Functional Linkage between the Glutaminase and Synthetase Domains of Carbamoyl-phosphate Synthetase

ROLE OF SERINE 44 IN CARBAMOYL-PHOSPHATE SYNTHETASE-ASPARTATE CARBAMOYLTRANSFERASE-DIHYDROOROTASE (CAD) *

(Received for publication, June 2, 1999, and in revised form, July 13, 1999)

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Mammalian carbamoyl-phosphate synthetase is part of carbamoyl-phosphate synthetase-aspartate carbamoyltransferase-dihydroorotase (CAD), a multifunctional protein that also catalyzes the second and third steps of pyrimidine biosynthesis. Carbamoyl phosphate synthesis requires the concerted action of the glutaminase (GLN) and carbamoyl-phosphate synthetase domains of CAD. There is a functional linkage between these domains such that glutamine hydrolysis on the GLN domain does not occur at a significant rate unless ATP and HCO₃⁻, the other substrates needed for carbamoyl phosphate synthesis, bind to the synthetase domain. The GLN domain consists of catalytic and attenuation subdomains. In the separately cloned GLN domain, the catalytic subdomain is down-regulated by interactions with the attenuation domain, a process thought to be part of the functional linkage. Replacement of Ser⁴⁴ in the catalytic Ser⁴⁴ domain and the Escherichia coli carbamoyl-phosphate synthetase large subunit had little effect on glutamine hydrolysis. In contrast, ATP and HCO₃⁻ did not stimulate the glutaminase activity, indicating that the interdomain linkage had been disrupted. In accord with this interpretation, the rate of glutamine hydrolysis and carbamoyl phosphate synthesis were no longer coordinated. Approximately 3 times more glutamine was hydrolyzed by the Ser⁴⁴→Ala mutant than that needed for carbamoyl phosphate synthesis. Ser⁴⁴, the only attenuation subdomain residue that extends into the GLN active site, appears to be an integral component of the regulatory circuit that phases glutamine hydrolysis and carbamoyl phosphate synthesis.

Glutamine + H₂O → glutamate + NH₃
ATP + HCO₃⁻ → carboxy phosphate + ADP
Carboxy phosphate + NH₃ → carbamate + P₁
ATP + carbamate → carbamoyl phosphate + ADP

REACTIONS 1–4

Mammalian carbamoyl-phosphate synthetase is part of a large multifunctional protein called CAD (3–5), which also has aspartate transcarbamoylase and dihydroorotase activities, enzymes that catalyze the second and third steps of the de novo pathway, respectively. The 243-kDa CAD polypeptide is organized into discrete structural domains each with a specific function (6–9). The 38-kDa glutaminase (GLN) domain located on the amino end of the polypeptide (10, 11) catalyzes the hydrolysis of glutamine and transfers the ammonia to the adjacent 120-kDa synthetase (CPS) domain, where the remaining partial reactions take place. The CPS domain of CAD (10) and all known CPSases (12, 13) consist of two homologous subdomains, CPS.A and CPS.B (10), that probably arose as a result of an ancestral gene duplication and fusion (12). Escherichia coli CPSase is a monofunctional protein (14, 15) consisting of a 42-kDa GLN subunit and a 120-kDa CPS subunit. Despite differences in structural organization, the sequence and domain structure of the mammalian and bacterial proteins are very similar. There is extensive evidence that the two different ATP-dependent partial reactions, the activation of bicarbonate (Reaction 2) and the phosphorylation of carbamate (Reaction 4), are catalyzed by CPS.A and CPS.B, respectively (16–22).

The x-ray structure of the E. coli enzyme (23–26) has been solved to a resolution of 1.8 Å. Remarkably, the active sites were found to be widely separated but connected by a narrow tunnel that passes through the interior of the molecule. The ammonia generated by hydrolysis of glutamine presumably diffuses through the tunnel to the active site of CPS.A, where it reacts with carboxy phosphate to form carbamate. The carbamate then diffuses through the tunnel to the active site of CPS.B, where carbamoyl phosphate is formed in the second ATP-dependent reaction.

The mechanism of glutamine hydrolysis by CPSase (27–31) and other trpG-type amidotransferases (32–34) is analogous to that of the thiol proteases. The reaction proceeds through a thioester intermediate, and there is evidence that a catalytic

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* This work was supported by United States Public Health Service Grant GM47399. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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¹ The abbreviations used are: CPSase, carbamoyl-phosphate synthetase activity; CAD, the multifunctional protein having glutamine-dependent carbamoyl-phosphate synthetase, aspartate transcarbamoylase, and dihydroorotase activities; CPS, the synthetase domain or

subunit of carbamoyl-phosphate synthetase; GLN, the amidotransferase or glutaminase domain or subunit of carbamoyl-phosphate synthetase; GLNase, glutaminase activity; GLN-CPS, the hybrid CPSase consisting of the mammalian GLN domain and the E. coli CPS domain; PCR, polymerase chain reaction; HPLC, high performance liquid chromatography; OPA, o-thiophthalaldehyde.

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Role of Ser<sup>44</sup> in CAD

The GLN subunit consists of two subdomains, the catalytic (dark shading) and the attenuation subdomain (light shading). The diagram shows the location of the active site residues Cys<sup>252</sup> and His<sup>336</sup>, but Glu<sup>383</sup> located in close proximity to His<sup>336</sup> is partially obscured in this orientation and was omitted for clarity. Ser<sup>44</sup>, which is located within the attenuation subdomain, extends into the active site region of the catalytic subunit. A part of the interface between the GLN domain and part of the CPS domain (shown in black) is also visible. The structure was modeled based on the E. coli CPSase x-ray structure (29).

The activity is expressed as nanomoles/min/mg of the GLN domain, and the mutant were isolated by the method described earlier (39). To form the hybrid CPSase (GLN-CPS), stoichiometric amounts of the wild type CAD GLN domain or its mutant and the E. coli CPSase large subunit were mixed together and incubated for 15 min (31, 39). The complex was then concentrated using either a Speed-Vac at room temperature or by centrifugation using Millipore Ultrafree-MC 30,000 NMWL filter units at 4 °C. Protein concentrations were determined by the Bradford dye binding method using bovine serum albumin as a standard (46). SDS-gel electrophoresis was carried out on 10% polyacrylamide gels (47).

**Molecular Modeling**—The structure of the CAD GLN-CPS domains was modeled with E. coli CPSase (Brookhaven Protein Data Bank; identification code 1JDB) serving as the tertiary template using the program Quanta version 4.0 (MSI). The alignment of CAD and E. coli CPSase sequences was carried out giving equal weight to secondary structure and sequence similarity. Undefined regions were regularized in two stages, 50 cycles of steepest descent, followed by 200 cycles of adopted basin set NR, prior to final energy minimization.

**Enzyme Assays**—The CPSase activity was assayed at 37 °C using a radiometric procedure described previously (6, 48) using a 2 mM excess of MgCl<sub>2</sub> over the concentration of ATP in the assay. The GLNase activity (31) of the isolated CAD GLN domain and the mammalian-E. coli CPSase hybrid complex was measured by an assay that involved separating the reactant glutamine from the product glutamate by HPLC as described below. The assay buffer contained 25 mM HEPES, pH 7.4, 0.5 mM dithiothreitol, 0.05 mM EDTA, 25 mM KCl (with or without 10 mM ATP, 12 mM MgCl<sub>2</sub>, 15 mM NaHCO<sub>3</sub>), and variable glutamine in a total volume of 0.35 ml. The reaction was initiated by adding 7–17 μg of the GLN domain in 50 μl, allowed to proceed for 1 h at 37 °C, and then quenched with 100 μl of 20% trichloroacetic acid. The samples stood on ice for 10 min prior to centrifugation for 5 min at 14,000 rpm to remove the precipitated protein. The supernatant (60 μl) was then neutralized with 10.8 μl of 1.2 M NaOH. For glutamine concentrations less than 10 mM, the samples were derivatized by directly adding 100 μl of OPA reagent to the neutralized sample. At higher glutamine concentrations, 200 μl of OPA reagent was added to 10 μl of the neutralized protein solution. Exactly 90 s following the addition of OPA, a 100-μl sample was injected onto the HPLC column. The activity is expressed as nanomoles/min/mg of the GLN domain, and the amounts assayed given in the legends represent micrograms of the GLN domain.

**Gel Filtration**—The formation of a complex between the isolated 38-kDa mammalian GLN domain and the mutant, with the 120-kDa E. coli CPSase synthetase subunit, was verified by gel filtration. The hybrid (45–50 μg) in 0.1 mM potassium phosphate, pH 7.6, 1 mM EDTA and 5% glycerol was applied to a 1.5 × 63-cm Sephacryl S-300 column. The column was pre-equilibrated and eluted at 0.2 ml/min with the same buffer. Column fractions were analyzed by assaying CPSase and by SDS-gel electrophoresis.

**EXPERIMENTAL PROCEDURES**

**Materials**—[14C]Glutamine was purchased from DuPont, HPLC-grade methanol was from Burdick and Jackson, and the o-phthalaldehyde (OPA) reagent solution and all other chemicals were purchased from Sigma.

**Plasmids and Strains**—The 7.1-kilobase plasmid pHGGLN52 (39) carries a 1.1-kilobase insert encoding the mammalian CAD GLN domain in a vector derived from pEKK1 (40). The expression of the protein is under control of the pyrBI promoter. The E. coli host strain, EK1104 (40), lacks the pyrB and pyrI genes and has a leaky pyrF mutation. The high copy number plasmid PHN12 (41), which carries the carB gene that encodes the large subunit of E. coli CPSase, was kindly provided by Dr. Carol Lusty (The Public Health Research Institute of the City of New York, New York, NY) as was the E. coli strain L673, which is defective in the carA and carB genes, encoding both E. coli carbamoyl-phosphate synthetase subunits, as well as the Lon protease.

**Cell Growth and Recombinant DNA Methods**—Cells harboring the recombinant plasmids were routinely grown from a single colony in 2 × YT medium supplemented with 50–100 mg/liter ampicillin. The expression of the mammalian GLN domain in EK1104 transformants was induced as described previously (39), while the E. coli CPSase subunit was expressed constitutively (42) in L673 cells transformed with pHN12, a plasmid encoding the E. coli CPSase, was kindly provided by Dr. Carol Lusty (The Public Health Research Institute of the City of New York, New York, NY) as was the E. coli strain L673, which is defective in the carA and carB genes, encoding both E. coli carbamoyl-phosphate synthetase subunits, as well as the Lon protease.

**Protein Methods**—CAD was isolated from an overproducing strain of Syrian hamster cells (BHK-21) as described previously (5, 45). The E. coli CPSase large subunit was isolated from pHN12 transformants using the methods of Robinio et al. (42). The wild type CAD GLN domain and
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TABLE I

| Substrates | $K_m$ (mM) | $V_{max}$ (nmol/min/mg) | $k_{cat}$ (s$^{-1}$) | $k_{cat}/K_m$ (s$^{-1}$) |
|-----------|------------|-------------------------|---------------------|---------------------|
| Wild type | GLN None | 4.03 ± 0.23 | 31.9 ± 0.71 | 20.2 ± 0.45 | 5.01 |
| GLN-CPS | None | 0.090 ± 0.015 | 487.9 ± 15.8 | 309 ± 10 | 3433 |
| GLN-CPS | ATP, HCO$_3^-$ | 0.113 ± 0.011 | 4914 ± 117 | 3110 ± 73.8 | 27,522 |
| CAD$^a$ | None | 0.096 | 34.6 | 140 | 1470 |
| CAD$^a$ | ATP, HCO$_3^-$ | 0.096 | 474.1 | 1920 | 20,000 |

$^a$ Data taken from Chaparian and Evans (28).

RESULTS

Mutation of Serine 44—The mammalian GLN domain was modeled using the E. coli CPSase structure (23) as a template. As expected, given the strong sequence similarity of the mammalian and bacterial proteins, the configuration of active site residues closely resembles that observed for the E. coli enzyme. Ser$_{44}^4$ (corresponding to Ser$_{47}^5$ in the E. coli structure) is located within the catalytic site of the GLN domain. The side chain extends into the site (Fig. 1) with its $\gamma$-oxygen atom positioned within 4.2 Å of the Cys$_{252}^5$ sulfur atom and 4.9 Å from the $\epsilon$ ring nitrogen of the catalytic histidine residue, His$_{336}^5$.

Ser$_{44}^4$ was replaced with alanine by PCR-directed mutagenesis using the plasmid pHGGLN52 (39) as a template. High levels of the 38-kDa wild type and mutant domains were expressed when the plasmids were transformed into the E. coli strain EK1104. Both proteins were purified to homogeneity as described (39) previously. Gel filtration on a Sephacryl S-300 column showed that, as in the case of the wild type protein, a stoichiometric mixture of the mammalian Ser$_{44}^4$ → Ala GLN domain and the E. coli CPSase CPS subunit formed a stable hybrid complex.

The Ser$_{44}^4$ → Ala Mutation Activates the Isolated GLN Domain—The isolated GLN domain had very low catalytic activity (Table I), a consequence of a high $K_m$ (4 mm) and a very low $k_{cat}$ (0.020 s$^{-1}$). The formation of a hybrid complex consisting of the mammalian GLN domain and the isolated E. coli CPS subunit restored the function of the GLN domain. The $K_m$ decreased 45-fold while the $k_{cat}$ increased 15-fold relative to the isolated domain. The steady state kinetic parameters of the hybrid are similar to those obtained for CAD.

Contrary to the results that would be expected if Ser$_{44}^4$ were a catalytic residue, the suppression of catalytic activity of the isolated domain was largely relieved by its replacement with alanine (Fig. 2 and Table I). Compared with the isolated wild type domain, the $K_m$ was reduced 7-fold, while the $k_{cat}$ increased 21-fold. Thus, the mutation appreciably activated the isolated GLN domain, increasing the $k_{cat}/K_m$ by a factor of 680.

The formation of the hybrid with the Ser$_{44}^4$ → Ala GLN domain gave a species with kinetic parameters similar to those obtained for the wild type complex, although the $k_{cat}$ and $V_{max}$ values are about 1.7-fold higher. Compared with the isolated Ser$_{44}^4$ → Ala GLN domain, the $K_m$ for glutamine was reduced another 6-fold in the mutant hybrid, but there was no appreciable change in $k_{cat}$.

Thus, Ser$_{44}^4$ does not participate in glutamine hydrolysis in either the isolated domain or the hybrid in the absence of ATP and bicarbonate.

The Mutant Hybrid Protein Catalyzes the Synthesis of Carbamoyl Phosphate—The Ser$_{44}^4$ → Ala hybrid protein can also catalyze the overall synthesis of carbamoyl phosphate. Saturation curves for the overall reaction (Fig. 3, A and B, and Table II) show that the $K_m$ for both glutamine and ATP are very similar to the values determined for the wild type hybrid. However, the $k_{cat}$ values obtained from both glutamine (0.193 s$^{-1}$) and ATP (0.164 s$^{-1}$) are approximately 14-fold lower for the mutant protein.

*ATP and Bicarbonate Do Not Activate the Glutaminase Reaction in the Mutant—The binding of ATP and bicarbonate to the CPS subunit stimulates the GLNase activity of the wild type hybrid and CAD 10- and 14-fold, respectively. The stimulation is the result of a corresponding increase in $k_{cat}$ without any significant change in the affinity for glutamine. In the absence of ATP and bicarbonate (Fig. 4, Table I), the $k_{cat}$ for glutamine hydrolysis of the mutant hybrid (0.525 s$^{-1}$) is only marginally increased compared with that of the wild type hybrid (0.309 s$^{-1}$) and it does not significantly change in the presence of saturating concentrations of these substrates (0.552 s$^{-1}$). Thus, the functional linkage that coordinates the reactions on the GLN and CPS domains is lost as a result of replacement of Ser$_{44}^4$ with alanine.

Coordination between the Activation of Bicarbonate and Glutaminase Hydrolysis Is Lost in the Mutant—In CAD and in the wild type hybrid complex, the rate of glutamine hydrolysis is closely matched to the rate of carbamoyl phosphate synthesis. The maximum velocity for glutamine hydrolysis in the presence of saturating ATP and bicarbonate (Table I, $k_{cat} = 3.11$ s$^{-1}$) parallels the overall rate of carbamoyl phosphate synthesis (Table II, $k_{cat} = 2.66$ s$^{-1}$) in the wild type hybrid. Moreover,
a replot (Fig. 3C) of the velocity data of carbamoyl phosphate synthesized (Fig. 3A versus glutamine hydrolyzed (Fig. 4) measured at various concentrations of glutamine gives a slope of $1.15 \pm 0.04$, in accord with the expected stoichiometry of the overall reaction at all concentrations of the substrate.

In contrast, the rate of glutamine hydrolysis exceeds the rate of carbamoyl phosphate synthesized in the mutant hybrid. The $k_{cat}$ for the GLNase reaction measured at saturating ATP and bicarbonate is $0.552 \text{ s}^{-1}$, whereas the $k_{cat}$ for the CPSase reaction is only $0.193 \text{ s}^{-1}$. Similarly, the plot of glutamine hydrolysis versus carbamoyl phosphate synthesized had a slope of $2.9 \pm 0.1$, indicating that the 1:1 stoichiometry observed for the reaction catalyzed by the wild type enzyme is not sustained in the mutant. This result would be expected if Ser44 plays a major role in phasing the reactions occurring on the two domains.

**DISCUSSION**

Carbamoyl phosphate synthesis involves the concerted action of two domains that must act in synchrony. We are especially interested in the mechanism of glutamine hydrolysis and the reciprocal interactions between the GLN and CPS domains of the mammalian multifunctional protein CAD. Although we have expressed each of the functional domains, we cannot as yet obtain sufficient amounts of CPS needed for the sorts of studies described here. However, previous steady state and presteady state kinetic studies (31, 39) showed that the hybrid consisting of the isolated mammalian GLN domain and the *E. coli* CPS subunit has catalytic parameters similar to CAD as well as a functional interdomain linkage. Thus, we have used this system to assess the role of Ser44 in carbamoyl phosphate synthesis.

Whereas the hydrolysis of glutamine by CAD can be easily measured, the isolated GLN domain has barely detectable activity as a result of an appreciable increase in the $K_m$ for glutamine and decrease in $k_{cat}$. However, the kinetic parameters are restored to the normal values found in CAD when a stoichiometric complex is formed by the non-covalent association of the isolated GLN domain and the CPS subunit of *E. coli* CPSase. Sequence comparisons (10, 11) showed that the 40-kDa CPSase GLN domain consists of two distinct regions. The carboxyl half of the domain is homologous to the amidotransferase domain of all trpG or triad type amidotransferases (33, 34, 49), while the amino half of the domain is unique to the CPSases. Since amidotransferase domains of the other biosynthetic enzymes have an average molecular mass of 20 kDa and do not have a chain segment corresponding to the amino half of the CPS GLN domain, it was reasonable to assume that all of the residues required for glutamine binding and catalysis would be found in the carboxyl half of the CAD GLN domain.

Support for this interpretation was obtained when we cloned and expressed (50) the two halves of the mammalian CPSase GLN domain and showed that they are autonomously folded subdomains with specific functions. The amino half has no catalytic activity but forms a stable complex with the CPS domain. The carboxyl half, the catalytic subdomain, binds glutamine with the same affinity and is more active ($k_{cat} = 5.7 \text{ s}^{-1}$) than the maximum glutaminase activity observed for intact CAD, even when assayed in the presence of saturating ATP and HCO$_3^-$ ($k_{cat} = 1.9 \text{ s}^{-1}$). We therefore suggested that a major function of the amino half of the GLN domain, the attenuation subdomain, was to modulate the intrinsically high catalytic activity of the catalytic subdomain and that this region of the molecule was instrumental in relaying the interdomain signal between the GLN and CPS domains. In accord with this hypothesis, the x-ray structure of *E. coli* CPSase (23) subsequently showed that the attenuation subdomain makes exten-
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**TABLE II**

Kinetic parameters for carbamoyl phosphate synthesis

| Enzyme                | Variable substrate | $K_m$ (mM) | $V_{max}$ (nmol/min/mg) | $k_{cat}$ (s^{-1}) | $k_{cat}/K_m$ (s^{-1} \times 10^2) |
|-----------------------|--------------------|------------|-------------------------|-------------------|-----------------------------------|
| Wild type GLN-CPS     | ATP                | 1.66 ± 0.07 | 3947 ± 109              | 2500 ± 69         | 1,506                             |
|                       | Glutamine          | 0.114 ± 0.015 | 4200 ± 175              | 2660 ± 111        | 23,333                            |
| Ser^{44}–Ala GLN-CPS  | ATP                | 1.83 ± 0.104 | 259.0 ± 10.1           | 164 ± 6           | 89.6                              |
|                       | Glutamine          | 0.111 ± 0.010 | 304.2 ± 13.2           | 193 ± 8           | 1,739                             |

a The ATP saturation curves are sigmoidal and were fit to the Hill equation, so these represent apparent $K_m$ values. The Hill coefficient is 2.08 ± 0.11 and 2.15 ± 0.15 for the wild type and mutant proteins, respectively. The glutamine saturation curves were fit to the Michaelis-Menten equation.

**Fig. 4. Glutaminase of the GLN-CPS hybrid in the presence and absence of saturating ATP and bicarbonate.** A hybrid complex of the mammalian GLN domain and the *E. coli* CPS subunit was formed as described under “Experimental Procedures.” The hybrid with the wild type GLN domain (7 μg of the GLN domain) was assayed in the presence of 10 mM ATP and 15 mM bicarbonate (●) and in the absence of these substrates (■). Similarly, the Ser^{44} → Ala hybrid complex (12 μg of the GLN domain) was also assayed in the presence (●) and absence of ATP and bicarbonate (■). The solid lines represent a least squares fit to the Michaelis-Menten equation.

Serine contacts with the CPS subunit in *E. coli* CPSase. The x-ray structure also showed that Ser^{44} is the only residue in the attenuation subdomain that extends into the GLN active site, making it a prime candidate for participation in the functional linkage.

The activation of the GLN domain that occurs upon association with the CPS subunit is almost entirely mimicked by the replacement of Ser^{44} with alanine in the isolated domain. The $k_{cat}$ increases 20-fold to 0.43 s^{-1} compared with a value of 0.02 s^{-1} for the wild type hybrid complex. The high activity of the isolated Ser^{44} → Ala GLN domain confirms that this serine residue is not involved in catalysis. The $K_m$ also decreases to 0.6 mM in the isolated Ser^{44} → Ala domain, but is still 6-fold higher than that of the wild type hybrid complex. One could argue that this residue is important for binding glutamine, but, when the Ser^{44} → Ala GLN domain associates with the *E. coli* CPS subunit, the $K_m$ is virtually the same as the wild type hybrid complex. Thus, it seems clear that the suppression of activity in the isolated GLN domain is, to a large extent, the result of the serine residue. Since alanine is nearly isosteric with serine, the depression of activity is unlikely to be a consequence of steric interference, suggesting that Ser^{44} may form a hydrogen bond with one of the active site residues that interferes with its normal function in catalysis.

When the wild type isolated domain combines with the CPS subunit, Ser^{44} is likely to be displaced to its position located in the electron density maps of *E. coli* CPSase (23). Since the serine residue has been replaced in the mutant, little change would be expected when the Ser^{44} → Ala domain combines with the CPS subunit. Consistent with this interpretation, the kinetic parameters for glutamine hydrolysis by the Ser^{44} → Ala hybrid are similar to the values measured for the wild type hybrid complex, suggesting that Ser^{44} is not required for glutamine hydrolysis by the hybrid in the absence of ATP and HCO$_3^{-}$.

The hydrolysis of glutamine and the activation of bicarbonate are parallel reactions that must occur in phase to avoid the wasteful hydrolysis of glutamine or ATP that would otherwise occur. This requirement is especially important since the active sites are far from one another, about 45 Å in the *E. coli* structure (23), and ammonia must be delivered to the active site of CPS A via a long interdomain tunnel. The coordination of these partial reactions requires that the GLNase activity be modulated so that it is not operating at or near its full catalytic potential unless the concentration of the other substrates needed for carbamoyl phosphate synthesis are saturating. A part of the interdomain functional linkage is the down-regulation of the glutaminase activity when ATP and bicarbonate are limiting. Steady state and presteady state kinetic studies of CAD (28, 31) showed that, in the absence of ATP and bicarbonate, the thioester intermediate accumulates and the rate constant for the breakdown of the thioester intermediate is the same as the $k_{cat}$ for glutamine hydrolysis, indicating that it is the rate-limiting step. When ATP and bicarbonate are present, the breakdown of the thioester is accelerated, the intermediate cannot be detected, and the $k_{cat}$ for the hydrolysis of glutamine increases 14-fold. Since the substrate induced acceleration of catalysis is due to an increase in $k_{cat}$ without an apparent change on glutamine binding, it is likely that the juxtaposition of catalytic residues is suboptimal in the absence of ATP and bicarbonate. Similar results have been reported (29, 30) for *E. coli* CPSase. The functional linkage is abolished in the Ser^{44} → Ala hybrid. The addition of ATP and bicarbonate has no significant effect on either the $K_m$ or the $k_{cat}$ of the hybrid, suggesting that Ser^{44} is essential for transmission of the allosteric signal that up-regulates the GLN domain. Since activation primarily involves an increase in the rate of breakdown of the thioester, it is possible that Ser^{44} promotes its hydrolysis, but only when ATP and bicarbonate are bound to the CPS domain.

If this functional linkage is important in phasing the reactions occurring on the GLN and CPS domains, then the rate of glutamine hydrolysis need no longer match the rate of carbamoyl phosphate synthesis if the linkage is disrupted. The stoichiometry of carbamoyl phosphate synthesis is tightly controlled in the wild type hybrid, with 1 mol of glutamine hydrolyzed/mol of carbamoyl phosphate synthesized. In contrast, the mutant hybrid hydrolyzed 3 times more glutamine than that needed for carbamoyl phosphate synthesis, with the excess presumably leaking out of the complex.

We conclude that serine 44 in the GLN attenuation domain is not a catalytic residue in the usual sense but rather is an
essential component in the regulatory linkage that phases glutamine hydrolysis and carbamoyl phosphate synthesis.

Acknowledgment—We thank Dr. Carol Lusty for the generous gifts of plasmids and strains.

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