Cloning, Expression, and Functional Interactions of the Amidotransferase Domain of Mammalian CAD Carbamyl Phosphate Synthetase*

Hedeel I. Guy and David R. Evans
From the Department of Biochemistry, Wayne State University School of Medicine, Detroit, Michigan 48201

(Received for publication, August 27, 1993, and in revised form, October 26, 1993)

The trpG-type amidotransferases, a homologous but structurally diverse family of molecules, catalyze glutamine hydrolysis to supply ammonia for many biosynthetic reactions. The amidotransferase or glutaminase (GLNase) domain of mammalian carbamyl phosphate synthetase (CPSase), part of a 243-kDa polypeptide that initiates de novo pyrimidine biosynthesis, has been cloned and expressed in Escherichia coli. Complementation studies showed that a functional protein was produced in vivo which could provide ammonia for carbamyl phosphate synthesis by the host CPSase synthetase subunit. The recombinant 38-kDa protein was identified by immunoblotting, but when purified to homogeneity, had marginal glutaminase activity. Titration of the E. coli CPSase synthetase subunit with the mammalian GLNase domain resulted in the formation of a fully active 1:1 stoichiometric stable complex which catalyzed the glutamine-dependent overall reaction. The hybrid, isolated by gel filtration, had kinetic parameters ($K_{\text{GLN}} = 102 \mu M$, $k_{\text{cat}} = 1.8 \text{ mmol} \cdot \text{min}^{-1}$) similar to those of the native E. coli CPSase. Thus, the amidotransferase activity of mammalian CPSase is carried by an autonomous domain which folds independently. However, optimal catalytic activity requires association of the glutaminase and synthetase domains. The conservation of this linkage in the mammalian E. coli hybrid suggests that the subunit interfaces must be nearly identical in the eukaryotic and prokaryotic proteins.

Glutamine serves as a nitrogen donor in many biosynthetic pathways (1). The enzymes which catalyze these reactions consist of an amidotransferase or glutaminase (GLNase) domain and a synthetase domain (2). Glutamine hydrolysis is catalyzed by the amidotransferase domain, and the resulting ammonia is used by the synthetase domain in the biosynthetic reaction. There are two classes of amidotransferases, trpG-type and purF-type, which have very different amino acid sequences (3). However, all of the members of both families have a conserved cysteine residue which participates in the formation of a γ-glutamylthioester intermediate during the hydrolysis of glutamine (4–12).

Carbamyl phosphate synthetase (CPSase, EC 6.3.5.5), a typical trpG-type amidotransferase, catalyzes the formation of carbamyl phosphate from glutamine, bicarbonate, and two ATP molecules (1). The CPSase glutaminase and synthetase activities can be associated with separate subunits as in Escherichia coli CPSase (13) or can be carried on a single polypeptide chain, sometimes in association with other enzymatic activities, as in the mammalian multifunctional protein CAD (14–18).

E. coli carbamyl phosphate synthetase consists of a large (118 kDa) subunit, which catalyzes ammonia dependent carbamyl phosphate synthesis, and a small (42 kDa) amidotransferase subunit (13). The CPSase subunits could be reversibly dissociated with thiocyanate and isolated by gel filtration (13, 19). The 120-kDa subunit catalyzes ammonia-dependent carbamyl phosphate synthetase, whereas the 40-kDa subunit hydrolyzes glutamine. Reconstitution of the native enzyme from the isolated subunits restored the ability of the enzyme to use glutamine as a nitrogen donor. Although the subunits can function autonomously, there is extensive evidence for a functional linkage between the glutamine binding site of the small subunit and the active site of the large synthetase subunit which modulates their activities (6–8, 13, 19–22). For example, in the absence of MgATP and bicarbonate, the glutaminase activity is only about 2% of that observed for the overall reaction (1).

CAD consists of a 243-kDa polypeptide (16–18) which associates to form hexamers and higher oligomers (18, 23). In addition to glutamine-dependent carbamyl phosphate synthesis, the first step in de novo pyrimidine biosynthesis, it also has aspartate transcarbamylase (EC 2.1.3.2) and dihydorotase (EC 3.5.2.3) activities, which catalyze the second and third steps of the pathway. The amino acid sequence of hamster CAD polypeptide has been determined by sequencing two partially overlapping cDNA clones (24–27), and the GLNase and CPSase domains have been identified (26, 27) by the strong similarity (40–50%) of their sequences to their counterparts in E. coli and other organisms. We have shown recently (28) that the mechanism of glutamine hydrolysis by CAD is analogous to that found for the thiol proteases and have determined the rate constants for each step in the process. The functional linkage, needed to avoid hydrolysis of glutamine when the other substrates are limiting, is also present in CAD. ATP and bicarbonate increase the $k_{\text{cat}}$ of the glutaminase 14-fold by increasing the rate of hydrolysis of the thioester intermediate.

To establish whether or not the region of the CAD polypeptide identified as the GLNase domain is a separate, autonomously folding functional entity and to further investigate interdomain signaling between the amidotransferase and synthetase domains, we have cloned and expressed in E. coli a cDNA fragment encoding the putative domain of hamster CAD.

EXPERIMENTAL PROCEDURES

Plasmids and Strains—The 4.2-kb plasmid pKB-BS, a subclone of pKB11 (27), consisted of pBlueScript with a 1203-base pair insert encoding the amino end of the hamster CAD polypeptide (amino acids 1–365) and part of the regulatory region of the CAD gene. The 6.0-kb plasmid pEK81 (a gift of Evan Kantrowitz, Boston College, Chestnut Hill, Mass.) contains the entire CAD coding region (amino acids 1–365). A C-terminal myc epitope tag was added in frame to the amino acid 365 of the CAD coding sequence by PCR and cloning of a myc epitope oligonucleotide into a PCR-amplified C-terminal coding fragment of pEK81. This C-terminal myc epitope tagged plasmid, pEK81-Myc, has been used to generate a myc epitope tagged CAD protein in mammalian cells. The generation of human placenta DNA and the purification of rhCAD using rhCAD antibodies are described in (29, 30).
Cloning of the CAD Amidotransferase Domain

Hill, MA) was derived from pEK2 (29) and consists of pUC119 with a pyrB gene encoding the ATCase catalytic, and regulatory subunits, respectively. Strains L673 and L798 were derived from pEK2 (29) and consists of pUC119 with a pyrB and pyr genes and has a leaky pyrF mutation, so that only low levels of orotidine phosphoribosyl transferase are produced. The E. coli mutants (30), L673, defective in carA and carB, L798, defective in carB, and Hill, MA) was derived from pEK2 (29) and consists of pUC119 with a phosphate synthetase, respectively. Strains L673 and L798 were defective in the lon protease (30).

Cell Growth and Recombinant DNA Methods—Cells harboring the recombinant plasmids were routinely grown from a single colony in 2 x YT media supplemented with 50–100 mg/ml ampicillin. For induction of recombinant proteins under control of the pyrBI promoter, the EKI104 cells were grown in a minimal media consisting of 6 g/liter Na2HPO4, 3 g/liter KH2PO4, 1 g/liter NH4Cl, 0.5 g/liter NaCl, 0.5 g/liter casamino acids (Difco 0220), 4 g/liter glucose, 0.1 mg/liter ZnSO4, 7H2O, 0.5 mg/liter FeSO4, 7H2O, 0.1 mg/liter CaCl2, 1 mg/liter MgSO4, 7H2O, 0.5 mg/liter riboflavin, and 10 mg/liter tryptophan supplemented with 12 mg/liter uracil and 100 mg/liter ampicillin. Under these conditions there is sufficient uracil to sustain growth for 16–20 h, after which time growth is arrested or slowed and the recombinant protein is expressed. Growth was monitored spectrophotometrically at 600 nm.

The cells were harvested, typically 20–22 h after inoculation, by centrifugation at 2,000 x g for 30 min in an Sorvall R3C centrifuge. Plasmids were isolated using either a lysosome miniprep procedure (31) or by CsCl gradient centrifugation (32). Transformation and preparation of competent E. coli cells was carried out by the Hanahan procedure (33). DNA fragments were gel-purified following electrophoresis in 0.8% agarose by extraction onto glass beads (Gene Clean kit) or by the low melting point agarose method (32). Restriction digests, ligations, and other DNA methods were carried out using standard protocols (32).

Preparative CAD GLN Domain—The sequence encoding the hamster CAD glutaminase domain was excised from the partial CAD cDNA clone, pKB-BS, constructed (27) by Kanthowitz (Boston College), unpublished result. The pEKB3 plasmid, which encodes the E. coli operon, pyrBI, and overproduces ATCase, served as the vector. The pEKB3 plasmid was cut with NdeI, a site which had been engineered at the exact start of the pyrBI coding sequence. The NdeI site of pEKB3 was then destroyed by mung bean nucleases and the CAD fragment, which included the CAD translation initiation codon, was inserted by blunt end ligation. The recombinant, pHG-GLN52, has the CAD GLNase coding sequence under control of the pyrBI promoter. Although pHG-GLN52 contains most of the pyrBI coding sequences, pyrB is out of frame and is not expressed. The mammalian domain begins at the CAD start and extends to residue 365. The carboxy end of the polypeptide has the sequence Asn-Pro-Asp-Gly-COOH where the last 2 amino acids are derived from the vector.

RESULTS

Construction of the CAD GLN Recombinant—The sequence encoding the hamster CAD glutaminase domain was excised from the partial CAD cDNA clone, pKB-BS, constructed (27) by Kanthowitz (Fig. 1). The plasmid pEKB3 which encodes the E. coli operon, pyrBI, and overproduces ATCase, served as the vector. The pEKB3 plasmid was cut with NdeI, a site which had been engineered at the exact start of the pyrBI coding sequence. The NdeI site of pEKB3 was then destroyed by mung bean nucleases and the CAD fragment, which included the CAD translation initiation codon, was inserted by blunt end ligation. The recombinant, pHG-GLN52, has the CAD GLNase coding sequence under control of the pyrBI promoter. Although pHG-GLN52 contains most of the pyrBI coding sequences, pyrB is out of frame and is not expressed. The mammalian domain begins at the CAD start and extends to residue 365. The carboxy end of the polypeptide has the sequence Asn-Pro-Asp-Gly-COOH where the last 2 amino acids are derived from the vector.

Complementation of CarA Mutants—The recombinant plasmid pHG-GLN02 was transformed in E. coli L132, a strain which is defective in CarA, the gene which encodes the amidotransferase subunit of E. coli carbamyl phosphate synthetase.
The CarA strain cannot grow in low ammonia media, but will grow in media supplemented with 100 mM NH4Cl, because when present in high concentrations, ammonia can replace glutamine as a nitrogen donor (Table I). Transformation of this strain with pHG-GLN52 conferred the ability to grow on low ammonia media. The recombinant GLN domain was extremely resistant to proteolysis. The purified protein remained intact after incubation with either trypsin or elastase at a ratio of protein to protease of 100:1 for 2 h at 37 °C. Similar results were obtained when the digestion was carried out in the presence of 100 mM glutamine or a stoichiometric amount of the E. coli CPSase synthetase subunit. Thus, the recombinant protein had a stable compactly folded structure.

**Titration of E. coli CPSase Synthetase Subunit with the CAD GLN Domain**—Although the isolated GLNase domain had extremely low catalytic activity, the complementation studies suggested that it was functional and formed a complex with the large subunit of E. coli carbamyl phosphate synthetase. The 120-kDa E. coli CPSase synthetase subunit was isolated (Fig. 4) from an overproducing strain using a modified version of the procedure of Rubinio et al. (42). The glutamine-dependent CPSase activity was measured as the bacterial synthetase subunit that was titrated with increasing amounts of the mammalian GLNase domain (Fig. 4). As expected, the E. coli synthetase subunit had no activity in the absence of the glutaminase domain. However, glutamine-dependent carbamyl phosphate synthase synthesis was gradually restored as the mammalian GLN domain was added reaching full activity of 3 μmol/min/mg at a molar ratio of GLNase/CPSase synthetase of 0.9. Thus, the mammalian GLNase domain and the bacterial CPSase synthetase subunit form a fully functional, one-to-one stoichiometric complex.

**Isolation of the Hamster E. coli CPSase Hybrid**—The formation of the mammalian bacterial hybrid was also demonstrated by sucrose gradient centrifugation. When the GLNase domain was mixed with a 2-fold molar excess of the E. coli CPSase
**DISCUSSION**

The region of the CAD cDNA identified by sequence homology as the amidotransferase domain was cloned and expressed in *E. coli*. Although the expressed protein had the expected size, 38 kDa, and formed a compactly folded species highly resistant to proteolysis, it had a very low catalytic activity. However, in *in vivo* complementation studies suggested not only that it was functional, but that it formed a complex with the *E. coli* CPSase synthetase subunit. The alternative explanation, no physical association but a functional GLNase domain which produces ammonia that diffuses to the synthetase subunit, is unlikely. Ammonia, not the ammonium ion, is the substrate for the biosynthetic reaction (44), and at physiological pH only 1% of the ammonia is in the unionized form. The $K_m$ for ammonia for *E. coli* CPSase is 170–180 mM (41), so that its intracellular concentration would have to reach high levels to give significant CPSase activity.

The putative complex was demonstrated by sucrose gradient centrifugation and by titration of the *E. coli* synthetase subunit with the purified mammalian GLNase domain. The titration showed that a stoichiometric complex was formed, but the curve was somewhat sigmoidal perhaps an indication that the complex is unstable at low GLNase/CPSase molar ratios. Nevertheless, once formed, the complex was stable and could be isolated by gel filtration.

The interaction with the *E. coli* synthetase subunit strongly activates the GLNase domain. Although the intrinsic activity of the isolated domain is too low for a detailed steady state kinetic study using our current methods, the difference in activity of the GLNase domain (0.0063 μmol/min/mg) compared with the hybrid (3.3 μmol/min/mg) represents a 2,200-fold activation when differences in the molecular mass are considered. In *E. coli* CPSase, optimal glutamine hydrolysis also requires the physical association of the amidotransferase and synthetase subunits, although the effect is less pronounced; the isolated subunit has only 10% of the glutaminase activity of the native synthetase subunit, a species formed which sedimented much more rapidly than either the CPSase subunit or the GLNase domain individually (Fig. 5). SDS-polyacrylamide gel electrophoresis confirmed the formation of the hybrid. The *E. coli* CPS subunit was found in fractions 19–23, whereas in the presence of the mammalian domain it appeared in fractions 15–18 along with the 40-kDa mammalian domain. When centrifuged alone, the GLNase domain, a much smaller species, was found in fractions 24–27.

The pure hybrid could also be isolated by gel filtration on a Sephacryl S-300 gel filtration column (data not shown). The elution volume of the hybrid on this column indicated that it is a dimer consisting of two copies of each type of subunit.

**State Steady Kinetics and Regulation of the Hybrid CPSase**—A steady state kinetic study of the purified hybrid molecule (Fig. 6, Table II) showed that the $K_m$ values for glutamine are similar for the mammalian, bacterial, and hybrid CPSases. The $S_{0.5}$ values for ATP for the mammalian (28, 39) and hybrid molecules were about 5-fold higher than the value reported (41, 43) for the *E. coli* enzyme. Although we have not tested purified *E. coli* CPSase, this discrepancy is undoubtedly due to differences in the assay conditions, since the glutamine and ATP saturation curves of wild type *E. coli* enzyme in cell extracts gave $S_{0.5}$ values which were very similar to those obtained for the purified hybrid molecule. The turnover number for the hybrid, 8.3 s$^{-1}$, is 13-fold higher than the value for the hamster protein, but close to the value observed for the *E. coli* enzyme.

The effect of the bacterial CPSase activator ornithine and inhibitor UMP as well as that of the mammalian activator 5-phosphoribosyl-1-pyrophosphate and inhibitor UTP on the glutamine-dependent carboxylate synthetase activity of the hybrid were also tested. The molecule was activated 2-fold by ornithine and inhibited 60% by UMP, whereas UTP was a much poorer inhibitor and 5-phosphoribosyl-1-pyrophosphate had no effect on the activity. The formation of the complex with the mammalian amidotransferase domain did not alter the regulatory properties of the synthetase domain.

---

**Fig. 2. Expression of pHG-GLN52.** A 100-ml culture of cells transformed with pHG-GLN52 was grown for 20 h in minimal media containing 12 mg/l uracil and 100 mg/l ampicillin. The cells were harvested, resuspended to a final volume of 1 ml in 50 mM Tris HCl, pH 7.4, 1 mM DTT, and 5% glycerol and broken by sonication. The extract was first centrifuged at low speed, 735 × g for 20 min, and the supernatant was then recentrifuged at high speed, 16,000 × g for 20 min. The pellets were resuspended in 1 ml of the buffer used for sonication. The extract, supernatant, and pellet fractions (50 μl) were analyzed by SDS-polyacrylamide gel electrophoresis (A) and by immunoblotting (B and C) with anti-CAD antibodies. For A and B: lane 1, high speed pellet; lane 2, high speed supernatant; lane 3, low speed pellet; lane 4, low speed supernatant; lane 5, extracts of the pHG-GLN52/EK1104 cells; lane 6, extracts of pEK91/EK1104 cells; and lane 7, purified CAD. For C, lane 1, cell extract; lane 2, purified CAD. (Note that the intermediate band in immunoblot C does not correspond to any protein, rather it is an artifact which sometimes develops during the staining procedure.)
Purification of the CAD GLN domain. The extract from a 600-ml culture of pHG-GLN62-transformed cells was applied to a 1.7 x 52-cm DEAE-Sephael column equilibrated in 0.05 M Tris-HCl, pH 7.0, 1 mM DTT, and 5% glycerol and eluted with 3-4 column volumes with the same buffer. The column was then eluted with a 0-0.5 M NaCl linear gradient in this buffer. The nearly homogeneous GLNase domain (GLN) eluted at approximately 0.15 M NaCl.

Titration of E. coli CPSase synthetase subunit with hamster GLNase domain. Increasing amounts (0.1-3.5 µg) of the purified mammalian GLNase domain were incubated with 2.4 µg of the purified E. coli CPSase synthetase subunit in 0.1 ml of 0.05 M Tris-HCl, pH 7.6, 1 mM DTT, and 5% glycerol for 5 min at room temperature. The samples were assayed for glutamine-dependent carbamyl phosphate synthetase activity as described under "Experimental Procedures." The molar ratio of GLNase domain to CPSase synthetase subunit (GLN/CPS) was calculated assuming a molecular mass of 40 and 120 kDa, respectively.

E. coli enzyme (19). The reduced activity of the GLNase subunit resulted from a low apparent affinity for glutamine; the \( K_m \) is 150 mM for the subunit compared to 0.4 mM for native E. coli CPSase (19). Similarly, there is a 30-fold stimulation of E. coli anthranilate synthetase component II, the amidotransferase, when it forms a complex with component I, the synthetase subunit (45, 46). The reduced activity of the isolated mammalian amidotransferase domain and its activation when the hybrid forms are consistent with these studies of bacterial amidotransferases.

The \( k_{cat} \) of the hybrid and E. coli enzymes are very similar,
indicating that the linkage is fully functional. On the other hand, the mammalian and hybrid proteins which were assayed under comparable conditions exhibit a 9-fold difference in $k_{cat}$. Thus, the potential rate at which the mammalian GLNase domain can catalyze glutamine hydrolysis is much greater than that required by the mammalian synthetase domain.

The restoration of glutaminase activity when the mammalian GLNase domain associates with the bacterial synthetase subunit indicates that not only are the residues responsible for physical association of the GLNase and CPSase domains conserved, but so are those residues which transmit the interdomain signals. Thus, the domain interfaces have been preserved to a remarkable degree, supporting a critically important role for the interdomain interactions. These results lend support to the idea that the CAD protein evolved by a stepwise fusion of genes encoding multifunctional proteins. The region of the polypeptide catalyzing the glutamine-dependent CPSase reaction appears to have been formed by fusion of ancestral genes that are remarkably similar to the E. coli carA and carB genes.

Moreover, these results demonstrate that although the GLNase and CPSase synthetase domains of CAD are covalently linked together on the same polypeptide chain, the physical association and functional linkage of the domains also involves an extensive network of noncovalent interactions. The connecting chain segment, which probably arose during the gene fusion from which this region of the molecule originated, is not a substitute for the noncovalent interactions between the domains. The consolidation of multiple activities of CAD on a single polypeptide ensures that the functional domains are simultaneously expressed in stoichiometric amounts. However, in addition to its role in coordinating the synthesis of the CAD domains, the linker region which bridges the GLNase and CPSase domains may influence their function in some unforeseen way. We are currently exploring this interesting possibility.

The allosteric effectors ornithine and UMP alter the apparent affinity of the E. coli CPSase for ATP (20, 47). These effectors also modulate carbamyl phosphate-dependent ATP synthesis by the isolated synthetase subunit (19), a result which clearly demonstrates that the allosteric sites are located on this subunit. The mammalian allosteric effectors are quite different and the observation that the regulation of the hybrid is the same as the wild type E. coli CPSase confirms that the synthetase domain is the sole locus of allosteric control. The functional linkage between the GLNase and CPSase domains or subunits, however, represents an important element of the control mechanism, since it provides a means of stimulating or attenuating glutamine hydrolysis as the activity of the synthetase subunit is modulated by allosteric effectors.

The expression of the mammalian GLNase domain showed that this region of the CAD cDNA molecule encodes an autonomous domain which can fold independently without the mediation of any other parts of the CAD polypeptide. Although the expressed protein assumes a stable tertiary structure, interactions with other regions of the CAD molecule, specifically the synthetase domain, are required for normal function. The clones described here will provide a source of the purified domain for further mechanistic studies of the mammalian CPSase amidotransferase reaction. Moreover, since the mammalian CPSase domain has proven difficult to express at workable levels in E. coli, the discovery that a fully functional complex is formed with E. coli CPSase synthetase subunit, should provide a useful system for studying the activation of GLNase domain and other aspects of interdomain signaling.

Acknowledgments—We thank Dr. Carol Lusty (The Public Health Research Institute of the City of New York, New York) and Dr. Evan Kantrowitz (Boston College, Chestnut Hill, MA) for the generous gifts of plasmids and strains.

REFERENCES

Cloning of the CAD Amidotransferase Domain