Purification and Properties of the Arginine-specific Carbamoyl-phosphate Synthase from *Saccharomyces cerevisiae*

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The arginine-specific carbamoyl-phosphate synthase of yeast was stabilized sufficiently to allow partial purification of the enzyme (30- to 40-fold). The synthase (mol. wt 115000) comprised two unequal subunits: a heavy subunit (mol. wt 80000) capable of catalysing synthesis of carbamoyl phosphate with ammonia as a nitrogen donor and a light subunit conferring upon the holoenzyme the ability to utilize glutamine. The enzyme had unusually high affinity for ATP ($K_m = 0.2$ mM) and atypical negative cooperativity for glutamine binding ($[S]_{0.5} = 0.25$ mM). Glutamine activity was not modulated by possible effectors such as arginine, ornithine or *N*-acetylglutamate. Thus, although the yeast arginine enzyme physically and functionally resembles the single enteric synthase, the systems differ substantially both in kinetic properties and in regulation of activity.

INTRODUCTION

Carbamoyl-phosphate synthase catalyses the formation of carbamoyl phosphate, an intermediate in the biosynthesis of both arginine and pyrimidines. *Escherichia coli* (Piérard *et al.*, 1973; Trotta *et al.*, 1974) and *Salmonella typhimurium* (Abdelal & Ingraham, 1975) possess a single synthase, common to both pathways, that is subject to regulation consistent with its dual function. This enzyme is composed of two subunits of unequal size (Trotta *et al.*, 1974; Abdelal & Ingraham, 1975). The heavy subunit alone catalyses the synthesis of carbamoyl phosphate from ammonia, ATP and bicarbonate, a reaction which is activated by ornithine. The light subunit confers the ability to utilize glutamine as the amide donor (Trotta *et al.*, 1974; Abdelal & Ingraham, 1975) as well as sensitivity to feedback inhibition by UMP (Abdelal & Ingraham, 1975).

In contrast to the single synthase of enteric bacteria, genetical and physiological evidence suggests the presence in *Saccharomyces cerevisiae* (Lacroute *et al.*, 1965) and *Neurospora crassa* (Davis, 1967) of two enzymes, each specific to either arginine or pyrimidine biosynthesis and each under separate control. In *S. cerevisiae* the pyrimidine-specific activity is encoded by the *cpu* locus, which is part of the *ura-2* genetic region; this region also includes loci that encode aspartate transcarbamylase and a UTP regulatory site controlling the feedback inhibition of both enzyme activities (Lacroute, 1968). Consistent with these genetic data, an aggregate of the yeast pyrimidine-specific carbamoyl-phosphate synthase and aspartate transcarbamylase has been partially purified and characterized (Lue & Kaplan, 1971; Aitken, Lue & Kaplan, 1975).

The activity of the yeast arginine-specific carbamoyl-phosphate synthase depends upon the integrity of two loci, *cpaI* and *cpaII*, which show no linkage to each other or to *cpu* (Lacroute *et al.*, 1965). Mutants lacking *cpaI* product retain the ability to synthesize carbamoyl phosphate from ammonia but lesions within the *cpaII* locus abolish both glutamine-dependent and ammonia-dependent arginine-specific activity (Piérard *et al.*, 1973). The arginine-specific synthase of *N. crassa* is also encoded by two unlinked loci (Davis,

1965*b*); preliminary characterization of this enzyme has been reported (Davis, 1965*a*; Williams & Davis, 1970). However, physical evidence for the yeast arginine-specific enzyme has been lacking due to the marked instability of the glutamine-dependent activity (Piérard *et al.*, 1973). In view of the arginine- and uracil-sensitive phenotypes described for the enteric system (Piérard *et al.*, 1973; Abdelal, Griego & Ingraham, 1976; Abdelal & Ingraham, 1975), which resemble the phenotypes of yeast *cpu* and *cpa* mutants, respectively, we feel that biochemical corroboration of the genetic evidence is desirable. In the present paper, we describe conditions that stabilized a novel arginine-specific activity sufficiently to allow partial purification and characterization of the enzyme.

METHODS

Growth of cells and preparation of cell extracts. Yeasts were grown in Yeast Nitrogen Base (Difco) with 2% (w/v) glucose and 50 mM-potassium phosphate buffer at pH 7.0. Cells were harvested in the exponential phase, washed once with water and suspended at a concentration of 2 g (wet wt) ml⁻¹ in 0.1 M-potassium phosphate buffer, pH 7.6, containing 0.5 mM-EDTA (dipotassium salt), 0.5 mM-glutamine, 3 mM-ATP, 4 mM-MgCl₂, 1 mM-UTP, 1 mM-phenylmethanesulphonyl fluoride and 5% (v/v) glycerol. These ligands and stabilizers were present in all buffers used during subsequent purification. Cells were disrupted by passage through a French press and the crude extract was centrifuged at 39000 *g* for 20 min. Protein was determined by the method of Lowry *et al.* (1951).

Enzyme assays. Carbamoyl-phosphate synthase [EC 2.7.2.5, ATP:carbamate phosphotransferase (dephosphorylating); and EC 2.7.2.9, ATP:carbamate phosphotransferase (dephosphorylating, amido-transferring)] activity was determined as previously described (Abdelal & Ingraham, 1975). The reaction mixture (final volume 0.5 ml) contained, unless otherwise stated: enzyme, 100 mM-triethanolamine buffer (pH 8.0), 100 mM-KCl, 12 mM-ATP, 16 mM-MgCl₂, 10 mM-NaH¹⁴CO₃ (1.5 × 10⁵ to 2.0 × 10⁵ c.p.m. μmol⁻¹), and either 10 mM-glutamine or 100 mM-NH₄Cl. For determination of synthase activity during purification, the reaction was coupled with excess homogeneous ornithine transcarbamylase [purified as previously described by Abdelal, Kennedy & Nainan (1977)] in the presence of 10 mM-ornithine. This coupled assay converted labile [¹⁴C]carbamoyl phosphate to thermostable [¹⁴C]citrulline, allowing an extended incubation period of 1 h. Prior to kinetic, inhibition or repression experiments, enzyme preparations were passed through a Sephadex G-25 column to remove ligands. Aspartate transcarbamylase (EC 2.1.3.2; carbamoyl-phosphate:L-aspartate carbamoyltransferase) activity was assayed by the determination of carbamoyl aspartate as described by Prescott & Jones (1969).

Enzyme purification. The arginine-specific enzyme was purified from both wild-type strain x2180-1A (from the Yeast Genetic Stock Center, University of California, Berkeley, U.S.A.) and strain 4031b (obtained from André Piérard, Université Libre de Bruxelles, Brussels, Belgium), which lacks the pyrimidine-specific synthase (*cpu*⁻). The protein concentration in the extract was adjusted to 35 mg ml⁻¹, then protamine sulphate [6 ml of 1% (w/v) solution in 0.1 M-potassium phosphate buffer, pH 7.6, per 10 ml of extract] was added with stirring at 0 °C and equilibrated for 10 min. After centrifuging at 39000 *g* for 30 min, the supernatant was adjusted to a protein concentration of 15 mg ml⁻¹ and then fractionated with ammonium sulphate. The fraction precipitating between 45 and 60% saturation was dissolved in 0.02 M-potassium phosphate buffer, pH 7.6, and applied to a DEAE-cellulose column (40 × 1.6 cm) equilibrated with the same buffer. Protein was eluted with a linear 300 ml gradient (0.02 to 0.5 M) of potassium phosphate buffer, pH 7.6. The arginine-specific enzyme eluted between 0.15 and 0.22 M with the peak of activity at 0.18 M. Fractions containing enzyme activity were combined, concentrated and applied in 0.1 M-potassium phosphate buffer, pH 7.6, to a Sephadex G-200 column (100 × 1.6 cm; void volume, 63 ml); the elution volume for the enzyme was 102 ml. These procedures resulted in a 30- to 40-fold purification. Further attempts to purify the synthase by chromatography on hydroxylapatite were successful in separating the enzyme from other proteins but resulted in appreciable loss of activity. Accordingly, peak fractions from gel filtration (following protamine sulphate and ammonium sulphate fractionation of extracts) were used to characterize the enzymes from wild-type and mutant strains. Work is in progress to improve the yield and stability of the enzyme by alternative purification methods.

Fig. 1

Fig. 1. Gel filtration pattern of 30 to 60 % saturated ammonium sulphate fraction of *S. cerevisiae* strain x2180-1A (wild type). A 3.5 ml sample (70 mg protein ml⁻¹) was applied to an upward-flow Sephadex G-200 column (110 × 1.6 cm; void volume, 79 ml) and eluted with 0.1 M-potassium phosphate buffer, pH 7.6, containing 1 mM-UTP and the ligands and stabilizers described in Methods. Fractions of 1.6 ml were collected and assayed for enzyme activity: ○, glutamine-dependent synthase activity; ●, ammonia-dependent synthase activity; △, aspartate transcarbamylase activity.

Fig. 2

Fig. 2. Calibration curve for the estimation of molecular weight by gel filtration on a Sephadex G-200 column (100 × 1.6 cm; void volume, 63 ml). Synthase was eluted with 0.1 M-potassium phosphate buffer, pH 7.6, containing ligands; fractions of 1.7 ml were collected and assayed for activity. Arrow indicates the elution volume of arginine-specific synthase. Molecular weight standards: fer, apoferritin; cat, catalase; adh, alcohol dehydrogenase; bsa, bovine serum albumin; ova, ovalbumin; chy, chymotrypsinogen.

RESULTS

Physical evidence for an arginine-specific synthase

Three distinct carbamoyl-phosphate synthase activities were evident in the gel filtration pattern of *S. cerevisiae* strain x2180-1A (wild type) (Fig. 1). The elution profile of another strain (Σ1278b, the wild type of André Piérard) of yeast was essentially the same. The physiological role of each of the activities is suggested by the biochemical, genetical and regulatory properties of the system.

The activity of peak I, which utilized either glutamine or ammonia as a nitrogen donor, eluted with aspartate transcarbamylase as an aggregate with a molecular weight of about 450000. This activity was strongly inhibited by UTP. Thus, peak I corresponds to the complex of pyrimidine-specific synthase and aspartate transcarbamylase activities described by Lue & Kaplan (1971) and Aitken *et al.* (1975). Peak II also possessed both glutamine and ammonia activities with substrate and cofactor requirements characteristic of carbamoyl-phosphate synthase. Peak III, which utilized ammonia but not glutamine as a substrate, appeared as a shoulder of peak II. Although the properties of peak III superficially resembled those of the catabolic carbamate kinase of *Streptococcus faecalis* (Raijman & Jones, 1973), elution profiles of synthase mutants (generously supplied by André Piérard) confirmed the model of carbamoyl phosphate synthesis deduced from genetic data by Lacroute *et al.* (1965). Peak II was absent from strain MG641 (*cpaI*⁻), and both peaks II and III were lost in strain MG642 (*cpaII*⁻). Neither peak II nor peak III was affected in strain 4031b (*cpw*⁻), which lacked only peak I. Thus, as suggested by Piérard *et al.* (1973), the *cpaII* locus encodes an ammonia-dependent subunit essential to the activity of an arginine-

Fig. [3. Carbamoyl-phosphate synthase activity as a function of glutamine concentration: (a) double-reciprocal plot; (b) Hill plot. Glutamine-dependent activity was determined as described in Methods but with the glutamine concentration varied as indicated and ATP and MgCl_2 concentrations held constant at 1 mM and 5 mM, respectively. The activity of the reaction in the presence of 5 mM-glutamine was 1.8 nmol carbamoyl-phosphate formed at 15 min.

specific synthase, and the *cpaI* locus specifies a subunit conferring upon the holoenzyme the ability to utilize glutamine. These data also corroborate the absence of functional interaction between the products of the *cpa* loci and those of the *ura-2* region, adduced from genetic analysis by Lacroute *et al.* (1965).

Properties of the partially purified synthase

A 0.4 ml sample of partially purified peak II was layered on a 12 ml linear sucrose gradient [7 to 30 % (w/v) in 0.1 M-potassium phosphate buffer with ligands, pH 7.6] and centrifuged (39000 rev. min⁻¹ for 20 h at 4 °C) in an IEC B60 preparative ultracentrifuge equipped with an IEC 404 angle rotor. The sedimentation coefficient and molecular weight of the holo-enzyme were estimated to be 7.4S and 150000 respectively, with the peak of synthase activity coinciding with the position of the yeast alcohol dehydrogenase marker (after Martin & Ames, 1961). The molecular weight was also estimated by gel filtration on Sephadex G-200 (Fig. 2) with apoferritin (480000), catalase (200000), yeast alcohol dehydrogenase (150000), bovine serum albumin (67000), ovalbumin (45000) and chymotrypsinogen (25000) as standards (Andrews, 1969). V_e/V_0 was 1.61, giving a calculated molecular weight of 115000. The molecular weight of the heavy subunit (ammonia-dependent activity of strain MG641, *cpaI*⁻) was estimated by gel filtration to be 80000.

The pH optimum for the partially purified synthase was 8.0 in triethanolamine buffer, and the enzyme was about 35 % inhibited at pH 7 and 9. Enzyme assayed in glycylglycine buffer at pH 9 had 30 % more activity than that assayed in triethanolamine while potassium citrate buffer inhibited activity by 80 % at pH 7.

Reaction rates were determined with ATP and glutamine as variable substrates. Based on a stability constant for MgATP^{2-} of 73000 M⁻¹ in triethanolamine buffer at pH 8.0 (see O'Sullivan & Perrin, 1964), the concentration of free Mg^{2+} in these experiments was 4 mM. The ATP saturation curve appeared hyperbolic with an apparent K_m of 0.2 mM at 1 mM-glutamine and 10 mM- HCO_3^- . The double-reciprocal plot for glutamine (Fig. 3a) was concave downward, and a Hill plot of the data (Fig. 3b) yielded an interaction coefficient of 0.6, indicating negative cooperativity in the binding of this substrate. The $[S]_{0.5}$ for glutamine binding was 0.25 mM.

Possible allosteric effectors of the arginine-specific enzyme were tested for modulation of the partially purified activity. Glutamine activity of the synthase was neither significantly inhibited nor activated by arginine, ornithine, UTP, *N*-acetylglutamate or glycine (glutamine-

dependent activity was determined as described in Methods, but with non-saturating ATP and MgCl_2 concentrations of 0.2 mM and 4 mM, respectively, and all effectors at 5 mM, except for UTP at 0.1 mM).

Transcriptional regulation of the arginine-specific synthase

Although activity of the yeast synthase was not regulated by feedback inhibition, gene expression was under negative control of arginine. With strain 4031b (*cpu*⁻), glutamine activity was repressed sixfold and ammonia activity twofold by growth in the presence of 1000 mg arginine ml⁻¹. Neither arginine-specific activity was affected by 200 mg uracil ml⁻¹ in the growth medium. These data are identical with the results of Piérard *et al.* (1973).

DISCUSSION

The yeast arginine-specific carbamoyl-phosphate synthase resembles the single enteric synthase in several of its physical properties. The molecular weight of 150000 as estimated by sucrose gradient ultracentrifugation is comparable to the values reported for the bacterial synthase (Trotta *et al.*, 1974; Abdelal & Ingraham, 1975). The analogous catalytic functions for the unequal subunits of the yeast arginine-specific enzyme and enteric synthase provide additional suggestion of structural conservation.

However, in contrast to these superficial similarities, the yeast arginine-specific enzyme differs substantially from other well-characterized carbamoyl-phosphate synthases with respect to its kinetic properties and in the apparent absence of regulation of its activity. The apparent K_m for ATP was 10-fold lower than that reported for the yeast pyrimidine-specific enzyme (Aitken *et al.*, 1975) and considerably lower than both the $[S]_{0.5}$ for ATP of the prokaryotic synthases (Anderson & Meister, 1966; Abdelal & Ingraham, 1975) and the K_m estimated for the arginine enzyme of *N. crassa* (Davis, 1965*a*). The difference between the two yeast enzymes in affinity for ATP is particularly difficult to reconcile in light of reports that *S. cerevisiae* lacks compartmentalization of the two activities (Urrestarazu, Vissers & Wiame, 1977). Also atypical was the glutamine saturation curve of the arginine enzyme; negative cooperativity for glutamine binding has not been noted in the kinetic analyses of other synthases.

Our inhibition data show that glutamine-dependent activity of the enzyme is not modulated by possible effectors such as arginine, ornithine, UTP or *N*-acetylglutamate. These data confirm the observations of Lacroute *et al.* (1965), who demonstrated that neither arginine nor UTP affected the activity of the arginine-specific synthase in crude extracts. The amount of enzyme present is regulated, however, by a mechanism that has several uncommon characteristics. Synthesis of the glutamine-dependent subunit (*cpaI* product) was shown to be under negative operonic control, but synthesis of the ammonia-dependent subunit (*cpaII* product) apparently lacks genetic control (Thuriaux *et al.*, 1972). The available repression data indicate that transcription of the *cpaII* locus must be regulated to some extent, though not as efficiently as that of the *cpaI* cistron [see Chamberlin (1974) for a discussion of possible mechanisms]. Because of this differential repression, Piérard *et al.* (1973) postulated that the ammonia-dependent subunit might be produced in excess. The presence of peak III in the elution profile of wild-type yeast (Fig. 1) supports this hypothesis.

Regulation comparable to the yeast system has been reported for *N. crassa* by Cybis & Davis (1975), who demonstrated that the arginine-specific synthase and acetylglutamate kinase catalysed the rate-controlling reactions in arginine biosynthesis. Although the arginine-specific synthase was completely insensitive to feedback inhibition in crude extracts, expression of glutamine-dependent activity was efficiently regulated at the level of transcription by stringent arginine repression. The authors suggested that regulation by feedback inhibition was not compatible with the organellar compartmentalization of the synthase and that the unusually high repressibility of the enzyme compensated for the

absence of feedback control. These arguments are less compelling for the yeast system, for which the synthase and kinase have also been shown to be the rate-limiting enzymes of the arginine pathway (Hilger *et al.*, 1973). The yeast synthase, which apparently also lacks feedback control, does not seem to be localized (Urrestarazu *et al.*, 1977) and has a markedly lower amplitude of repression (6-fold as compared to 60-fold) than the corresponding *Neurospora* enzyme.

Williams & Davis (1970) have reported a precursory molecular weight of 250000 for the *Neurospora* arginine-specific carbamoyl-phosphate synthase. This value is appreciably greater than the 150000 molecular weight of other synthases, including the yeast arginine-specific enzyme described here. Because no additional physical or kinetic data for the *Neurospora* enzyme has been forthcoming, it is not yet possible to infer which features of the fungal arginine-specific synthases have been conserved and which have diverged. Evidence suggesting structural similarity between the yeast arginine-specific enzyme and the enteric synthase is already available, although the striking differences in kinetic behaviour between the two systems make it essential to purify the yeast enzyme to homogeneity to confirm its subunit composition and unusual kinetic properties. Also, the question of the structure of the yeast pyrimidine-specific enzyme and its evolutionary relationship to the arginine-specific synthase remains unresolved.

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