Requirement for the Carboxyl-terminal Domain of Saccharomyces cerevisiae Carbamoyl-phosphate Synthetase*

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The arginine-specific carbamoyl phosphate synthetase of Saccharomyces cerevisiae is a heterodimeric enzyme, with a 45-kDa CPA1 subunit binding and cleaving glutamine, and a 124-kDa CPA2 subunit accepting the ammonia moiety cleaved from glutamine, binding all of the remaining substrates and carrying out all of the other catalytic events. CPA2 is composed of two apparently duplicated amino acid sequences involved in binding the two ATP molecules needed for carbamoyl phosphate synthesis and a carboxy-terminal domain which appears to be less tightly folded than the remainder of the protein. Using deletion mutagenesis, we have established that essentially all of the carboxy-terminal domain of CPA2 is required for catalytic function and that even small truncations lead to significant changes in the CPA2 conformation. In addition, we have demonstrated that the C-terminal region of CPA2 can be expressed as an autonomously folded unit which is stabilized by specific interactions with the remainder of CPA2. We also made the unexpected finding that, even when ammonia is used as the substrate and there is no catalytic role for CPA1, interaction with CPA1 led to an increase in the V\textsubscript{max} of CPA2 in crude extracts.

Although CPSases function to catalyze the same overall reaction (Equation 1), they differ in various properties as shown in Table I (1, 2). However, all of the CPSases thus far sequenced display marked sequence identity and similarity (5, 6). The bacterial CPSases and the arginine-specific CPSase of yeast are heterodimeric, with the small subunit (glutamine amidotransferase) binding and cleaving glutamine (Equation 2) whereas the large subunit (synthetase) accepts the ammonia moiety cleaved from glutamine, binds all of the remaining substrates and effectors, and carries out all of the other catalytic events (Equations 3–5). In the single subunit CPSases, the glutamine amidotransferase moiety is fused to the NH\textsubscript{2} terminus of the synthetase moiety. Some of the CPSases are also fused to additional enzymes involved in pyrimidine biosynthesis; syrian hamster CAD, with the structure N-CPSase-dihydrorotase-aspartate transcarbamoylase-C, is the best characterized member of this group (5, 6). CPSase I, which cannot utilize glutamine as a substrate and has no detectable glutaminase activity, also contains the NH\textsubscript{2}-terminal glutamine amidotransferase homology region but residues essential for glutamine amidotransferase activity have been replaced (7, 8). Analysis of the CPSase sequences has also shown that the synthetase moiety consists of two homologous halves that appear to have resulted from an ancestral gene duplication (9).

Each synthetase half contains the consensus primary sequence motifs identified for ATP-utilizing enzymes (9, 10). Studies utilizing ATP analog localization (4, 10–12) and site-directed mutagenesis (3) have demonstrated that each synthetase half contains a functional ATP binding site and that the NH\textsubscript{2}-terminal half binds ATP\textsubscript{1} (Equation 3) while the COOH-terminal half binds ATP\textsubscript{2} (Equation 5).

Mild proteolysis studies with rat liver CPSase I (13–15) demonstrated the existence of three independently folded globular domains corresponding to the NH\textsubscript{2}-terminal glutamine amidotransferase moiety (domain A) and to the two ATP-utilizing moieties (domains B and C), and also identified a fourth COOH-terminal structural domain (domain D). The 20-kDa domain D appears to be much less tightly folded than domains A-C since it undergoes cleavage to small peptides under the mild proteolysis conditions. The A-D domain organization determined for the rat liver CPSase I appears to be common to all CPSases since various features of it have subsequently been observed in the CPSase unit of CAD (16) and in the Escherichia coli CPSase (17).

Binding of the allosteric effectors IMP (18), UMP (17), UTP (19, 20), PRPP (19), and N-acetylglutamate (21) has been localized to domain D. Affinity labeling studies with the rat liver CPSase I (4) have strongly suggested that domain D is also involved in binding of both molecules of ATP. Domain D appears to fold back to complement the ATP-binding regions of globular domains B and C. It is possible that amino acid residues from domain D participate in catalytic processing of the ATP molecules and/or in excluding water from the active site.
although they may participate directly in ATP binding. A feasible model for the transmission of allosteric effects within CPSase would be that the binding of effectors to domain D alters the positioning of this domain at the ATP sites and thereby affects the affinity of the CPSase for ATP.

While it is known, from studies on rat liver CPSase I (13–15) and E. coli CPSase (19, 22), that elimination of the entire domain D yields inactive CPSase, the effects of less drastic C-terminal truncation of CPSase have not been determined. Several lines of evidence suggest that at least limited COOH-terminal truncation might yield stable and functional CPSase: (a) analysis of amino acid identities among the synthetase moieties of six CPSases (5) revealed 28% identical residues throughout domains B and C, but only 8% identical residues for the 191-residue domain D, with only 5 of the 167 COOH-terminal residues identical; (b) deletion analysis of the E. coli CPSase (22), aimed at defining the domains necessary for interaction between the small and large subunits, revealed that domain D was not necessary for heterodimer formation; (c) a hybrid construction with domains A-C from E. coli CPSase and domain D from Syrian hamster CAD retained catalytic activity (19); and (d) deletions would be less likely to exert long-range perturbations of the rather loosely-folded domain D structure, with its multiple sites available for proteolysis, than would be expected for a tightly folded globular domain, with no sites susceptible to proteolysis under nondenaturating conditions. To further define the role of domain D in the structure and function of CPSase, we have carried out deletion mutagenic analysis of CPA2, the large synthetase subunit of the yeast arginine-specific CPSase (9). Since CPA2 has no known allosteric effectors (Table I), its use should allow a clearer definition of the relationship between protein structure and catalytic function than would be possible if allosteric effects at domain D were also involved. Furthermore, it is possible to carry out an in vivo assay for the functioning of CPA2 deletion mutants in addition to determining their properties in vitro.

**EXPERIMENTAL PROCEDURES**

**Materials—**Oligonucleotides were synthesized at the Tufts University Analytical Core Facility. Restriction and DNA modifying enzymes were from either New England Biolabs or Boehringer Mannheim. Plasmid DNA was obtained from Stratagene. Gendecain (BIO-101) and Wizard (Promega) kits were used for the isolation of DNA fragments and for plasmid purification. Long range gel solution and (α-32P)ATP used for DNA sequencing were from J. T. Baker and DuPont NEN, respectively. Citrulline, amino acids, aprotinin, benzamidine, e-aminocaproic acid, chymostatin, p-chloromercuriphenylsulfonate, and phenanthridine were purchased from Sigma. Pefabloc and leupeptin were from Boehringer Mannheim and pepstatin was from Peptides Int.

**Strains—**The E. coli strains used for transformation and propagation of plasmid DNA were XL1-Blue and J1010. E. coli BMH71–18mutS was used for site-directed mutagenesis by the unique-site elimination method (23). BL21 (DE3) was used for overexpression of protein in E. coli (24). Saccharomyces cerevisiae strain BJ2168 (MATa prc1-407 prb1-1122 pep4-34i2 trp1 ura3-52 gal2) was used to construct the CPA2-disrupted strain LPL26 (MATa prc1-407 prb1-1122 pep4-34i2 trp1 ura3-52 gal2 CPA2::hisG).

**Plasmids—**Cloned CPA2 and CPA1 were kindly provided by Dr. Carol Lusky (New York Public Health Research Institute) on the Yep13 plasmids pL2/T5 (26) and pL113/ST4 (27), respectively. To facilitate further manipulations of cloned CPA2, pUC19/CPA2 was constructed by inserting at the Sal I site of pUC19 a 4.8-kb Sal I fragment containing the 3.36-kb CPA2 gene plus flanking sequences upstream and downstream of the CPA2 coding region (0.95 and 0.5 kb, respectively). Yeast shuttle vectors pRS315 and pRS316 (low copy number vectors with LEU and URA selection markers, respectively, 28) and pRS424 and pRS425 (high copy number vectors, with TRP and LEU selection markers, respectively, 29) were kindly provided by Dr. Philip Hieter (Johns Hopkins University) and Dr. Thomas Christianson (Southern Illinois University), respectively. pNKYS1, used for gene disruption, was the generous gift of D. Eric Alani (Dana Farber Cancer Institute). The bacterial expression plasmid pET11-d was purchased from Novagen.

**Media—**E. coli was grown in LB media (30). S. cerevisiae LPL26 was grown in one of two rich media, YEPD or SC (synthetic complete), or in SD (synthetic dextrose) minimal medium, as indicated for individual experiments. Media components are (31): (a) for YEPD, 1% yeast extract, 2% Bacto-peatone, 2% glucose; (b) for SC, 0.67% yeast nitrogen base without amino acids, 2% glucose, and 0.2% of an amino acid mixture (2 g each of the 20 common amino acids, an additional 2 g of leucine, 2 g of uracil, 0.5 g of adenine, 0.2 g of p-aminobenzoic acid); where indicated one or more of the amino acid mixture components is omitted; (c) for SD, 0.67% yeast nitrogen base without amino acids and 2% glucose; and (d) for SC, 0.67% yeast nitrogen base without amino acids and 2% glucose, plus indicated supplements (857 μg/ml each) to satisfy auxotrophic requirements for growth. For in vivo screening for CPA2 functionality, transformants were selected for ability to grow without added leucine on SC minus leucine and screened for ability to grow without added arginine on SD plus uracil, to repress inherent URA2, and plus tryptophan, since LPL26 is auxotrophic for tryptophan.

**General Procedures—**All molecular biological procedures, including agarose gel electrophoresis, restriction enzyme digestion, ligation, and bacterial transformation, were performed essentially as described by Sambrook et al. (30). Standard yeast genetic and molecular biological procedures were performed as described previously (31, 32). Yeast transformation using lithium acetate was performed according to Gietz et al. (33). In vivo homologous recombination in yeast and bacteria as alternative methods to in vitro ligation were as described by Muhle et al. (34) and Bubeck et al. (35), respectively. DNA sequences were
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Determined by diode array DNA sequencing using Sequenase version 2 and following the protocol recommended by the manufacturer (U. S. Biochemical Corp.). The fidelity of all PCR products was confirmed by sequencing.

Determination of In Vitro Enzymatic Activity and Protein Concentration—In order to attain the maximum possible units of enzymatic activity with the CPA2 deletions, they were cloned into the high copy number shuttle vector pRS424 (which has a TPR selection marker; 29) and LPL26 was co-transformed with pRS424/cpa2 and pL113/ST4 (a high copy number shuttle vector containing cloned CPA1 and a LEU selection marker; 27). The co-transformed LPL26 cells were grown from single colonies in synthetic dextrose medium plus 0.085% glutaraldehyde or uracil at 30°C until late logarithmic phase. In preliminary experiments where CPA2 activity was determined in the absence of CPA1, only pRS424/CPA2 (or the low copy number plasmid pRS315/CPA2) was used for transformation of LPL26 and 1 mg/ml arginine was added to the media to achieve repression of chromosomal CPA1 expression (36). The cells were harvested by centrifugation at 2,500 × g for 15 min at 4°C, washed once in deionized water, and pelleted as before. Cells were broken by resuspending in 2 volumes of lysis buffer (50 mM Tris/HCl, 20 mM glycine, and 2 mM EDTA, pH 8.3) at 4°C and vortexing with an equal volume of cold glass beads for four 60-s pulses, with 60-s cooling intervals between pulses. After a 15-min centrifugation at 100,000 × g at 4°C, the supernatant was placed into assay buffer (50 mM Hepes, pH 7.0) and the final pH (8.3) were as optimized for CPA2 (38). Protein concentration was determined by the method of Bradford (39).

SDS-Polyacrylamide Gel Electrophoresis and Western Blot Analysis—SDS-polyacrylamide gel electrophoresis was performed on 8% gels as described by Laemmli (40). crude extracts for electrophoretic and Western blot analysis were prepared as described above for enzymatic assay except that the following lysis buffer was used in order to minimize proteolysis: 50 mM potassium phosphate, 2 mM EDTA, 10% glycerol, 1 mM e-aminocaproic acid, 0.77 μM aprotinin, 2 mM benzamidine, 50 μM p-chloromercuriphenylsulfonate, 1.65 μM chymostatin, 6.3 μM leupeptin, 5 mM β-mercaptoethanol, 4.2 mM Pefabloc, 21.9 μM pepstatin, and 1 mg/ml phenanthroline, final pH 7.4. Aliquots of the crude extracts were added to an equal volume of 2 × gel sample buffer (68 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 5 mM β-mercaptoethanol, and 0.0005% bromophenol blue) and heated to 95°C for 5 min immediately after the bead beating step. The amounts of proteins were quantitated by scanning the gels, stained with Coomassie Brilliant Blue R-250, with a Hoefer GS300 scanning densitometer, and integrating the peaks with the Hoefer GS365W system.

For immunoblot analysis, proteins were separated by SDS-polyacrylamide gel electrophoresis and then electrophoretically transferred to a Polyvinylidene difluoride membrane (DuPont NEN) with a Hoefer TE42 unit. Transfer was conducted at 80 V for 30 min in 25 mM Tris-HCl, 192 mM glycine, pH 8.3, 25% methanol. The membrane was blocked with 5% dry milk in PBS-T (1.4 mM Na2HPO4, 8.1 mM NaH2PO4, 150 mM NaCl, 0.05% Tween 20) for 1 h at room temperature (the temperature for all subsequent immunoblot steps). After two 5-min PBS-T washes, the membrane was incubated for 1 h with a 1:10,000 dilution of the primary antibody (rabbit anti-CPA2) in PBS-T, 1% bovine serum albumin. After one 15-min and four 5-min PBS-T washes, the membrane was incubated for 1 h with a 1:3,000 dilution of horseradish peroxidase-antirabbit IgG conjugate (Bio-Rad) in PBS-T, 1% bovine serum albumin. After one 15-min and four 5-min PBS-T washes, the membrane was incubated for 1 h with a 1:3,000 dilution of horseradish peroxidase-antirabbit IgG conjugate (Bio-Rad) in PBS-T, 1% bovine serum albumin. After one 15-min and four 5-min PBS-T washes, the membrane was incubated for 1 h with a 1:3,000 dilution of horseradish peroxidase-antirabbit IgG conjugate (Bio-Rad) in PBS-T, 1% bovine serum albumin. After one 15-min and four 5-min PBS-T washes, the membrane was incubated for 1 h with a 1:3,000 dilution of horseradish peroxidase-antirabbit IgG conjugate (Bio-Rad) in PBS-T, 1% bovine serum albumin. After one 15-min and four 5-min PBS-T washes, the membrane was incubated for 1 h with a 1:3,000 dilution of horseradish peroxidase-antirabbit IgG conjugate (Bio-Rad) in PBS-T, 1% bovine serum albumin. After one 15-min and four 5-min PBS-T washes, the membrane was incubated for 1 h with a 1:3,000 dilution of horseradish peroxidase-antirabbit IgG conjugate (Bio-Rad) in PBS-T, 1% bovine serum albumin. After one 15-min and four 5-min PBS-T washes, the membrane was incubated for 1 h with a 1:3,000 dilution of horseradish peroxidase-antirabbit IgG conjugate (Bio-Rad) in PBS-T, 1% bovine serum albumin. After one 15-min and four 5-min PBS-T washes, the membrane was incubated for 1 h with a 1:3,000 dilution of horseradish peroxidase-antirabbit IgG conjugate (Bio-Rad) in PBS-T, 1% bovine serum albumin. After one 15-min and four 5-min PBS-T washes, the membrane was incubated for 1 h with a 1:3,000 dilution of horseradish peroxidase-antirabbit IgG conjugate (Bio-Rad) in PBS-T, 1% bovine serum albumin. After one 15-min and four 5-min PBS-T washes, the membrane was incubated for 1 h with a 1:3,000 dilution of horseradish peroxidase-antirabbit IgG conjugate (Bio-Rad) in PBS-T, 1% bovine serum albumin. After one 15-min and four 5-min PBS-T washes, the membrane was incubated for 1 h with a 1:3,000 dilution of horseradish peroxidase-antirabbit IgG conjugate (Bio-Rad) in PBS-T, 1% bovine serum albumin. After one 15-min and four 5-min PBS-T washes, the membrane was incubated for 1 h with a 1:3,000 dilution of horseradish peroxidase-antirabbit IgG conjugate (Bio-Rad) in PBS-T, 1% bovine serum albumin. After one 15-min and four 5-min PBS-T washes, the membrane was incubated for 1 h with a 1:3,000 dilution of horseradish peroxidase-antirabbit IgG conjugate (Bio-Rad) in PBS-T, 1% bovine serum albumin. After one 15-min and four 5-min PBS-T washes, the membrane was incubated for 1 h with a 1:3,000 dilution of horseradish peroxidase-antirabbit IgG conjugate (Bio-Rad) in PBS-T, 1% bovine serum albumin.

Fig. 1. Scheme for the construction of CPA2-disrupted protocase-deficient strain LPL26. Restriction sites used in constructing the CPA2:hisG-URA3-hisG fragment are: B, BamHI; Bg, BglII; E, EcoRI; and X, Xhol. The filled, hatched, and open boxes represent CPA2, URA3, and hisG sequences, respectively.

RESULTS

Construction of the CPA2-disrupted Proteinase-deficient Yeast Strain LPL26—The strain LPL26 (MATα prc1–407 prbl–1122 pep4–3 leu2 trpl ura3–52 gal2 CPA2:hisG) was constructed (Fig. 1) by disrupting the chromosomal copy of CPA2 in the proteinase-deficient S. cerevisiae strain B1268 (25). The gene disruption strategy developed by Alani et al. (41) was used: insertion of a hisG-URA3-hisG fragment into cloned CPA2; integrative transformation of the disrupted CPA2 fragment into the BJ2168 genome, with stable integrants selected for Ura+; and vegetative growth of the hisG-URA3-hisG integrants to allow recombination between the hisG fragments and popping out of the URA3 marker so that Ura+ selection can be used in subsequent transformations of LPL26. As shown in Fig. 1, an internal BglII fragment (0.93 kb) was removed from...
pUC19/CPA2 and replaced with a BglII-BamHI fragment (3.8 kb) containing the hisG-URA3-hisG fragment from pNKY51. A linearized 5.8-kb CPA2::hisG-URA3-hisG fragment, resulting from digestion with EcoRI and XhoI, was transformed into BJ2168. Transformants with the hisG-URA3-hisG fragment inserted in the chromosomal copy of CPA2 were selected by ability to grow in the absence of uracil. Transformant colonies were patched out on 5-fluoroorotic acid plates (31) to select for the ura3 genotype which would result from homologous recombination of the flanking hisG repeats and resultant excision of the URA3 fragment. Enzymatic and Western blot analysis of LPL26 crude extracts, as well as growth requirements for the strain (see below), confirmed the disrupted CPA2 genotype.

Construction of the CPA2 Expression Vector and Development of the Functional Complementation Screen for in Vivo CPA2 Activity—The yeast CPA2 gene was originally cloned in the YEp13 plasmid pJL2/T5 by Lusty and Lu (26). We have inserted CPA2, including the promoter region that allows independent expression in yeast, into pRS315, a YCp vector designed by Sikorski and Hieter (28) to allow highly efficient DNA manipulations, to create pAL6, a low copy number CPA2 expression plasmid. pAL6 was constructed as follows (Fig. 2):

(a) a 3.3-kb Sall-ApaI fragment of CPA2 was isolated from pUC19/CPA2; (b) a 941-base pair fragment of CPA2 extending from position 2380 (upstream of the ApaI at 2416) to the translation termination codon was amplified by PCR (with the downstream primer incorporating a BamHI restriction site) and digested with ApaI and BamHI; and (c) the fragments constructed in steps a and b were ligated to each other and to the Sall and BamHI sites in the pRS315 polylinker region.

The functional effects of deletion mutations within domain D were analyzed by in vivo screening with the LPL26 and pAL6 system. Since carbamoyl phosphate synthesized by either CPA2 or URA2 enters a common pool in yeast (42), URA2 of LPL26 was repressed and feedback-inhibited (42) by including uracil in the medium used for screening so that URA2 could not substitute for CPA2 in providing carbamoyl phosphate for arginine synthesis. Under these conditions, LPL26 is dependent for growth on the presence of arginine in the medium or on transformation with a plasmid carrying wild-type CPA2 or a functional cpa2. To determine whether cpa2 deletion constructions could produce carbamoyl phosphate, their ability to functionally complement CPA2-disrupted LPL26 was assessed. LPL26 was transformed with pAL6 or its derived deletion constructions and transformants were selected by ability to grow without added leucine. The transformants were sequenced for functional pRS315/cpa2 by ability to grow without added arginine, and extent of growth was compared to that of pAL6 transformants.

Interaction between Individually Expressed Domains B+C and D—If domain D (the COOH-terminal 191 amino acid residues of CPA2) and domains B+C (the NH₂-terminal 926 amino acid residues of CPA2) can act as independent folding units, then individually expressed domain D should associate with individually expressed domains B+C to form a stable CPA2. As outlined in Fig. 3, pRS316/AC191, designed to express domains B+C only, was constructed by PCR amplification (primers P6 and P7, Table II) of a DNA fragment extending
from the unique ApaLI site (nucleotide 2416) to the end of domain C (nucleotide 2779), with a stop codon and BamHI site incorporated in the downstream primer, and ligation of the ApaLI- and BamHI-digested PCR product with the Sall-ApaLI fragment of CPA2 and the Sall+BamHI-digested pRS316 vector. pRS315D, designed to express domain D only, was constructed by recombinant PCR, as outlined in Fig. 4. The primer pair P1 and P19 was used to amplify the promoter and riboso-
mal binding sequences upstream of CPA2, the ATG start site and the first 9 nucleotides of domain D sequence; the primer pair P18 and P9 was used for amplifying sequences encoding domain D, the ATG start site, and 9 nucleotides upstream of the start codon (complementary to sequences at positions 2782–3357 and at −9 to +3). The primary PCR products were purified and used for secondary PCR with primers P1 and P9. The secondary PCR product, containing the domain D sequence with an additional methionine residue at the NH2 terminus and also containing upstream CPA2 transcriptional and translational recognition sites, was digested with Sall and BamHI and cloned into the corresponding sites of the pRS315 polylinker region.

**Fig. 4.** Construction of low and high copy number plasmids for autonomous expression of domain D. The positions of the oligonucleotides used for primary PCR amplifications of the sequences encoding the entire domain D and the regulatory sequence upstream of CPA2 are indicated. The restriction sites that were used for cloning are: B, BamHI and S, Sall. BamHI and Sall sites are also in the pRS424 and pRS315 polylinker regions.

In vivo screening in CPA2-disrupted S. cerevisiae LPL26 was used to determine whether individually expressed domain D could associate with individually expressed domains B+C to form a stable and/or functional CPA2. Transformants of LPL26 containing pAL6 or pRS315/D were identified by ability to grow in the absence of exogenous leucine (via the pRS315 LEU selection marker) whereas transformants of LPL26 containing pRS316/ΔC191 were identified by ability to grow in the absence of exogenous uracil (via the pRS316 URA selection marker). Co-transformants of LPL26 containing both pRS316/ΔC191 and pRS315/D were identified by ability to grow in media from which both leucine and uracil were omitted. When tested for ability to produce carbamyl phosphate and thus grow in the absence of exogenous arginine, only pAL6 transformants, expressing wild-type CPA2, showed cell growth. pRS316/ΔC191 transformants, expressing domains B+C, did not show functional complementation, consistent with previous findings with rat liver CPSase I (13–15) and E. coli CPSase (19, 22) that elimination of the entire D domain yields inactive CPSase. Neither pRS316/ΔC191 plus pRS315/D co-transformants, expressing domains B+C independently of expressed domain D, nor pRS315/D transformants, expressing only domain D, could functionally complement the LPL26 cells.

Western blot analysis was carried out to confirm that the individual domains D and B+C were expressed under the conditions of the complementation assay. Although intact CPA2 was expressed from a pAL6 transformant of LPL26, and the domain B+C polypeptide was expressed from a pRS316/ΔC191 transformant and from a pRS316/ΔC191 plus pRS315/D co-transformant of LPL26, there was no detectable domain D in the crude extracts prepared from a pRS315/D transformant or from a pRS316/ΔC191 plus pRS315/D co-transformant (data not shown). In order to increase the potential yield of expressed domain D, the high copy number plasmids pRS424/D (parallel construction to that of pRS315/D, Fig. 4) and pRS425/ΔC191 (parallel construction to that of pRS316/ΔC191, Fig. 3) were utilized. When LPL26 was transformed with these high copy number plasmids and the crude extracts subjected to Western blot analysis (Fig. 5), intact CPA2 and the domain B+C polypeptide were again found to be stably expressed from their corresponding plasmids whereas domain D was not stably expressed. However, domain D, as well as the domain B+C polypeptide, was stably expressed in LPL26 co-transformed with high copy number plasmids pRS424/D and pRS425/ΔC191. In a control co-transformation where intact CPA2 and domain D were expressed independently from high copy number plasmids, there was no domain D detected in the Western blot, although wild-type CPA2 was present (data not shown). These findings very strongly suggest that there is a specific interaction between individually expressed domain D and domains B+C which can stabilize domain D. In contrast, when domain D is expressed alone or in the presence of intact CPA2, where the regions of domain D interaction on domains B+C are already occupied by the cognate domain D, the conformation of the 20-kDa domain D must be extremely susceptible to prote-
Figure 5. Western blot analysis of autonomous domain D expression. Crude extracts were prepared from LPL26 transformants grown in media containing arginine, fractionated on an SDS-12% polyacrylamide gel, and subjected to Western blot analysis, as described under "Experimental Procedures." Each lane contained 25 μg of total protein with the exception of the untransformed host cells (lane 1, 75 μg). The transformed cells contained the following plasmids: lane 2, pRS425/CPA2; lane 3, pRS425/ΔC101 CPA2 + pRS424; lane 4, pRS425 + pRS424/D; lane 5, pRS425/ΔC191 CPA2 + pRS424/D.

Figure 6. Schematic diagram of domain D deletions. The filled box of the top line corresponds to domain D of CPA2. Each deletion included the 12 nucleotides immediately preceding subsite D1-ATP (open boxes) or subsite D2-ATP (hatched boxes).
In Vitro Activity of CPA2 Truncations—Although the LPL26 screen was invaluable for observing the behavior of CPA2 deletions in a native environment, in vitro analysis was used to provide additional, complementary information on the effects of deletion on CPA2 activity. In preliminary experiments with pAL6 transformants of LPL26, only low ammonia-dependent carbamoyl phosphate synthesis activity was observed in the crude extract (0.10 nmol of carbamoyl phosphate synthesized/min/mg total protein) and the activity was even lower (0.058 nmol/min/mg of total protein) when arginine was included in the growth medium to repress CPA1 formation. In order to confirm this apparent effect of CPA1 on the enzymatic activity resident in CPA2, we varied the amounts of CPA2 present by using low or high copy number vectors and varied the amounts of CPA1 present by using only the chromosomal copy of CPA1, using a high copy number vector in addition to the chromosomal copy, or by using growth on arginine to repress expression from the chromosomal CPA1. Western blot analysis was used to determine the amount of CPA2 present in each case. The absence of CPA1 after growth in arginine and its presence after growth in the absence of arginine were confirmed by the absence or presence, respectively, of glutamine-dependent carbamoyl phosphate synthesis activity; CPA1 quantitation by Western blot was not possible since no antibodies are available. The specific activities (nanomole of carbamoyl phosphate synthesized per min/unit CPA2) for the indicated transformants were as follows: 0.28 for low copy number pAL6 (CPA1 repressed); 0.37 for pAL6 + chromosomal CPA1 expression; 0.44 for high copy number pRS424/CPA2 (CPA1 repressed); 0.60 for pRS424/CPA2 + chromosomal CPA1 expression; 0.36 for pRS424/CPA2 + pl L113/ST4 (a high copy number plasmid expressing wild-type CPA1 and a LEU selection marker; 27) grown in arginine to repress CPA1 expression from both the chromosome and the plasmid; and 1.11 for pRS424/CPA2 + pl L113/ST4. It thus appears that even though ammonia is being used as the substrate in the assay and there is no catalytic role for the amidotransferase activity of 45-kDa CPA1, interaction with this small subunit must affect the conformation of the 124-kDa CPA2 and thereby increase the rate of carbamoyl phosphate synthesis.

In order to amplify the potentially small activities of truncated forms of CPA2, all in vitro assays were carried out on preparations from LPL26 co-transformed with high copy number plasmids expressing CPA1 and the truncated forms of CPA2. As shown in Table IV, carbamoyl phosphate synthesis activity was observed only with wild-type CPA2 and with CPA2 from which 5 amino acids had been deleted. Although the more extensive deletions (20, 25, and 30 amino acids) could produce sufficient carbamoyl phosphate in vivo to support the growth of LPL26, their in vitro activity was below the limits of detection. As expected for the truncations (41 and 191 amino acids) which could not support LPL26 growth, there was no detectable in vitro activity. Also shown in Table IV are the activity levels observed when 20 mM glycine was included in the assay mixture. Previous studies (38) have shown that glycine increases by 2-3-fold the rate of ammonia-dependent carbamoyl phosphate synthesis, presumably by occupying the glutamine site of CPA1 and inducing a more active conformation of CPA2. The addition of glycine caused an unexpectedly large increase, 12-fold, in the activity of the ΔC5 CPA2. Thus, although ΔC5 CPA2 was only 22% as active as wild-type CPA2 in the absence of glycine, their activities were essentially the same when glycine was added. These findings strongly suggest that, at least in vitro, the ΔC5 CPA2 conformation is significantly different from that of wild-type CPA2, but that interaction with the glycine-CPA1 complex can force ΔC5 CPA2 into an essentially wild-type conformation. The conformations of the ΔC20, ΔC25, and ΔC30 deletions of CPA2 appear to be so different from wild-type that they cannot maintain an interaction with CPA1 when extracted into a necessarily more dilute solution and/or they are extremely susceptible to proteolysis when the cell is broken; either situation would result in significant in vivo activity yet undetectable in vitro activity.

Table III

| Domain D deletion | 21°C | 30°C | 37°C | Generation time (h) |
|-------------------|------|------|------|----------------------|
|                   | Day 1 | Day 2 | Day 3 | Day 1 | Day 2 | Day 3 | Day 1 | Day 2 | Day 3 | 30°C | 37°C |
| Wild type CPA2    | +++  | +++  | +++  | +++  | +++  | +++  | +++  | +++  | +++  | 4.1  | 5.7  |
| ΔC5               | +++  | +++  | +++  | +++  | +++  | +++  | +++  | +++  | +++  | 3.9  | 6.3  |
| ΔC20              | +++  | +++  | +++  | +++  | +++  | +++  | +    | +    | +    | 7.3  | 19.3 |
| ΔC25              | +++  | +++  | +++  | +++  | +++  | +    | +    | +    | +    | 5.6  | 14.5 |
| ΔC30              | +++  | +++  | +++  | +++  | +++  | +    | +    | +    | +    | 9.8  | NG*  |
| ΔC41              | -    | -    | -    | -    | -    | -    | -    | -    | -    | ND*  | ND*  |
| ΔC57              | -    | -    | -    | -    | -    | -    | -    | -    | -    | ND*  | ND*  |
| ΔC66              | -    | -    | -    | -    | -    | -    | -    | -    | -    | ND*  | ND*  |
| ΔC113             | -    | -    | -    | -    | -    | -    | -    | -    | -    | ND*  | ND*  |
| ΔC166             | -    | -    | -    | -    | -    | -    | -    | -    | -    | ND*  | ND*  |
| ΔC191             | -    | -    | -    | -    | -    | -    | -    | -    | -    | ND*  | ND*  |
| ΔN1–120           | -    | -    | -    | -    | -    | -    | -    | -    | -    | ND*  | ND*  |

*NG, indicates no detectable change in turbidity.
*ND, no determination.

Yield Differences in Truncated Forms of CPA2 Expressed in S. cerevisiae—Western blot analysis was carried out to confirm the expression of the CPA2 truncations that were apparently inactive in vitro yet active in vivo (ΔC20, ΔC25, and ΔC30), as well as those that were inactive under all conditions (ΔC41, ΔC57, ΔC66, ΔC113, ΔC166, ΔC191, and ΔN1–120), and to allow calculation of the relative specific activity for CPA2 and ΔC5 CPA2. The less extensively truncated forms of CPA2 (ΔC5, ΔC20, ΔC25, and ΔC30), as well as ΔC191 (with the entire domain D deleted), could be detected in crude extracts prepared from pRS315 (low copy number) transformants or from pRS424 (high copy number) transformants (data not shown). When expressed from the high copy number pRS424 plasmid, the
DISCUSSION

Our analysis of C-terminal deletions in yeast CPA2 has demonstrated that essentially all of domain D is required for full catalytic function. Deletion of only 5 amino acids, out of a total of 1128, led to a decreased ability to synthesize carbamoyl phosphate. Enzymatic function was further decreased with increased truncation and was completely abolished with deletion of 41 or more amino acids. Functional analysis of the deletions also yielded the unexpected finding that the ability of CPA2 to carry out ammonia-dependent carbamoyl phosphate synthesis was very strongly influenced by the presence of CPA1. It thus appears that even though ammonia is being used as the substrate in the assay and there is no catalytic role for the amidotransferase moiety of CPA1, interaction with this small subunit must affect the conformation of CPA2 and thereby greatly increase the rate of carbamoyl phosphate synthesis. In contrast, it is known (43, 44) that the large subunit of E. coli CPSase (carB) can function to carry out ammonia-dependent carbamoyl phosphate synthesis in the absence of its corresponding small subunit (carA); the V_{max} is similar for carB and the carA/carB holoenzyme although the K_{m} values for bicarbonate and ATP are increased 2–3-fold in carB. For both the yeast CPA2/CPA1 system (38) and the E. coli carA/carB system (1), occupancy of the glutamine site or the small subunit by a non-hydrolyzable glutamine analog such as glycine is known to cause a 2–3-fold increase in the rate of ammonia-dependent carbamoyl phosphate synthesis by the large subunit. A number of previous studies have established that there is a functional coupling between the two subunits so that occupancy of the glutamine site on the small subunit, by either glutamine or glycine, causes a conformational change in the large subunit, leading to an increased ability to synthesize carbamoyl phosphate (1).

Several lines of evidence suggested that interaction with CPA1 causes CPA2 to assume a conformation with much greater catalytic activity and/or stability. The ability of CPA2 to carry out ammonia-dependent carbamoyl phosphate synthesis in vitro was greatly enhanced by the presence of CPA1: high copy number expression of both CPA1 and CPA2 yielded a 3 times greater specific activity than high copy number expression of CPA2 only. Four of the truncated CPA2 polypeptides (ΔC41, ΔC57, ΔC113, and ΔN1–120) could be detected by Western blot analysis only when CPA1 was present. Additional support for the importance of CPA1/CPA2 interaction came from analysis of the ΔC20–30 mutants, where the inactivity of these truncations in vitro apparently resulted from their inability to maintain an interaction with CPA1. This proposed activation and/or stabilization of CPA2 by interaction with CPA1 and the apparently ready dissociation of the two subunits would explain the previously observed lability of this enzymatic activity (38, 45) and might also serve as a basis for regulation of carbamoyl phosphate synthesis in yeast. As noted in Table I, all other known CPSases are subject to allosteric regulation. However, in spite of extensive screening (38, 45), no allosteric effectors have been identified for the S. cerevisiae arginine-specific CPA2. Transcription of both CPA1 and CPA2 can be induced by the GCN4 protein involved in the general control of amino acid biosynthesis system, and expression of CPA1 can be completely repressed by arginine at a post-transcriptional level (36). If, as proposed, CPA2 is only fully active when interacting with CPA1, then more severe repression of CPA2 would be unnecessary since the arginine-induced repres-

![Fig. 7](http://www.jbc.org/)

**C-terminal Domain of Carbamoyl-phosphate Synthetase**

**Table IV**

| CPA2 enzyme | Carbamoyl phosphate synthesis | CPA2 expression |
|-------------|-------------------------------|----------------|
| Wild type  | 1.113 3.154 10.70             |                |
| ΔC5        | 0.246 2.989 5.41              |                |
| ΔC20       | U U 0.39                     |                |
| ΔC25       | U U 1.10                     |                |
| ΔC30       | U 0.99                       |                |
| ΔC41       | U U 0.67                     |                |
| ΔC191      | U U 1.45                     |                |

ΔC166 construction also yielded a detectable CPA2 fragment of the expected molecular weight; however, ΔC166 could not be detected when expressed from the low copy number pRS315 plasmid. The corresponding CPA2 fragments were not observed for the ΔC41, ΔC57, ΔC66, ΔC113, or ΔN1–120 constructions when either low or high copy number plasmids were used for LPL26 transformation.

Since in vitro activity determinations had indicated that interaction with CPA1 yielded an increased V_{max} for CPA2, we carried out Western blot analysis of crude extracts prepared from LPL26 co-transformed with high copy number plasmids expressing CPA2 truncations and CPA1 (pRS424 and pJ L113/ST4, respectively). As shown in Fig. 7, under these conditions all of the constructions, except ΔC66, could be detected. The mass of the active ΔC5 construction present in the crude extract was about half that of the wild-type CPA2 (Table IV). The mass recoveries of the ΔC20, ΔC25, and ΔC30 constructions, which showed activity in vivo but not in vitro, were 3.6–10.3% of the wild-type CPA2 recovery. Given these amounts of truncated CPA2 protein and the detection limits of the in vitro assay, any carbamoyl phosphate synthesis activity of the truncation mutants would have to be less than 10% of the wild-type activity. Thus, truncation of even 5 amino acids affected both the activity and the stability of CPA2, and truncations of 20 and greater amino acids led to very large effects on activity and stability. It is also noteworthy that the yields (Fig. 7) of the extensively truncated CPA2 forms were not simply a function of the extent of truncation: ΔC57 − ΔC166 > ΔC191 > ΔN1–120 − ΔC113. This finding, plus the fact that the very extensive ΔC191 and ΔC166 truncations could be stably expressed in the absence of CPA1 (data not shown), strongly suggests that incorrectly folded domain D truncations can destabilize the overall CPA2 structure even more than total removal of domain D does.

**Fig. 7.** Western blot analysis of CPA2 deletions expressed from the high copy number plasmid pRS424 in the presence of CPA1 expressed from the high copy number plasmid pJ L113/ST4. Each lane contained 50 μg of total cell protein. A: lanes 1, LPL26 host cells; lanes 2–7, transformant cells expressing ΔC5, ΔC20, ΔC25, ΔC30, ΔC41, and wild-type CPA2, respectively. B: lanes 1–7, LPL26 transformed with ΔC191, ΔC57, ΔC166, ΔC66, ΔN1–120, ΔC113, and wild-type CPA2, respectively.
sion of CPA1 would be effectively communicated to CPA2. Thus, instead of the allosteric control utilized by other CPSases, the activity of the yeast arginine-specific CPSase could be regulated by the combination of activation of CPA2 by CPA1 and repression of CPA1 by arginine.

Prior to the present studies, the CPSase domain structure had been studied only in rat liver CPSase I (13–15) and syrian hamster CAD (16). From those studies, using mild proteolysis, domains A, B, and C could be defined as independent folding units. The status of domain D, however, was not clear since it was observed to undergo cleavage to small peptides even under the mild proteolysis conditions where domains A, B, and C were stable. Our present findings allow definition of yeast CPA2 domain D as an autonomous folding unit that is stabilized by interaction with domains B+C. Thus, we found that individually expressed domain D was detectable, but only when the polypeptide comprising domains B+C was co-expressed. Although independently folded domain D could interact with independently folded domains B+C to yield a stable structure, this reconstituted CPA2 was not able to synthesize carbamoyl phosphate, indicating that it was not identical in final folded form to the wild-type CPA2 conformation.

The present studies have also indicated that essentially all of domain D is required for correct folding of CPA2. The amount of ΔC5 CPA2 obtained in crude extracts was about half that obtained for wild-type CPA2. Western blot analysis of the more extensive truncations generally revealed much less CPA2 recovery than for ΔC5 CPA2. It cannot be ruled out that the lack of expression, or lowered level of expression, of truncations was due to variable mRNA synthesis and/or stability. However, since the wild-type CPA2 and truncation mutants were all expressed from plasmids with identical upstream and downstream sequences, it is unlikely that significant differences occurred in mRNA synthesis and/or stability. It therefore seems most likely that the lower yields of various CPA2 truncated forms resulted from conformational changes that made the truncated proteins more susceptible to proteolysis than the wild-type CPA2. This increased susceptibility to proteolysis was not simply due to smaller size of the truncated products since the ΔC113 and ΔN1–120 deletions yielded less detectable product than the shorter polypeptides resulting from the ΔC166 and ΔC191 truncations. Functional analysis of the ΔC5 mutant further indicated that its conformation was significantly different from wild-type: ΔC5 was only 22% as active as wild-type CPA2 in the absence of glycine, but binding of glycine to CPA1 resulted in wild-type catalytic behavior of the truncated protein than the shorter polypeptides resulting from the ΔC5 CPA2.

We have demonstrated that domain D is much more integral to CPA2 structure and function than its lack of sequence conservation might suggest. It thus appears that a common domain D structure can be formed from many different sequences of amino acids, as is known to be the case, for instance, for the dinucleotide-binding fold (48), where only a relatively few amino acid residue changes are crucial for maintaining the common structural framework and are therefore conserved, while many different combinations of the remaining amino acid residues can form the bulk of the common structural framework. The significant effects on CPA2 structure and function observed with even small carboxyl-terminal truncations precluded use of domain D deletion analysis to assign specific functions to the specific D1-ATP and D2-ATP structural regions. However, the integral role of domain D in overall CPA2 structure and function would be consistent with its involvement in substrate binding and/or catalysis, as well as in constituting the locus for allosteric effector interaction in the CPSases which undergo allosteric regulation.

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