Activation by Fusion of the Glutaminase and Synthetase Subunits of *Escherichia coli* Carbamyl-phosphate Synthetase* 

(Received for publication, February 11, 1997, and in revised form, May 22, 1997)

**HedeeI. Guy, Andrea Rotgeri, and David R. Evans**

*From the Department of Biochemistry and Molecular Biology, Wayne State University School of Medicine, Detroit, Michigan 48201*

*Escherichia coli* carbamyl-phosphate synthetase consists of two subunits that act in concert to synthesize carbamyl phosphate. The 40-kDa subunit is an amidotransferase (GLN subunit) that hydrolyzes glutamine and transfers ammonia to the 120-kDa synthetase subunit (CPS subunit). The enzyme can also catalyze ammonia-dependent carbamyl phosphate synthesis if provided with exogenous ammonia. In mammalian cells, homologous amidotransferase and synthetase domains are carried on a single polypeptide chain called CAD. Deletion of the 29-residue linker that bridges the GLN and CPS domains of CAD stimulates glutamine-dependent carbamyl phosphate synthesis and abolishes the ammonia-dependent reaction (Guy, H. I., and Evans, D. R. (1997) *J. Biol. Chem.* 272, 19906–19912), suggesting that the deletion mutant is trapped in a closed high activity conformation. Since the catalytic mechanisms of the mammalian and bacterial proteins are the same, we anticipated that similar changes in the function of the *E. coli* protein could be produced by direct fusion of the GLN and CPS subunits. A construct was made in which the intergenic region between the contiguous *carA* and *carB* genes was deleted and the sequences encoding the carbamyl-phosphate synthetase subunits were fused in frame. The resulting fusion protein was activated 10-fold relative to the native protein, was unresponsive to the allosteric activator ornithine, and could no longer use ammonia as a nitrogen donor. Moreover, the functional linkage that coordinates the rate of glutamine hydrolysis with the activation of bicarbonate was abolished, suggesting that the protein was locked in an activated conformation similar to that induced by the simultaneous binding of all substrates.

**Escherichia coli** carbamyl-phosphate synthetase (CPSase; EC 6.3.5.5) initiates both *de novo* pyrimidine and arginine biosynthesis (1) and is regulated by metabolites from both pathways. The enzyme consists of a small 40-kDa subunit (GLN subunit), encoded by *carA*, that hydrolyzes glutamine and transfers ammonia to the large 120-kDa subunit (CPS subunit) (2, 3). The *carB* gene encodes the large synthetase subunit (CPS subunit), which catalyzes the synthesis of carbamyl phosphate from ammonia, 2 ATP molecules, and bicarbonate (1, 4, 5). Ammonia, not ammonium ion, is the substrate (1), and thus, ammonia generated *in situ* must therefore be seques- tered within the complex to escape protonation in the aqueous phase. Ammonium chloride can also serve as a nitrogen donor for carbamyl phosphate synthesis by the intact enzyme or the isolated synthetase subunit, but only if high enough concentrations are used so that, at neutral pH, there is sufficient ammonia to sustain catalysis at a significant rate.

Mammalian carbamyl phosphate synthesis is catalyzed by CAD (6–8), a multifunctional protein that also has aspartate transcarbamylase and dihydroorotase activities. All of the CAD functions are associated with specific domains and subdomains (9–14) of a 254-kDa polypeptide. Carbamyl phosphate is synthesized by the concerted action of the contiguous GLN and CPS domains (15, 16), which are connected by a 29-residue chain segment, the GC linker. The amino acid sequences of the mammalian domains and the subunits of bacterial CPSase are clearly homologous, suggesting a common tertiary structure (15–25). Moreover, the mechanism of carbamyl phosphate synthesis (1) by both proteins is thought to be identical.

In the accompanying report (26), we showed that deletion of the linker to produce a protein in which the GLN and CPS domains are fused in frame alters the catalytic and regulatory properties of mammalian CPSase. The *k*<sub>cat</sub> for glutamine-dependent carbamyl phosphate synthesis is increased 10-fold, whereas the ammonia-dependent reaction is abolished. These observations suggested a model in which the enzyme cycles between two conformations, an open inactive conformation that can bind exogenous ammonia and a catalytically active conformation in which access to the ammonia site on the synthetase subunit is restricted. These conformations are likely to involve changes in the juxtaposition of the GLN and CPS domains made possible by the linker that serves as a spacer, allowing the covalently linked domains to move relative to one another. Deletion of the GC linker was postulated to trap the enzyme in the catalytic conformation. Since the GLN and CPS domains are separate subunits in the *E. coli* enzyme (2), there are no constraints on the relative position of the domains. To test the idea that the mammalian multifunctional protein arose during the course of evolution by gene fusion, Kern *et al.* (27) linked the *E. coli* CPSase subunits together via the mammalian GC linker. The resulting protein was found to be fully functional. Thus, the GC linker is long enough and flexible enough to accommodate any changes in the juxtaposition of the domains that may occur in both mammalian and bacterial proteins.

Based on the similarities of the structure and catalytic mechanism, we anticipated that functional changes analogous to those observed in the mammalian CPSase deletion mutant
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Fig. 1. Structural organization of E. coli CPSase. E. coli CPSase consists of a 40-kDa amidotransferase subunit (GLN subunit) and a 120-kDa synthetase subunit (CPS subunit). A fusion protein (GLN-CPS) was constructed in which the carboxyl-terminal end of the GLN subunit was linked to the amino-terminal end of the CPS subunit. The sequences of the carboxyl-terminal end of the GLN subunit and the amino-terminal end of the CPS subunit are shown. The fusion protein was designed to be exactly homologous to the CAD deletion mutant (26). Eight residues on the amino-terminal end of the E. coli synthetase subunit, which have no counterpart in CAD, were deleted along with the 4 residues of the core CPS domain (15). The result suggested that the changes in juxtaposition of the GLN and CPS domains, with concomitant changes in catalytic activity and accessibility to exogenous ammonia, are a common feature of this family of enzymes.

EXPERIMENTAL PROCEDURES

Plasmids and Strains—pLLK12, a plasmid encoding the small and large subunits of E. coli CPSase, was kindly provided by Dr. Carol Lusty (The Public Health Research Institute of the City of New York, New York), as were E. coli strains RC50 and L673 (28), which are defective in the carA and carB genes encoding the E. coli CPSase subunits. Strain L673 also lacks the lon protease. The recombinant proteins, which are expressed constitutively under the control of the carAB promoter, were isolated from cells grown to stationary phase (28).

Construction of the Recombinant Plasmids—Overlap extension by polymerase chain reaction (29) was used to delete residues 1620–1675 from pLLK12. The resulting 298-base pair insert was ligated to the large fragment of pLLK12 cleaved with the same enzymes. The resulting 1.9-kb insert that contained the restriction sites for BclI and ApaI was ligated to the large fragment of pLLK12 cleaved with the same enzymes. The resulting plasmid (pAR14) contained the coding sequences for the fused E. coli glutaminase-synthetase domains under the control of the carAB promoter.

RESULTS

Fusion of the CPSase GLN and CPS Subunits—The carA and carB genes encoding the subunits of the E. coli CPSase GLN and CPS domains (Fig. 1) were fused in frame (see “Experimental Procedures” and Fig. 2) at residues that were exactly homologous to those used in the construction of the mammalian deletion mutant lacking the GC linker (26). The resulting construct, pAR14, encodes a fusion protein in which the carboxyl-terminal end of the GLN subunit is directly linked to the amino-terminal end of the CPS subunit. When the recombinant plasmid was transformed into E. coli strain L673, the fusion protein was expressed (Fig. 3) at a level comparable to that of the wild-type E. coli protein (70% of the total cellular protein) and could be readily purified (see “Experimental Procedures”).

Molecular Mass of the Fusion Protein—The molecular mass of the fused protein was determined by SDS-polyacrylamide gel electrophoresis to be 160 kDa, close to the value calculated from the amino acid sequence. The protein eluted on a calibrated Sepharose S-300 column as a single species with a calculated molecular mass of 648 kDa, indicating that it is a tetramer.

Function of the Fusion Protein—Whereas the Km of the fusion protein obtained from the ATP saturation curve of the glutamine-dependent CPSase activity was comparable to that of the wild-type protein (Table I), the V max was 10-fold higher. (68 kDa), and alcohol dehydrogenase (150 kDa). The void volume was determined with blue dextran. The column was eluted with the equilibration buffer following application of 0.5 ml (14–120 mg) of the test proteins. The elution volume was determined by measuring the CPSase activity, by SDS-gel electrophoresis, or by determining the absorbance at 280 nm.

**Gel Filtration Chromatography**—The molecular masses of the recombinant proteins were determined by gel filtration (36) on a 1.9 × 42-cm Sepharose S-300 column equilibrated in 0.05 M Tris-HCl, pH 7.4, 1 mM dithiothreitol, 5% glycerol, and 25 mM MgCl2. Substrate saturation curves were obtained by assaying 6.8 μg of protein with the concentrations of sodium bicarbonate, glutamine, and ATP, when fixed, held at 5, 3.5, and 10 mM, respectively. A spectrophotometric coupled enzyme assay (35) was used to assay the glutaminase activity.

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Similar results were obtained for the bicarbonate and glutamine saturation curves. Thus, direct fusion of the GLN and CPS domains results in activation of both bacterial and mammalian proteins. In contrast to the mammalian deletion mutant, the E. coli protein could still catalyze the ammonia-dependent reaction. However, the ammonia-dependent activity was so low (0.1% (Table I) to 2% in various preparations) that it was not possible to obtain a reproducible saturation curve.

ATP saturation curves conducted in the presence of the CPSase allosteric effectors, the inhibitor UMP and the activator ornithine (Fig. 4), showed that the regulatory properties of the fusion protein were also altered. UMP clearly inhibits the protein, but the major effect is on the $V_{max}$, not the $K_m$, as observed with the native protein. The $V_{max}$ was reduced by 40% in the presence of UMP. The kinetic parameters obtained for the fusion protein in the presence of ornithine were virtually identical to the control values, indicating that it could no longer be allosterically activated.

Coupling of the Glutaminase and Synthetase Activities—The glutaminase activity of the fusion protein was 10-fold higher than the corresponding activity of the wild-type protein when all of the substrates needed for carbamyl phosphate synthesis were present (Table II). The rate of glutamine hydrolysis by native E. coli CPSase is normally suppressed in the absence of all of the other substrates needed for carbamyl phosphate synthesis (38), but is appreciatively activated by ATP and bicarbonate (Table II). Under conditions of saturating bicarbonate and glutamine, the glutaminase activity of the native enzyme increased with increasing ATP concentrations (Fig. 5) to a maximum extrapolated value of 1204 ± 0.131%. The concentration of ATP that produces half-maximal activation is 5.6 ± 1.2 mM. This coupling mechanism is thought to spare glutamine hydrolysis when the concentration of ATP is limiting. However, in the case of the fusion protein (Fig. 5 and Table II), the glutaminase activity is high whether or not ATP and bicarbonate are present, and the addition of these substrates had little further stimulatory effect on the glutaminase activity.

Elastase Digestion of the Fusion Protein—The low residual ammonia-dependent activity of the fusion protein could be explained by limited proteolytic cleavage into separate GLN and CPS subunits. The trace amounts of a 40-kDa and a 120-kDa species observed in various preparations are consistent with this explanation. To test this idea, the purified fusion protein was subjected to limited elastase digestion (Fig. 6). As expected, the protein initially exhibited high glutamine-dependent activity and barely detectable ammonia-dependent activity. As digestion proceeded, the protein was progressively cleaved into 40- and 120-kDa fragments, suggesting that cleavage occurred at the fusion junction of the GLN and CPS subunits. Proteolysis was correlated with a decrease in the glutamine-dependent activity and a parallel increase in the ammonia-dependent CPSase activity. On complete cleavage, the rates of the glutamine- and ammonia-dependent reactions were comparable to those observed for the wild-type protein.

DISCUSSION

The GLN and CPS domains of CAD are separated by a 29-residue chain segment that is believed to function as a spacer that allows the domains to move relative to one another (28). Deletion of this bridging segment produced profound changes in the catalytic and regulatory properties. In contrast, the domains of E. coli CPSase are separate subunits (2), and there are therefore no covalent constraints on quaternary structural changes. When the carboxyl-terminal end of the GLN subunit was fused to the amino-terminal end of the CPS subunit in a manner exactly analogous to the mammalian deletion mutant, similar functional changes were observed. Whereas the $K_m$ values for ATP, bicarbonate, and glutamine

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**Table I**

| Protein Variable substrate | $K_m$ (mM) | $V_{max}$ (μmol/min/mg) |
|---------------------------|------------|-------------------------|
| CPSase                    | ATP        | 0.43 ± 0.02             |
|                           | Bicarbonate| 3.66 ± 0.32             |
|                           | Glutamine  | 0.11 ± 0.02             |
|                           | NH$_4$Cl   | 5.33 ± 1.23             |
| CPSase fusion protein     | ATP        | 1.37 ± 0.16             |
|                           | Bicarbonate| 5.22 ± 1.11             |
|                           | Glutamine  | 0.12 ± 0.02             |
|                           | NH$_4$Cl   | ND$^b$                  |

$^a$ The wild-type protein isolated from RC50 cells transformed with pLLK12; $^b$ ND, not determined.

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**Fig. 3.** Expression of the linked E. coli CPSase domains. A 4-ml culture of RC50 cells transformed with pAR14 was grown overnight to stationary phase in LB medium containing 0.1% glucose and 150 g/liter ampicillin. The cells were harvested, resuspended to a final volume of 1 ml in 50 mM Tris-HCl, pH 7.4, 1 mM dithiothreitol, and 5% glycerol; and broken by sonication. The extract was centrifuged at 16,000 g for 20 min, and the supernatant (5 μl) was analyzed by SDS-polyacrylamide gel electrophoresis (lane 1). Lanes 2 and 3 show induced supernatants of RC50 cells transformed with plasmid pAB1, which expresses the E. coli CPSase small subunit. Lanes 4 and 5 show the wild-type E. coli CPSase protein expressed in RC50 cells transformed with pLLK12. Extracts of wild-type CPSase and the small subunit of E. coli CPSase were prepared as described for the fused protein. Standard proteins (SD) that served as molecular mass markers are also shown.

**Fig. 4.** Effect of UMP and ornithine on the E. coli CPSase fusion protein. ATP saturation curves obtained using 0.15 ml of extracts of RC50 cells expressing the fusion protein were assayed in the absence of allosteric ligands (●) and in the presence of 5 mM UMP (△) or 12 mM ornithine (○). Note that the curves from the nonlinear least-squares fit in the absence of ligands and in the presence of ornithine are superimposable.

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TABLE II

Effect of ATP and bicarbonate on the glutaminase activity

| Parameter                                      | E. coli CPSase | E. coli fusion protein |
|------------------------------------------------|---------------|-----------------------|
| Activity in absence of ligands (μmol/min/mg)  | 0.314         | 39.0                  |
| Activity in presence of 10 mM ATP and 5 mM NAHCO₃ (μmol/min/mg) | 2.24          | 43.0                  |
| -Fold activation by ATP and NaHCO₃            | 7.1           | 1.1                   |

**Fig. 5. Coupling of the partial reactions of E. coli CPSase native and fusion proteins.** The glutaminase activities of native E. coli CPSase (○) and of the fusion protein (●) were assayed at a fixed concentration of glutamine (3.5 mM) and sodium bicarbonate (5 mM) and increasing concentrations of ATP. The glutaminase activity of the fusion protein in the absence of ATP was much higher than the corresponding value for the native protein, but is expressed as a percent of the value obtained in the absence of ATP (0.31 and 39 μmol/min/mg for the native and fusion proteins, respectively) to facilitate comparison. The data for native CPSase were fit by least-squares analysis to the expression \( v = V_0 + \left( V_{max}\text{ATP}/K + \text{ATP} \right) \), where \( v \) is the observed glutaminase activity, \( V_0 \) is the activity in the absence of ATP (100%), \( V_{max} \) is the calculated maximum activity in the presence of saturating ATP, and \( K \) is the concentration of ATP that gives half-maximum activation.

These results suggest that E. coli CPSase, like its mammalian counterpart, can exist in an open inactive conformation in which the ammonia-binding site on the CPS subunit is accessible and a closed catalytic conformation that cannot bind ammonia, but in which the active-site residues are optimally positioned. The question then becomes, what is the functional advantage of the inactive conformation? The catalytically active conformation can still bind ATP, bicarbonate, and glutamine, and the intermediates (ammonia and carboxyl phosphate) are effectively sequestered within the complex (Fig. 7). While ammonia-dependent activity was unmeasurable, whereas in others, a small but significant activity could be detected. We attribute this residual ammonia-dependent activity to the presence of trace amounts of the unlinked subunits produced by endogenous proteases. Controlled elastase digestion showed that the artificial junction between the domains was the most susceptible region of the fusion protein to proteolysis. Upon cleavage into individual subunits, the hyperactivity was diminished, and the ammonia-dependent activity was restored. The catalytic properties of the cleaved fusion protein were indistinguishable from those of the native enzyme. This experiment clearly showed that the fusion of the two subunits per se produced the observed functional changes.

**Fig. 6. Controlled proteolysis of the E. coli fusion protein.** The partially purified fusion protein (10 μg) was subjected to limited digestion by elastase at a protease/protein ratio of 1:100 at 37 °C in 50 mM Tris-HCl, pH 7.4, 1 mM dithiothreitol, and 5% glycerol, and the time-dependent changes in glutamine-dependent (●) and ammonia-dependent (○) CPSase activities were assayed (A). The samples were also analyzed by SDS-gel electrophoresis along with molecular mass standards (SD) and purified E. coli CPSase (WT) (B).
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fig. 7. model of the two conformational states of e. coli cpsase. the diagram is a schematic representation of the two conformational states of e. coli cpsase. a shows a low activity state that binds atp and bicarbonate and either glutamine or ammonia. when all of the substrates are bound, the enzyme is proposed to assume the conformation shown in b, a high activity conformation that sequesters the substrates and intermediates. the conformation shown in b can still bind glutamine, atp, and bicarbonate, but not ammonia. carboxy phosphate formed from the atp-dependent activation of bicarbonate reacts with ammonia, produced in situ or exogenously available, to form carbamate. a second atp molecule then phosphorylates carbamate to form carbamyl phosphate. when the gln and cps subunits are fused, the enzyme is effectively trapped in the conformation shown in b.

these studies also suggest a possible role for the nonfunctional gln domain of mitochondrial cpsase i. this enzyme has a functional cps domain fused to an inactive gln domain via a 26-residue connecting chain segment (20, 46). whereas the gln domain is clearly homologous to the active amidotransferases, it lacks the active-site cysteine (20, 42) and probably has other, as yet unidentified, mutations (29) that render it inactive. thus, ammonia is the only nitrogen-donating substrate. long considered a vestige of the evolutionary history of this family of proteins, there may well have been selective pressures responsible for its retention. however, assuming that an analogous series of conformational changes occur in this enzyme, an open low activity conformation produced by changes in the juxtaposition of the gln and cps domains would be necessary for ammonia binding. once all of the substrates are bound, the enzyme assumes the closed high activity conformation, which promotes catalysis and sequesters the intermediates. in this scheme, the gln domain, despite its lack of catalytic activity, is an essential component involved in mediating conformational changes that occur during the catalytic cycle.

in summary, the direct fusion of the gln and cps domains traps the enzyme in a closed high activity conformation thought to be similar to that induced by the binding of atp and bicarbonate. the conformational changes, revealed by studies of these constructs, may thus be an important element of the interdomain signaling that coordinates parallel reactions occurring at different sites of the molecule. the observation that similar changes in catalytic and regulatory properties occur in the mammalian deletion mutant and the e. coli fusion protein suggests that a common mechanism may be operative throughout this family of proteins.

acknowledgment—we thank dr. carol lusty for the generous gift of plasmids and strains.

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