Allosteric Control of the Oligomerization of Carbamoyl Phosphate Synthetase from *Escherichia coli*†

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ABSTRACT: Carbamoyl phosphate synthetase (CPS) from *Escherichia coli* is allosterically regulated by the metabolites ornithine, IMP, and UMP. Ornithine and IMP function as activators, whereas UMP is an inhibitor. CPS undergoes changes in the state of oligomerization that are dependent on the protein concentration and the binding of allosteric effectors. Ornithine and IMP promote the formation of an $(\alpha\beta)_4$ tetramer while UMP favors the formation of an $(\alpha\beta)_2$ dimer. The three-dimensional structure of the $(\alpha\beta)_4$ tetramer has unveiled two regions of molecular contact between symmetry-related monomeric units. Identical residues within two pairs of *allosteric* domains interact with one another as do twin pairs of *oligomerization* domains. There are thus two possible structures for an $(\alpha\beta)_2$ dimer: an elongated dimer formed at the interface of two allosteric domains and a more compact dimer formed at the interface between two oligomerization domains. Mutations at the two interfacial sites of oligomerization were constructed in an attempt to elucidate the mechanism for assembly of the $(\alpha\beta)_4$ tetramer through disruption of the molecular binding interactions between monomeric units. When Leu-421 (located in the oligomerization domain) was mutated to a glutamate residue, CPS formed an $(\alpha\beta)_2$ dimer in the presence of ornithine, UMP, or IMP. In contrast, when Asn-987 (located in the allosteric binding domain) was mutated to an aspartate, an $(\alpha\beta)$ monomer was formed regardless of the presence of any allosteric effectors. These results are consistent with a model for the structure of the $(\alpha\beta)_2$ dimer that is formed through molecular contact between two pairs of allosteric domains. Apparently, the second interaction, between pairs of oligomerization domains, does not form until after the interaction between pairs of allosteric domains is formed. The binding of UMP to the allosteric domain inhibits the dimerization of the $(\alpha\beta)_2$ dimer, whereas the binding of either IMP or ornithine to this same domain promotes the dimerization of the $(\alpha\beta)_2$ dimer. In the oligomerization process, ornithine and IMP must exert a conformational alteration on the oligomerization domain, which is ∼45 Å away from their site of binding within the allosteric domain. No significant dependence of the specific catalytic activity on the protein concentration could be detected, and thus the effects induced by the allosteric ligands on the catalytic activity and the state of oligomerization are unlinked from one another.

Carbamoyl phosphate synthetase (CPS)[1] from *Escherichia coli* is a heterodimeric protein $(\alpha\beta)$, consisting of a 42 kDa amidotransferase subunit and a larger subunit of molecular mass 118 kDa (Figure 1). Glutamine binds to the small subunit where it is subsequently hydrolyzed to ammonia and glutamate. The large subunit is composed of four major domains. The *carboxy phosphate* domain is found at the N-terminus (residues 1−400), and it harbors the binding site for the ATP that is used to phosphorylate bicarbonate. An additional molecule of ATP binds to the homologous *carbamoyl phosphate* domain (residues 553−933) where it functions in the phosphorylation of the intermediate, carbamate. The two phosphorylation domains are connected by the *oligomerization* domain (residues 400−553). At the extreme C-terminus is the *allosteric* domain, and it contains the binding sites for the metabolic effectors, ornithine, IMP, and UMP. The three active sites contained within CPS are connected to one another by an intermolecular tunnel, through which intermediates are transferred from one site to the next (*1*).

The overall reaction for the formation of carbamoyl phosphate from bicarbonate, glutamine, and two molecules of ATP is presented in eq 1 and the chemical mechanism is summarized in Scheme 1 (*2*). In addition to the overall reaction, CPS also catalyzes three partial reactions, which are shown in eqs 2−4 (*3*):

$$2\text{MgATP} + \text{HCO}_3^- + \text{Gln} \rightarrow 2\text{MgADP} + \text{carbamoyl-P} + \text{Glu} + \text{P}_i \quad (1)$$

$$\text{glutamine} + \text{H}_2\text{O} \rightarrow \text{glutamate} + \text{NH}_3 \quad (2)$$

$$\text{MgATP} + \text{H}_2\text{O} \rightarrow \text{MgADP} + \text{P}_i \quad (3)$$

$$\text{MgADP} + \text{carbamoyl-P} \rightarrow \text{MgATP} + \text{NH}_2\text{CO}_2^- \quad (4)$$

In *E. coli*, CPS is subject to allosteric control by specific metabolic effectors (*3*−*6*). Ornithine and IMP activate CPS,

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[1] Abbreviations: CPS, carbamoyl phosphate synthetase; OTCase, ornithine transcarbamoylase; ATCase, aspartate transcarbamoylase.

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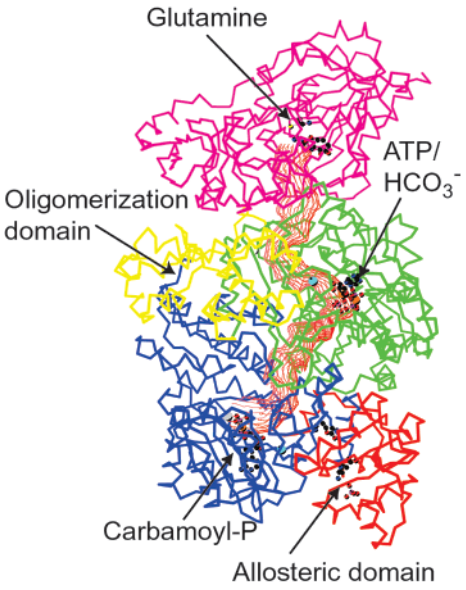
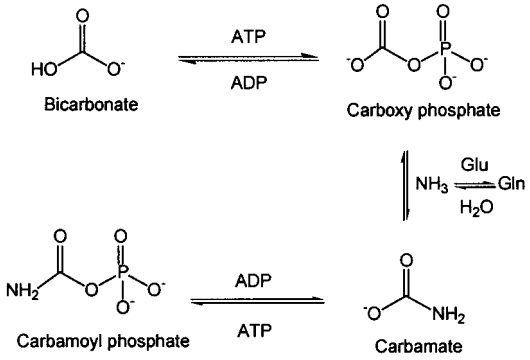


FIGURE 1: An α-carbon trace of the heterodimeric (αβ) structure of wild-type CPS from *E. coli*. Binding sites of substrates are indicated, and the molecular tunnel connecting the three active sites is represented in red. Coordinates taken from Thoden et al. (*1*).

Scheme 1



whereas UMP is an allosteric inhibitor. These effectors have been shown to modulate the oligomerization of the (αβ) heterodimer to higher ordered species. The formation of an $(\alpha\beta)_4$ tetramer is facilitated by the presence of ornithine or IMP, whereas the $(\alpha\beta)_2$ dimer is stabilized with UMP (*7*−*11*). The functional relationship between the specific catalytic activity and the various oligomeric states of CPS is unclear. Both Powers et al. (*10*) and Anderson (*11*) have reported an increase in the specific catalytic activity at protein concentrations >0.10 mg/mL. Powers et al. (*10*) have proposed that the higher ordered oligomeric forms of CPS are more active than the (αβ) monomer, whereas Anderson (*11*) has postulated an alteration in the equilibrium distribution between an inactive and active monomer. Structural explanations for these changes in specific catalytic activity are lacking.

The tetrameric form of CPS, solved in the presence of the allosteric activator ornithine, is illustrated in Figure 2 (*1*). Each of the four (αβ) monomeric units interacts with its nearest neighbor at two distinct sites. There is direct molecular contact between identical residues within the allosteric domains and an additional interfacial site between pairs of oligomerization domains. Two structurally different forms of an $(\alpha\beta)_2$ dimer are possible. A *side-by-side* dimer could form through dimerization at the oligomerization domain interface. Alternatively, an *end-to-end* dimer may

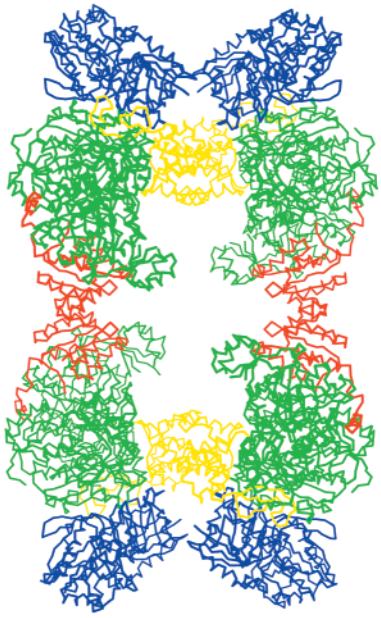


FIGURE 2: Three-dimensional structure of the wild-type CPS tetramer. The interface between pairs of allosteric domains is shown in red. The interface between pairs of oligomeric domains is shown in yellow. Coordinates taken from Thoden et al. (*1*).

form through interfacial contact between homologous residues within the allosteric domain. However, the three-dimensional structure of an $(\alpha\beta)_2$ dimer of CPS, formed in the presence or absence of UMP, has not been solved. In this paper we have elucidated the molecular pathway for the assembly of the $(\alpha\beta)_4$ tetramer from the (αβ) monomeric species through the construction and characterization of mutant proteins that have been structurally perturbed at the sites of interfacial contact. Specific single site mutants at the oligomeric domain interface are $(\alpha\beta)_2$ dimers in the presence or absence of allosteric ligands, whereas single site mutants at the allosteric domain interface are (αβ) monomers under all solution conditions. The protein interface between pairs of allosteric domains must form prior to the interface between the oligomerization domains during the assembly of the higher ordered oligomers of this enzyme.

MATERIALS AND METHODS

Materials. All chemicals and coupling enzymes were purchased from either Aldrich or Sigma, unless otherwise stated. [γ-^{32}P]ATP was obtained from Amersham Pharmacia Biotech. Restriction enzymes were purchased from New England Biolabs, and *pfu* DNA polymerase was acquired from Promega.

Mutagenesis and Protein Purification. Site-directed mutagenesis with CPS was performed as described previously (*12*). Oligonucleotide synthesis and DNA sequencing reactions were performed by the Gene Technology Laboratory, Texas A&M University. The plasmids containing the *car*AB genes were transformed in the RC50 cell line of *E. coli* for expression of the wild-type and mutant forms of CPS. The wild type and mutant variants of CPS were purified as previously described (*13*).

Kinetic Measurements and Analysis. The rate of ADP formation was measured using a pyruvate kinase/lactate dehydrogenase coupling system (*13*). The reaction mixtures contained 50 mM Hepes (pH 7.6), 20 mM $MgCl_2$, 100 mM KCl, 40 mM $KHCO_3$, 10 mM glutamine, 1.0 mM phospho-

enolpyruvate, 0.2 mM NADH, 20 units of pyruvate kinase, 30 units of lactate dehydrogenase, and varying amounts of ATP and allosteric effectors in a final volume of 2.5 mL. The rate of ATP synthesis was measured with a hexokinase/glucose-6-phosphate dehydrogenase coupling system (*12*). The assay solution included 50 mM Hepes (pH 7.6), 15 mM $MgCl_2$, 100 mM KCl, 0.75 mM NAD, 0.04 mg/mL hexokinase, 10 mM glucose, 0.0016 mg/mL G6DPH, 2.0 mM carbamoyl phosphate, and varying amounts of ADP and allosteric effectors in a final volume of 2.0 mL. The kinetic parameters were determined by fitting the experimental data to eq 5, where k_{cat} is the turnover number, K_m is the Michaelis constant, and A is the substrate concentration.

$$v/E_t = k_{cat}A/(K_m + A) \quad (5)$$

Rapid Quench Experiments. The rapid quench experiments were conducted with a Kintek RQF-3 rapid quench instrument, as described previously (*14*). The steady-state rate of ADP formation was measured at various concentrations of CPS. Assay solutions contained 50 mM Hepes (pH 7.6), 20 mM $MgCl_2$, 100 mM KCl, 40 mM $KHCO_3$, 10 mM glutamine, 1.0 mM ATP, and various concentrations of CPS and allosteric effectors. The reactions were initiated by mixing 15 μL of CPS with 15 μL of a solution containing the radiolabeled substrate. The reactions were subsequently quenched with 1.0 M HCl, vortexed, and then incubated on ice. The rate of formation of acid-labile phosphate was measured using [γ-^{32}P]ATP with a specific radioactivity of 89 000 cpm/nmol. One microliter of the quenched reaction sample was spotted onto a polyethylenimine TLC plate (Selecto Scientific) and then developed using a buffer of 0.75 M phosphate, pH 3.6. The TLC plates were dried overnight, and then the radioactive images transferred to a phosphorimaging plate (Molecular Dynamics). The plates were developed for 12 h before analysis using a Molecular Dynamics Storm 860 PhosphorImager System with ImageQuant software.

Sedimentation Velocity Experiments. Sedimentation velocity experiments were performed with a Beckman Optima XL-A centrifuge and an An 60 Ti rotor at 25 °C. Protein samples were dialyzed at 4 °C against a solution containing 50 mM Hepes (pH 7.6) and 100 mM KCl with the addition of either 10 mM ornithine, 0.2 mM UMP, or 0.2 mM IMP. The 3 or 12 mm double sector charcoal-filled Epson centerpieces were used with a sample capacity of 75 or 300 μL, respectively. All experiments were conducted at a rotor speed of 42 000 rpm, and scans were taken at 4 min intervals. The sedimentation of CPS was monitored by the absorption of light at 230 or 280 nm. The sedimentation data were analyzed with the use of the computer program SVEDBERG (*15*).

RESULTS

Previous investigations have demonstrated that the oligomerization state of the wild-type CPS is dependent on the protein concentration and the presence of specific allosteric effectors (*7*−*11*). To identify the structural and thermodynamic parameters that dictate the quaternary structure of CPS, the sedimentation coefficient was measured as a function of the enzyme concentration in the presence and absence of the known allosteric effectors for the bacterial CPS. Site-directed mutations were constructed in an attempt to specifically disrupt the protein−protein interactions at the sites of the two known molecular interfaces between the four heterodimeric units ($\alpha\beta$) found in the crystal structure of the tetrameric protein $(\alpha\beta)_4$.

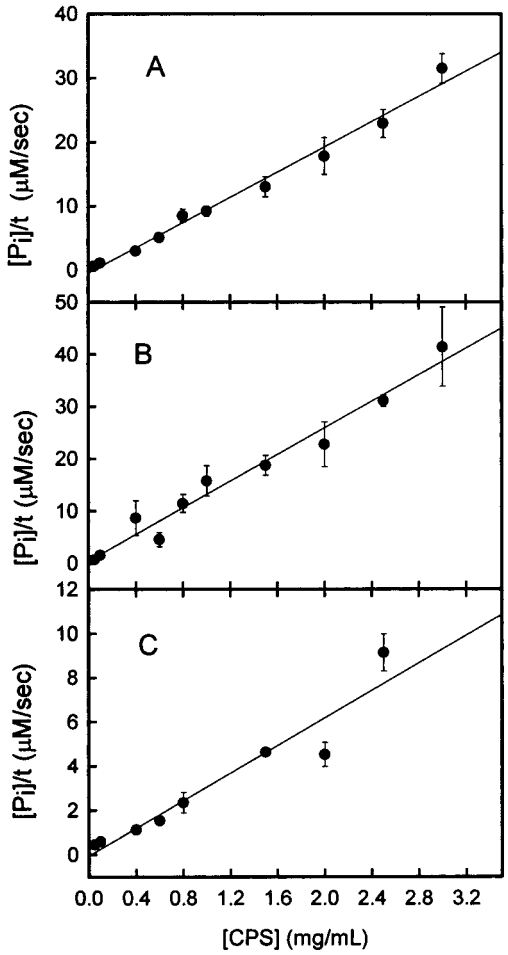


FIGURE 3: Catalytic activity of CPS as a function of protein concentration: (A) no added allosteric effectors; (B) 10 mM ornithine and; (C) 0.1 mM UMP. Additional details are provided in the text.

Specific Activity of Wild-Type CPS as a Function of Enzyme Concentration. The catalytic activity of wild-type CPS was measured at various concentrations of the enzyme (0.05−2.9 mg/mL) by assaying the rate of ADP formation. The steady-state rate at the elevated protein concentrations was measured at 1.0 mM ATP using a rapid-quench method as described in the Materials and Methods section. The rate of ADP formation was a linear function of the protein concentration over the range of protein concentrations examined (Figure 3A). The addition of saturating amounts of either ornithine or UMP did not alter the linearity of these plots (Figure 3B,C). The specific catalytic activity must therefore be identical for all of the oligomeric forms of CPS that are present in this protein concentration range.

Sedimentation Velocity Experiments with the Wild-Type CPS. The sedimentation coefficient, $s_{20,w}$, of the wild-type enzyme was determined as a function of the protein concentration in the presence and absence of the various allosteric effectors of CPS. At the lowest protein concentration attainable (0.05 mg/mL) the sedimentation coefficient has a value of ~8 S and then gradually increases without a plateau to a value that exceeds 11 S at a protein concentration of ≥3 mg/mL (Figure 4). In the presence of 0.2 mM UMP,

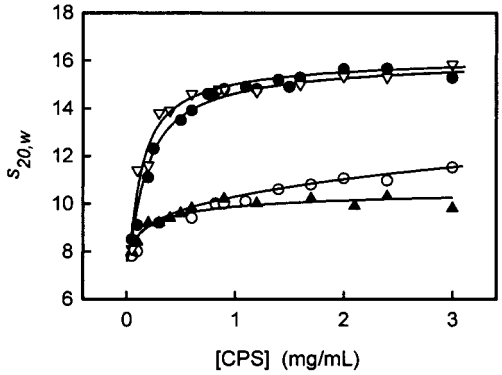


FIGURE 4: Sedimentation coefficient profile of the wild-type CPS as a function of the enzyme concentration. CPS was dialyzed prior to the ultracentrifugation experiments in a solution containing 50 mM Hepes (pH 7.6), 100 mM KCl, and either 10 mM ornithine (●), 0.2 mM IMP (▽), 0.2 mM UMP (▲), or none (○). The solid lines represent a nonlinear fit of the data to either eq 6 or 7.

the value of $s_{20,w}$ increases up to ~10 S as the concentration of CPS becomes higher. The sedimentation coefficient of the wild-type CPS in the presence of either 10 mM ornithine or 0.2 mM IMP increases to a value of ~16 S. These values are in good agreement with previous results (*10*), and they verify that the allosteric inhibitor UMP favors the formation of a dimer while the two allosteric activators, ornithine and IMP, promote the formation of a tetramer. The value of $s_{20,w}$ for the native enzyme at high protein concentrations, in the absence of any added allosteric effectors, exceeds the sedimentation coefficient of CPS in the presence of UMP. Apparently, the $(\alpha\beta)_2$ dimeric complex of CPS, formed in the absence of allosteric effectors, can form a tetramer only at very high protein concentrations.

Disruption to the Oligomerization Domain Interface. A close-up view of the domain interface between two oligomerization domains within the tetrameric form of CPS is presented in Figure 5A. A close inspection of the X-ray crystal structure of CPS reveals that there are several amino acid residues that are apparently critical for direct protein contact between two pairs of oligomerization domains within the tetrameric structure. In particular, there is a close hydrophobic interaction between Leu-421 and the identical residue in the adjacent subunit. This residue was mutated to a glutamate in an attempt to disrupt the hydrophobic interaction through charge−charge repulsion. The mutant, L421E, was as active as the wild-type enzyme, and the binding of the allosteric effectors was confirmed through the measurement of the kinetic effects exhibited by ornithine and UMP on the catalytic properties of the mutant enzyme (Tables 1 and 2). The sedimentation velocity experiments with the L421E mutant demonstrated that the formation of the tetramer was not observed under any of the solution

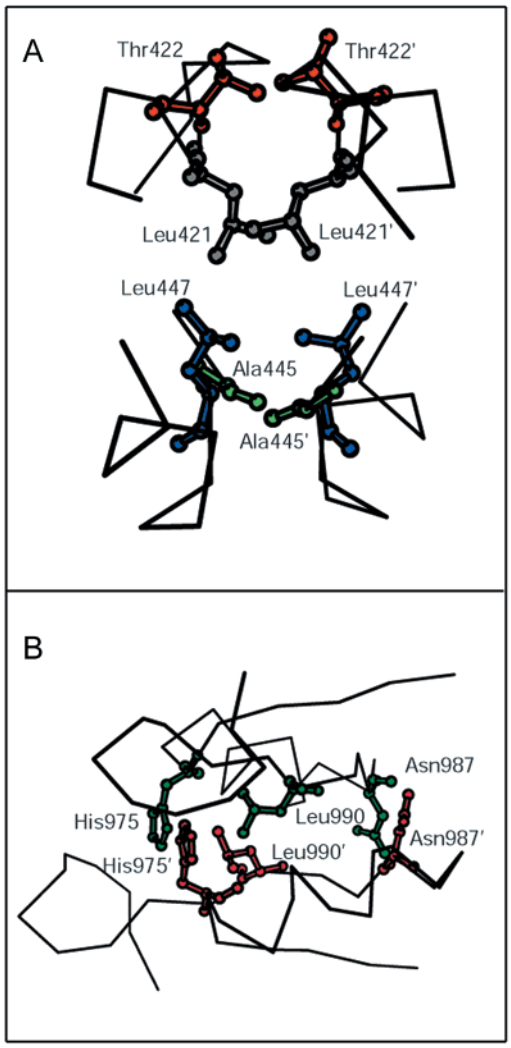


FIGURE 5: Molecular interactions within the tetrameric form of CPS: (A) the interface between oligomerization domains and (B) the interface between the allosteric domains in the wild-type CPS. The key residues are highlighted in ball-and-stick format. Coordinates taken from Thoden et al. (*1*).

conditions examined (Figure 6A). The sedimentation coefficient increased up to a value of ~10 S, which is very similar to the magnitude of $s_{20,w}$ for the UMP-induced dimer observed with the wild-type enzyme. Therefore, a perturbation at the subunit interface within the oligomerization domain totally disrupted the formation of the $(\alpha\beta)_4$ tetramer from a dimeric intermediate.

Disruption to the Allosteric Domain Interface. At the allosteric domain interface in the tetrameric structure of CPS, three pairs of amino acid residues make the major intermolecular contacts with one another, as illustrated in Figure

Table 1: Kinetic Parameters for the Glutamine-Dependent Turnover of ATP by the Wild-Type and Mutant Enzymes[a]

	WT		L421E		N987D		L421E/N987D	
effector[b]	V_{max} [μmol/(min·mg)]	$K_{m,ATP}$ (mM)	V_{max} [μmol/(min·mg)]	$K_{m,ATP}$ (mM)	V_{max} [μmol/(min·mg)]	$K_{m,ATP}$ (mM)	V_{max} [μmol/(min·mg)]	$K_{m,ATP}$ (mM)
none	2.7 ± 0.1	0.47 ± 0.06	2.5 ± 0.1	1.1 ± 0.1	3.1 ± 0.1	1.1 ± 0.1	3.2 ± 0.1	1.1 ± 0.1
ornithine	2.4 ± 0.1	0.038 ± 0.002	2.2 ± 0.1	0.14 ± 0.01	2.4 ± 0.1	0.081 ± 0.010	2.2 ± 0.1	0.084 ± 0.005
UMP	1.4 ± 0.1	1.6 ± 0.2	1.1 ± 0.1	1.4 ± 0.5	2.2 ± 0.1	1.4 ± 0.1	2.3 ± 0.1	1.5 ± 0.2
IMP	2.4 ± 0.1	0.37 ± 0.04	2.9 ± 0.1	0.49 ± 0.05	2.9 ± 0.1	0.44 ± 0.04	3.4 ± 0.1	0.72 ± 0.03

[a] Reaction conditions: 50 mM Hepes (pH 7.6), 100 mM KCl, 20 mM $MgCl_2$, 20 mM $KHCO_3$, 10 mM glutamine, and variable ATP. [b] Effector concentrations: ornithine, 10 mM; UMP, 100 μM; IMP, 1.0 mM.

Table 2: Kinetic Parameters for the ATP Synthesis Reaction by the Wild-Type and Mutant Enzymes[a]

	WT		L421E		N987D		L421E/N987D	
effector[b]	V_{max} [μmol/(min·mg)]	$K_{m,ADP}$ (mM)	V_{max} [μmol/(min·mg)]	$K_{m,ADP}$ (mM)	V_{max} [μmol/(min·mg)]	$K_{m,ADP}$ (mM)	V_{max} [μmol/(min·mg)]	$K_{m,ADP}$ (mM)
none	0.21 ± 0.01	0.23 ± 0.04	0.24 ± 0.01	0.16 ± 0.01	0.17 ± 0.01	0.82 ± 0.08	0.23 ± 0.01	0.72 ± 0.01
ornithine	0.17 ± 0.01	0.016 ± 0.002	0.22 ± 0.01	0.014 ± 0.002	0.16 ± 0.01	0.065 ± 0.007	0.19 ± 0.01	0.063 ± 0.006
UMP	0.13 ± 0.02	4.8 ± 1.6	0.15 ± 0.02	4.1 ± 0.9	0.071 ± 0.001	1.2 ± 0.03	0.090 ± 0.002	1.1 ± 0.1
IMP	0.24 ± 0.01	0.30 ± 0.04	0.26 ± 0.01	0.24 ± 0.03	0.19 ± 0.01	0.63 ± 0.09	0.25 ± 0.03	1.0 ± 0.4

[a] Reaction conditions: 50 mM Hepes (pH 7.6), 100 mM KCl, 15 mM $MgCl_2$, 2.0 mM carbamoyl phosphate, and variable ADP. [b] Effector concentrations: ornithine, 10 mM; UMP, 100 μM; IMP, 1.0 mM.

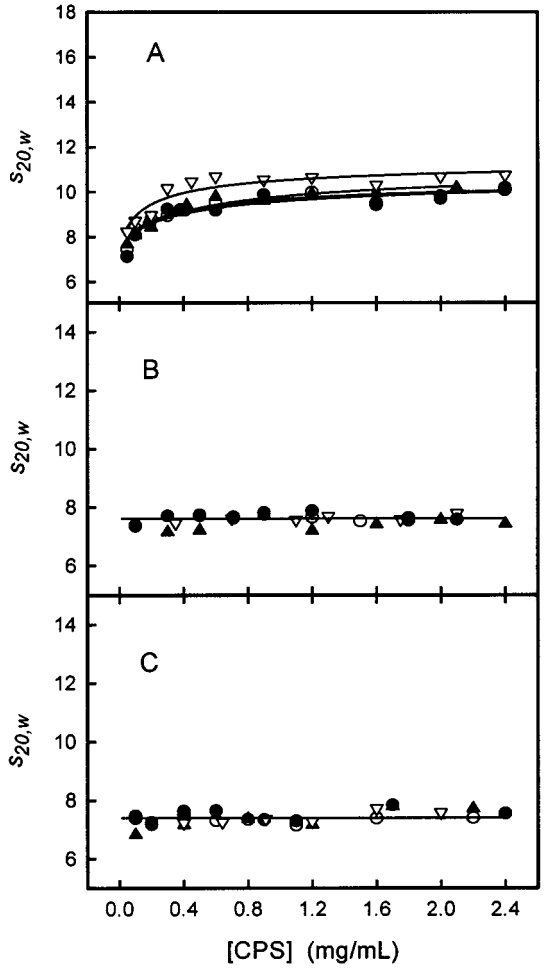


FIGURE 6: Sedimentation coefficient profiles for CPS mutants: (A) L421E, (B) N987D, and (C) L421E/N987D. Each enzyme was dialyzed prior to the ultracentrifugation experiment in a solution containing 50 mM Hepes (pH 7.6) and 100 mM KCl. Symbols: (●) 10 mM ornithine, (▽) 0.2 mM IMP, (▲) 0.2 mM UMP, or (○) none.

5B. In particular, there are two hydrogen-bonded pairs of residues, Asn-987 and His-975, which apparently play an important role in the maintenance of the domain interface. The side chain of His-975 pairs with its nearest neighbor to form a very short hydrogen bond, whereas Asn-987 forms two pairs of intermolecular hydrogen bonds between the carbonyl oxygen of the protein backbone and the amide nitrogen from the side chain carboxamide group. Asn-987 was selected as the amino acid residue to be mutated within the allosteric domain interface because it is located the furthest away from the allosteric binding site for UMP/IMP. Asn-987 was mutated to aspartate. The N987D mutant did not oligomerize, even in the presence of ornithine or IMP (Figure 6B). The sedimentation coefficient remained constant at ~7.6 S over the entire range of protein concentrations tested. The magnitude of the sedimentation coefficient for N987D was very similar to what has been reported previously for the monomeric form of CPS (*7*, *10*). Therefore, the mutant remained as a monomer under all conditions examined. This single mutation completely disrupted the formation of the tetrameric and dimeric forms of CPS. The kinetic data with this mutant suggest that the binding of the allosteric effectors has not been perturbed (Tables 1 and 2).

Perturbation at Both Domain Interfaces. A double mutant, L421E/N987D, was constructed in order to simultaneously hinder the interface interactions between the allosteric and oligomerization domains. The double mutant did not undergo oligomerization under any of the conditions tested (Figure 6C). In this case the value of $s_{20,w}$ was constant at ~7.4 S, confirming the monomeric state of the protein. The kinetic properties of L421E/N987D were very similar to those displayed by the wild-type enzyme and other mutants created for this investigation (Tables 1 and 2).

DISCUSSION

Specific Activity of Oligomeric States. The rate of ADP formation was measured as a function of the concentration of the wild-type CPS at a fixed level of ATP. Within experimental error the catalytic turnover was found to be a linear function of the protein concentration. Additionally, no deviation from linearity was detected in the presence of either ornithine or UMP at concentrations of these effectors that have been previously shown to alter the oligomeric state of the bacterial protein. Thus, the oligomeric state (monomer, dimer, or tetramer) of the wild-type CPS has no significant effect on the catalytic turnover of this protein. Moreover, the oligomerization-impaired mutants, L421E, N987D, and L421E/N987D, have essentially the same catalytic activity as the wild-type enzyme and are allosterically regulated by UMP and ornithine. Therefore, the allosteric effects on the kinetic constants, induced via the binding of IMP, UMP, or ornithine to the allosteric binding domain of CPS, must be mediated by direct conformational changes to the active sites and are functionally unrelated to the conformational changes that modulate oligomerization to higher ordered complexes. Moreover, there is no experimental evidence for the existence of substrate cooperativity in CPS from *E. coli* (*16*).

Oligomerization of Carbamoyl Phosphate Synthetase. The sedimentation velocity experiments have shown that the oligomerization state of CPS is dependent on the protein concentration. At the lowest protein concentration feasible for these experiments, the protein sediments as an ($\alpha\beta$) monomer in the presence or absence of added allosteric effectors. In the presence of the allosteric inhibitor UMP,

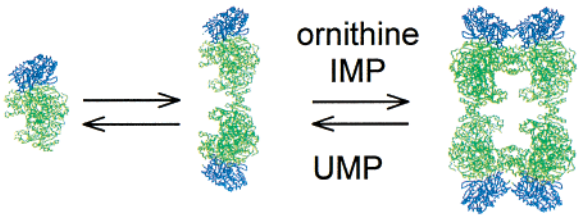
CPS forms an $(\alpha\beta)_2$ dimer at protein concentrations up to ~3 mg/mL. In contrast, the two allosteric activators, IMP and ornithine, induce the formation of an $(\alpha\beta)_4$ tetrameric form of CPS over a similar range of protein concentrations. The sedimentation coefficient for the wild-type protein in the absence of an added allosteric effector was measurably larger than that observed in the presence of UMP. These results indicate that the $(\alpha\beta)_4$ tetramer may form in the absence of ornithine or IMP but with a much higher dissociation constant than in the presence of either ornithine or IMP.

Manipulation of Oligomer Assembly. Mutants of CPS were constructed in an attempt to disrupt the two interfacial sites that have been shown to be formed during the assembly of the $(\alpha\beta)_4$ tetramer. When Leu-421 was mutated to a glutamate residue, the protein was unable to form a tetrameric species in the presence of either ornithine or IMP. The binding of ornithine and IMP to the allosteric domain was not disrupted, since these ligands were able to induce the allosteric activation effects on the kinetic constants possessed by the mutant enzyme. Therefore, the dimeric species of the L421E mutant that does form in the presence of ornithine or IMP must have an oligomeric structure that resembles an end-to-end dimer where the single interfacial site must be limited to interactions between a pair of allosteric domains. However, these data alone do not exclude the formation of a side-by-side dimer during the oligomerization of the wild-type protein.

When Asn-987 at the allosteric domain interface was mutated to an aspartate residue, CPS remained as an $(\alpha\beta)$ monomer under all of the solution conditions tested. Since an $(\alpha\beta)_2$ dimer was unable to form in the presence of UMP, it can be concluded that the dimeric state of the wild-type CPS that forms in either the presence or absence of UMP must exclusively be an end-to-end dimer. An $(\alpha\beta)$ monomeric species was also formed in the presence of ornithine or IMP. These results clearly indicate that the side-by-side dimer is not on the assembly pathway to the formation of the $(\alpha\beta)_4$ tetramer. It can also be concluded that the assembly of the $(\alpha\beta)_4$ tetrameric species, induced by the binding of ornithine or IMP to the allosteric domain, must be triggered through a conformational change to the oligomerization domain. The interface between oligomerization domains in the $(\alpha\beta)_4$ tetramer is approximately 45 Å away from the binding sites for ornithine, IMP, and UMP within the allosteric domain.

Association Constants for Oligomer Assembly. The association constants for oligomer assembly were estimated by simulation of the $s_{20,w}$ profiles as a function of the CPS concentration, as previously described (*7*). It is assumed in this analysis that the association/dissociation of CPS is rapid and without the resolution of boundaries between the various oligomeric species (*17*). The sedimentation velocity data for the wild-type CPS fit reasonably well to the model presented in Scheme 2, which involves an equilibrium between monomeric, dimeric, and tetrameric forms of CPS. In this model, it was initially assumed that K_{12}, the association constant for the dimerization of CPS, is independent of the presence of allosteric effectors, but that K_{24}, the association constant for formation of the tetramer, is dependent on the presence of ornithine, IMP, or UMP. It was also assumed that the values of $s_{20,w}$ for the monomeric or dimeric forms of CPS are constant, regardless of the identity of the specific

Scheme 2



effector bound to the allosteric domain. In addition, the value of $s_{20,w}$ for the tetramer was assumed to be equal for the IMP- or ornithine-bound forms of CPS. The apparent sedimentation coefficient, s_{app}, can be expressed as a weight average of $s_{20,w}$ of the various oligomeric forms of CPS as shown in eq 6, where s_n and c_n are the limiting sedimentation coefficient and the concentration of an n-mer, respectively. K_{12} is the association constant for the monomer−dimer equilibrium shown in Scheme 2, whereas K_{24} is the association constant for the dimer−tetramer equilibrium.

$$s_{app} = \frac{\sum n s_n^2 c_n}{\sum n s_n c_n} = \frac{s_1^2 c_1 + 2 s_2^2 c_2 + 4 s_4^2 c_4}{s_1 c_1 + 2 s_2 c_2 + 4 s_4 c_4} = \frac{s_1^2 c_1 + 2 s_2^2 c_1^2 K_{12} + 4 s_4^2 c_1^4 K_{12}^2 K_{24}}{s_1 c_1 + 2 s_2 c_1^2 K_{12} + 4 s_4 c_1^4 K_{12}^2 K_{24}} \quad (6)$$

$$s_{app} = \frac{\sum n s_n^2 c_n}{\sum n s_n c_n} = \frac{s_1^2 c_1 + 2 s_2^2 c_2}{s_1 c_1 + 2 s_2 c_2} = \frac{s_1^2 c_1 + 2 s_2^2 c_1^2 K_{12}}{s_1 c_1 + 2 s_2 c_1^2 K_{12}} \quad (7)$$

The value of K_{12} was initially estimated through a nonlinear fit of the sedimentation velocity data for the wild-type CPS in the presence of UMP using eq 7, where the formation of the tetramer was neglected. A fixed value of 7.5 S, obtained by averaging the $s_{20,w}$ values from the sedimentation velocity data of the N987D and L421E/N987D mutants, was used for s_1. A fit of the data presented in Figure 4 for the wild-type enzyme in the presence of UMP yielded values of 10.8 ± 0.2 S for s_2 and $(3.7 \pm 1.1) \times 10^5$ M for K_{12}. These values were then used to fit the $s_{20,w}$ profiles for the ornithine- and IMP-bound forms of CPS by using eq 6. The minimization of the sum of squared residuals occurred at $s_4 = 16.4$ S and $K_{24} = 6 \times 10^6$ M for IMP and 3×10^6 M for ornithine. When sedimentation profiles for the wild-type enzyme in the absence of added effector were fit to eq 6, the value of K_{24} was found to be equal to 2×10^4 M. Thus, ornithine and IMP increase the value of K_{24} by ~100-fold relative to the situation when no effector is bound to the allosteric domain. UMP must decrease the value of K_{24} significantly since there was no evidence to indicate that a tetramer could form at the highest protein concentrations attainable.

The values of the sedimentation coefficient were also measured for the L421E mutant in the presence and absence of added allosteric effectors. As with the wild-type CPS in the presence of UMP, K_{12} and s_2 were estimated by fitting the data to eq 7. In the absence of added allosteric effectors the value of s_2 was 10.9 ± 0.3 S, whereas in the presence of ornithine, UMP, or IMP the values were found to be 11.0 ± 0.6 S, 11.6 ± 0.4 S, and 11.7 ± 0.3 S, respectively. The average value for s_2 was 11.3 ± 0.4 S. In the absence of

added allosteric effectors the value of K_{12} for the L421E mutant was $2.0 \pm 0.8 \times 10^5$ M. In the presence of ornithine, UMP, or IMP, the values for K_{12} were found to be $(1.6 \pm 0.9) \times 10^5$, $(1.2 \pm 0.4) \times 10^5$, and $(3.5 \pm 1.4) \times 10^5$ M, respectively. The average value of K_{12} for the L421E mutant was $(2.1 + 1.1) \times 10^5$ M. Within experimental error, the equilibrium constant for the dimerization of CPS was independent of the presence or absence of allosteric effectors bound to the enzyme.

Anderson has previously proposed a model where active and inactive monomers are in thermodynamic equilibrium with one another (*8*, *9*, *11*). Our results are consistent with the model proposed by Powers et al. in which the UMP-induced dimer had an elongated shape (*10*). Nevertheless, we have found no evidence to support any model that suggests that the various oligomeric forms of CPS have different catalytic activities. It appears that the kinetic effects exhibited by the allosteric ligands, through conformational changes at the active sites, are separate from the conformational changes that modulate alterations in the state of oligomerization. There are, however, examples of other oligomeric proteins where the specific catalytic activity is modulated through changes in the state of association. Thus, when the tetrameric form of rabbit muscle phosphofructokinase dissociates to a dimer, the specific catalytic activity is reduced by at least 20-fold (*18*). The opposite trend is observed with phosphorylase *a* where the specific catalytic activity of the dimer is about five times greater than the tetramer at 25 °C (*19*).

Potential Metabolic Role for Oligomerization. Our experimental observation of an insignificant dependence of catalytic activity on the state of association of CPS suggests a potential metabolic role for the oligomerization of CPS. The intracellular concentration of CPS in *E. coli*, estimated to be 1 mg/mL (*20*), implies that the oligomerization of CPS may have physiological significance. Recently, the channeling of carbamoyl phosphate in prokaryotes, especially among hyperthermophilic organisms such as *Pyrococcus furious* (*21*), *Thermus* ZO5 (*22*), and *Pyrococcus abyssi* (*23*), has been reported, where the protection of the thermolabile carbamoyl phosphate is particularly crucial. These reports have suggested the formation of a direct molecular complex between CPS and either ATCase and/or OTCase as a mechanism for metabolic channeling of carbamoyl phosphate from one enzyme to another. If channeling is operative in *E. coli*, then it is likely that the quaternary structure of CPS would play an important role in the formation of a complex with either ATCase or OTCase. Since separate compartments for the biosynthesis of pyrimidine and arginine are unavailable in *E. coli*, then the partitioning of carbamoyl phosphate would have to be achieved by an alternate mechanism. This may be accomplished by modulating the formation of a protein−protein complex. For example, the tetrameric form of CPS may have a higher affinity for OTCase than for ATCase when the concentration of ornithine achieves a certain concentration in cells. A greater fraction of carbamoyl phosphate would then be available for the synthesis of arginine. However, channeling of carbamoyl phosphate in *E. coli* has not been reported.

Summary. CPS from *E. coli* has been shown to undergo oligomerization to higher ordered species that is dependent on the protein concentration and the binding of ligands to the allosteric domain of the large subunit. The ($\alpha\beta$) monomer forms a dimer with an association constant of $\sim 3 \times 10^5$ M that is essentially independent of the binding of allosteric ligands to the large subunit. The dimerization occurs at the interface between two pairs of allosteric domains. The end-to-end $(\alpha\beta)_2$ dimer associates to an $(\alpha\beta)_4$ tetramer through interfacial contact of homologous pairs of oligomerization domains. The formation of the tetramer is suppressed by the binding of UMP whereas the binding of either IMP or ornithine enhances tetramer formation ~100-fold. The catalytic activity is dependent on the presence or absence of specific allosteric ligands but independent of the oligomeric state of the protein. The allosteric effects on catalysis are thus independent of the effects on the state of oligomerization for CPS.

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