Unmasking a Functional Allosteric Domain in an Allosterically Nonresponsive Carbamoyl-phosphate Synthetase*

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Although carbamoyl-phosphate synthetases (CPSs) share sequence identity, multidomain structure, and reaction mechanism, they have varying physiological roles and allosteric effectors. Escherichia coli CPS (eCPS) provides CP for both arginine and pyrimidine nucleotide biosynthesis and is allosterically regulated by metabolites from both pathways, with inhibition by UMP and activation by IMP and ornithine. The arginine-specific CPS from Saccharomyces cerevisiae (sCPS), however, apparently responds to no allosteric effectors. We have designed and analyzed a chimeric CPS (chCPS, in which the C-terminal 136 residues of eCPS were replaced by the corresponding residues of sCPS) to define the structural basis for the allosteric nonresponsiveness of sCPS and thereby provide insight into the mechanism for allosteric selectivity and responsiveness in the other CPSs. Surprisingly, ornithine and UMP each had a significant effect on chCPS activity, and did so at concentrations that were similar to those effective for eCPS. We further found that sCPS bound both UMP and IMP and that chCPS bound IMP, although none of these interactions led to changes in enzymatic activity. These findings strongly suggest that the nonresponsive sCPS is not able to communicate occupancy of the allosteric site to the active site but does contain a latent allosteric interaction domain.

Carbamoyl phosphate (CP)1 is a high energy phosphate compound that plays a key role in the introduction of both ammonia and single carbon units into the metabolic pool. CP formation is catalyzed by carbamoyl-phosphate synthetase (CPS) and the labile metabolite is subsequently utilized in two distinct biosynthetic pathways, one producing pyrimidine nucleotides and the other producing arginine and/or urea (1). Feedback inhibition and feed forward activation serve as major control features for almost all CPSs (2). Elucidating the structural basis for this allosteric regulation and for its varying specificity to fit the different physiological roles of CPSs is critical for understanding the overall structure/function relationship for CPSs and for potentially altering their activity in pathological conditions.

Escherichia coli and other enteric bacteria have a single enzyme that provides CP for both arginine and pyrimidine biosynthesis. E. coli CPS (eCPS) is allosterically regulated by metabolites from both pathways. Pyrimidine nucleotides, with UMP the most effective, serve as feedback inhibitors for eCPS (3, 4). Because coordinated synthesis of purine and pyrimidine nucleotides is necessary for nucleic acid synthesis, purine nucleotides, with IMP the most effective, serve as allosteric activators for eCPS (3, 4). Ornithine, the co-substrate with CP for the subsequent arginine pathway enzyme ornithine transcarbamoylase, is also an allosteric activator for eCPS (4, 5).

The arginine-specific CPSs from Saccharomyces cerevisiae, Bacillus subtilis, and Neurospora crassa are unique in their apparent total lack of allosteric response (6–8). The regulation of the S. cerevisiae CPS (sCPS) has been especially well studied in vivo and in vitro and tight regulation at both the transcriptional (9, 10) and translational (9, 11) levels have been demonstrated. Despite extensive screening, however, there have been no allosteric regulators identified so far, and it is clear that sCPS does not respond to any of the known regulators for other CPSs (6–8). To define the structural basis for this allosteric nonresponsiveness and thereby provide insight into the mechanism for allosteric selectivity and responsiveness in the other CPSs, we have, in the present work, designed and analyzed a chimeric CPS (chCPS) in which the C-terminal 136 residues of eCPS were replaced by the corresponding segment of sCPS. We have found that the chimera retains partial allosteric response, indicating that while the nonresponsive sCPS contains a latent allosteric interaction domain, it is not able to communicate occupancy of the allosteric site to the active site.

EXPERIMENTAL PROCEDURES

Materials—Oligonucleotides were synthesized at the Tufts University Analytical Core Facility. Restriction and modifying enzymes were from New England BioLabs or Roche Molecular Biochemicals. Pfu DNA polymerase, Edinburgh minimal medium, and yeast extract plus supplements were obtained from Stratagene. Wizard PCR preparation and Miniprep kits were obtained from Promega. Isopropyl-β-D-thiogalactoside was from Gold BioTechnology, Inc. Ornithine, IMP, and AGA were purchased from Fuka. All other nucleotides, carbamoyl glutamate, and ornithine transcarbamoylase were purchased from Sigma. [8-3H]IMP (6.5 Ci/mmol), L-[2,3-3H]ornithine (38 Ci/mmol), and [5-3H]UMP (19 Ci/mmol) were purchased from Moravek Biochemicals, Inc.

Plasmids, Strains, and Growth Conditions—The plasmid pUCABI, encoding both the small and large subunits of eCPS as well as ornithine transcarbamoylase, was provided by Mendel Tuchman (Washington Children’s Hospital); in this plasmid, expression of eCPS was placed under the control of the trc promoter and the second amino acid of the small subunit was changed from isoleucine to valine (12). Yeast shuttle vector pRS315 was a gift from Phil Hieter (Johns Hopkins University). Cloned CPA2 (coding for the large subunit of sCPS) was provided by Carol Lustig (New York Public Health Research Institute) and our laboratory previously cloned the gene into pH820 (15). The plasmid pER81, which expresses both the catalytic and regulatory chains of E. coli ATCase, and the E. coli strain EK1104, from which the genes encoding these subunits have been deleted, were gifts from Evan R.
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The e. coli strain XLI-Blue was used for transformation and propagation of plasmid DNA. E. coli strain L673, which lacks both CPS subunits and is defective in the Lon protease, was generously provided by Carol Lusty. The CPA2-disrupted S. cerevisiae strain LPL26 and S. pombe strain SP-Q01 were used, respectively, for functional analyses and expression of sCPS.

E. coli strains L673 and XLI-Blue were grown in Luria-Bertani or Terrific broth, as described by Sambrook et al. (YES) or minimal medium (EMM) supplemented with 5 mM thiamine to induce expression.

Recombinant DNA Methods—Bacterial transformations and recombinant DNA techniques were carried out as described in Sambrook et al. (15). Site-directed mutants were generated using the QuikChange method (Stratagene) or by the recombinant PCR method (18). Each mutagenesis cassette was sequenced to verify that no undesired changes were incorporated into the nucleotide sequence.

To create pES (the expression plasmid for chCPS), a 523-bp fragment encoding domain D of sCPS was amplified by PCR (pRS315/CPA2 as template, primer P1 introduced a Spel site at nucleotide 3654 and P2 introduced a HindIII site and removed the existing SpeI site at nucleotide 3154) and digested with HindIII and SpeI to give a 506-bp fragment. A unique SpeI restriction site was introduced into pUCABI at the junction between eCPS C and D domains (nucleotides 5458, primers P3 and P4). Digestion of the modified pUCABI with HindIII and SpeI removed the 1468-bp fragment coding for domain D of eCPS and ornithine transcarbamoylase (also present in the original expression vector) and allowed ligation of the 506-bp CPA2 fragment. Mutagenic primers P5 and P6 eliminated the SpeI site from the final construction and yielded pES.

CPS Expression and Purification—eCPS and chCPS were expressed in E. coli strain L673 as described (19). The purification protocol for eCPS and chCPS was adapted from one developed for recombinant eCPS (20) and was carried out as described (19). sCPS was expressed from the plasmid pRS315 in S. cerevisiae and crude extract was prepared as described (15). To enhance the yield of sCPS and to facilitate its purification, sCPS was expressed in S. pombe as a glutathione S-transferase (GST) fusion protein. The sCPS coding region was subcloned from pRS315 to the expression vector pESP-1 to yield expression plasmid pESP-S that encodes a 151-kDa protein in which the 27-kDa GST domain is fused to the 124-kDa sCPS. The construct was verified by restriction mapping, DNA sequencing, and expression analysis. The sCPS fusion protein was expressed in S. pombe and purified by affinity chromatography according to the protocol provided by the manufacturer (Stratagene).

Enzyme Assays and Protein Analysis—CP synthesis was determined in a two-step assay by coupling the CPS reaction to that of ornithine transcarbamoylase and then quantitating the resulting citrulline (20). The reaction mixtures contained 50 mM Hepes, 10 mM ATP, 5 mM excess of MgSO4 over the total concentration of all nucleotides, 100 mM KCl, 20 mM NaHCO3, 10 mM glutamine (or 300 mM NH4Cl), 1 mM dithiothreitol, 5 mM ornithine, and 0.2 unit of ornithine transcarbamoylase. The reaction was terminated by the addition of cold PBS (pH 7.6, 37 °C, 100 μl final volume). After varying times of incubation, citrulline was determined colorimetrically with diacylmonoxime, as previously described (21). To compare CP synthesis in the absence and presence of ornithine, the CPS reaction was coupled to the aspartate transcarbamoylase reaction and the amount of carbamoyl aspartate was determined (14). Analysis of the 0.684 mg of aspartate transcarbamoylase used for substitution of ornithine and ornithine transcarbamoylase in the reaction mixtures and the diacylmonoxime treatment was as described by Prescott and Jones (22). ATCase used in this assay was expressed from the EGER1 plasmid in E. coli strain EK1104 and purified as described (14).

ATPase activities were determined by using a pyruvate kinase/lactate dehydrogenase coupled assay to follow cleavage of ATP (19). To determine bicarbonate-dependent ATPase activity, no ammonia source was added to the assay mixture. To determine ammonium-dependent ATPase activity, 300 mM NH4Cl was included in the reaction mixture and to determine the dependence of glutamine-dependent ATPase activity, 10 mM glutamine was included in the mixture. ATP synthesis from ADP and CP was determined by a hexokinase/glucose-6-phosphate dehydrogenase-coupling system (19).

Protein concentrations were measured using the dye binding assay of Bradford (25) with bovine serum albumin as the standard or from A280 with 1 mg/ml eCPS yielding an E280 of 0.855 (21). Proteins were separated on 4–15% Tris-HCl criterion SDS-PAGE gels, following the protocol recommended by the manufacturer (Bio-Rad) and stained with Coomassie Brilliant Blue R-250.

Ornithine, UMP, and IMP Binding—Binding of ornithine, UMP, and IMP to CPSs was determined in a radiometric assay as previously described (20, 26). 100 μM of CPS was allowed to interact for 10 min at 25 °C with 0.1 mM [3H]-IMP (0.44 Ci/mmol), 0.5 mM l-(12,3-H)ornithine (18 mCi/mmol), or 0.05 mM [5-3H]IMP (0.8 Ci/mmol) in 50 mM Hepes, pH 7.6 (final volume, 0.1 ml). Enzyme-bound ligand was separated from free ligand on NICK™ columns (Amersham Biosciences, Inc). The column effluent was added to 10 ml of ScintiSafe Plus 50% (Fisher), and counts were recorded in a Beckman model LS6500 scintillation counter.

Circular Dichroism (CD) Spectra—CD spectra were obtained at room temperature on a Jasco J-715 spectropolarimeter. The concentrations of eCPS and chCPS were 0.7–1.6 μM for far UV analysis and 7–10 μM for near UV analysis. The proteins were placed in 0.01 m potassium phosphate, pH 7.6, containing 0.15 mM sodium fluoride by centrifugal desalting on Sephadex G-50 (27) and the solutions were filtered (0.2-μm low protein binding filters, Gelman Sciences). Spectra were recorded at 100 nm/min in the spectral range from 200 to 250 nm and at 10 nm/min in the spectral range 250 to 325 nm, using 0.1-cm path length cuvettes. The protein spectra were corrected by subtracting the spectrum of the buffer solution before converting the data to molar ellipticities.

Data Analysis—Kinetic data were collected on a Beckman DU 640 spectrophotometer and were fit by nonlinear regression (Grafit, version 5.01; Beckman Enzyme Mechanism software) to the equation: v = Vmax[S/(Km + S)], where v is the initial velocity, Vmax is the maximal velocity, S is the substrate concentration, and Km is the Michaelis constant. The multiple sequence alignment was produced by the Protein Information Resource (pir.georgetown.edu) and the three-dimensional visualization of the eCPS crystal structure (Swiss Protein Data base accession number 1CE8; Ref. 28) by Swiss-Model (GlauxSmithKline).

RESULTS AND DISCUSSION

Design of an E. coli/S. cerevisiae chCPS—Although CPSs vary in physiological role and mode of regulation, all catalyze the same overall reaction: formation of CP, Pi, and two ADP from HCO3-, ammonia (free or cleaved from glutamine), and two ATP. Additionally all CPSs appear to share a multidomain structure that is best defined in the solved crystal structure (29, 30) of eCPS. Domain A, which exists as a separate 95-kDa amidotransferase subunit in both eCPS and sCPS, is responsible for the binding and cleaving of glutamine to yield ammonia (1). The ~120-kDa eCPS synthetase subunit, which is responsible for binding all other substrates and for catalyzing all other reactions (1), is comprised of four domains. Domains B and C (403 and 382 residues, respectively) are regions of internal duplication (31) and each domain contains one ATP binding site (29, 30). The 149-residue domain D is involved in oligomerization (29, 30, 32).

Domain D, the ~160-residue C-terminal region of the eCPS synthetase subunit, termed the “allosteric domain” (29, 30), is critical for CP synthesis (13), and is also involved in oligomerization (29, 30). IMP binding has been localized to domain D by photoaffinity labeling (33), site-directed mutagenesis (34–39), and crystal structure analysis (28). UMP binding has also been localized to domain D by photoaffiliation (40) and site-directed mutagenesis (35–39). Studies with a number of UMP and IMP

1 Primers (with mutated residues in bold): P1, 5′-AATGACACAGCTATAGCATTACC-3′; P2, 5′-GCCGGCGCTGTCGCTGC-3′; P3, 5′-GGGCGCAGAACATGCTGAGAAG-3′; P4, 5′-CTTCATGGA-CATGTTGCTGCC-3′; P5, 5′-GCGCTGCGGCAACATCTTCCATGTACACTAC-3′; P6, 5′-GTAGTGGTACATGGGAAGTGTT-TGCTGTCGCGACTG-3′.
analogs, especially dUMP, have suggested that IMP and UMP bind to overlapping sites (41). Ornithine binding has been localized to the interface of the C/D domains by crystal structure analysis (29, 30) and site-directed mutagenesis (35, 36, 42, 43). Domain D also appears to be the allosteric domain for the other CPSs that have been studied thus far (44, 45).

Domain boundaries for construction of chCPS were based on the solved eCPS crystal structure and on sequence alignment, with domain C of eCPS 39% identical to that of sCPS and domain D of eCPS 26% identical to that of sCPS (46). In the eCPS crystal structure (29, 30), domain D starts at residue 937 and extends to the C-terminal end of the protein (residue 1073). Sequence alignment indicated that the corresponding region in sCPS extends from residue 959 to the C-terminal end of the protein (residue 1118). Therefore, for the present studies, we created an expression vector pEs to yield chCPS in which the separate eCPS 41-kDa amidotransferase subunit is retained unchanged and in which residues 937–1073 of the eCPS synthetase subunit are replaced by residues 959–1118 of the sCPS synthetase subunit.

We also constructed the complementary chimeric CPS in which residues 1–958 were derived from the sCPS synthetase subunit and residues 937–1073 from the eCPS subunit. However, this construction yielded inactive CPS when expressed in *S. cerevisiae* (using the pRS315 expression vector used for sCPS expression) or in *E. coli* (using the pUCAB expression vector used for eCPS and chCPS expression). The only other chimeric CPS construction of which we are aware was designed on the basis of limited proteolysis data because the eCPS crystal structure was not yet available and was comprised of the first 901 residues of the eCPS synthetase subunit fused to residues 1273–1461 of the pyrimidine-specific hamster CPS synthetase subunit (47). This construction was active and its analysis showed that domain D of the hamster CPS was sufficient to confer on the chimera the hamster CPS response to allosteric nucleotides.

**Expression, Purification, and Characterization of Wild Type and Chimeric CPSs**—To determine whether the chCPS construction could produce CP, its ability to functionally complement *E. coli* strain L673 was assessed. L673 is deficient in eCPS and is therefore dependent for growth on the presence of arginine and uracil in the medium or on transformation with a plasmid carrying a functional CPS (13). L673 cells expressing either eCPS or chCPS grew at approximately the same rate on solid minimal medium, indicating that chCPS was able to effectively catalyze the synthesis of CP.

eCPS and chCPS were expressed in L673 and purified as described under “Experimental Procedures.” The purity of the preparations was assessed by SDS-PAGE (Fig. 1). This analysis also revealed the expected subunit composition for the purified proteins, with the same 41-kDa amidotransferase subunit for each protein and with 121- and 118-kDa synthetase subunits for chCPS and eCPS, respectively. Comparison of circular dichroism spectra of eCPS and chCPS revealed no significant differences and the spectra were very similar to that previously reported for eCPS (20).

The kinetic parameters of chCPS were assessed by previously reported assays (24) that reflect the utilization of only ATP_{B}, only ATP_{C}, or both molecules of ATP (Table I). In the bicarbonate-dependent ATPase reaction, which reflects usage of only ATP_{B}, chCPS displayed behavior very similar to that of eCPS. The chCPS *K_{m}* for ATP_{B} was about twice that of eCPS and the chCPS *V_{max}* was about half of the eCPS value. In the ATP synthesis reaction, which reflects usage of only ATP_{C}, similar *ATP_{C} K_{m}* values were observed for chCPS and eCPS, with the former value 1.5 times greater than the latter. There was a larger distinction in *V_{max}* for this partial reaction, with chCPS displaying a 5.6-fold reduction. In the glutamine-dependent ATPase reaction, which reflects the coupled utilization

![Figure 1](image-url)  
**Fig. 1.** SDS-PAGE of purified eCPS, chCPS, and sCPS. Proteins were separated on a 4–15% gel and stained with Coomassie Brilliant Blue R-250. eCPS and chCPS each have a ~40-kDa eCPS amidotransferase subunit (corresponding well to the value of 41,431 calculated from the amino acid sequence). The apparent size of the GST/sCPS fusion protein is 140 kDa, corresponding well to the calculated molecular mass of 151 kDa (27-kDa GST and 124-kDa sCPS); the amidotransferase subunit is not included in this construction.
of both ATP molecules to form CP, the $K_m$ for ATP was very similar for chCPS and eCPS. Also, chCPS displayed a ratio of CP produced to ATP utilized close to the 1:2 value for eCPS. With glutamine as the nitrogen source, chCPS produced 1 μmol of CP for each 2.88 ATP cleaved and, with ammonia as a nitrogen source, chCPS produced 1 μmol of CP for each 2.30 ATP cleaved. However, the chimera $V_{\text{max}}$ for the overall reaction was the kinetic parameter most distinct from eCPS parameters, with a 23-fold reduction. Domain D is known to be critical for enzymatic activity (13, 35) and might participate in conformational changes at the active site and/or might function to exclude water from the active site (13). Although the overall architecture of eCPS and sCPS appears to be conserved, allowing the construction of an active chimera, the folding of domain D of sCPS cannot be expected to precisely replicate that of the 26% identical eCPS domain D, with alterations presumably decreasing the catalytic effectiveness. Because allosteric effectors bound at domain D primarily affect ATPC, and apparently thereby affect the overall activity (1), alterations of domain D would be expected to have parallel effects on the assays that reflect usage of ATPC only and usage of both ATPs. However, this was not the case in the present study (Table I) nor in previous studies with eCPS (35). Alterations in domain D have a larger effect on the ability of the enzymes to catalyze the overall coupled reaction than on their ability to carry out the ATPC partial reaction, indicating that the interactions between domain D and the ATP sites that are required for the allosteric response are not identical to the interactions involved in the catalytic events.

**Screening of Potential Allosteric Effectors with chCPS**—The known allosteric effectors for CPSs vary with the physiological role of the corresponding CPS. Higher organisms have two CPSs, with each dedicated to either the pyrimidine nucleotide or arginine/urea pathway. These pyrimidine-specific CPSs are allosterically inhibited by pyrimidine nucleotides (48–50), with UTP the most effective (in contrast to eCPS where UMP is the most effective) and a subset of them are also allosterically activated by 5-phosphorylribosyl 1-pyrophosphate (a substrate for purine nucleotide biosynthesis). Alternatively, the arginine/urea-specific CPSs require the allosteric activator N-acetylglutamate (AGA, which serves as a sensor of excess amino acids to be detoxified by the urea cycle; Ref. 51).

Compounds known to be allosteric effectors for any of the CPSs (AGA, IMP, ornithine, 5-phosphorylribosyl 1-pyrophosphate, UMP, and UTP) were screened for effects on the glutamine-dependent ATPase activity of chCPS and eCPS. Neither chCPS nor eCPS showed any response to the addition of AGA (5 mM) or 5-phosphorylribosyl 1-pyrophosphate (0.1–1 mM). In contrast to eCPS, chCPS failed to show any response to IMP (Fig. 2A). However, two of the eCPS effectors (UMP and ornithine) had a significant effect on chCPS activity, and did so at concentrations that were similar to the concentrations effective for eCPS (Fig. 2A and B). The response to the addition of these allosteric effectors was smaller for chCPS than for eCPS. The addition of ornithine resulted in a 1.6-fold maximal increase in chCPS activity versus a 4.4-fold maximal increase in eCPS activity. The addition of UMP caused a 30% decrease in chCPS activity versus a 72% decrease for eCPS. The chCPS response to ornithine was not surprising because ornithine is known to bind at the domain C/D interface (29, 30, 35, 36, 42, 43), and because domain C of chCPS is derived from the ornithine-responsive eCPS. However, the chCPS response to UMP was unexpected because the binding site for pyrimidine nucleotides resides entirely in domain D (29, 30, 35–40). We therefore screened a second pyrimidine nucleotide, UTP, which is known

**Table II**

| CPS | Bound dpm determined | mmol ligand/mmol CPS |
|-----|----------------------|----------------------|
|     | $[^{3}H]UIMP$ | $[^{3}H]IMP$ | $[^{3}H]Ornithine$ | $[^{3}H]UIMP$ | $[^{3}H]IMP$ | $[^{3}H]Ornithine$ |
| eCPS | 10705 | 7350 | 2176 | 8.3 | 11.6 | 85.1 |
| chCPS | 3584 | 2335 | 1021 | 3.6 | 4.3 | 47.3 |
| sCPS | 2766 | 2054 | 143 | 1.7 | 2.4 |
to inhibit eCPS but only at 100 times higher concentrations than required for UMP (3). As shown in Fig. 2B, similar concentrations of UTP yielded similar effects for chCPS and eCPS, with 42 and 55% decreases in activity, respectively. Because the eCPS sites for UMP/UTP and IMP appear to overlap (41), and because UMP/UTP affected chCPS activity, we tested additional conditions for effects of IMP on chCPS. Uniquely among the CPS allosteric effectors, the IMP effect on eCPS is dependent on temperature, with weak inhibition observed at 25 °C in contrast to the weak activation observed at 37 °C (52). Incubation of chCPS with IMP at 25 °C, however, yielded the same absence of response that was observed in the 37 °C incubation. It thus appears that the modest IMP response of eCPS is not reflected in chCPS, but that the more robust UMP and ornithine responses of eCPS do have chCPS counterparts.

The allosteric nonresponsiveness of sCPS to metabolic intermediates and end products of the arginine/urea and pyrimidine nucleotide pathways has been observed in a number of laboratories using a variety of biochemical and genetic probes (6–10). However, given our unexpected findings with chCPS, we expressed sCPS from the plasmid pRS315 in S. cerevisiae (13), and confirmed (using the glutamine-dependent CP synthesis assay procedure described under “Experimental Procedures”) that its activity is not affected by the compounds (IMP, UMP, and ornithine) that serve as allosteric effectors for eCPS. To directly test this hypothesis, we examined the allosteric effectors for eCPS as a positive control for the sCPS and eCPS (Table I). The effects on eCPS were as previously reported (1). The chCPS allosteric responses to ornithine and UMP followed the same trend as eCPS but were generally smaller. For the overall reaction (glutamine-dependent ATPase), addition of ornithine caused a 10-fold decrease in the eCPS $K_{\text{m}}$ and a 3-fold decrease in the chCPS $K_{\text{m}}$ whereas addition of UMP led to equivalent 1.6–1.7-fold increases in $K_{\text{ATP}}$. The effects of ornithine and UMP on ATPC usage (determined in the ATP synthesis assay) were much stronger in eCPS than chCPS. The effects of ornithine and UMP on the utilization of only ATPB (as determined in the bicarbonate-dependent ATPase assay) were similar for eCPS and chCPS but did not appear to reflect the primary regulatory role of these effectors because decreases in both the $K_{\text{m}}$ and $V_{\text{m}}$ for ATPB were observed in the presence of both ornithine and UMP.

**Binding of Allosteric Effectors to eCPS, sCPS, and chCPS**—For eCPS, domain D is known to be the sole (IMP/UMP) or partial (ornithine) locus for binding allosteric effectors (29, 30, 33–42). Occupancy of the effector site is then communicated principally to the ATPC active site and secondarily to the ATPB active site (1). The present findings strongly suggest that the nonresponsive sCPS can bind UMP (and possibly ornithine) at domain D but cannot communicate occupancy of the allosteric site to the active site(s). When the sCPS domain D is placed in the chimeric context, however, it is able to at least partially carry out the communication between allosteric and active sites.

To directly test this hypothesis, we examined the allosteric effector binding abilities of chCPS and sCPS. The synthetase subunit of sCPS was expressed as a GST fusion protein to enhance the yield of sCPS and to facilitate its purification, and the amidotransferase subunit of sCPS was omitted from this construction. It was not possible to cleave the GST moiety because the CPS moiety is also subject to partial thrombin proteolysis. However, the purified fusion protein had a specific activity of 0.64 μmol of ATP cleaved per min/mg (25 °C) in the ammonia-dependent ATPase assay, consistent with the reported value for sCPS (53) of 1.21 μmol of ATP cleaved per min/mg (37 °C). We used eCPS as a positive control for the binding studies. To minimize potential nonspecific binding (e.g., binding of the nucleotides to either of the ATP sites), we...
utilized concentrations of UMP and ornithine (0.05 and 0.5 mM, respectively) that yielded a half-maximal effect on chCPS activity and the concentration of IMP (0.1 mM) that yielded a half-maximal effect on eCPS activity. eCPS displayed low but significant binding of all three effectors (Table II). Presumably the low levels of binding result from the limiting ligand concentrations utilized and from rates of ligand dissociation that are rapid relative to the free/bound ligand separation protocol.

The observation of significant binding of UMP and ornithine by chCPS (Table II) is expected given the demonstration that these ligands can alter the activity of chCPS (Fig. 2). The stoichiometry of UMP binding was 2.3-fold less for chCPS than for eCPS, consistent with the observation (Fig. 2) that the maximal effect of UMP on chCPS glutamine-dependent ATPase activity was 2.4-fold less than the maximal effect of UMP on eCPS activity. The stoichiometry of ornithine binding was 1.8-fold less for chCPS than for eCPS, consistent with the 2.8-fold lower ornithine effect on chCPS activity than on eCPS activity. The apparent inability of scPS to bind ornithine (Table II) strongly suggests that the eCPS moiety of chCPS is entirely responsible for the interaction of the chimera with ornithine. The findings (Table II) that scPS bound both UMP and IMP and that chCPS bound IMP were unexpected because none of these interactions had been revealed by effects on enzymatic activity.

**CPS Residues Interacting with Allosteric Effectors**—eCPS residues interacting directly with ornithine or IMP have been identified from crystal structures (28–30) and their extent of conservation in chCPS is shown in Fig. 3. Fig. 4A presents the three-dimensional arrangement of these eCPS residues and Fig. 4B provides a visualization of the structural changes in the ornithine and IMP sites that occur in chCPS. eCPS has not been crystallized in the presence of UMP. However, a number of studies strongly suggest that the IMP and UMP sites overlap (41).

The main core of eCPS domain D is a modified "Rossmann"-fold that is characterized by a five-stranded parallel β-sheet flank ed on either side by two and three α-helices, respectively. Each of these α-helices ends in a type-1 turn. The ornithine binding pocket is formed by the fifth β-strand and the fifth α-helix of domain D and three β-strands from domain C (29, 30). The carboxylate and α-amino groups of ornithine interact with domain D, forming hydrogen bonds with O1 of Thr-1042 and with O of Tyr-1040, respectively. The α-amino group of ornithine interacts with domain C, forming hydrogen bonds with O1 of Glu-783, O of Asp-791, and O1 of Glu-892. In confirmation of these proposed roles, site-directed mutation of Glu-783, Asp-791, and Glu-892, and Tyr-1042 yielded greatly diminished binding of ornithine without significantly affecting UMP/IMP binding (42, 43).

In chCPS (which binds and responds to ornithine), the domain C residues are maintained and therefore all of the interactions with the α-amino group of ornithine (Glu-783, Asp-791, and Glu-892) are maintained. However, in scPS (which fails to bind ornithine), one of these hydrogen bonding groups is lost because of the substitution of Glu-892 by valine. This substitution presumably explains the absence of ornithine interaction in scPS. In domain D of chCPS, Thr-1042 is substituted by asparagine, which also has hydrogen bonding potential but might be spatially arranged so that the bonding to the carboxylic acid group of ornithine is weakened or possibly eliminated. The additional substitution of Tyr-1040 by leucine in chCPS eliminates the hydrogen bonding to the α-amino group of ornithine. These alterations of Thr-1042 and Tyr-1040 in the chimera could well account for the decreased binding of ornithine relative to eCPS.

In eCPS, IMP is localized to the C-terminal end of the domain D β-sheet and is completely contained within domain D (29, 30). Four eCPS residues appear to interact with the phospho moiety of IMP: Lys-954, Thr-974, Thr-977, and Lys-993. In chCPS, two of these are conserved (Thr-974 and Thr-977) and Lys-954 is conservatively substituted by arginine. However, the positive charge of Lys-993 is eliminated by a valine substitution, which also introduces novel hydrophobic character at that site. Five eCPS residues appear to interact with the IMP hypoxanthine ring: Val-994, Ile-1001, Asp-1025, Val-1028, and Ile-1029. In chCPS, three of these are conservatively substituted (leucine replaces Ile-1001, isoleucine replaces Val-1028, and methionine replaces Ile-1029). However, Val-994 is replaced by serine and Asp-1025 is replaced by valine. Both substitutions introduce significant change by interchanging a large hydrophobic side chain with a smaller polar or charged one. Four eCPS residues appear to interact with the ribose moiety of IMP: Ser-948, Asn-1015, Asn-1017, and Ser-1026. In chCPS, two of these interactions are maintained (Asn-1015 conserved and Thr-1017 conservatively substituted by serine) but two are radically altered (Ser-948 substituted by leucine and Ser-1026 substituted by aspartate). Because Ser-1026 is the only residue identified in site-directed mutagenesis studies as being more important for interaction with IMP than UMP (39), its substitution with aspartate in scPS could explain the inability of the chimera to functionally respond to the presence of IMP. The diminished UMP interaction of chCPS (binding and activity effects relative to eCPS) as well as the diminished IMP binding are most likely based on one or more of the other substitutions in residues occurring at the IMP/UMP site of eCPS. It should be noted, however, that alterations within the chCPS three-dimensional arrangement of the residues essential for allosteric interaction might also contribute to the diminished allosteric interactions.

**Regulation and Evolution of scPS**—The present data indicate that the nonresponsive scPS can bind IMP and UMP at domain D but cannot communicate occupancy of the allosteric site to the active site(s). Prior to these chimera studies, analysis of multiple CPS domain D alignments (e.g. Fig. 3) had yielded an enigma: conservation in CPSs of the residues involved in binding nucleotides at the allosteric effector site was more generalized than the ability to respond to the nucleotide effectors with a change in catalytic activity. Because the amount of scPS available is subject to several layers (9–11) of transcriptional and translational regulation (in contrast to the much less tight transcriptional regulation for eCPS), there has been no evolutionary pressure to retain allosteric regulation of CPSs. Nevertheless, scPS has retained the ability to bind IMP and UMP, presumably under pressure to maintain stability of the overall structure and/or pressure to maintain the contributions of domain D to catalytic effectiveness.

**Acknowledgments**—We thank Carol Lusty for the CPS II-deficient *E. coli* strain L673, Mendel Tuchman for the plasmid pUCABI, Phil Hieter for the plasmid pBR325, Evan Kantrowitz for the plasmid pEK81 and the *E. coli* strain EK1104, and Michael Kothe and Amna Saeed Kothe for valuable discussions.

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J. Biol. Chem. 2002, 277:45466-45472.
doi: 10.1074/jbc.M208185200 originally published online September 19, 2002

Access the most updated version of this article at doi: 10.1074/jbc.M208185200

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