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Role of the Hinge Loop Linking the N- and C-Terminal Domains of the Amidotransferase Subunit of Carbamoyl Phosphate Synthetase

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Carbamoyl phosphate synthetase from *Escherichia coli* catalyzes the formation of carbamoyl phosphate from bicarbonate, glutamine, and two molecules of ATP. The enzyme consists of a large synthetase subunit and a small amidotransferase subunit. The small subunit is structurally bilobal. The N-terminal domain is unique compared to the sequences of other known proteins. The C-terminal domain, which contains the direct catalytic residues for the amidotransferase activity of CPS, is homologous to other members of the *Triad* glutamine amidotransferases. The two domains are linked by a hinge-like loop, which contains a type II β turn. The role of this loop in the hydrolysis of glutamine and the formation of carbamoyl phosphate was probed by site-directed mutagenesis. Based upon the observed kinetic properties of the mutants, the modifications to the small subunit can be separated into two groups. The first group consists of G152I, G155I, and Δ155. Attempts to disrupt the turn conformation were made by the deletion of Gly-155 or substitution of the two glycine residues with isoleucine. However, these mutations only have minor effects on the kinetic properties of the enzyme. The second group includes L153W, L153G/N154G, and a *ternary* complex consisting of the intact large subunit plus the separate N- and C-terminal domains of the small subunit. Although the ability to synthesize carbamoyl phosphate is retained in these enzymes, the hydrolysis of glutamine is partially uncoupled from the synthetase reaction. It is concluded that the hinge loop, but not the type-II turn structure of the loop per se, is important for maintaining the proper interface interactions between the two subunits and the catalytic coupling of the partial reactions occurring within the separate subunits of CPS. © 2000 Academic Press

Key Words: carbamoyl phosphate synthetase; *Triad* glutamine amidotransferase; site-directed mutagenesis; type-II β turn.

Carbamoyl phosphate synthetase (CPS)[2] is a member of the *Triad* class of glutamine amidotransferases, which also includes anthranilate synthase, GMP synthetase, CTP synthetase, PABA synthetase, and aminodeoxychorismate synthase, among others (1). The glutamine-binding site of this family of amidotransferases contains a strictly conserved Cys–His–Glu triad (1). This class of enzymes initiates the hydrolysis of glutamine at one active site and then transfers the ammonia product to another active site within the same protein (1). The CPS from *Escherichia coli* is a heterodimer. The small subunit (~42 kDa) hydrolyzes glutamine though the intermediacy of a thioester with the catalytic Cys-269 (2, 3). The large subunit (~118 kDa) assembles carbamoyl phosphate from ammonia, bicarbonate, and two molecules of ATP (4–6). Based upon isotopic labeling studies and the discovery of three partial reactions, Anderson and Meister proposed that carbamoyl phosphate is constructed within the active site(s) of CPS via four distinct chemical steps as illustrated in Scheme 1 (7). The three-dimensional structure of the CPS from *E. coli* has confirmed that the large subunit contains separate sites for the phosphorylation of bicarbonate and carbamate while the small subunit contains the active site for the hydrolysis

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[2] Abbreviations used: CPS, carbamoyl phosphate synthetase; CAD, the trifunctional mammalian enzyme consisting of carbamoyl phosphate synthetase, aspartate transcarbamoylase and dihydroorotase; PABA, *para*-aminobenzoate; PCR, polymerase chain reaction; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis.
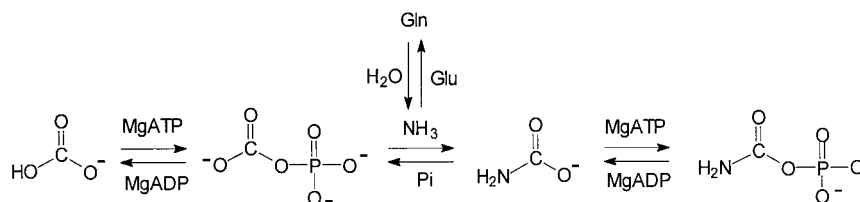
SCHEME I

of glutamine (8, 9). Remarkably, a molecular tunnel of nearly 100 Å has been identified within the interior of the protein, connecting the binding site for glutamine to the two phosphorylation sites within the large subunit (8, 9).

The architecture of the small subunit of CPS is distinctly bilobal. The amino acid sequence of the N-terminal domain does not share any significant identity to sequences of other proteins. The three-dimensional structure of this domain appears to share some common characteristics with several known proteins.[3] The C-terminal domain is, however, homologous to the other members of the *Triad* class of glutamine amidotransferases. The active site of the small subunit is located at the interface between the N- and C-terminal domains of the small subunit (8). These two domains are linked together by a hinge-like loop, which contains a type-II β turn (Fig. 1). The four residues of this β turn are Leu-153, Asn-154, Gly-155, and Met-156. The role of this hinge-like loop in the overall structure and function of the small subunit of CPS is uncertain. This loop may be important in the maintenance of critical catalytic interactions at the molecular interface between the N- and C-terminal domains of the small subunit. Since the amino acid residues at the interface of the N- and C-terminal domains constitute the interior walls of the molecular tunnel within the small subunit, this loop may also play a structural role for the architectural integrity of the ammonia tunnel within CPS. A fully functional tunnel is essential for the catalytic coupling of the four sequential and parallel chemical reactions occurring within the three active sites contained within the small and large subunits of this enzyme (10).

MATERIALS AND METHODS

Materials. All chemicals and coupling enzymes used for activity measurements were purchased from either Sigma or Aldrich unless

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[3] The N-terminal domain of the small subunit of CPS contains two four-stranded orthogonal β-sheets. One is parallel and the other is antiparallel. This structural feature is also present in the C-terminal domain of aconitase, the apical domain of the transferrin receptor and the central domain of pyruvate phosphate dikinase. The overall structure of the N-terminal domain of the small subunit of CPS appears most similar to the C-terminal domain of aconitase.

otherwise stated. Restriction enzymes, Vent and *Pfu* DNA polymerase were purchased from Promega. Oligonucleotide synthesis and DNA sequencing were performed by the Gene Technologies Laboratory, Texas A&M University. The RC50 cell line, which does not express CPS (11), was a generous gift from Dr. Carol J. Lusty.

Preparation of the G152I, G155I, Δ155, L153W, and L153G/N154G mutant enzymes. Site-directed mutagenesis for the construction of G152I, G155I, Δ155, L153W, and L153G/N154G within the small subunit of CPS was performed as described previously (12) using the polymerase chain reaction and the method of overlap extension (13). The modified plasmids were transformed in the RC50 cell line for expression and purification of the mutant proteins.

Deletion of the hinge loop. A CPS expression vector, which was designed to coexpress the intact large subunit, the N-terminal domain (1–154) and the C-terminal domain (155–380) of the small subunit, was prepared by the method of overlap extension. A termination codon (TAA), a copy of the ribosome-binding site (TCAGGAG-TAAAAGAGCC) from the start of the *carB* gene, and an initiation codon (ATG) were inserted between the codon for Asn-154 and the codon for Gly-155 from the small subunit (Scheme II). The resulting plasmid was transformed in the RC50 cell line for coexpression of the large subunit, the C-terminal domain, and N-terminal domain of the small subunit as a heterotrimeric protein.

Subcloning of the large subunit and the C-terminal domain from the small subunit. The region of DNA which codes for the complete large subunit and the C-terminal domain of the small subunit of CPS was obtained from sequential digestion of the above-described *ternary* vector with the restriction enzymes *Hin*dIII and *Eco*RV. The resulting blunt-ended 4580-bp fragment was ligated with the *Sma*I-
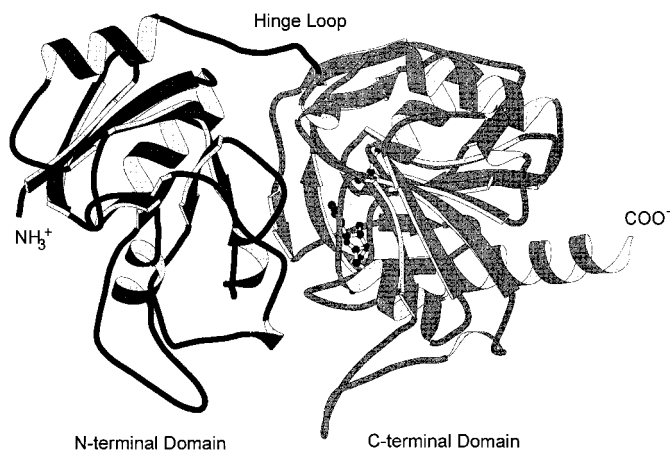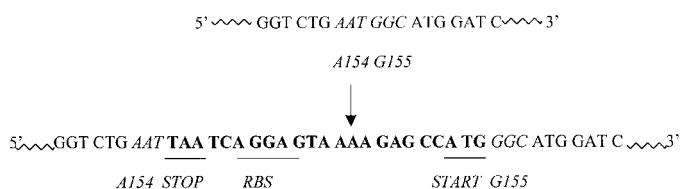

FIG. 1. Ribbon diagram for the small subunit of CPS. The N- and C-terminal domains are linked with a hinge loop (Gly152–Met156). The catalytic triad residues (Cys-269, His-353, and Glu-355) are drawn in a ball-and-stick format. The coordinates are taken from Ref. 9.

5' ⌇⌇⌇ GGT CTG *AAT GGC* ATG GAT C ⌇⌇⌇ 3'

A154 G155

↓

5' ⌇⌇⌇GGT CTG *AAT* **TAA TCA GGA GTA AAA GAG CCA TG** *GGC* ATG GAT C ⌇⌇⌇3'

A154 STOP   *RBS*   *START G155*

SCHEME II

digested pBS+ expression vector. The desired vector with the gene sequence inserted downstream of the *lac* promoter in the correct orientation was obtained following blue/white screening and sequence verification of the ligation sites. The vector (pL/CS) was transformed into BL21 cells for coexpression of the large subunit and the C-terminal domain of the small subunit.

Subcloning of the C-terminal domain from the small subunit. The DNA sequence, which codes for the C-terminal domain of the small subunit and an upstream ribosome-binding site, was obtained from amplification of the above-described *ternary* vector with the primers 5′ GAT AAC CC<u>G GAT CC</u>G GCG CTG 3′ and 5′ CAC CCA <u>GAA TTC</u> GGA TAC TTT 3′. These primers contain a *Bam*HI and an *Eco*RI restriction site, respectively. The resulting PCR fragment was digested with the restriction enzymes *Bam*HI and *Eco*RI, and then ligated into the pBS+ vector between the *Bam*HI and the *Eco*RI sites downstream of the *lac* promoter. The sequence of the insert was verified. The resulting vector (pCS) was transformed into BL21 cells for expression of the C-terminal domain of the small subunit.

Expression and purification of the mutant proteins. The mutant proteins G152I, G155I, Δ155, L153W, and L153G/N154G, were expressed and purified as described previously (14). For expression of the C-terminal domain of the small subunit or coexpression of the large subunit and the C-terminal domain of the small subunit, BL21 cells containing the vector pCS or pL/CS were grown at 30°C in TB medium (15) until the OD_{590} of the culture reached 0.5. Protein expression was induced with the addition of 0.4 mM IPTG. The cells were harvested after induction for 4 to 20 h. For BL21 cells expressing the C-terminal domain of the small subunit, the desired protein was found only in the pellet following cell lysis and centrifugation, and was not pursued further. For BL21 cells coexpressing the large subunit and the C-terminal domain of the small subunit, both polypeptides were present in the supernatant solution following cell lysis and centrifugation. Standard purification procedures for the wild-type CPS were followed (14).

Statistical analysis of kinetic data. Kinetic measurements were carried out as previously described (16). The catalytic parameters, V_{max} and K_m, were determined by fitting the data to Eq. [1], where ν is the initial velocity, V_{max} is the maximal velocity, K_m is the Michaelis constant, and A is the substrate concentration. The data for the enhancement of ATP hydrolysis in the presence of a nitrogen source were fitted to Eq. [2] (17). In this equation, V_o is the initial enzyme velocity in the absence of a nitrogen source I, K_a is the apparent activation constant, and α is the ratio of the velocities at saturating and zero concentration of the nitrogen source. In this case, V_{max} is expressed as αV_o:

$$\nu = V_{max}A/(K_m + A) \qquad [1]$$

$$\nu = V_o(K_a + \alpha I)/(K_a + I). \qquad [2]$$

RESULTS

The C-terminal and N-terminal domains of the small subunit of carbamoyl phosphate synthetase from *E.*

coli, are linked together by a hinge-like loop that contains a type II β turn. This investigation aims to dissect the functional role of the hinge loop during the hydrolysis of glutamine and the formation of carbamoyl phosphate. Amino acid residues within the hinge loop were modified in order to perturb the type II turn structure. The mutants G152I, G155I, L153W, Δ155, and L153G/N154G were prepared and purified to greater than 95% homogeneity as judged by SDS–polyacrylamide gel electrophoresis. The *ternary* construction, which was designed to coexpress the N-terminal domain (1–154) of the small subunit, the C-terminal domain (155–380) of the small subunit, and the large subunit, was found to elute as a single peak during size exclusion chromatography. This *ternary* complex is estimated to be more than 95% homogenous by SDS–PAGE electrophoresis (Fig. 2). The overall effects on the catalytic properties of CPS mutants by these modifications were determined for each protein by measuring the rate of formation of ADP, glutamate, and carbamoyl phosphate under a variety of assay formats. The kinetic parameters, K_m and V_{max}, obtained for the wild-type and the mutant enzymes are summarized in Tables I–III.

Expression of the C-terminal domain of the small subunit alone led to the production of an insoluble form of the protein, most likely as inclusion bodies. This was not pursued further. When co-expressed with the large subunit, both the large subunit and the C-terminal domain of the small subunit were produced as soluble proteins, based on the results of SDS–PAGE electrophoresis (not shown). However a binary complex of the C-terminal domain of the small subunit and the large subunit was absent in fractions eluted during size exclusion chromatography. Instead, the homogenous large subunit of CPS was obtained as a single peak. This indicates that interactions between the large subunit and the C-terminal domain of the small subunit are relatively weak compared to those observed between the large subunit and the intact small subunit. The C-terminal domain of the small subunit could not
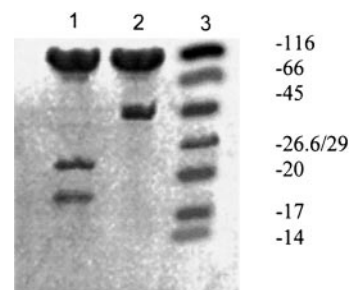

FIG. 2. SDS–polyacrylamide gel electrophoresis of the *ternary* complex of CPS. The gel electrophoresis was performed on a PhastSystem from Pharmacia with a 20% homogenous gel. Lane 1, the *ternary* complex; lane 2, wild-type CPS; lane 3, molecular markers.

TABLE I

Kinetic Parameters for the ATPase Reactions of the Wild-Type and Mutant Enzymes

	HCO_3^--dependent[a], V_{max} (s^{-1})	NH₃-dependent[b]		Gln-dependent[c]	
		V_{max} (s^{-1})	K_m (NH_4^+) (mM)	V_{max} (s^{-1})	K_m (Gln) (mM)
WT	0.45 ± 0.04	8.0 ± 0.5	296 ± 32	6.9 ± 0.08	0.098 ± 0.001
G155I	0.13 ± 0.01	ND	>600[d]	5.3 ± 0.02	0.61 ± 0.07
Δ155	0.11 ± 0.01	ND	>600[d]	4.9 ± 0.005	0.74 ± 0.03
G152I	0.15 ± 0.01	4.5 ± 0.2	295 ± 26	3.3 ± 0.1	0.77 ± 0.08
L153W	0.022 ± 0.002	ND	>600[d]	6.5 ± 0.2	10.5 ± 0.9
L153G/N154G	0.052 ± 0.008	ND	>600[d]	5.1 ± 0.2	1.3 ± 0.1
Ternary complex	0.014 ± 0.002	ND	>600[d]	5.2 ± 0.1	6.9 ± 0.4

[a] Rate constant for ADP formation monitored for the bicarbonate-dependent ATPase, ammonia-dependent ATPase, or glutamine-dependent ATPase reaction. Reaction conditions for the bicarbonate-dependent ATPase: pH 7.6, 25°C, 5.0 mM ATP, 40 mM bicarbonate, 20 mM Mg^{2+}, 100 mM K^+, and 10 mM ornithine.

[b] Reaction conditions: pH 7.6, 25°C, variable amounts of NH_4Cl, 5.0 mM ATP, 40 mM bicarbonate, 20 mM Mg^{2+}, 100 mM K^+, and 10 mM ornithine.

[c] Reaction conditions: pH 7.6, 25°C, variable amounts of glutamine, 5.0 mM ATP, 40 mM bicarbonate, 20 mM Mg^{2+}, 100 mM K^+, and 10 mM ornithine.

[d] Not saturated at 600 mM NH_4Cl, pH 7.6.

be recovered from any of the chromatographic fractions. It is likely that this polypeptide is unstable when separated from the large subunit during the size exclusion chromatography.

Kinetic properties of G155I, Δ155, and G152I. Gly-155 is the third residue within the type-II turn of the hinge loop from the small subunit of CPS. This residue position is most frequently occupied by a glycine or asparagine in type-II turns because of the inherent ability of these two residues to adopt the required backbone angles (18). Replacement of this glycine residue by a bulky isoleucine residue and deletion of this residue were both intended to disrupt the normal type-II turn conformation. A hinge loop without the type II turn structure will most likely be extended in

length and structurally less rigid. These two mutants displayed almost identical kinetic properties. In the glutaminase partial reaction, the maximal rates of hydrolysis were elevated four- to sixfold, relative to the wild-type value, while the K_m values for glutamine were unaltered. For the overall synthesis of carbamoyl phosphate, using glutamine as the nitrogen source, there were minimal effects on the value of V_{max}, while the K_m values for glutamine were six- to sevenfold higher. In reactions using ammonia as the nitrogen source, the K_m values for ammonia were approximately two- to threefold higher than the wild-type value while the net effects on V_{max} were significantly smaller. Gly-152 is the amino acid residue that immediately precedes the first residue of the type II turn within the

TABLE II

Kinetic Parameters for the Glutaminase Reaction of the Wild-Type and Mutant Enzymes

	Glutaminase in the absence of ATP and HCO_3^-[a]		Glutaminase in the presence of saturating ATP and HCO_3^-[b]	
	V_{max} (min^{-1})	K_m (Gln) (mM)	V_{max} (s^{-1})	K_m (Gln) (mM)
WT	0.25 ± 0.01	0.083 ± 0.006	2.9 ± 0.1	0.069 ± 0.007
G155I	1.0 ± 0.05	0.13 ± 0.03	2.5 ± 0.1	0.43 ± 0.03
Δ155	1.8 ± 0.03	0.072 ± 0.006	2.4 ± 0.1	0.48 ± 0.09
G152I	0.93 ± 0.01	0.084 ± 0.005	1.9 ± 0.1	0.43 ± 0.05
L153W	21 ± 0.4	0.20 ± 0.02	6.8 ± 0.1	9.5 ± 0.6
L153G/N154G	3.6 ± 0.1	0.070 ± 0.006	3.8 ± 0.3	2.2 ± 0.4
Ternary complex	11 ± 0.1	0.089 ± 0.002	5.4 ± 0.2	6.0 ± 0.5

[a] Rate constant for formation of glutamate monitored for the glutaminase reaction in the absence or presence of ATP and bicarbonate. Reaction conditions: pH 7.6, 25°C, variable amounts of glutamine, and 100 mM K^+.

[b] Reaction conditions: pH 7.6, 25°C, variable amounts of glutamine, 5.0 mM ATP, 40 mM bicarbonate, 20 mM Mg^{2+}, 100 mM K^+, and 10 mM ornithine.

Kinetic Parameters for the Carbamoyl Phosphate Synthesis Reaction of the Wild-Type and Mutant Enzymes

	NH$_3$-dependent[a]		Gln-dependent[b]	
	V_{max} (s^{-1})	K_m (NH$_4$Cl) (mM)	V_{max} (s^{-1})	K_m (Gln) (mM)
WT	2.9 ± 0.1	211 ± 23	3.2 ± 0.1	0.075 ± 0.003
G155I	1.9 ± 0.2	650 ± 100	2.1 ± 0.1	0.51 ± 0.01
Δ155	1.9 ± 0.2	444 ± 67	2.0 ± 0.1	0.68 ± 0.01
G152I	2.0 ± 0.1	329 ± 32	1.7 ± 0.1	0.64 ± 0.01
L153W	0.64 ± 0.05	467 ± 88	2.4 ± 0.1	7.1 ± 0.3
L153G/N154G	1.8 ± 0.1	818 ± 104	2.3 ± 0.1	1.7 ± 0.1
Ternary complex	0.6 ± 0.05	855 ± 110	2.1 ± 0.1	6.1 ± 0.2

[a] Rate constant for formation of carbamoyl phosphate. Reaction conditions: pH 7.6, 25°C, variable amounts of NH$_4$Cl, 5.0 mM ATP, 40 mM bicarbonate, 20 mM Mg^{2+}, 100 mM K$^+$, and 10 mM ornithine.

[b] Reaction conditions: pH 7.6, 25°C, variable amounts of glutamine, 5.0 mM ATP, 40 mM bicarbonate, 20 mM Mg^{2+}, 100 mM K$^+$, and 10 mM ornithine.

loop. Although Gly-152 is not a part of the turn structure, this residue adopts atypical dihedral angles of 90.2° (ϕ) and 151.1° (ψ). Therefore, the replacement of Gly-152 with an isoleucine at this position may also affect the conformation of the turn. The kinetic properties of G152I were very similar to those of G155I and Δ155, except that the reactions with ammonia as the nitrogen source were not affected by this particular modification.

Kinetic properties of L153W. A space-filling model of the three-dimensional structure of CPS indicates that the side chain of Leu-153, the first residue of the type II turn, is buried between the interface of the N-terminal and C-terminal domains of the small subunit. Therefore, replacement of this leucine residue with a bulkier tryptophan may wedge the two domains apart, thus disrupting interactions at the interface between the two domains. This is schematically shown in Fig. 3. In the absence of added ATP and bicarbonate, L153W hydrolyzes glutamine almost two orders of magnitude faster than does the wild-type enzyme. During the overall reaction with saturating levels of ATP and bicarbonate, the maximal rate of glutamine hydrolysis was 2.3-fold higher than the wild-type value while the K_m values for glutamine were two orders of magnitude greater than that of the wild-type enzyme. This enhancement in the glutaminase activity has apparently led to a partial uncoupling between the hydrolysis of ATP and glutamine during the overall synthesis of carbamoyl phosphate. To form one equivalent of carbamoyl phosphate, L153W hydrolyzes 2.8 equivalents of glutamine. In addition, L153W displays significantly altered kinetic properties for reactions occur-

ring at the large subunit. For the carbamoyl phosphate synthesis reaction using ammonia as the nitrogen source, the K_m value for ammonia was twice the wild-type value while the maximal rate was reduced by 5-fold. For the bicarbonate-dependent hydrolysis of ATP, the maximal rate was reduced to 5% that of the wild-type value.

Kinetic properties of the ternary complex. When coexpressed, the large subunit, the N-terminal domain (1–154) and the C-terminal domain (155–380) of the small subunit of CPS, formed a tight heterotrimeric complex. This *ternary* complex retained the ability to form carbamoyl phosphate from both nitrogen sources and to catalyze the partial reactions occurring on both subunits. Interestingly, the kinetic parameters of this mutant are very similar to those observed with L153W for both the partial reactions and the overall reaction. For the glutaminase partial reaction, L153W hydrolyzes glutamine 44-fold faster than does the wild-type CPS. During the overall synthesis of carbamoyl phosphate, the k_{cat} value for glutamine hydrolysis was 1.9-fold higher than the wild-type value, and the K_m value for glutamine was 87-fold higher than observed with the wild-type CPS. The precise coupling between the partial reactions is also affected in the ternary complex. In order to form one equivalent of carbamoyl phosphate, 2.6 equivalents of glutamine are hydrolyzed. The *ternary* complex also possesses very similar kinetic properties for the partial reactions occurring within the large subunit.

Kinetic properties of L153G/N154G. An additional strategy to disrupt the type II turn structure within the hinge loop of the amidotransferase subunit in CPS is the double replacement with glycine residues at positions 153 and 154, which are, respectively, the first and second residues of the turn. Since residues 153 and 154 are flanked by Gly-152 and Gly-155, the hinge loop of the L153G/N154G mutant contains a tetraglycine segment, which should render this loop more confor-
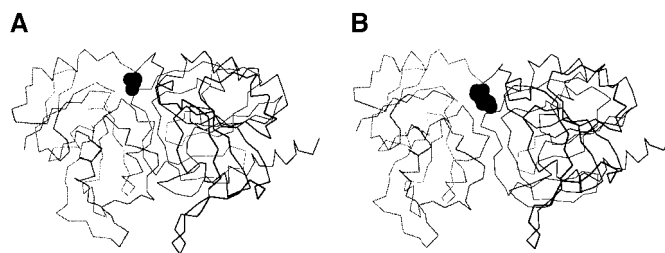

FIG. 3. Schematic presentation of the putative effects on the structure of the small subunit of CPS by L153W. (A) Wild-type CPS. (B) L153W. The N-terminal and C-terminal domains of the small subunit of CPS are shown as an α-carbon trace. The side chain of residue 153 is drawn in CPK mode. The coordinates for the wild-type CPS are taken from Ref. 9. In B, the structure is not generated by any calculation but is presented solely for the purpose of illustration.

mationally flexible than that observed in the wild-type CPS. In the glutaminase partial reaction, L153G/N154G hydrolyzed glutamine 14-fold faster than the wild-type enzyme. For the overall synthesis of carbamoyl phosphate, the maximal rate of glutamine hydrolysis was 30% higher than with the wild-type value, while the K_m value for glutamine was elevated by an order of magnitude. The K_m value for ammonia in the ammonia-dependent synthetase reaction was 4-fold higher than the wild-type value. This modification also reduced the maximal rate of the bicarbonate-dependent hydrolysis of ATP by 9-fold.

DISCUSSION

In the crystal structure of CPS from *E. coli,* the N-terminal domain and the C-terminal domain of the small subunit are linked by a hinge-like loop that contains a type II β turn (8). Compared to carbamoyl phosphate synthetases from other sources, the residues of this hinge-like loop are not strictly conserved. However, it is not clear whether the type-II turn structure within the loop observed in the *E. coli* enzyme is preserved among other carbamoyl phosphate synthetases. The active site for the amidotransferase activity is located at the interface of the two domains of the small subunit. Residues from both domains contribute to the formation of the ammonia channel, which leads from the active site within the small subunit to the bicarbonate phosphorylation site within the large subunit (9). Both domains also make molecular contacts with the large subunit. Since CPS catalyzes multiple reactions via both linear and parallel processes, the partial reactions occurring on the two subunits must be synchronized via both intra- and intersubunit communication. In the current study, the function of this hinge loop connecting the two domains of the small subunit was examined by site-directed mutagenesis.

Mutations were designed with the intent of disrupting the type-II turn structure within the hinge loop. Based upon the observed kinetic effects, the modifications can be separated into two groups. The first group consists of G152I, G155I, and Δ155. The deletion of Gly-155 prevents the formation of the type-II turn. The replacement of Gly-152 or Gly-155 with an isoleucine was also intended to disrupt this turn conformation. Indeed, G155I and Δ155 display very similar kinetic properties. Nevertheless, these three modifications have only minor effects on the kinetic properties of CPS. The partial reactions that occur on the two subunits are fully coupled to one another as found with the wild-type enzyme. Therefore, it appears that the type-II turn structure of the hinge loop per se is not important for the overall catalytic function of CPS.

The second group of modifications includes L153W, L153G/N154G, and the *ternary* complex. L153W was intended to wedge the two domains of the small subunit slightly apart (Fig. 3) while the double glycine replacement at positions 153 and 154 would replace the original type-II turn with a conformationally more flexible tetraglycine segment. For the *ternary* complex, it is formed without assistance or restraint by the hinge loop. The overall effects of this group of modifications on the kinetic properties of CPS are more pronounced than those of the first group. In the overall synthesis reaction with glutamine as the nitrogen source, the K_m values for glutamine are elevated by 30- to 140-fold while the V_{max} values for glutamine hydrolysis exceed that of the wild-type enzyme by 35 to 135%. There is a good correlation between the increase in the glutaminase activity in the overall reaction and the increase (14 to 84-fold) in the activity of the partial glutaminase reaction. The kinetic effects are also reflected in the altered overall stoichiometry of these mutants. To form one molecule of carbamoyl phosphate, these mutant enzymes hydrolyze 1.7–2.8 molecules of glutamine instead of one molecule of glutamine as with the wild-type CPS. Apparently, there is some leakage of the internally derived ammonia, which is not fully captured by the carboxy phosphate intermediate formed within the large subunit. These results are thus consistent with the hinge loop playing a role in the structural integrity of the ammonia tunnel. The perturbations to the tunnel structure in these modified enzymes are likely responsible for the observed catalytic uncoupling between the partial reactions occurring within the small and large subunits.

Since the overall kinetic parameters of L153W and the *ternary* complex are very similar, it is highly improbable that the overall molecular contacts among the three polypeptides in the *ternary* complex will differ significantly from the ones between the small and the large subunits in L153W. We have also observed that expression of the C-terminal domain of the small subunit alone leads to the formation of inclusion bodies, whereas coexpression with the large subunit produces soluble proteins. This is consistent with a heterotropic association between the large subunit and the C-terminal domain of the small subunit. However, no binary complex of the two polypeptides could be detected during size exclusion chromatography. These results suggest that the interface between the large subunit and the C-terminal domain of the small subunit alone, as observed in the wild-type CPS, is not sufficient for the formation of a tight binary complex. Therefore, it appears that the interface interactions between the N-terminal and C-terminal domains of the small subunit are also important for subunit interactions between the small subunit and the large subunit. It has been reported that the separately cloned amidotransferase subdomain of the mammalian CPS, which is a part of the trifunctional CAD polypeptide, hydrolyzes

glutamine 3-fold faster than CAD in the carbamoyl phosphate synthesis reaction and 40-fold faster than CAD in the glutaminase partial reaction (19). This amidotransferase subdomain is the structural equivalent of the C-terminal domain of the small subunit of CPS from *E. coli.* The magnitude of this elevation for the glutaminase activity by the amidotransferase subdomain is thus comparable to that found for the *ternary* complex of CPS reported here. The amidotransferase subdomain from CAD does not form a stable hybrid complex with the large subunit of CPS from *E. coli.* However, the authors contend that a transient complex was formed if the amidotransferase subdomain was in large excess of the large subunit of CPS from *E. coli,* since the product, carbamoyl phosphate, was detected when glutamine was used as the nitrogen source (19). In light of the X-ray crystal structure of CPS from *E. coli* and the lack of tight complex formation between the amidotransferase domain and the large subunit, we find it difficult to rationalize the formation of carbamoyl phosphate from a transient complex without the participation of the N-terminal domain. This domain contributes significantly to the formation of the ammonia channel in CPS from *E. coli.* An alternative explanation is that the formation of carbamoyl phosphate is the result of the synthetase reaction using the *external* ammonia from the bulk solution, which is initially derived from the hydrolysis of glutamine by the amidotransferase subdomain and then released into the bulk solvent.

To summarize, the C-terminal and N-terminal domains of the small subunit of CPS from *E. coli* are connected with a hinge loop that contains a type II β turn. Through modification of amino acid residues within the hinge loop, we have demonstrated that the type II β turn structure per se is not important for the overall function of CPS. However, our results are consistent with the overall hinge loop playing a significant role in the maintenance of the structural integrity of the molecular tunnel within the small subunit of CPS and the synchronization of the partial reactions occurring on the separate subunits of CPS.

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