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Structure-Function Relationships in the Arginine Pathway Carbamoylphosphate Synthase of *Saccharomyces cerevisiae*

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The arginine pathway carbamoylphosphate synthase (CPSase A) from *Saccharomyces cerevisiae* was shown to be highly unstable and could not be substantially purified. In spite of this instability, a number of important properties of this enzyme were determined with crude preparations. A molecular weight of 140,000 (7.9S) was estimated for the native enzyme by sucrose gradient centrifugation; a significantly higher value, 175,000, was obtained by gel filtration on Sephadex. The enzyme is an aggregate consisting of two protein components, coded for by the unlinked genes *cpaI* and *cpaII*. These components were separated by diethylaminoethyl-cellulose chromatography. Their molecular weights, estimated by Sephadex gel filtration, were 36,000 and 130,000. The large component catalyzed the synthesis of carbamoylphosphate from ammonia. The small component was required in addition to the large one for the physiologically functional glutamine-dependent activity. Apparent Michaelis constants at pH 7.5 of 1.25 mM for glutamine and 75 mM for NH_4Cl were measured with the native enzyme. The use of various glutamine analogs, including 2-amino-4-oxo-5-chloropentanoic acid, indicated that binding of glutamine to a site located on the small component was followed by transfer of its amide nitrogen to the ammonia site on the heavy component. This ammonia site was able to function independently of the utilization of glutamine. However, binding of glutamine was conjectured to cause a conformational change in the heavy component that greatly increased the rate of synthesis of carbamoylphosphate from ammonia. Glutamine, which was also shown to stabilize the aggregation of the two components, appeared to be a major effector of the catalytic and structural properties of CPSase A. In view of these observations, the CPSase A of yeast appears to share a number of structural and catalytic properties with the *Escherichia coli* enzyme. Obviously, the unlinked *cpaI* and *cpaII* genes of yeast are homologous to the adjacent *carA* and *carB* genes that code for the two subunits of the bacterial enzyme.

The study of the biosynthesis of carbamoylphosphate (CP) in bacteria and fungi has revealed two markedly different ways of controlling the production of this common precursor of arginine and the pyrimidines (18). Bacteria have a single CP synthase (EC 2.7.2.9); it is subject to control by end products from both pathways. Examples of this bacterial control of CP synthesis are provided by *Escherichia coli* and *Salmonella typhimurium* (1, 18, 28). Fungi, on the other hand, possess two independently regulated CP synthases. *Neurospora crassa* and *Saccharomyces cerevisiae* illustrate this type of organization (5, 11, 18).

The two CP synthases of *S. cerevisiae* (Fig. 1) have been distinguished by their respective patterns of regulation (11). One is linked to the pyrimidine pathway and is designated CPSase P; it is feedback inhibited and repressed by pyrimidines. *cpu* mutations affect this enzyme; they have been mapped in the *ura2* region, which codes for an aggregate of CPSase P and aspartate carbamoyltransferase (EC 2.1.3.2). A second synthase, CPSase A, is related to the arginine pathway, since it is repressed by this amino acid. Control of its activity has not been observed. Mutations affecting CPSase A are distributed among two unlinked loci, *cpaI* and *cpaII*. A double auxotrophy for arginine and pyrimidines results from the presence of both a *cpa* and a *cpu* mutation in the same cell. Strains harboring *cpa* or *cpu* mutations alone grow well on minimal medium; however, the growth of *cpu* mutants is inhibited by arginine, whereas pyrimidines prevent the growth of *cpa* mutants.

Accordingly, the two synthases appear to con-

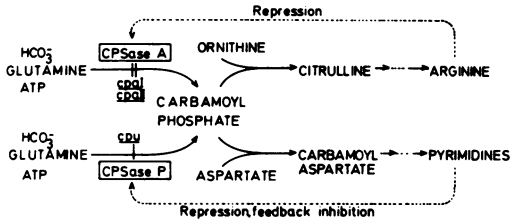

FIG. 1. *Scheme of carbamoylphosphate synthesis in S. cerevisiae (modified from reference 11).*

tribute to the formation of a single cellular pool of CP (11). In this respect, *S. cerevisiae* is different from *Neurospora*, in which the CP synthesized by each synthase is "channeled" to its respective pathway (5, 6). The localization of the arginine pathway CP synthase in mitochondria seems to play a major role in channeling (4). This conclusion is confirmed by the observation that in yeast, which displays little channeling of CP, the homologous enzyme, CPSase A, is cytosolic (29).

Like other glutamine-dependent CP synthases, CPSase A catalyzes in vitro the synthesis of CP from either glutamine or ammonia as the nitrogen donor (11). *cpaII* mutations abolish the CP-synthesizing activity of CPSase A with both nitrogen donors, whereas the glutamine-dependent activity alone is affected by *cpaI* mutations (18, 32). Restoration of the glutamine-dependent activity by in vitro complementation between extracts of mutants harboring *cpaI* and *cpaII* mutations has been shown (11). These various findings provide evidence that the product of gene *cpaII* is able to catalyze the synthesis of CP from ammonia, whereas the product of gene *cpaI* is necessary in addition to that of *cpaII* for the utilization of glutamine as a nitrogen donor.

The present report is concerned with the structural and catalytic properties of CPSase A and with a more detailed description of the roles of genes *cpaI* and *cpaII* in specifying this glutamine-dependent CP synthase. Preliminary accounts of some of the data presented here have appeared previously (18, 32).

MATERIALS AND METHODS

Organisms. All strains used in this work are derivatives of the wild-type strain Σ1278b. Strains 4031c (*cpu2*), MG641 (*cpaI3*), MG642 (*cpaII3*), 6028d (*cpaI3 cpu2*), and MG701 (*cpaII3 cpu2*) have been previously described (25). Strains 6974a (*cpu2 argJ*), 6987a (*cpaI3 argJ*), and 9040a (*cpaII3 argJ*), kindly provided by Marcelle Grenson, have been constructed by mating strains 4031c, MG641, and MG642, respectively, with strain MG409 (*argJ*). The leaky *argJ* mutation of strain MG409, affecting a nonidentified step of arginine biosynthesis, results in slow growth on minimal medium and achieves derepression of the expression of all the *arg* genes as well as that of *cpaI* and *cpaII* genes (22; A. Piérard et al., in preparation). This property was used in some of the experiments to obtain high yields of CPSase A and its components. Specific activities of CPSase A as high as 2.5 U/mg of protein were obtained for strain 6974a, as compared with about 0.3 U/mg for strain 4031c. Except for strains 6987a and 9040a, which need a functional pyrimidine pathway synthase (CPSase P) to grow on minimal medium, all strains used in this work harbored *cpu* mutations to avoid any contamination of the extracts by CPSase P. Yet, the contamination of extracts of strains 6987a and 9040a by CPSase P appeared negligible, probably because CPSase P activity was lost during breakage of the cells in the French pressure cell.

Growth of cells and preparation of cell extracts. Cells were grown aerobically on a rotary shaker at 29°C in minimal medium 149 [containing 0.02 M $(NH_4)_2SO_4$ as the nitrogen source] which has been described previously (7). This medium was supplemented with 3% glucose (wt/vol), biotin (0.25 μg/ml), thiamine (1 μg/ml), inositol (10 μg/ml), calcium panthotenate (2 μg/ml), and pyridoxine (1 μg/ml). Cultures in volumes of 250 to 2,500 ml were harvested by centrifugation during exponential-phase growth (optical density at 660 nm, between 0.400 and 0.500). The cells were washed with distilled water and stored frozen at −20°C. Frozen cells were suspended in 0.02 M imidazole buffer (pH 7.5) containing 0.5 mM dipotassium-magnesium ethylenediaminetetraacetate (Magnesium-Titriplex, E. Merck AG, Darmstadt, Germany) and crushed in a French pressure cell. The extract was centrifuged at 2,500 × *g* for 15 min and passed over Sephadex G-25 equilibrated with the same buffer before being assayed.

Enzyme assay. CP synthase activity was estimated by coupling it with ornithine carbamoyltransferase (EC 2.1.3.3) in the presence of ornithine as previously described (8). The nitrogen substrate was either 0.01 M glutamine or 0.1 M NH_4Cl. Protein was estimated by the method of Lowry et al. (13). One enzyme unit catalyzes the formation of 1 μmol of CP per h at 30°C. Specific activities are expressed as enzyme units per milligram of protein.

Detection of CPSase A components by in vitro complementation. Reconstitution of glutamine-dependent CPSase A activity by mixing cell extracts of mutants *cpaI* and *cpaII*, as described previously (11), was used to detect the isolated products of genes *cpaI* and *cpaII* during separation experiments. Cell extracts of mutant strains 6987a (*cpaI3 argJ*) and 9040a (*cpaII3 argJ*) bearing *cpaI* and *cpaII* mutations that were shown to complement each other efficiently in vitro were used for this purpose. Fresh concentrated extracts (5 to 10 mg of protein per ml) of these mutants, after treatment on Sephadex G-25, were mixed with the preparation to be tested and incubated at 30°C. After 5 min of incubation, glutamine-dependent CPSase A activity was determined as described above.

Density gradient centrifugation. Sucrose density gradient centrifugation was performed by the method of Martin and Ames (14). The sedimentation constants were calculated with a molecular weight of 240,000 and a sedimentation coefficient of 11.3S for catalase.

Partial purification. A crude extract of *S. cerevisiae* cells from 1 to 2 liters of culture on minimal medium was prepared as described above and treated with streptomycin sulfate (1%, wt/vol) for 30 min at 30°C. All subsequent operations were carried out at this temperature. The precipitate was removed by centrifugation, and the pellet was discarded. The supernatant was brought to 80% saturation by solid ammonium sulfate, adjusted to pH 7.5, and allowed to stir gently for 15 min. The precipitate was separated by centrifugation, suspended in a minimal volume of 0.5 M potassium phosphate buffer (pH 7.5) containing 0.5 mM Magnesium-Titriplex, and desalted by passage over Sephadex G-25 equilibrated with the same buffer. The resulting solution was applied to a column (1 by 16 cm) of diethylaminoethyl-cellulose equilibrated with the same buffer. The column was first washed with 40 ml of this equilibration buffer and then eluted with a linear gradient of 0.05 to 0.5 M potassium phosphate buffer (pH 7.5) containing 0.5 mM Magnesium-Titriplex.

Gel filtration on Sephadex columns. The molecular weights of CPSase A and its components were estimated by the gel filtration technique of Andrews (2) with columns of Sephadex superfine G-100 and G-200 calibrated with proteins of known molecular weights. Filtration on Sephadex G-200 was performed on columns (2.5 by 90 cm) containing silicon-coated glass beads (diameter, 6 mm) as described by Sachs and Painter (23). Such columns provided markedly increased flow rates and proved useful for reducing CPSase A inactivation during filtration. Filtration on Sephadex G-100 was performed in the usual column (2.5 by 40 cm). Both types of column were equilibrated with 50 mM potassium phosphate buffer (pH 7.5) containing 0.5 mM Magnesium-Titriplex, and were eluted with the same buffer.

Chemicals. Marker enzymes and biochemicals used in this work were commercial preparations from Sigma Chemical Co., St. Louis, Mo., or Boehringer, Mannheim, Germany.

$Na_2{}^{14}CO_3$ was purchased from the Radiochemical Centre, Amersham, England.

L-2-Amino-4-oxo-5-chloropentanoic acid (chloroketone) was prepared from *N*-carbobenzoxy-L-aspartyl-α-benzyl ester as described by Khedouri et al. (10). The latter compound was synthesized from aspartate as described by Bergmann and Zervas (3).

RESULTS

Attempts to stabilize and purify CPSase A. Crude *S. cerevisiae* extracts were found to lose their glutamine-dependent CPSase A activity rapidly. The half-life of this glutamine activity was 8 h at 0°C in the presence of imidazole buffer (pH 7.5). The ammonia-dependent activity was less unstable (half-life, 20 h). The stability of the glutamine-dependent activity could not be improved with other buffers [phosphate, citrate-phosphate, tris(hydroxymethyl)aminomethane-hydrochloride, triethanolamine, borax-NaOH, sodium glycine, or $NH_4{}^+$-NH_3] tried over a total pH range of 5 to 10. The stability of the ammonia-dependent activity was markedly higher at pH 8.5 in the presence of 0.05 M tris(hydroxymethyl)aminomethane-hydrochloride (3% loss in 24 h).

The addition of various stabilizing agents including 2-mercaptoethanol, dithiothreitol, ethylene glycol, and a protease inhibitor such as phenylmethylsulfonyl fluoride had no significant effect on the stability of CPSase A. Of a number of arginine pathway intermediates and substrates or cofactors of CPSase A that were tried, including glutamate, *N*-α-acetylglutamate, ornithine, *N*-α-acetylornithine, citrulline, arginine, and HCO_3^- and K^+ ions, only glutamine, ATP, and Mg^{2+} ions were found to afford substantial protection of CPSase A activity in crude cell extracts. Whereas the optimal conditions for stabilizing the ammonia-dependent activity were the presence of 10 mM Mg-ATP plus a 2 mM excess of Mg^{2+} ions, the best results for the glutamine-dependent activity were achieved with 5 mM glutamine plus 2 mM Mg^{2+} ions.

Nevertheless, such stabilizing conditions were found to provide negligible protection of CPSase A during attempts at purifying this enzyme. All purification steps that were investigated resulted in large losses of CPSase A activity, in spite of the presence of glutamine and Mg^{2+} ions. For example, a relatively simplified and rapid purification scheme involving streptomycin sulfate precipitation, followed by chromatography on a column of diethylaminoethyl (DEAE)-cellulose and gel filtration on Sephadex G-200, led to a final yield of only 3% of the initial glutamine-dependent activity, and no significant purification was achieved.

The precise nature of CPSase A inactivation has not been determined. Since the glutamine-dependent activity was lost more rapidly than the ammonia-dependent activity, we were led to suspect that inactivation might be a result of the dissociation of CPSase A into its components. However, this possibility appears unlikely since little glutamine-dependent activity can be regained by adding excesses of extracts of mutants *cpaI* or *cpaII* to an inactivated CPSase A preparation. In spite of the difficulty encountered in attempts at purifying CPSase A, considerable information concerning the subunit structure of this enzyme was obtained with crude or very partially purified preparations, as will be reported in the following sections.

Sucrose density gradient centrifugation of CPSase A. A first estimation of the size of CPSase A was provided by sucrose density gradient centrifugation. Crude extracts of mutant 6974a (*cpu2 argJ*), displaying specific CPSase activities of approximately 2.5 U/mg of protein with glutamine and 0.3 U/mg with ammonium

chloride as the nitrogen donor, were first subjected to gradient centrifugation (see Materials and Methods). Both glutamine- and ammonia-dependent activities were recovered in a single peak corresponding to an average sedimentation coefficient of 7.9S and a calculated molecular weight of 140,000. This value was not significantly affected by the presence of glutamine, ammonium chloride, or Mg-ATP during centrifugation.

Extracts of mutant 6987a (*cpaI3 argJ*) were inactive when glutamine was provided as the amino group donor in the CPSase A reaction but were active with ammonia (specific activities, 0.1 to 0.2 U/mg of protein). This ammonia-dependent CPSase A activity sedimented with a constant of 6.7S (corresponding to a molecular weight of 109,000) during gradient centrifugation. Extracts of mutant 9040a (*cpaII3 argJ*) were devoid of any CPSase A activity. However, they complemented in vitro with extracts of mutant 6987a in restoring glutamine-dependent CPSase A activity. The complementing CPSase A fraction of such extracts was shown to sediment at almost the same rate (sedimentation constant, 7.6S) as native CPSase A. These various findings are consistent with the view that mutation *cpaI3* abolishes the synthesis of a small component of CPSase A that is only required for glutamine-dependent activity, whereas mutation *cpaII3* affects a larger component that is necessary for both activities. The latter inactive component, as shown by the behavior of extracts of mutant 9040a during centrifugation, retained the ability to bind firmly to the small component.

Chromatography on DEAE-cellulose. Extracts of these various mutants were subjected to DEAE-cellulose chromatography after a few preparation steps (see Materials and Methods). Although this procedure usually provided poor activity yields (less than 20% of the initial glutamine-dependent CPSase A activity), it allowed the characterization of the two components of CPSase A. This was facilitated by the finding that dissociation of CPSase A occurs during chromatography on DEAE-cellulose. Figure 2 shows the dissociation of wild-type CPSase A into its two components. The small component was not retained by the column; it had no CPSase A activity per se but complemented actively with extracts of mutant 6987a (*cpaI3 argJ*) in restoring glutamine-dependent activity. The large component was eluted shortly after the linear phosphate gradient was applied and was active with ammonia; it displayed efficient complementation with an extract of mutant 9040a (*cpaII3 argJ*). Superimposed on the ammonia-dependent activity peak was a small glutamine-dependent activity peak. The latter peak was considerably increased when glutamine was present in the buffers used for DEAE-cellulose chromatography of CPSase A (Fig. 2B). Since, at the same time, the small component peak practically disappeared, it may be concluded that glutamine prevented the dissociation of CPSase A into its components. This finding also establishes that the glutamine-dependent activity present in the second peak corresponds to

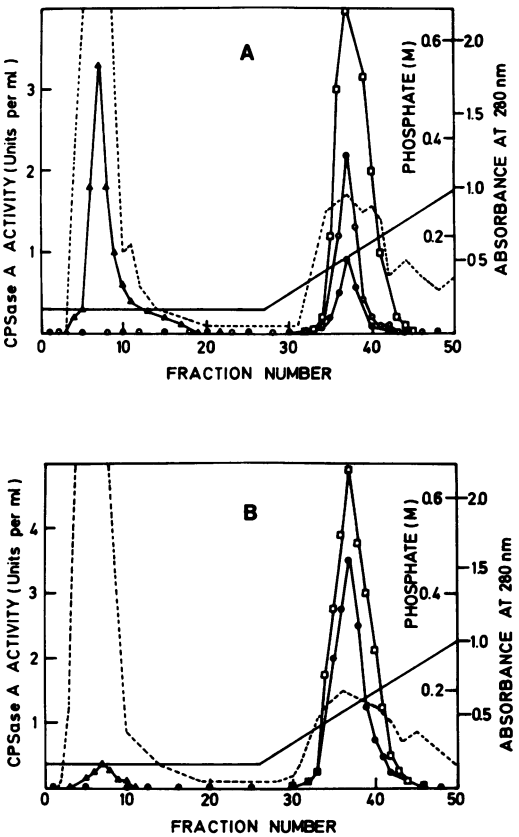

FIG. 2. *DEAE-cellulose chromatography of wild-type CPSase A from mutant 6974a (cpu argJ) grown on minimal medium. (A) Elution profile obtained when equilibration and elution buffers without glutamine were used. The sample applied on the column contained 220 U of glutamine-dependent CPSase A activity. (B) Elution profile obtained when buffers containing 20 mM glutamine were used. The sample applied on the column contained 260 U of glutamine-dependent CPSase activity. Symbols: - - -, Absorbance at 280 nm; ●, glutamine-dependent CPSase A activity; ○, ammonia-dependent CPSase A activity; △, glutamine-dependent CPSase A activity in the presence of added extract of mutant 6987a (cpaI 3 argJ); □, glutamine-dependent CPSase A activity in the presence of added extract of mutant 9040a (cpaII3 argJ); ——, potassium phosphate molarity.*

undissociated enzyme. Mg^{2+} ions alone were found to partially protect CPSase A against dissociation but to a much lesser degree than glutamine. Ammonium ions were without effect.

That the small and large components are respectively specified by genes *cpaI* and *cpaII* was established unambiguously by DEAE-cellulose chromatography of extracts of mutants 6987a (*cpaI3 argJ*) and 9040a (*cpaII3 argJ*). The elution pattern obtained for mutant 6987a showed that the large component alone was present in extracts of this mutant (Fig. 3A). Chromatography of an extract of mutant 9040a provided two peaks that complemented in vitro with an extract of mutant 6987a (Fig. 3B): a major complementing peak behaved like a free small component, whereas a smaller peak was eluted at approximately the same ionic strength as native CPSase A. Obviously this peak contained the small component in a complex with the inactive large component.

Estimation of molecular weights by gel filtration. The molecular weights of CPSase A and its two components were estimated by gel filtration on columns of Sephadex. A molecular weight of 175,000 was obtained by gel filtration on Sephadex G-200 of wild-type CPSase A from mutant 6974a (Fig. 4A). Similar conditions, when applied to the large component from mutant 6987a (*cpaI3 argJ*), yielded a value of 130,000 (Fig. 4C). The free small component was separated from the large component and from native CPSase A by DEAE-cellulose chromatography of extracts of mutant 6974a or 9040a (see above); its molecular weight, estimated by filtration on a column of Sephadex G-100, was 36,000 (Fig. 4B).

Substrate dependence of CPSase A. Synthesis of CP by CPSase A requires glutamine, ATP, bicarbonate, and Mg^{2+} ions (11). Potassium ions also are an absolute requirement for the reaction; the optimal concentration is 100 mM, higher concentrations being inhibitory. Ammonium ions have been shown to substitute for glutamine as the source of nitrogen for the synthesis of CP (11). Figure 5 shows the saturation of the enzyme by the two nitrogen donors. The apparent Michaelis constants were 1.25 mM for glutamine and 75 mM for NH_4Cl (corresponding to a K_m of 2.3 mM for free NH_3).

Whereas the saturation curves of CPSase A by glutamine, ammonium chloride, and bicarbonate (apparent K_m, 4.3 mM) were hyperbolic, the saturation of the enzyme by equimolar concentrations of ATP and Mg^{2+} ions followed a slightly sigmoid curve (Fig. 6). This sigmoid character was increased by the presence of an excess of free ATP and reduced by an excess of Mg^{2+} ions. Such behavior does not necessarily imply regulatory properties but might simply be because the Mg-ATP complex is the active substrate (12). In this respect, it should be recalled that no evidence for a control of CPSase A activity has been obtained.

Separate binding sites for glutamine and ammonia. L-2-Amino-4-oxo-5-chloropentanoic acid, the chloroketone analog of glutamine synthesized by Khedouri et al. (10) during their study of *E. coli* CPSase, was used to demonstrate the separation of the glutamine- and am-

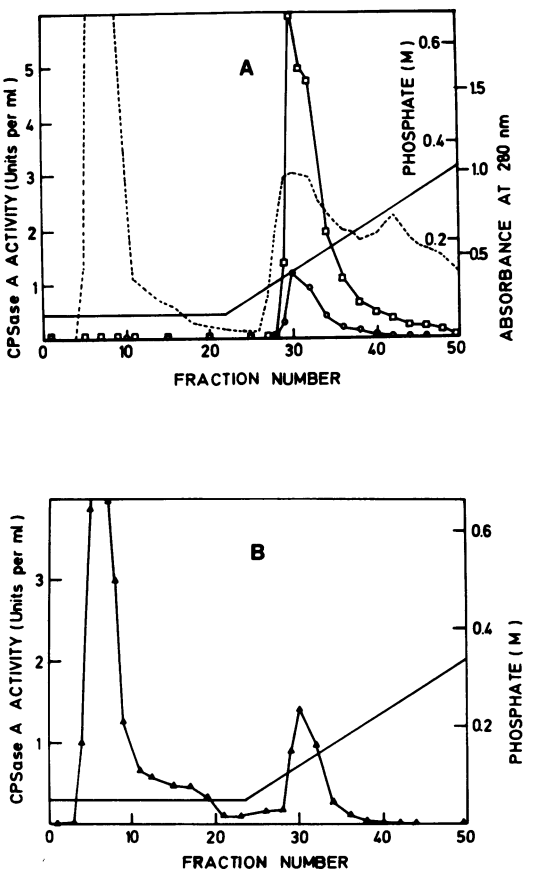

FIG. 3. *DEAE-cellulose chromatography of CPSase A components from mutants 6987a (cpaI3 argJ) and 9040a (cpaII3 argJ). All conditions and symbols are as described in the legend to Fig. 2. Equilibration and elution buffers did not contain glutamine. (A) Elution profile obtained with an extract of mutant 6987a. The sample applied on the column contained 180 U of large component (as estimated by in vitro complementation with mutant 9040a). (B) Elution profile obtained with an extract of mutant 9040a. The sample applied on the column contained 110 U of small component (as estimated by in vitro complementation with mutant 6987a). Absorbance at 280 nm was not recorded in this experiment.*

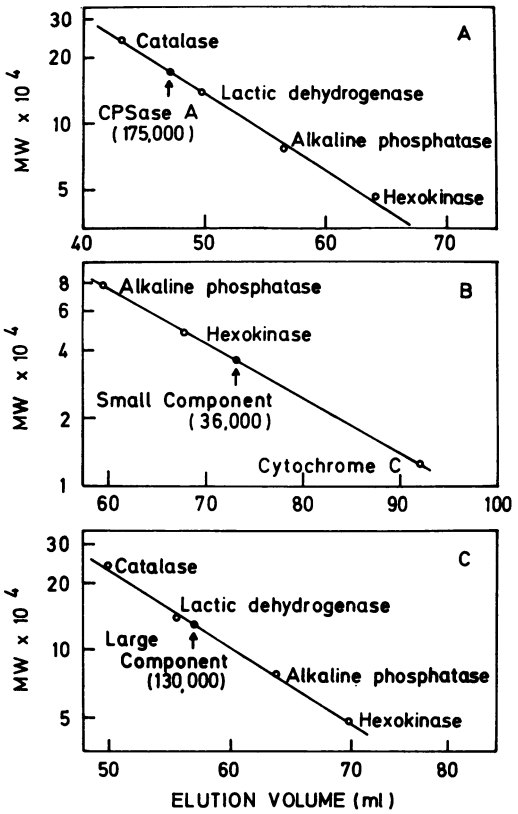

FIG. 4. *Estimation of molecular weights of S. cerevisiae CPSase A and its components by Sephadex gel filtration. The markers used were beef liver catalase (240,000 daltons), beef heart lactic dehydrogenase (140,000 daltons), E. coli alkaline phosphatase (78,000 daltons), yeast hexokinase (48,000 daltons) and horse cytochrome c (12,400 daltons). (A) Molecular weight of native CPSase A on Sephadex G-200; (B) molecular weight of small component of CPSase A on Sephadex G-100; (C) molecular weight of large component of CPSase A on Sephadex G-200.*

monia-binding sites of CPSase A. This reagent was found to inhibit glutamine-dependent activity markedly; at the same time, a substantial increase in the ammonia-dependent activity was observed (Table 1). The effects of 2-amino-4-oxo-5-chloropentanoic acid were irreversible, since the activities of the native enzyme could not be restored by dialysis of the treated enzyme. The inhibition with time of glutamine-dependent activity is shown in Fig. 7. A significant inactivation of the enzyme occurred during incubation at 30°C, but the rate of decay was considerably faster in the presence of the inhibitor. Significant protection of CPSase A against chloroketone inhibition was provided by glutamine (Fig. 7).

These experiments indicate that 2-amino-4-oxo-5-chloropentanoic acid reacts with a glutamine-binding site that is distinct from the ammonia site. Whereas the ammonia site is located on the large component, the glutamine site is

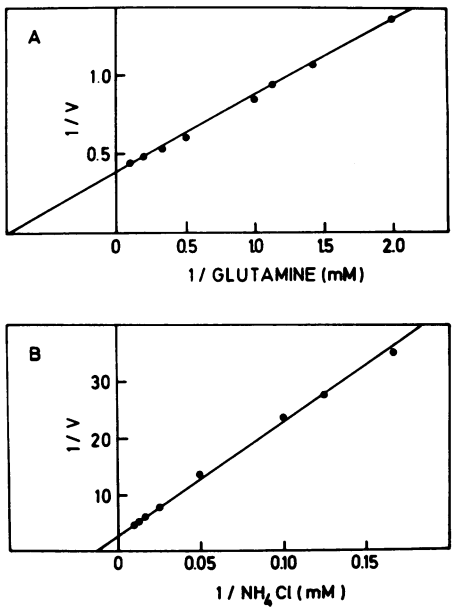

FIG. 5. *Activity of CPSase A with glutamine and ammonium chloride as nitrogen donors. Double-reciprocal plots of CPSase A activity as a function of glutamine and ammonium chloride concentrations. A crude extract of mutant 6974a was used as the source of CPSase A. All conditions except the concentration of nitrogen donors were as described in the text. Velocities are specific activities (units of activity per milligram of protein).*

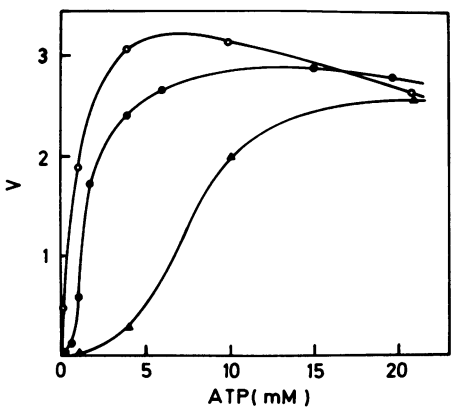

FIG. 6. *Glutamine-dependence activity of CPSase A as a function of Mg-ATP concentrations. A crude extract of mutant 6974a was used. Symbols: ●, ATP as indicated with $MGCl_2$ equimolar with ATP; ○, equimolar ATP and $MgCl_2$ as indicated plus a 5 mM excess of $MgCl_2$; △, equimolar ATP and $MgCl_2$ as indicated plus a 5 mM excess of ATP.*

TABLE 1. *Effect of L-2-amino-4-oxo-5-chloropentanoic acid on the activity of wild-type CPSase A of S. cerevisiae with glutamine and ammonia as nitrogen donors*

Nitrogen donor	Sp act[a] (U/mg of protein) after incubation in the presence of:	
	No addition	0.5 mM 2-amino-4-oxo-5-chloropentanoic acid
Glutamine (10 mM)	2.31	0.03
NH_4Cl (100 mM)	0.29	0.91

[a] Samples of an extract (3 mg of protein per ml) of mutant 6974a were incubated for 10 min at 30°C with the addition indicated in the table. The extracts were dialyzed by filtration on Sephadex G-25 and assayed for CPSase A as described in the text.

provided by the small component. The location of the glutamine site on the small component has been confirmed by the observation that in vitro complementation between extracts of mutants 9040a (*cpaII3 argJ*) and 6987a (*cpaI3 argJ*) is abolished by a pretreatment of the 9040a extract with the chloroketone analog, but not by a similar treatment of the 6987a extract (data not shown).

The nature of the activation of the ammonia-dependent activity that results from the binding of the chloroketone analog was studied (Fig. 8). It consisted in an increase of the reaction velocity (by a factor of 2 or more), with little variation of the affinity for ammonia.

Activation of the ammonia-dependent activity of CPSase A through interaction of glycine with the glutamine site. Studies on the pH dependence of CPSase A stability have led to the fortuitous observation that glycine, used as a buffer component, exerts a marked stimulatory effect on the ammonia-dependent activity of CPSase A. A four- to fivefold stimulation of this activity was observed in the presence of 20 mM glycine.

The nature of the interaction of glycine with CPSase A was studied. Glycine influenced mainly the velocity of the reaction (Fig. 9a); the K_m of the enzyme for NH_4Cl was not significantly affected. Similarly, the reciprocal plots of activation ($v - v_0$) versus glycine concentration for various NH_4Cl concentrations yielded a family of lines that cut the base line at the same point (Fig. 9B), providing an activation constant of 7.5 mM. This behavior indicates that glycine combines with the enzyme independently from NH_4Cl.

We obtained several lines of evidence supporting the view that glycine acts by binding at the glutamine site. (i) The stimulating effect of glycine was absent in most strains harboring *cpaI* mutations. As seen above, such strains lack a functional small component and are unable to utilize glutamine for CP synthesis. (ii) Glycine, like glutamine, protected CPSase A against 2-amino-4-oxo-5-chloropentanoic acid (Fig. 7). (iii) Kinetically, glycine was found to behave as a competitive inhibitor with respect to glutamine. An inhibition constant of 25 mM was calculated for glycine.

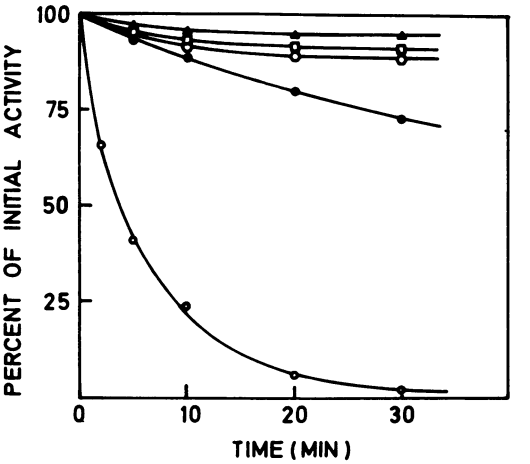

FIG. 7. *Effect of 2-amino-4-oxo-5-chloropentanoic acid as a function of time on the glutamine-dependent activity of CPSase A. A crude cell extract of mutant 6974a was incubated at 30°C with 0.075 mM 2-amino-4-oxo-5-chloropentanoic acid. At the indicated intervals, samples were removed, immediately added to the assay mixture containing 20 mM glutamine, and assayed for glutamine-dependent CPSase A activity. Symbols: ●, Control without inhibitor; ○, 0.075 mM inhibitor; △, control plus 20 mM glutamine; or 0.075 mM inhibitor plus 20 mM glutamine; □, control plus 20 mM glycine; ◯, 0.075 mM inhibitor plus 20 mM glycine.*

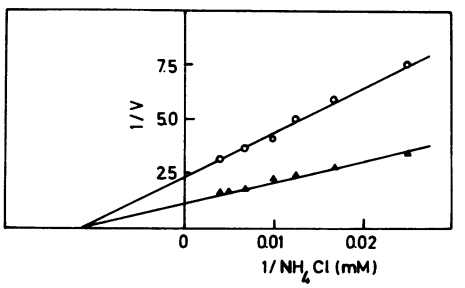

FIG. 8. *Effect of 2-amino-4-oxo-5-chloropentanoic acid on the ammonia-dependent activity of CPSase A. Double-reciprocal plots of activity as a function of ammonium chloride concentrations. Symbols: ○, Native CPSase A from mutant 6974a; △, the same enzyme after a 10-min incubation at 37°C in the presence of 0.2 mM 2-amino-4-oxo-5-chloropentanoic acid.*

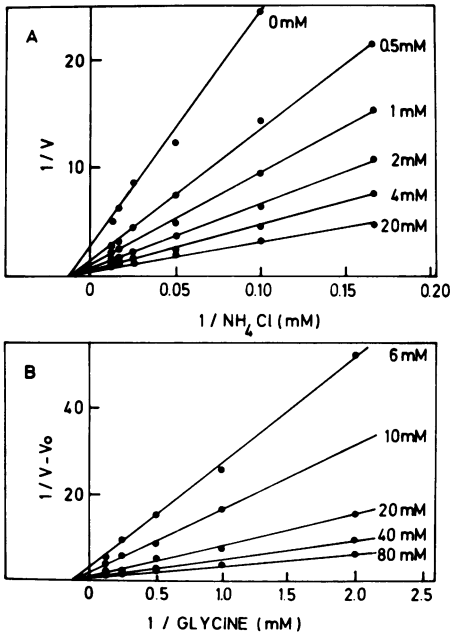

FIG. 9. *Activation of ammonia-dependent activity by glycine. (A) Double-reciprocal plots of velocity against NH_4Cl concentrations for various concentrations of glycine. (B) Double-reciprocal plots of the difference in velocity with and without glycine ($v - v_0$) against glycine concentrations for various NH_4Cl concentrations. Mutant 6974a was the enzyme source.*

Specificity of the activation. The specificity of the activation of the ammonia-dependent activity of CPSase A was studied. Various compounds, structurally related to glycine, were examined for their ability to replace glycine as an activator. Only L-α-alanine was found to achieve a significant activation. All other compounds tested, including L-valine, L-serine, glycyl-glycine, glyoxylic acid, L-α-aminobutyrate, and L-methionine, were without effect.

In contrast, we observed that three closely related analogs of glutamine, namely L-γ-glutamylhydroxamic acid, *O*-carbamoyl-L-serine, and L-2-amino-3-ureido-propionic acid were able to stimulate the ammonia-dependent activity of CPSase A. Azaserine (*O*-diazoacetyl-serine), L-glutamic acid, asparagine, and *N*-α-acetylglutamine displayed no stimulating effect. Neither glycine nor alanine, or any of the three other activators mentioned above, could be used as a nitrogen donor for CP synthesis, but all were competitive inhibitors of the glutamine-dependent activity (Table 2). The effect on the rate of the reaction (a fivefold increase of the velocity at saturating activator concentrations in the presence of 10 mM NH_4Cl) was of the same order for all five activators. However, L-2-amino-3-ureido-propionic acid and *O*-carbamoyl-L-serine were shown to exhibit markedly more favorable activation constants than the other effectors (Table 2).

Activation of the ammonia-dependent activity by glutamine. That some glutamine analogs could induce a substantial stimulation of the ammonia activity by interacting with the glutamine-binding site also suggested that glutamine itself was able to bring about such stimulation. This suggestion could of course not be tested directly but has received support from the study of the *cpaI2* mutation. The properties of this particular mutation are compared in Table 3 with those of the *cpaI3* mutation. Like other *cpaI* mutations, *cpaI2* abolished the glu-

TABLE 2. *Apparent kinetic constants of glycine, alanine, and certain glutamine analogs as competitive inhibitors of the glutamine-dependent activity and activators of the ammonia-dependent activity of CPSase A*

Compound	Inhibition constant[a] (mM)	Activation constant[b] (mM)
Glycine	25	7.5
L-α-Alanine	30	5.0
L-γ-Glutamylhydroxamic acid	12	4.0
O-Carbamoyl-L-serine	10	1.5
L-2-Amino-3-ureido-propionic acid	10	0.7

[a] Apparent inhibition constant toward the glutamine-dependent activity.

[b] Activation constant toward the ammonia-dependent activity were obtained from plots similar to those shown in Fig. 9 for glycine.

TABLE 3. *Stimulation of ammonia-dependent CPSase A activity by glutamine in a cpaI mutant*

Strain[a]	Sp act (U/mg of protein) of CPSase A in the presence of:			
	Glutamine	NH_4Cl	Glutamine (10 mM) + NH_4Cl (100 mM)	Glycine (20 mM) + NH_4Cl (100 mM)
Σ1278b (wild type)	0.364	0.049	0.294	0.211
MG696 (*cpaI2*)	0.003	0.021	0.050	0.051
MG641 (*cpaI3*)	0.002	0.017	0.017	0.017

[a] Strain Σ1278b was grown in minimal medium plus uracil (50 μg/ml) to repress the pyrimidine enzyme CPSase P maximally. Strains MG696 and MG641 were grown in the same medium containing uracil (50 μg/ml) and a limiting concentration of arginine (5 μg/ml). The cells were harvested after the plateau corresponding to arginine exhaustion had been reached. Enzyme assay was performed as described in the text.

tamine-dependent activity of CPSase A and did not impair the ammonia-dependent activity. The latter activity, however, was increased 2.5-fold when glutamine or glycine was present in the reaction mixture. It is thus proposed that the *cpaI2* mutation prevents the use of glutamine as a nitrogen donor but leaves intact a second function of glutamine, which consists of facilitating the formation of CP from its amide nitrogen or from ammonia by mediating an adequate conformational change.

DISCUSSION

The main finding of this study is that the native enzyme (molecular weight, 185,000, as estimated by gel filtration) can be dissociated into two unequal components, whose the respective functions have been established. The large component (molecular weight, 140,000) is only capable of catalyzing the synthesis of CP from ammonia, whereas the native enzyme utilizes either glutamine or ammonia as the nitrogen donor. The smaller component (molecular weight, 36,000) is required in addition to the large one for the utilization of glutamine. This is a type of organization that has been previously established for diverse glutamine amidotransferases (for a review, see various papers in reference 20), including carbamoylphosphate synthase from other sources. The arginine pathway CPSase of *Neurospora* may be expected to exhibit similar properties (5), but at present the closest resemblance to *S. cerevisiae* CPSase A is found in the single CPSase of *E. coli*. The monomer of this bacterial enzyme (molecular weight, 170,000) has been reversibly dissociated to heavy (molecular weight, 130,000) and light (molecular weight, 42,000) subunits that have been thoroughly characterized (15, 25, 27). The heavy subunit catalyzes all the steps required for the synthesis of CP from ammonia, whereas the light subunit is necessary for the glutamine-dependent activity and bears a glutamine-binding site (19). The only catalytic activity associated with the isolated light subunit is glutaminase activity (27, 31). In addition, significant intersubunit interactions have been shown to occur and seem to play a major role in the functioning of the enzyme (19, 30, 31). In contrast with yeast CPSase A, the activity of the *E. coli* enzyme is under the control of several allosteric effectors, which bind to the heavy subunit (18, 25, 27).

Our conclusions concerning the structure and roles of the components of CPSase A in the synthesis of CP rest on partially indirect evidence, since these components, being unstable, have not been purified and are poorly characterized biochemically. Nevertheless, some of the features that have been established for the bacterial enzyme can be extrapolated to the yeast enzyme. For example, we have been unable to determine whether the small component exhibits glutaminase activity. Yet, the extensive structural and catalytic similarities between the two enzymes suggest identical roles for the small CPSase A component and the light bacterial subunit. Likewise, we have not established whether these components are each composed of one single polypeptide chain, but this is strongly suggested by the comparison of the molecular weights of yeast CPSase A and its components with those of *E. coli* and its subunits. It should be noted, however, that whereas the bacterial enzyme has been shown to undergo reversible self-association to yield dimeric or possibly higher associated forms (26), no evidence of the occurrence of higher aggregated species of yeast CPSase A has been obtained.

Glutamine, in addition to being the preferred nitrogen substrate of CPSase A, played an important role in determining the structure and properties of the enzyme. Binding of glutamine to the enzyme led to several important modifications of the properties of the enzyme. Its stability was improved, and dissociation into its components was made more difficult. Similar effects of glutamine have been noted earlier with anthranilate synthetase (9, 21). In addition, glutamine analogs for the wild-type CPSase A and glutamine itself for the enzyme of one particular *cpaI* mutant stimulated the ammonia-dependent activity. These results are interpreted to indicate that glutamine should be considered as a positive effector of CPSase A.

Finally, it should be emphasized the CPSase A is coded by two unlinked genes, *cpaI* and *cpaII*, and consequently cannot benefit from the operon type of organization that has been proposed for the homologous genes of *E. coli* and *S. typhimurium* (1, 16, 17). Previous observations have shown that the synthesis of CPSase A obeys a negative type of control similar to that known in bacteria (24). The mechanism by which such a control influences the expression of genes *cpaI* and *cpaII* will be the subject of a further report (Piérard et al., in preparation).

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LITERATURE CITED

1. **Abdelal, A. T. H., and J. L. Ingraham.** 1975. Carbamylphosphate synthetase from *Salmonella typhimurium*. Regulation, subunit composition, and function of the subunits. J. Biol. Chem. **250**:4410–4417.
2. **Andrews, P.** 1965. The gel-filtration behaviour of proteins related to their molecular weights over a wide range. Biochem. J. **96**:595–606.
3. **Bergmann, M., and L. Zervas.** 1932. Über ein allgemeines Verfahren der Peptid-Synthese. Ber. Dtsch. Chem. Ges. **65**:1192–1201.
4. **Bernhardt, S. A., and R. H. Davis.** 1972. Carbamylphosphate compartmentation in *Neurospora*: histochemical localization of aspartate and ornithine transcarbamylase. Proc. Natl. Acad. Sci. U.S.A. **72**:989–993.
5. **Davis, R. H.** 1967. Channeling in *Neurospora* metabolism, p. 303–322. *In* H. J. Vogel, J. O. Lampen, and V. Bryzon (ed.), Organizational biosynthesis. Academic Press Inc., New York.
6. **Davis, R. H.** 1972. Metabolite distribution in cells. Two carbamylphosphate gradients and their sources can be discerned in *Neurospora*. Science **178**:835–840.
7. **Grenson, M., M. Mousset, J. M. Wiame, and J. Bechet.** 1966. Multiplicity of the amino acid permeases in *Saccharomyces cerevisiae*. I. Evidence for a specific arginine-transporting system. Biochim. Biophys. Acta **127**:325–338.
8. **Hilger, F., M. Culot, M. Minet, A. Piérard, M. Grenson, and J. M. Wiame.** 1973. Studies on the kinetics of the enzyme sequence mediating arginine synthesis in *Saccharomyces cerevisiae*. J. Gen. Microbiol. **75**:33–41.
9. **Kane, J. F., W. M. Holmes, K. L. Smiley, Jr., and R. A. Jensen.** 1973. Rapid regulation of an anthranilate synthase aggregate by hysteresis. J. Bacteriol. **113**:224–232.
10. **Khedouri, E., P. M. Anderson, and A. Meister.** 1966. Selective inactivation of the glutamine binding site of *Escherichia coli* carbamylphosphate synthetase by 2-amino-4-oxo-5-chloropentanoic acid. Biochemistry **5**:3552–3556.
11. **Lacroute, F., A. Piérard, M. Grenson, and J. M. Wiame.** 1965. The biosynthesis of carbamoylphosphate in *Saccharomyces cerevisiae*. J. Gen. Microbiol. **40**:127–142.
12. **London, W. P., and T. L. Steck.** 1969. Kinetics of enzyme reactions with interaction between a substrate and a (metal) modifier. Biochemistry **8**:1767–1779.
13. **Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall.** 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193**:265–275.
14. **Martin, R. G., and B. N. Ames.** 1961. A method for determining the sedimentation behavior of enzymes: application to protein mixtures. J. Biol. Chem. **236**:1372–1379.
15. **Matthews, S. L., and P. M. Anderson.** 1972. Evidence for the presence of two nonidentical subunits in carbamylphosphate synthetase of *Escherichia coli*. Biochemistry **11**:1176–1183.
16. **Mergeay, M., D. Gigot, J. Beckmann, N. Glansdorff, and A. Piérard.** 1974. Physiology and genetics of carbamoylphosphate synthesis in *Escherichia coli* K-12. Mol. Gen. Genet. **133**:299–316.
17. **Piérard, A., N. Glansdorff, D. Gigot, M. Crabeel, P. Halleux, and L. Thiry.** 1976. Repression of *Escherichia coli* carbamoylphosphate synthase: relationships with enzyme synthesis in the arginine and pyrimidine pathways. J. Bacteriol. **127**:291–301.
18. **Piérard, A., M. Grenson, N. Glansdorff, and J. M. Wiame.** 1973. A comparison of the organization of carbamylphosphate synthesis in *Saccharomyces cerevisiae* and *Escherichia coli*, based on genetical and biochemical evidences, p. 483–503. *In* S. Prusiner and E. R. Stadtman, (ed.), The enzymes of glutamine metabolism. Academic Press, New York.
19. **Pinkus, L. M., and A. Meister.** 1972. Identification of a reactive cysteine residue at the glutamine binding site of carbamoylphosphate synthetase. J. Biol. Chem. **247**:6119–6127.
20. **Prusiner, S., and E. R. Stadtman (ed.).** 1973. The enzymes of glutamine metabolism. Academic Press, Inc., New York.
21. **Queener, S. W., S. F. Queener, J. R. Meeks, and I. G. Gunsalus.** 1973. Anthranilate synthase from *Pseudomonas putida*. Purification and properties of a two-component enzyme. J. Biol. Chem. **248**:151–161.
22. **Ramos, F., P. Thuriaux, J. M. Wiame, and J. Béchet.** 1970. The participation of ornithine and citrulline in the regulation of arginine metabolism in *Saccharomyces cerevisiae*. Eur. J. Biochem. **12**:40–47.
23. **Sachs, D. H., and E. Painter.** 1972. Improved flow rates with porous Sephadex gels. Science **175**:781–782.
24. **Thuriaux, P., F. Ramos, A. Piérard, M. Grenson, and J. M. Wiame.** 1972. Regulation of the carbamoylphosphate synthetase belonging to the arginine biosynthetic pathway of *Saccharomyces cerevisiae*. J. Mol. Biol. **67**:277–287.
25. **Trotta, P., M. E. Burt, R. H. Haschemeyer, and A. Meister.** 1971. Reversible dissociation of carbamylphosphate synthetase into a regulated synthesis subunit and subunit required for glutamine utilization. Proc. Natl. Acad. Sci. U.S.A. **66**:1027–1035.
26. **Trotta, P. P., L. F. Estis, A. Meister, and R. H. Haschemeyer.** 1974. Self-association and allosteric properties of glutamine-dependent carbamylphosphate synthetase. Reversible dissociation to monomeric species. J. Biol. Chem. **249**:482–491.
27. **Trotta, P. P., L. M. Pinkus, R. H. Haschemeyer, and A. Meister.** 1974. Reversible dissociation of the monomer of glutamine-dependent carbamylphosphate synthetase into catalytically active heavy and light subunits. J. Biol. Chem. **249**:492–499.
28. **Trotta, P. P., L. M. Pinkus, V. P. Wellner, L. Estis, R. H. Haschemeyer, and A. Mesiter.** 1973. Structure-function relationships in glutamine-dependent carbamylphosphate synthetase, p. 431–482. *In* S. Prusiner and E. R. Stadtmann (ed.), The enzymes of glutamine metabolism. Academic Press Inc., New York.
29. **Urrestarazu, L. A., S. Vissers, and J. M. Wiame.** 1977. Change in location of ornithine carbamoyltransferase and carbamoylphosphate synthetase among yeasts in relation to the arginase/ornithine carbamoyltransferase regulatory complex and the energy status of the cells. Eur. J. Biochem. **79**:473–481.
30. **Wellner, V. P., P. M. Anderson, and A. Meister.** 1973. Interaction of *Escherichia coli* carbamylphosphate synthetase with glutamine. Biochemistry **12**:2061–2066.
31. **Wellner, V. P., and A. Meister.** 1975. Enhancement of the glutaminase activity of carbamylphosphate synthetase by alterations in the interaction between the heavy and light subunits. J. Biol. Chem. **250**:3261–3266.
32. **Wiame, J. M., V. Stalon, A. Piérard, and F. Messenguy.** 1973. Control of transcarbamoylation in microorganisms. Symp. Soc. Exp. Biol. **27**:333–363.