1,P^5-di(adenosine 5′)-pentaphosphate; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

phosphate, pH 7.6, 1.0 mM EDTA, applied to an Ultrogel AcA-34 column (5.0 × 200 cm) and eluted with 0.1 M potassium phosphate. In the purification of the wild-type enzyme, the fractions containing active enzyme were identified by assaying for carbamoyl phosphate synthetase activity. For the purification of the mutant enzymes, the fractions containing mutant carbamoyl phosphate synthetase were identified by SDS-PAGE gels (Laemmli, 1970) and immunodot or Western blots (Burnette, 1981). The fractions containing carbamoyl phosphate synthetase were pooled (~200 ml) and applied to a Pharmacia FPLC Mono-Q 10/10 column. Carbamoyl phosphate synthetase was eluted with a 100-ml gradient of 0.1 M potassium phosphate, pH 7.6, containing 0–1.0 M KCl. Wild-type and mutant strains of carbamoyl phosphate synthetase eluted at a concentration of 0.15 M KCl. Fractions of carbamoyl phosphate synthetase were pooled and stored at 4 or −70 °C.

Determination of Enzyme Activity—Glutamine-dependent carbamoyl phosphate synthesis was determined by measuring the rate of citrulline formation in a coupled assay containing ornithine transcarbamoylase and ornithine (Snodgrass and Parry, 1969). Each 3.0-ml reaction mixture contained 50 mM Hepes, pH 7.6, 100 mM KCl, 10 mM ATP, 20 mM $MgCl_2$, 20 mM $NaHCO_3$, 40 mM glutamine, 10 mM ornithine, and 0.4 units of ornithine transcarbamoylase. Upon addition of carbamoyl phosphate synthetase (10–40 μg), the reaction was incubated at 25 °C for specified times and then quenched by adding an acid reagent and diacetylmonoxime as described by Rubino *et al.* (1987). Citrulline concentration was measured from the absorbance at 464 nm using an absorption coefficient of 37,800 M^{-1} cm^{-1} (Snodgrass and Parry, 1969).

Carbamoyl phosphate synthesis was also determined by measuring the amount of MgADP formed using a pyruvate kinase/lactate dehydrogenase coupling system. The reaction was followed spectrophotometrically at 340 nm. Each 3.0-ml reaction mixture contained 25 mM Hepes, pH 7.5, 1.0 mM P-enolpyruvate, 0.2 mM NADH, 100 mM KCl, 20 mM $MgCl_2$, 10 mM ATP, 10 mM glutamine, 40 mM $NaHCO_3$, 20 units of pyruvate kinase, and 30 units of lactate dehydrogenase. Assays were conducted at 25 °C and carbamoyl phosphate synthetase (20–100 μg) was added last to initiate the reaction. For reactions where MgATP was the varied substrate, MgATP concentrations ranged from 15 to 250 μM in the presence of 10 mM ornithine, and 30–500 μM in the absence of ornithine.

For determination of the bicarbonate-dependent ATPase reaction, the formation of MgADP from MgATP and HCO_3^- was determined using the pyruvate kinase/lactate dehydrogenase coupled assay with the omission of glutamine from the reaction mixture. Assays were performed at 25 °C and carbamoyl phosphate synthetase (20–40 μg) was added to initiate the reaction which was then followed at 340 nm as described above. In assay conditions where MgATP was the varied substrate, MgATP concentrations varied from 30 to 500 μM both in the absence and presence of 10 mM ornithine.

For determination of MgATP synthesis from MgADP and carbamoyl phosphate, the reaction was followed by using a hexokinase/glucose-6-phosphate dehydrogenase coupled assay. The reaction was measured spectrophotometrically at 340 nm. Each reaction mixture contained 25 mM glycylglycine, pH 7.6, 125 mM KCl, 0.5 mM NAD^+, 10 mM ADP, 10 mM carbamoyl phosphate, 1.0 mM glucose, 15 mM magnesium acetate, 20 units of hexokinase, and 10 units of glucose-6-phosphate dehydrogenase. The reaction mixture was assayed at 25 °C upon addition of carbamoyl phosphate synthetase (20–100 μg). The reaction was also followed as a function of MgADP, with MgADP concentrations ranging from 60 to 900 μM in the absence of ornithine, and 5–90 μM in the presence of 10 mM ornithine.

RESULTS

Construction of Site-directed Carbamoyl Phosphate Synthetase Mutants—Site-directed mutants of carbamoyl phosphate synthetase were constructed by standard methods (Kunkel *et al.*, 1987). Glycine residues at positions 176, 180, and 722 were individually changed to isoleucine residues. An aspartic acid residue at position 733 was also changed to an alanine. The complementary oligonucleotide primers that were utilized to effect these site-specific changes are presented in Table I along with the designations for the new plasmids and the mutant proteins. Verification of the desired changes in the DNA was done by dideoxy sequencing of a 300-nucleotide segment containing the mutation site. The large size of the *car*AB operon (6.3 kilobases) precluded the practical resequencing of the entire plasmid for each new mutation. Confirmation of a single codon change at the desired location was made by a second round of mutagenesis on the new plasmids back to the wild-type sequence. The revertants acted as wild-type enzymes (see below).

Functional Effects of the Carbamoyl Phosphate Synthetase Mutations in Cells—Carbamoyl phosphate synthetase is essential for cell growth in minimal media since the arginine and pyrimidine biosynthetic pathways both require carbamoyl phosphate as a key intermediate. The effects of the four site-directed mutations within carbamoyl phosphate synthetase on cell growth in minimal media are presented in Table II. The *E. coli* strain RC50 (which lacks a functional carbamoyl phosphate synthetase) does not grow in a minimal media but does grow when supplemented with 100 μg/ml arginine and 50 μg/ml uracil. The transformation of RC50 with plasmid pMC41 permits growth in the absence of added arginine and uracil because of the production of a functional carbamoyl phosphate synthetase enzyme. Mutant strains, pDP415/RC50, pDP414/RC50, and pDP417/RC50, did not grow in minimal media unless supplemented with arginine and uracil. Strain pDP418/RC50 grew as did pMC41/RC50 in either minimal media or minimal media supplemented with arginine and uracil. All three of the revertant plasmids, pLP415, pLP414, and pLP417, were indistinguishable from pMC41.

Purification of Wild-type and Mutant Enzymes—The wild-type carbamoyl phosphate synthetase as well as the four individual site-directed mutants were purified in order to assess the catalytic characteristics of the expressed enzymatic activity. The five enzymes were purified to greater than 95% homogeneity using a simple combination of ammonium sulfate fractionation and chromatography on Ultrogel AcA-34

TABLE I

Site-directed changes in carbamoyl phosphate synthetase

Plasmid	DNA and protein sequences	Enzyme
	- Thr Met Gly **Ile** Ser Gly Gly -	G176I
pDP415	- CC ATG GGC **ATT** AGC GGC GGC -	
	173 - Thr Met Gly **Gly** Ser Gly Gly **Gly** Ile Ala Tyr - 183	Wild-type
pMC41	- CC ATG GGC **GGT** AGC GGC GGC **GGT** ATC GCT TAT -	
	- Ser Gly Gly **Ile** Ile Ala Tyr -	G180I
pDP414	- GC GGC GGC **ATT** ATC GCT TAT -	
	- Val Leu Gly **Ile** Arg Ala Met -	G722I
pDP417	- GTT CTC GGC **ATT** CGG GCG ATG -	
	719 - Val Leu Gly **Gly** Arg Ala Met Glu Ile Val Tyr Asp Glu Ala **Asp** Leu Arg Arg - 736	Wild-type
pMC41	- GTT CTC GGC **GGT** CGG GCG ATG GAA ATC GTC TAT GAC GAA GCT **GAC** CTG CGT CGC	
	- Glu Ala **Ala** Leu Arg Arg -	D733A
pDP418	- GAA GCT **GCC** CTG CGT CGC -	

TABLE II

Effects of mutations on cell growth in selective media

The growth rate (μ/h) of cultures was determined during log phase of growth, using the relationship: $\mu = (\log x_2 - \log x_1)/t_2 - t_1 \log 2$ (Monod, 1958), where x is the optical density at time t.

Strain	Mutation	Growth rate	
		Minimal media[a]	Arginine and uracil[b]
RC50	CPS^-	<0.01	0.15
pMC41/RC50	CPS^+	0.11	0.17
pDP415/RC50	G176I	<0.01	0.10
pDP414/RC50	G180I	<0.01	0.12
pDP417/RC50	G722I	<0.01	0.16
pDP418/RC50	D733A	0.11	0.16
pLP415/RC50	I176G	0.10	0.12
pLP414/RC50	I180G	0.13	0.13
pLP417/RC50	I722G	0.12	0.11

[a] Minimal media 132 (Glansdorff, 1965).
[b] Arginine (100 μg/ml) and uracil (50 μg/ml) were added to minimal media 132.

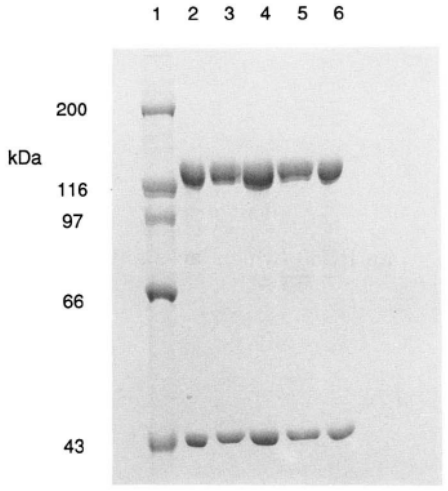

FIG. 1. **SDS-PAGE of wild-type and mutant carbamoyl phosphate synthetase.** Samples were subjected to electrophoresis on a 10% SDS-PAGE gel. *Lane 1*, molecular weight standards; *Lane 2*, wild-type carbamoyl phosphate synthetase purified from pMC41/RR1 (20 μg); *Lane 3*, G176I carbamoyl phosphate synthetase purified from pDP415/RC50 (15 μg); *Lane 4*, G180I carbamoyl phosphate synthetase purified pDP414/RC50 (20 μg); *Lane 5*, G722I carbamoyl phosphate synthetase purified from pDP417/RC50 (15 μg); *Lane 6*, D733A carbamoyl phosphate synthetase purified from pDP418/RC50 (20 μg).

and Mono-Q. The SDS-PAGE of the purified wild-type and mutant proteins is illustrated in Fig. 1 and shows the two bands corresponding to the large and small subunits.

Kinetic Properties of Carbamoyl Phosphate Synthetase and Selected Site-directed Mutants—The kinetic parameters, V_{max} and K_m, for the four site-directed mutants and wild-type carbamoyl phosphate synthetase are presented in Table III. The observed effects have been obtained for the ATPase, ATP synthesis, and carbamoyl phosphate synthesis reactions in both the presence and absence of the allosteric activator ornithine. Except for some rather modest increases in the Michaelis constant for ATP and ADP, the replacement of the aspartate at position 733 with an alanine residue has relatively little effect on the activity of carbamoyl phosphate synthetase. However, the substitution of any of the selected glycine residues with the bulkier isoleucine results in an order of magnitude reduction in the overall rate of carbamoyl phosphate synthesis. The V_{max} for the G180I mutant is reduced at least 3 orders of magnitude.

The kinetic effects on the two partial reactions are different depending on whether the altered glycine was located on the NH_2 or COOH-terminal half of the large subunit. The ATP synthesis reaction velocities for the G176I and G180I mutants are reduced by about 50%, whereas the ATPase reaction velocities are reduced by 1–2 orders of magnitude. Conversely, with the G722I mutant, in which the mutation is on the COOH-terminal half, the ATPase reaction is reduced by 50% but the ATP synthesis reaction is reduced by 1 order of magnitude relative to the wild-type specific activity.

DISCUSSION

The primary objective for the initiation of this study was to assign catalytic and/or regulatory functions to the amino- and carboxyl-terminal halves of the large subunit of carbamoyl phosphate synthetase from *E. coli*. The substantial similarity in the primary structure of these two halves suggests that each domain may be responsible for catalyzing separate reactions of the rather complex chemical mechanism of carbamoyl phosphate synthesis (see Scheme I). The most likely possibility appears to be that each domain contains a binding site for one of the two molecules of ATP required for the overall synthesis of carbamoyl phosphate. This hypothesis is supported in part by sequence homologies within the amino- and carboxyl-terminal halves of the large subunit of carbamoyl phosphate synthetase and the putative glycine-rich loops of many mononucleotide-binding proteins. Some of these comparisons are presented in Table IV. Additional support is found in the substantial sequence homology of both regions with the NH_2-terminal domain of pyruvate carboxylase (Lim *et al.*, 1988). This enzyme also utilizes ATP to activate bicarbonate to carboxyphosphate in a reaction that is identical to the first step in Scheme I.

The lack of a suitable crystal structure for carbamoyl phosphate synthetase forced us to consider the amino acid residues within these glycine-rich loops as the primary targets for site-directed mutagenesis. Our goal was to selectively inhibit the function of one ATP site without modification to the integrity of the remaining site by replacing individual amino acids with residues that would diminish the catalytic activity. With this strategy we have made the single explicit assumption that any functional and structural changes induced by these point mutations will be significantly greater on the domain containing the altered amino acid than on the other domain with the original wild-type sequence.[2] Parsonage *et al.* (1987) has shown that replacement of Gly-154 in the β subunit of F_1-ATPase with an isoleucine residue resulted in the reduction of the ATPase activity of the mutant to 17% of the wild-type activity. Therefore, it is feasible to significantly disrupt the catalytic activity by making site-specific changes to this putative loop in related proteins.

In this study the glycine residues at positions 176, 180, and 722 were mutated to isoleucine residues. These residues were chosen because each resides within the putative glycine-rich loop in the *E. coli* carbamoyl phosphate synthetase and are strictly conserved in the enzymes from *Bacillus subtilus*, rat, and yeast (Table V). Two of these residues are within the NH_2-terminal domain while the remaining glycine is in the

[2] Preliminary support for this hypothesis is obtained from the observation that truncation mutants composed of only the amino- or carboxy-terminal half of the large subunit can be expressed in *E. coli* and purified (L. Post, unpublished experiments). This suggests that these regions of the large subunit of carbamoyl phosphate synthetase can fold independently of the other domain.

TABLE III

Kinetic parameters of the wild-type and mutant enzymes

Enzyme	Ornithine	Reaction monitored: ATP synthesis[a] K_m	ATP synthesis[a] V_{max}	ATP hydrolysis[b] K_m	ATP hydrolysis[b] V_{max}	Glutamine-dependent ATP hydrolysis[c] K_m	Glutamine-dependent ATP hydrolysis[c] V_{max}	Carbamoyl phosphate synthesis[c], V_{max}
	mM	μM	μmol/min·mg	μM	μmol/min·mg	μM	μmol/min·mg	μmol/min·mg
Wild-type	0	90	0.39	20	0.43	350	3.6	
	10	5	0.39	7	0.40	34	3.3	1.8
G176I	0	80	0.33	230	0.03	200	0.38	
	10	8	0.27	66	0.04	34	0.48	0.22
G180I	0	160	0.17	800	0.001	200	0.002	
	10	10	0.14	10	0.002	20	0.002	0.0004
G722I	0	160	0.034	120	0.33	95	0.36	
	10	200	0.040	100	0.21	90	0.34	0.20
D733A	0	340	0.45	100	0.38	400	1.8	
	10	23	0.43	43	0.53	70	2.0	1.1

[a] pH 7.5, variable ADP, 10 mM carbamoyl phosphate, 20 mM Mg^{2+}.
[b] pH 7.5, variable ATP, 40 mM bicarbonate, 20 mM Mg^{2+}.
[c] pH 7.5, variable ATP, 40 mM bicarbonate, 20 mM Mg^{2+}, 10 mM glutamine.

TABLE IV

Alignment of carbamoyl phosphate synthetase (CPSase) and related proteins

The boxed regions depict sequences that are identical to the NH_2- or COOH-terminal half of *E. coli* CPS.

Enzyme	Range	Sequence	Reference
F_1-ATPase	136–164	M C P F A K G G K V G L F G G A G V G K T V N M M E L I R	*a*
Adenylate kinase	2–30	E E K L K K S K I I F V V G G P G S G K G T Q C E K I V Q	*b*
Pyruvate carboxylase	171–199	Y G Y P V I I K A A F G G G G R G M R V V R E G D D V A D	*c*
CPSase (*E. coli*)	162–190	V G F P C I I R P S F T M G G S G G G I A Y N R E E F E E	*d*
CPSase (*E. coli*)	708–736	I G Y P L V V R P S Y V L G G R A M E I V Y D E A D L R R	*d*
Carbamate kinase	170–198	K W L L E K G T I V I C A G G G G I P T M Y D E A G K K L	*e*

[a] Kanazawa *et al.*, 1982.
[b] Heil, *et al.*, 1974.
[c] Lim *et al.*, 1988.
[d] Nyunoya and Lusty, 1983.
[e] Baur *et al.*, 1989.

TABLE V

Sequence comparisons for carbamoyl phosphate synthetase (CPSase) from various sources at the glycine-rich loop

The boxed regions identify the conserved amino acids that were mutated in this study.

Source	Range	Sequence	Reference
CPSase (*E. coli*)	162–190	V G F P C I I R P S F T M G G S G G G I A Y N R E E F E	*a*
CPSase (*B. subtilis*)	162–190	I G F P V I V R P A Y T L G G T G G G I C S N E T E L K	*b*
CPSase (yeast)	182–208	V K Y P V I V R S A Y A L G G L G S G F A N N A S E M E	*c*
CPSase (rat)	580–608	I G Y P V M I R S A Y A L G G L G S G I C P N K E T L M	*d*
CPSase (*E. coli*)	708–736	I G Y P L V V R P S Y V L G G R A M E I V Y D E A D L R	*a*
CPSase (*B. subtilis*)	699–727	I G Y P V L V R P S Y V L G G R A M E I V Y H E E E L L	*b*
CPSase (yeast)	726–754	V N Y P V L I R P S Y V L S G A A M S V V N N E E E L K	*c*
CPSase (rat)	1122–1150	V G Y P C L L R P S Y V L S G S A M N V V F S E D E M K	*d*

[a] Nyunoya and Lusty (1983).
[b] C. Quinn and R. Switzer, University of Illinois, personal communication.
[c] Lusty *et al.*, 1983.
[d] Nyunoya *et al.*, 1985.

COOH-terminal domain. All three isoleucine mutants reduced the V_{max} for carbamoyl phosphate synthesis by at least 1 order of magnitude. The two mutants in the NH_2-terminal domain demonstrated relatively little effect on the ATP synthesis reaction but a substantial effect on the bicarbonate-dependent ATPase reaction. The converse was true for the mutant in the COOH-terminal domain. A very small effect was observed on the ATPase reaction but an order of magnitude reduction in the ATP synthesis reaction was produced.

The two partial reactions catalyzed by carbamoyl phosphate synthetase are believed to represent the functions of the two separate ATP sites. The bicarbonate-dependent ATPase reaction results from the hydrolysis of carboxyphosphate to CO_2 and P_i in the absence of a nitrogen source while the ATP synthesis reaction is the reverse of the step for the phosphorylation of the carbamate intermediate. Therefore, the kinetic effects of the glycine to isoleucine mutants demonstrate that portions of the NH_2-terminal domain of carbamoyl phosphate synthetase catalyze the phosphorylation of bicarbonate to carboxy phosphate while part of the COOH-terminal domain catalyzes the phosphorylation of carbamate to carbamoyl phosphate. At this time it is not clear as to the

degree of interaction between these sites nor is the distance known between the bound nucleotides. Further reconstructions of the large subunit of carbamoyl phosphate synthetase are in progress that are directed at answering these questions about a very complex enzyme.

These functional assignments are also supported to some extent by sequence alignments with pyruvate carboxylase and carbamate kinase (see Table IV). Pyruvate carboxylase is a biotin enzyme which utilizes ATP to activate bicarbonate through a mechanism that has been proposed to involve carboxy phosphate (Knowles, 1989). Alignment of the appropriate portion of the pyruvate carboxylase sequence with the NH_2- and COOH-terminal domains of carbamoyl phosphate synthetase exhibit 26% identical residues with the NH_2-terminal domain (residues 1–401) and 29% identical residues with the COOH-terminal domain (residues 553–933). The sequence alignment with carbamate kinase (which catalyzes the phosphorylation of ADP by carbamoyl phosphate) is not as strong but the sequence homology with the COOH-terminal domain is greater than with the NH_2-terminal domain. With this protein 14% of the residues are identical with amino acids 556–845 of carbamoyl phosphate synthetase, whereas only 7% of the amino acids are identical with residues 1–306 (Baur *et al.*, 1989).

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REFERENCES

Anderson, P. M. & Meister, A. (1965) *Biochemistry* **4,** 2803
Anderson, P. M. & Meister, A. (1966) *Biochemistry* **5,** 3164
Baur, H., Luethi, E., Stalon, V., Mercenier, A. & Haas, D. (1989) *Eur. J. Biochem.* **179,** 53
Bullock, W. O., Fernandez, J. M. & Short, J. M. (1987) *Biotechniques* **5,** 376
Burnette, W. N. (1981) *Anal. Biochem.* **112,** 195
Dreusicke, D. & Schulz, G. (1986) *FEBS Lett.* **208,** 301
Egner, U., Tomaselli, A. G. & Schulz, G. (1987) *J. Mol. Biol.* **195,** 649
Fry, D. C., Kuby, S. A. & Mildvan, A. S. (1985) *Biochemistry* **24,** 4680
Glansdorff, N. (1965) *Genetics* **51,** 167
Glansdorff, N., Dambly, C., Palchaudhuri, S., Crabeel, M., Pierard, A. & Halleux, P. (1976) *J. Bacteriol.* **127,** 302
Heil, A., Muller, G., Noda, L. H., Pinder, T., Schirmer, R. H. & von Zabern, I. (1974) *Eur. J. Biochem.* **43,** 131
Joyce, C. M. & Grindley, N. F. (1984) *J. Bacteriol.* **158,** 636
Jurnak, F. (1985) *Science* **230,** 32
Kanazawa, H., Kayano, T., Kiyasu, T. & Futai, M. (1982) *Biochem. Biophys. Res. Commun.* **105,** 1257
Knowles, J. R. (1989) *Annu. Rev. Biochem.* **58,** 195
Kunkel, T. A., Roberts, J. D. & Zakour, R. A. (1987) *Methods Enzymol.* **154,** 367
Laemmli, U. K. (1970) *Nature* **227,** 680
la Cour, T. F., Nyborg, J., Thirup, S. & Clark, B. F. (1985) *EMBO J.* **4,** 2385
Lim, F., Morris, C. P., Occhiodoro, F. & Wallace, J. C. (1988) *J. Biol. Chem.* **263,** 11493
Lusty, C. J., Widgren, E. E., Broglie, K. E. & Nyunoya, H. (1983) *J. Biol. Chem.* **258,** 14466
Maniatis, T., Fritsch, E. F. & Sambrook, J. (1982) *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
Meister, A. (1983) *Trans N. Y. Acad. Sci.* **2,** 117
Meister, A. (1989) *Adv. Enzymol.* **55,** 315
Monod, J. (1958) *Recherches sur la Croissance des Cultures Bacteriennes*, Hermann-Editeurs des Sciences et des Arts, Paris
Norrander, J., Kempe, T. & Messing, J. (1983) *Gene (Amst.)* **26,** 101
Nyunoya, H. & Lusty, C. J. (1983) *Proc. Natl. Acad. Sci. U. S. A.* **77,** 2119
Nyunoya, H., Broglie, K. E., Widgren, E. E. & Lusty, C. J. (1985) *J. Biol. Chem.* **260,** 9346
Pai, E. F., Sachsenheimer, W., Schirmer, R. H. & Schulz, G. E. (1977) *J. Mol. Biol.* **114,** 37
Pai, E. F., Kabsch, W., Krengel, U., Holmes, K., John, J. & Wittinghofer, A. (1989) *Nature* **341,** 209
Parsonage, D., Wilke-Mounts, S. & Senior, A. E. (1987) *J. Biol. Chem.* **262,** 8022
Piette, J., Nyonya, H., Lusty, C. J., Cunine, R., Weyens, G., Crabeel, M., Charlier, D., Glansdorff, N. & Pierard, A. (1984) *Proc. Natl. Acad. Sci. U. S. A.* **81,** 4134
Powers, S. G., Griffith, O. W. & Meister, A. (1977) *J. Biol. Chem.* **252,** 3558
Powers-Lee, S. G. & Corina, K. (1987) *J. Biol. Chem.* **262,** 9052
Raushel, F. M., Anderson, P. M. & Villafranca, J. J. (1978) *Biochemistry* **17,** 5587
Rubino, S. D., Nyunoya, H. & Lusty, C. J. (1987) *J. Biol. Chem.* **262,** 4382
Sachsenheimer, N. & Schulz, G. E. (1977) *J. Mol. Biol.* **114,** 23
Sanger, F., Nicklen, S. & Coulsen, A. R. (1977) *Proc. Natl. Acad. Sci. U. S. A.* **74,** 5463
Snodgrass, P. J. & Parry, D. J. (1969) *J. Lab. Clin. Med.* **73,** 940
Trotta, P. P., Burt, M. E., Haschemeyer, R. H. & Meister, A. (1971) *Proc. Natl. Acad. Sci. U. S. A.* **68,** 2599
Vieira, J. & Messing, J. (1987) *Methods Enzymol.* **153,** 3
Walker, J. E., Sarastee, M., Runswicke, M. J. & Gay, N. J. (1982) *EMBO J.* **1,** 945

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