Pressure-induced Dissociation of Carbamoyl-Phosphate Synthetase Domains
THE CATALytICALLY ACTIVE FORM IS DIMERIC

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Carbamoyl-phosphate synthetase consists of an amidotransferase domain or subunit (GLN) that hydrolyzes glutamine and transfers the ammonia to the synthetase component (CPS) where the biosynthetic reaction occurs. The CPS domain is composed of two homologous subdomains, CPS.A and CPS.B, that catalyze different ATP-dependent reactions involved in carbamoyl phosphate synthesis. When the individual CPS.A and CPS.B subdomains were individually cloned and expressed in Escherichia coli (Guy, H. I., and Evans, D. R. (1996) J. Biol. Chem. 271, 13762–13769), they were found to be functionally equivalent and could each independently catalyze carbamoyl phosphate synthesis. The proposal was advanced that, although the monomers could catalyze the individual partial reactions, overall synthesis of carbamoyl phosphate required a homodimer of CPS.A or CPS.B. To test this hypothesis, the GLN-CPS.B dimer was reversibly dissociated at 1500 bar in a high pressure cell. Dissociation was accompanied by a loss of both glutamine- and ammonia-dependent CPSase activity. Activity was recovered once the protein was returned to atmospheric pressure. If the sample was cross-linked before exposure to high pressure, there was no dissociation and no loss of biosynthetic activity. In contrast, the bicarbonate-dependent ATPase and the carbamoyl phosphate-dependent ATP synthetase activities were largely unaffected by pressure-induced dissociation. These experiments confirmed the hypothesis that the synthesis of carbamoyl phosphate requires the concerted action of the two active sites within the homodimer.

The synthesis of carbamoyl phosphate occurs in a series of four partial reactions (1–10) catalyzed by the individual domains and subdomains of carbamoyl-phosphate synthetase.

Glutamate + H₂O → Glutamine + NH₃

REACTION 1

O O O

\[ \text{HO--C--O}^- + \text{P--O}^- + \text{ADP} \rightarrow \text{HO--C--O}^- + \text{P--O}^- + \text{ADP} \]

REACTION 2

O O O

\[ \text{NH₂C--O}^- + \text{ATP} \rightarrow \text{NH₂C--O}^- + \text{P--O}^- + \text{ADP} \]

REACTION 3

O O O

\[ \text{HO--C--O}^- + \text{NH₃} \rightarrow \text{NH₂C--O}^- + \text{P} \]

REACTION 4

The enzymes typically consist of a 120-kDa CPS domain or subunit and a 40-kDa GLN domain or subunit (11). The GLN domain hydrolyzes glutamine and transfers ammonia to the CPS domain (11, 12), where all of the subsequent partial reactions occur. In Escherichia coli CPSase, the GLN and CPS domains are separate subunits, whereas the mammalian pyrimidine biosynthetic enzyme is part of a multifunctional protein CAD (13–15) in which the GLN and CPS domains are fused and carried on the same polypeptide chain as the aspartate transcarbamoylase (ATC) and dihydroorotase (DHO) domains, which catalyze the second and third steps in the de novo pathway. Although the structural organization of carbamoyl-phosphate synthetase is diverse, all of these molecules have a similar sequence and subdomain structure (16–25). The CPS domain consists of two homologous halves designated CPS.A and CPS.B, which have been shown to catalyze the two different ATP-dependent partial reactions (26–31) involved in the synthesis of carbamoyl phosphate. The CPS.A and CPS.B domains are in turn composed of three subdomains (32, 33) designated A1, A2, A3, and B1, B2, B3, respectively. The recent x-ray structure of E. coli CPSase (34) confirmed the subdomain structure of the synthetase domain and showed that the tertiary structure of CPS.A and CPS.B is very similar. The A1-A2 and B1-B2 subdomains have an almost identical fold, whereas the structure of A3 and B3 is distinctly different. CPS.A and CPS.B interact extensively and are related within the complex by an approximate 2-fold axis of symmetry. The most remarkable aspect of the structure is a 96-Å long tunnel that leads from the active site of the GLN domain to the active site of CPS.B, where carbamoyl phosphate is formed and released from the complex.

We recently made the unanticipated discovery (35) that...
when the CAD CPS.A and CPS.B were separately cloned and expressed in *E. coli*, the individual domains are functionally equivalent and can catalyze both ATP-dependent partial reactions. Remarkably, the isolated domains also catalyze the overall synthesis of carbamoyl phosphate using ammonia as a nitrogen donor. The CPS.A and CPS.B domains were each fused to the mammalian CAD GLN domain in these recombinants. Both GLN-CPS.A and GLN-CPS.B could also use glutamine as a nitrogen donor, demonstrating that not only was glutamine hydrolyzed but the resulting ammonia was efficiently transferred to the synthetase domain. We subsequently cloned and expressed the *E. coli* CPS A2 and B2 subdomains (33) and found that these species could also catalyze both partial reactions and the overall ammonia-dependent synthesis of carbamoyl phosphate. Moreover, both A2 and B2 are hyperactive with turnover numbers appreciably higher than that of the intact molecule, confirming that they are the catalytic subdomains of CPS.A and CPS.B.

To reconcile these results with previous studies which showed that the two CPS domains catalyze the two different ATP-dependent partial reactions, we proposed that the only active species is the homodimer. In this scheme, one of the monomers has the same function as CPS.A in the parent molecule, whereas the other assumes the role of CPS.B. In support of this idea, mammalian GLN-CPS.A and GLN-CPS.B, as well as the *E. coli* CPS subdomain B2, were found to be dimeric. However, definitive proof of this proposal requires that intact, correctly folded monomer be isolated and tested for catalytic activity. The CPSase activity is relatively labile, and conventional methods designed to dissociate oligomeric proteins might also be expected to disrupt the tertiary structure of the monomer. Previous pressure studies (36–40) showed that when oligomeric proteins are subjected to pressures exceeding about 1000 bar, they frequently dissociate into subunits. In general, no major perturbations of the tertiary structure occur until pressures in excess of 2000–3000 bar are attained. Consequently, we elected to use elevated pressure to dissociate the CPS.B and B2 dimers into monomers. This approach confirmed the hypothesis that the only species that can catalyze the overall synthesis of carbamoyl phosphate is the homodimer.

**EXPERIMENTAL PROCEDURES**

**Plasmids and Strains**—The 6.0-kilobase plasmid pHG-GCB consists of the sequences coding for the mammalian GLN domain fused directly to the sequences encoding the CPS.B subdomain (35) and is under control of the pyrB1 promoter (41). The 3.5-kilobase plasmid pHGB2 consists of the sequences coding for the *E. coli* B2 subdomain (33) under control of the carB promoter. The *E. coli* strain, L673, a uridine auxotroph (42) that lacks the *E. coli* carA and carB genes, encoding the *E. coli* CPSase GLN and CPS subunits, respectively, as well as the *Lon* protease, was kindly provided by Dr. Carol Lusty (The Public Health Research Institute of the City of New York, New York).

**Cell Growth and Recombinant DNA Methods**—Transformed L673 cells were grown, and the expression of the recombinant mammalian GLN-CPS.B protein was induced as described previously (43). The recombinant protein B2 was isolated from 300-ml cultures of transformed L673 cells grown to stationary phase (42). The cells were resuspended in 20 ml of 50 mM Tris-HCl, pH 7.4, 10% glycerol and broken by sonication for 2 min at 4 °C. The sonicate was centrifuged at 29,000 × g for 30 min at 4 °C. The supernatant was centrifuged at 300,000 × g for 30 min at 4 °C. The supernatant was dialyzed against 0.1 M. The extent of cross-linking was determined by denaturation of SDS-polyacrylamide electrophoresis gels.

**RESULTS**

**The Effect of Pressure on GLN-CPS.B—GLN-CPS.B, a recombinant protein in which the mammalian GLN domain is fused directly to CPS.B (Fig. 1), has previously been shown (35) to be a dimer composed of 94-kDa subunits. Cell extracts of pHG-GCB transformants expressing GLN-CPS.B, prepared as described under “Experimental Procedures,” were placed in the pressure apparatus and brought to 1500 bar within a few seconds. Samples were periodically withdrawn over the next hour at this pressure (Fig. 2A) and immediately assayed for GLN-dependent CPSase activity. After a short lag time, the initial high GLN-dependent CPSase activity began to gradually decrease. The half-life of inactivation was 20 min, and the activity was completely abolished after 1 h. In contrast, there was no loss of activity of an identical sample maintained at ambient pressure. After 1 h, the pressure was released, and the CPSase activity began to recover. The final sample taken after 120 min was as active as the control kept at atmospheric pressure, indicating that inactivation was completely reversible.

The reversible inactivation-reactivation was interpreted as a pressure-induced dissociation of the GLN-CPS.B dimer. The effect of protein concentration on the rate of recovery of catalytic activity (Fig. 3) supported this explanation. Although the loss of activity at elevated pressure was independent of protein concentration (data not shown), the rate of recovery following release of the pressure was proportional to the concentration of the protein, as expected for a second order reassociation process. However, restoration of activity exhibited cooperativity, especially apparent at lower protein concentrations, and did not exactly conform to second order reaction kinetics.

**Effect of Pressure on Ammonia-dependent CPSase Activity and Oligomeric Structure of GLN-CPS.B**—An alternative explanation for the pressure-induced loss of glutamine-dependent CPSase activity would be the disruption of the interactions between the GLN and CPS.B domains. Pressure-induced coupling glutamine hydrolysis with carbamoyl phosphate synthesis. Uncoiling might occur even though the GLN and CPS.B domains are fused and carried on a single polypeptide chain, as there are multiple noncovalent interactions between the domains. However, if this interpretation is correct, then the recovery phase would be expected to be first order and thus independent.
of protein concentration because the restoration of the linkage would be an intramolecular reaction.

To further rule out the uncoupling of the GLN and CPS domains as an explanation for the loss of activity, a similar experiment (Fig. 2B) was carried out using the purified GLN-CPS.B in which the ammonia-dependent CPSase activity was measured. The CPS.B domain, independent of the GLN domain, can use ammonia directly as the nitrogen donor for the synthesis of carbamoyl phosphate. As in the case of the Gln-dependent reaction, the NH₃-CPSase (Fig. 2B) exhibited reversible, pressure-induced inactivation. Although the shape of the curve differed somewhat, the half-life for inactivation was again 20 min.

To confirm that the GLN-CPS.B dimer was dissociated, samples were also taken throughout this experiment (data not shown) for chemical cross-linking and analysis by SDS-polyacrylamide gel electrophoresis. At atmospheric pressure, the protein was dimeric and remained dimeric during the 2-h incubation. However, the concentration of the monomer steadily increased at the expense of the dimer during the incubation at 1500 bar. After 1 h, all of the dimer had been converted to a monomer. The half-life for dissociation, where the protein existed as 50% monomer and 50% dimer, was approximately 20 min, in agreement with the time required for 50% inactivation. The pressure was released, and after 60 min at atmospheric pressure, the dimer had completely reformed and the activity was restored.

Effect of Pressure on Cross-linked GLN-CPS.B—If the loss of catalytic activity is exclusively coupled to the dissociation of the dimer, then a previously cross-linked dimer that is unable to dissociate should remain active after exposure to elevated pressure. Purified GLN-CPS.B cross-linked, as described under “Experimental Procedures,” was found by SDS-gel electrophoresis to be exclusively dimeric. Cross-linking resulted in little loss (<10%) of the NH₃-dependent CPSase activity. In contrast to the untreated protein, the NH₃-dependent catalytic activity of cross-linked GLN-CPS.B was largely unaffected by high pressure. The slight loss of catalytic activity, about 8% observed after 1 h at 1500 bar, was completely restored when the protein was brought back to atmospheric pressure. Thus, the pressure-induced inactivation of GLN-CPS.B can, for the most part, be attributed to the dissociation of the dimer.

Effect of Pressure on the CPSase Partial Reactions—These experiments were designed to test the hypothesis that the monomer can catalyze the partial reactions, but only the dimer can catalyze the overall reaction. The results reported above could also be explained by a pressure-induced disruption of the tertiary structure of the monomer, which abolished one or both of the ATP-dependent partial reactions (Reactions 2 and 4). The loss of either activity would result in the concomitant loss of carbamoyl phosphate synthesis activity. Consequently, the ATP-dependent partial reactions were measured during the pressure-induced dissociation of GLN-CPS.B. Following an experimental design identical to that described above, the rate of ATP hydrolysis was first measured when all of the substrates required for carbamoyl phosphate synthesis were present. When subjected to elevated pressure, the rate of ADP production (Fig. 4, solid circles) decreased by half, from 0.4 nmol/min to 0.2 nmol/min, indicating that only a single ATP was being consumed by the monomer. This result suggested that the overall reaction was abolished but that ATP continued to be hydrolyzed as a result of formation and subsequent hydrolysis of carboxyl phosphate. This interpretation was confirmed by directly assaying the bicarbonate-dependent ATPase activity of GLN-CPS.B (Reaction 2, Fig. 4, solid squares). This reaction measures the rate of the first partial reaction, the activation of bicarbonate. At atmospheric pressure, the rate of ATP formation was 0.2 nmol/min. Pressure-induced dissociation decreased the ATPase activity slightly to 0.13 nmol/min, indicating that although the catalytic activity of the monomer was slightly impaired, it could still catalyze the partial reaction at a comparable rate. The bicarbonate-dependent ATPase activity was restored to close its initial value when the molecule was returned to atmospheric pressure. The second partial reaction (Reaction 4), the phosphorylation of carbamate, was measured in the reverse direction as a carbamoyl phosphate-dependent
Throughout the pressure cycle, ATP formation continued to proceed at a rate comparable with that observed at atmospheric pressure (Fig. 4, solid triangles), although there was again a small, reversible pressure-induced loss of activity in the fully dissociated molecule. No significant change in the catalytic activity of any of these reactions was observed when the same measurements were made on a control protein sample (open symbols) incubated at atmospheric pressure.

Effect of Pressure on E. coli CPSase B2 Domain—A similar experiment (Fig. 5) was carried out on extracts of pHGB2 transformants, which express the B2 catalytic domain of *E. coli* CPSase. The B2 domain of the *E. coli* protein, a dimer of 28-kDa polypeptides, is not as stable as the mammalian GLN-CPS.B domain as indicated by the 23% loss of ammonia-dependent CPSase activity that occurred during the 120-min incubation period at atmospheric pressure. However, when subjected to 1500 bar, the activity was rapidly abolished. In contrast to the situation with the larger molecules, the activity experiment (Fig. 5) was carried out on extracts of pHGB2 transformants, which express the B2 catalytic domain of *E. coli* CPSase. The B2 domain of the *E. coli* protein, a dimer of 28-kDa polypeptides, is not as stable as the mammalian GLN-CPS.B domain as indicated by the 23% loss of ammonia-dependent CPSase activity that occurred during the 120-min incubation period at atmospheric pressure. However, when subjected to 1500 bar, the activity was rapidly abolished. In contrast to the situation with the larger molecules, the activity...
Under "Experimental Procedures." The sample (18 ml) was subjected to a pressure of 1500 bar. Samples were taken at the indicated times, and the NH$_4$CPSase activity was assayed. After 1 h, the protein solution was returned to atmospheric pressure, and sampling was continued. The activity of control samples of the B2 subdomain (C) incubated at atmospheric pressure was also assayed.

**DISCUSSION**

Previous studies have shown that oligomers generally dissociate when subjected to elevated pressure because of a decrease in the volume of the system. Moreover, the tertiary structure of the monomer is usually preserved provided that pressures do not exceed 2500–3000 bars. We have taken advantage of high pressure methodology to study the function of monomers of carbamoyl-phosphate synthetase domains under conditions least likely to disrupt the integrity of the monomer. Although the instrumentation allows assaying enzymatic activity at elevated pressure, all measurements were carried out after the sample had been returned to atmospheric pressure to avoid ambiguities associated with pressure-induced changes in the rate of the chemical reaction.

Carbamoyl-phosphate synthetase has a complex domain structure consisting of 40-kDa GLN and 120-kDa CPS domains or subunits. Each of the partial reactions involved in carbamoyl phosphate synthesis is catalyzed by different subdomains that have specialized functions and that interact to coordinate the reactions occurring at different sites within the molecule. Mutagenesis studies (26, 30, 31) have provided compelling evidence that CPS.A catalyzes the activation of bicarbonate (Reaction 2), whereas CPS.B phosphorylates carbamate (Reaction 4) to form the final product carbamoyl phosphate. The x-ray structure of *E. coli* CPSase supports this sequential scheme for carbamoyl phosphate synthesis. The GLN domain, the site of glutamine hydrolysis, is in contact with CPS.A where carboxyl phosphate is formed and presumably reacts with ammonia. In contrast, CPS.B, where carbamoyl phosphate is formed, is located at the other end of the molecule. The tunnel that threads its way through the center of the complex provides a means of sequestering the intermediates formed in the sequential mechanism, ammonia and carbamate, within the complex.

Although the sequential mechanism represents the generally accepted view, an intriguing new mechanism has recently been proposed (52) which suggests that synthesis occurs at a single site. Their proposal was prompted by the structural (34) and functional (35) equivalence of the CPS.A and CPS.B subdomains. In this alternative mechanism, carboxyl phosphate is formed in a reaction that is identical to the reaction (Reaction 2) proposed for the sequential mechanism. Ammonia then reacts with carboxyl phosphate to form a transient tetrahedral intermediate, which collapses to carbamoyl phosphate with the release of water. The most interesting aspect of this proposal is that ATP binding to the CPS.B domain acts as a "nucleotide switch," which triggers the active conformation at the site of synthesis. The same reaction, formation of carbamoyl phosphate, occurs on CPS.B, but because CPS.B cannot assume the activated conformation, the intermediate is subsequently hydrolyzed. The coupling of the hydrolysis of a second ATP to carbamoyl phosphate synthesis would make the biosynthetic reaction thermodynamically favorable. The authors argue that all of the data gathered in support of the sequential mechanism can be explained equally well or better by the nucleotide switch mechanism.

Both models require the concerted action of two catalytic sites located on two distinct subdomains. Thus, the observation (35) that CPS.A and CPS.B domains alone are able to catalyze the overall synthesis of carbamoyl phosphate may initially seem paradoxical. Moreover, the catalytic subdomains of CPS.A and CPS.B, which have a mass of only 31 and 28 kDa, respectively, are also fully functional (33) and can catalyze ammonia-dependent carbamoyl phosphate synthesis. The recently characterized CPSases from the archaeabacteria, *Pyrococcus furiosus* (53) and *Pyrococcus abyssii* (54), have a molecular mass of only 34 kDa and may be naturally occurring analogs of the recombinant CPS.A and CPS.B domains of mammalian carbamoyl-phosphate synthetase.

To reconcile our findings with studies which showed that both CPS domains participate in carbamoyl-phosphate synthetase, we proposed that the CPS.A and CPS.B monomers could each catalyze all of the partial reactions, but catalysis of the overall synthesis of carbamoyl phosphate requires a dimer composed of two of the equivalent domains. We found that the molecules were in fact dimeric, but to definitively prove this proposal, it was necessary to show that the monomers can catalyze the partial reactions but not the overall synthesis of carbamoyl phosphate.

The pressure studies reported here show that both glutamine- and ammonia-dependent CPSase activity is lost when the GLN-CPS.B was subjected to elevated pressure. The loss of glutamine-dependent activity cannot be solely attributed to disruption of the functional interactions between the GLN and CPS domains, as the ammonia-dependent activity that occurs independently of the GLN domain is also abolished at elevated pressure. On the other hand, we cannot dismiss the possibility that GLN-CPS interactions are diminished, as the domains are part of the same polypeptide chain, and these interactions would be expected to be quickly restored when the protein is returned to atmospheric pressure. However, the dissociation of the dimer was confirmed by measuring the dependence of the recovery on protein concentration and by chemical cross-linking. Moreover, there was no loss of catalytic activity if the molecule was prevented from dissociating by chemical cross-linking before exposure to high pressure.

The monomers are still able to catalyze both partial reactions at an appreciable rate, indicating that they remain functional. Dissociation resulted in only a 35% decrease in activity of both the bicarbonate-dependent ATPase and the carbamoyl phos-
phosphate-dependent ATP synthetase reactions. The modest loss of activity could indicate that there is a small, reversible perturbation of the tertiary structure of the monomer when subjected to elevated pressure. Alternatively, there may be interdomain interactions between the monomers in the dimer that stimulate catalytic activity. The loss of these interactions on dissociation could also account for the small decrease in the rate of the partial reactions catalyzed by the monomer.

Similar experiments suggest that the dimeric catalytic subdomain, B2 of \textit{E. coli} CPSase also dissociates at elevated pressure with loss of catalytic function. However, as the loss of activity was irreversible, we cannot be sure whether more profound structural changes were induced in the monomer. We have not as yet assayed the partial reactions in B2, an experiment that may provide a partial answer to this question.

Although the nucleotide switch model was based in part on the functional equivalence of CPS.A and CPS.B, both this model and the sequential mechanism can account for the results reported here. In fact, the initial interpretation (35) of the functional equivalence of CPS.A and CPS.B was made within the context of the sequential mechanism. Functional equivalence of CPS.A and CPS.B alone cannot discredit the sequential model because the substrates and the two ATP-dependent reactions catalyzed by the two domains are very similar.

Regardless of the mechanism, the two monomers of the CPS.B homodimer are assumed to occupy the same location and serve the same function as CPS.A and CPS.B in the native molecule. This proposal is plausible given the similarity of sequence and tertiary structure and the approximate 2-fold axis of symmetry that relates the CPS.A and CPS.B in the parent enzyme. The 2-fold symmetry is especially apparent at the interface between the two domains. In the sequential mechanism, formation of the CPS.B dimer reconstitutes the tunnel through which carbamoyl traverses the molecule from its site of formation to the site where carbamoyl phosphate is formed on the other domain. In the nucleotide switch model, one of the monomers serves as the site of synthesis, whereas the other uses ATP to drive the conformational change. In both mechanisms, only the dimer would be expected to catalyze the overall reaction. On the other hand, given the tenuous contacts between B2 and A2 in \textit{E. coli} CPSase and the fact that the A1 and B1 subdomains participate in forming the tunnel, it is more difficult to visualize a carbamoyl channel in the B2 homodimer. Perhaps further studies of the isolated catalytic subdomains, A2 and B2, would help distinguish between the two mechanisms. Another unexpected result was the failure of the GLN CPS.B monomer to catalyze a carbamoyl kinase-like reaction. Bicarbonate and ammonia, substrates used to measure the ammonia-dependent CPSase activity, are in equilibrium with carbamoyl. Carbamoyl kinase, an enzyme in the arginine deammonia-dependent CPSase activity, are in equilibrium with loss of catalytic function. However, as the loss of activity by default, assumes the role of carbamoyl phosphorylation. In this regard, the domains within the homodimer are reminiscent of mitochondrial ATPase catalytic subunits (61) in that although intrinsically identical, they have different functions at any particular moment. We attribute (35) this result to juxtapositional specialization of function in which the specific reaction catalyzed by the domain is determined by its location within the complex and its interactions with neighboring domains. Specifically, the domain closest to the GLN domain catalyzes bicarbonate activation. This interpretation is consistent with the recent x-ray structure (34) of \textit{E. coli} CPSase, which shows that the GLN domain is in intimate contact with CPS.A and far from CPS.B. We are currently constructing hybrid mutants of CPS.A and CPS.B to test this idea.

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