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MUTATIONAL ANALYSIS OF TWO PUTATIVE DOMAINS WITHIN THE LARGE SUBUNIT OF CARBAMOYL PHOSPHATE SYNTHETASE FROM *ESCHERICHIA COLI*

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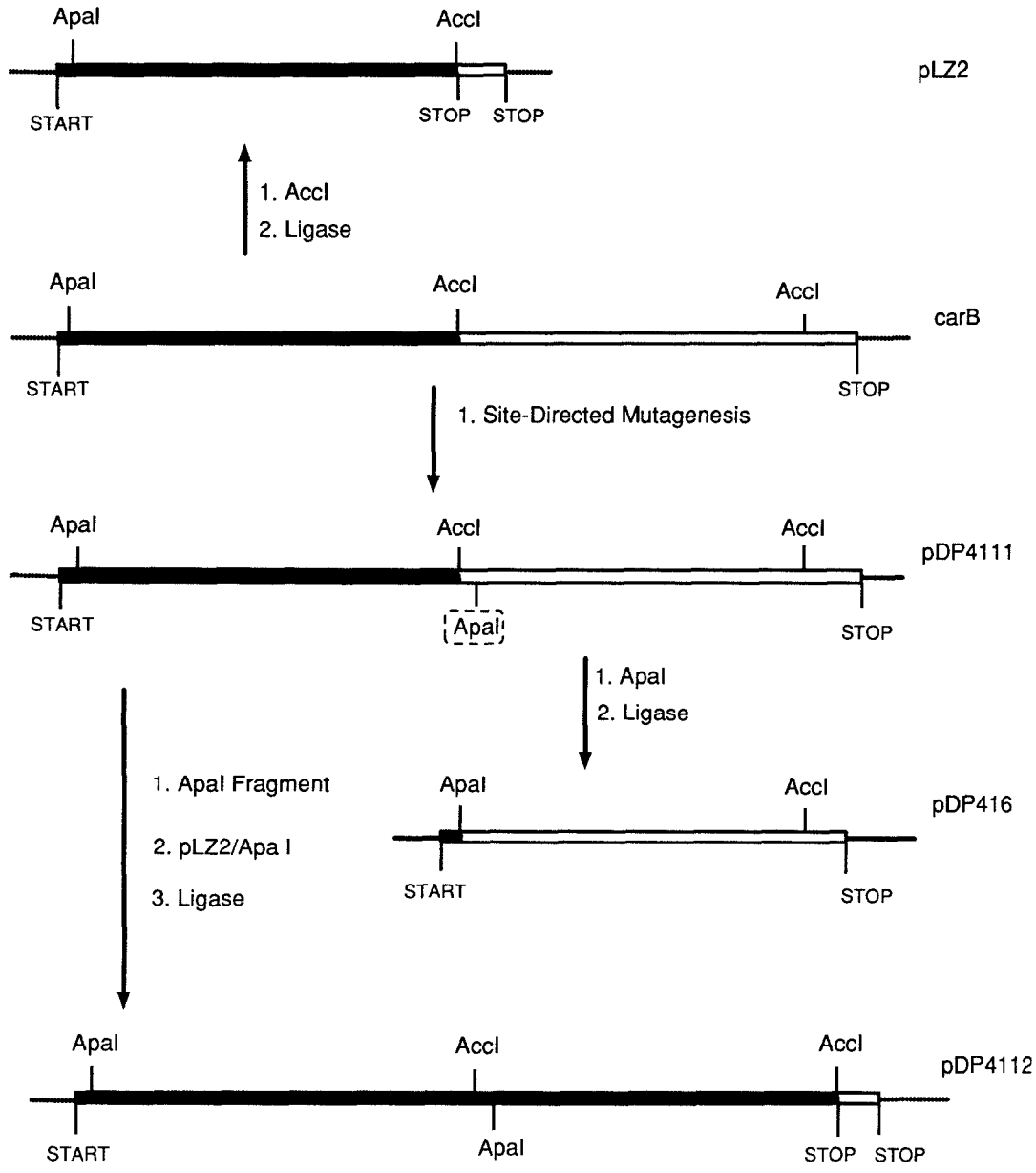
Abstract: There is a high degree of homology between the N-terminal and C-terminal halves of the large subunit of carbamoyl phosphate synthetase from *E. coli*. Selected regions were isolated through genetic manipulations and the mutant proteins purified. None of the truncated mutants or chimeric proteins possessed any full or partial catalytic activities indicating that these two domains cannot function independently of each other.

Carbamoyl phosphate synthetase (CPS) of *Escherichia coli* catalyzes the synthesis of carbamoyl phosphate from glutamine, bicarbonate and MgATP as shown in Equation 1.

$$\text{Glutamine} + HCO_3^- + 2\text{MgATP} \longrightarrow \text{Glutamate} + 2\text{MgADP} + P_i + \text{Carbamoyl Phosphate} \quad [1]$$

The protein is heterodimeric with a small subunit encoded by the *car*A gene[1] which catalyzes the hydrolysis of glutamine to ammonia and a large subunit encoded by the *car*B gene[2] which catalyzes the synthesis of carbamoyl phosphate and binds the allosteric effectors.[3] The chemical mechanism for carbamoyl phosphate formation is thought to involve the formation of carboxy-phosphate and carbamate as reaction intermediates. The large subunit has two physically distinct ATP binding sites[3,4] and a high degree of internal homology between the amino-terminal and the carboxyl-terminal halves, particularly between the regions 1-400 and 553-933.[2] The considerable sequence identity (39%) between these two regions strongly suggests that this protein may have evolved via a gene duplication and fusion of an ancestral protein.[2] Site-directed mutagenesis of glycine residues at positions 176, 180 and 722 to isoleucine residues has indicated that the amino-terminal domain is primarily involved in the phosphorylation of bicarbonate whereas the carboxyl terminal half is involved in the phosphorylation of carbamate to form carbamoyl phosphate.[4] In light of this evidence, it is reasonable to propose two putative domains, each containing an ATP binding site with the amino-terminal domain catalyzing the phosphorylation of bicarbonate and the carboxyl-terminal domain catalyzing the phosphorylation of carbamate. The isolation of these homologous domains of the large subunit, coupled with an assessment of the remaining catalytic activity, would help to more completely identify substrate binding and catalytic sites of this remarkable enzyme. In this study, isolated portions of the large subunit were genetically constructed and rearranged based on the location of the internal homology within the large subunit of carbamoyl phosphate synthetase.

The initial genetic construction undertaken was to create a mutant protein that contained only the N-terminal half of the large subunit. The vector pHN12 which contains a nonfunctional fraction of the *car*A gene and the entire *car*B gene[6] was obtained from Dr. Carol J. Lusty (Public Health Research Institute of New York). This plasmid was digested with the restriction enzyme *Acc*I and religated, creating a vector (pLZ2) with a 844



SCHEME I: PLASMID RECONSTRUCTION

base deletion and a new stop codon at the site of religation as illustrated in Scheme I. This construction produces a truncated version of the large subunit containing amino acid residues 1 through 556.

Since it has been proposed that carbamoyl phosphate synthetase has evolved by a duplication and fusion of an ancestral gene,[2,7] a genetic construction was undertaken where the N-terminal domain was duplicated, creating a mutant containing a tandem repeat of the complete amino-terminal half of the large subunit. Since the N-terminal domain has a similar protein composition to the C-terminal domain, the two domains are expected to exhibit nearly identical folding patterns. The replacement of the C-terminal domain with a duplicate of the N-terminal domain would then result in a protein very similar to the wild-type protein. The first step in the creation of this chimeric protein was to create an additional *Apa*I site in the *car*B gene by mutating the cytosine at nucleotide position 1700 to a guanine using site-directed mutagenesis.[8] This mutant plasmid (pDP4111) was subsequently digested with the restriction enzyme *Apa*I and the fragment containing the nucleotides 49 through 1704 (corresponding to amino acid residues 17 through 568) was inserted into the *Apa*I site of the vector pLZ2 as illustrated in Scheme I. The resultant vector (pDP4112) encodes for amino acid residues 1 through 568 followed by 17 through 556.

The protein containing only the carboxyl-terminal domain was genetically created by digesting the plasmid pDP4111 with the restriction enzyme *Apa*I and religating the vector. This creates a plasmid (pDP416) which encodes for the amino acid residues 1-16 followed by 569 through 1073 of the large subunit.

All three plasmids, pLZ2, pDP4112 and pDP416 were transformed into a CPS deficient *E. coli* strain, RC50 which was obtained from Dr. Carol J. Lusty. None of the three mutant enzymes produced in the RC50 strain were able to sustain growth on minimal media (Table I). Since CPS is essential for cell growth in minimal media, all three of these mutants are deficient in carbamoyl phosphate synthesis. The truncated and chimeric mutants were partially purified using the protocol for purification of the wild type CPS enzyme[9] with an additional chromatographic step.[10] The mutant proteins were identified by immunodot blots.[11]

The relative rates of ATP synthesis and ammonia-dependent ATP hydrolysis reactions[4] were determined for the mutant enzymes and are shown in Table I. The C-terminal domain was found to be too unstable to purify and none of the other mutant proteins was capable of catalyzing either reaction at a significant rate. Since none of the mutants contained either full or partial enzymatic activity, additional portions of the protein may be required for catalytic activity. The truncated and chimeric proteins constructed for this study may be catalytically inactive for a variety of reasons. For example, the nucleotide and/or bicarbonate binding sites may be partially shared between these two putative domains and thus an isolated domain would be expected to show no catalytic activity. Alternatively, the separation of the domains may cause small but critical conformational changes in the three-dimensional structure of the isolated protein fragments. Even though the N- and C-terminal halves of the large subunit have a high degree of sequence identity there are still substantial differences. These differences may be critical for the maintenance of function. The precise truncation and religation points for the mutant proteins were governed in part by the location of convenient restriction sites within the *car*B gene. Active proteins may be possible if alternative sites are selected. Current experiments are now being directed at replacement of only the highly homologous sections in an attempt to overcome some of these potential problems.

TABLE I

Characteristics of the domain constructions of carbamoyl phosphate synthetase.

	Ammonia-dependent ATP Hydrolysis	ATP Synthesis	Growth on Minimal Media
Wild-type CPS	100%	100%	+
*Car*B (1-556)	<0.04%	<2.0%	-
*Car*B [(1-568)-(17-556)]	<0.01%	<1.0%	-
*Car*B [(1-16)-(569-1073)]	ND	ND	-

pH 7.6, 25 °C

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10. The truncated mutants were purified by following a modification of the Anderson[9] protocol with an additional chromatography step. After elution of the protein from the Ultrogel AcA-34 column the fractions containing the truncated protein were applied to a green A (Amicon Corp.) dye matrix column (2.5 x 10.0 cm). The truncated proteins were eluted with a 100 ml gradient of 0.1 M phosphate buffer, pH 7.6 containing 0-1.0 M KCl.
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