THE ASPARTATE TRANSCARBAMYLASE AND CARBAMOYL PHOSPHATE SYNTHETASE OF YEAST: A MULTI-FUNCTIONAL ENZYME COMPLEX

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SUMMARY: A purification procedure for yeast aspartate transcarbamylase (ATCase) resulted in co-purification of the carbamoyl phosphate synthetase (CPSase) of the pyrimidine pathway; indeed, the CPSase specific activity rose by a considerably higher factor during the purification than did the ATCase. Both activities were highly sensitive to feedback inhibition (FI) by UTP. Sucrose density gradient centrifugation in presence of UTP established that both activities co-sedimented in a single peak of molecular weight 600,000. Omission of UTP caused dissociation into a complex of molecular weight 300,000 with both enzyme activities but of reduced sensitivity to FI. Chromatography on DEAE-Sephadex caused loss of CPSase, increase in ATCase and disappearance of FI of the latter; the ATCase recovered had a molecular weight of 140,000.

In yeast the first two enzymes in the pyrimidine pathway are a pyrimidine-specific carbamoyl phosphate synthetase (CPSase) and aspartate transcarbamylase (ATCase). Both enzymes are under the control of the ur-2 gene in yeast; both are sensitive to feedback inhibition (FI) by UTP and the synthesis of both is repressed by UTP as co-repressor (1,2,3). Genetic complementation studies (1) and the combined effects of temperature and dilution on ATCase (4) have suggested that this enzyme has a subunit structure. Other studies from this laboratory have shown that FI of ATCase is rapidly lost during heating of semi-purified preparations at 50°C (5) and that the feedback site of this enzyme is stabilized during extraction from the cell, and possibly within the cell, by UTP (6). We now present evidence to show that both these enzyme activities are associated with a single complex in which form they are co-purified, together with the feedback site.

METHODS: The strains of S. cerevisiae used have been previously described (2, 5), as has the minimal medium (7, as modified (5)) and the ATCase assay

(8, as modified (9, 6)). CPSase assay was performed using 1 ml. of a reaction mixture made up in 0,1 M phosphate buffer, pH7.6, containing 5 x 10^{-4}M glutamine, 5 x 10^{-2}M $NaHCO_3$, 3 x 10^{-2}M $MgSO_4$, 7.5 x 10^{-3}M ATP and 6 x 10^{-3}M ornithine; an excess of a purified preparation of E. coli ornithine transcarbamylase (10) was added in 5 µl., to convert all carbamyl phosphate formed to citrulline. The reaction was started by adding 20-200 µl of the preparation of CPSase to be tested. After an appropriate incubation period (20-60 min.) at 25°C the reaction was stopped by adding 5 ml. of the mixture described by Koritz and Cohen (8) as modified by Gerhart and Pardee (9). Colour was maximal after 18 min. at 30°C when it was read in the spectrophotometer at 560 mµ; citrulline was used as a standard. In the assay of the crude extract and of the supernatant fraction after protamine sulphate treatment, high blanks, due to endogenous carbamoyl derivatives, made it necessary to put these preparations through a column of G-25 Sephadex prior to testing. The enzyme was then eluted with 0.1 M phosphate buffer pH 7.6. This treatment was not performed after subsequent steps in the purification.

Purification of ATCase: Crude extract (step 1) was obtained as described elsewhere (6). Solid protamine sulfate was added to a concentration of 1% and the supernatant collected (step 2). At -10°C, isopropanol was added dropwise to a concentration of 12%, and the precipitate was collected and redissolved in 0.1 M phosphate buffer, pH 6.8, containing 30% $(NH_4)_2SO_4$. After centrifugation, the supernatant was brought to 45% $(NH_4)_2SO_4$ and the precipitate collected and dissolved in 10 ml. of 0.1 M phosphate buffer, pH 7.4 (step 3). To this was added 3 ml. calcium phosphate gel (37 mg. dry weight per ml.); the gel was washed once with the same buffer and then twice eluted with 10 ml. of 0.2 M phosphate buffer, pH 7.4, to yield 20 ml. enzyme eluate. To this was added 20 ml. of a solution of saturated $(NH_4)_2SO_4$; after 20 min. at 0°C, the precipitate was collected and redissolved immediately before use in these experiments (step 4).

RESULTS: Our ATCase preparations were generally purified by a factor of

Table I

Purification of the ATCase and CPSase of FL 233-3C, a derepressed haploid strain. The specific activities of CPSase (mμM citrulline formed/hr./mgm. protein) and ATCase (mμM ureidosuccinate formed/min./mgm. protein) are shown.

Purification Steps	Column 1 ATCase		Column 2 CPSase	
	Specific Activity	Purification	Specific Activity	Purification
1. Crude extract	299.2	1.0	36.2	1.0
2. Protamine Supernate	320.3	1.1	97.9	2.7
3. Isopropanol $(NH_4)_2SO_4$	2359.0	7.9	363.2	10.0
4. $Ca_3(PO_4)_2$ $(NH_4)_2SO_4$	6767.8	22.6	2361.8	65.0

Table II

Purification of the ATCase and CPSase of Fl 80-2A, a haploid strain lacking the CPSase of the arginine pathway. The procedure was the same as in Table I

Purification Steps	Column 1 ATCase		Column 2 CPSase	
	Specific Activity	Purification	Specific Activity	Purification
1. Crude Extract	48.6		4.5	
2. Protamine Supernate	66.5	1.4	15.7	3.5
3. Isopropanol $(NH_4)_2SO_4$	190.8	3.9	30.5	6.7
4. $Ca_3(PO_4)_2(NH_4)_2SO_4$	626.0	12.9	401.2	88.6

20-50 times; one such purification is illustrated in Table I. Col. 1 shows that the specific ATCase activity of the crude extract of the derepressed strain FL-233-3C was 299, approx. 3 X that of wild type strains (6); after purification, the specific activity rose by a factor of 23 X. Col. 2 shows that the CPSase activity was also purified by this procedure, in this case by a factor of 65X. In all our experiments, purification of the CPSase

was considerably greater than that of the ATCase, despite the fact that the procedure was worked out for the latter. Table II shows the purification of the enzymes extracted from strain FL 80-2A, a haploid strain lacking the CPSase activity of the arginine pathway (cpa-)(3). The ATCase was purified by a factor of 13X, the CPSase by 89X. Thus it is clear that it is the CPSase of the uracil pathway that is being purified.

Purified enzyme from strain FL 233-3C was subjected to sucrose density gradient centrifugation in the presence of 3 x 10^{-3} M UTP and of crystalline beef liver catalase as an internal marker. Fig. 1 shows that ATCase and

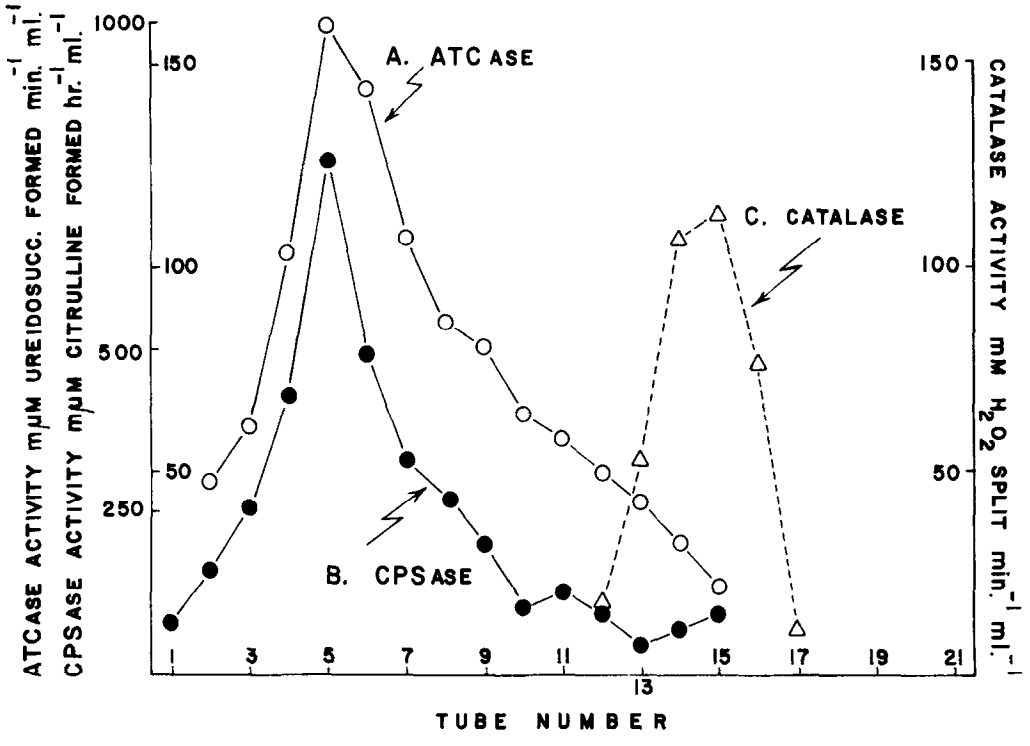

Figure 1. Sucrose density gradient centrifugation of a purified preparation spun at 60,000 rpm for 3 hours at 5°C. Linear gradients (4.6ml) using 5 and 20% (w/v) sucrose in 0.02M phosphate buffer pH7.6 containing 0.01M $MgSO_4.7H_2O$, 4 x 10^{-4}M glutamine, 3 x 10^{-3}M UTP and 10^{-3}M β-mercaptoethanol were prepared as described by Martin and Ames (13). Sample (0.1 ml.) was layered on the gradient with catalase as an internal marker. After centrifugation in the SW-65 rotor of the Beckman model L2-65 ultracentrifuge, fractions containing 13 drops (0.22 ml) were collected. Catalase activity was estimated by a modification of the method of Chantrenne (14). The activities are expressed as mM H_2O_2 split/min./ml. The CPSase activities were measured (see methods) using 0.1 ml. aliquots and ATCase 10 μl.

CPSase co-sedimented in a single peak corresponding to a molecular weight of 600,000. When UTP was omitted from the gradient, the two activities co-sedimented but in this case the molecular weight of the complex was 300,000; the ATCase had a considerably reduced sensitivity to FI (approx. 30% inhibition by 2 x 10^{-3}M UTP, as opposed to approx. 75% in the case of the preparation of molecular weight 600,000).

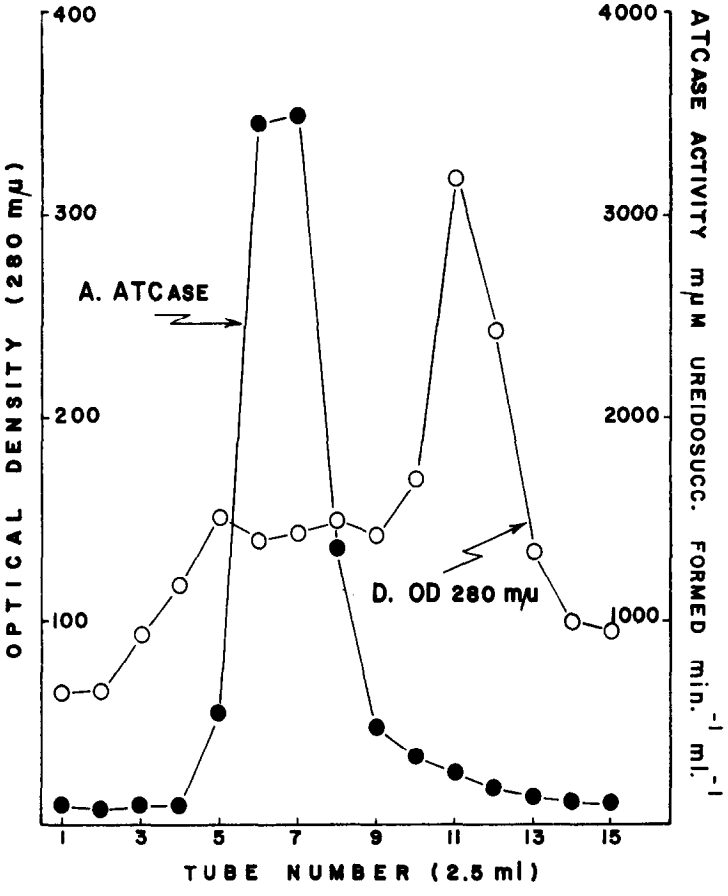

Figure 2. DEAE - Sephadex column chromatogram. The purified preparation was dissolved in 0.5 ml of 0.2M phosphate buffer, pH 6.85, containing 10^{-3}M β-mercaptoethanol and 2 x 10^{-4}M $MgSO_4$. This sample was applied to a 1.5 x 27 cm. column of DEAE-Sephadex and eluted with the same buffer at room temperature. Each fraction contained 2.5 ml.

Chromatography of the purified preparations on columns of DEAE-Sephadex yielded an eluate which showed but a single band upon electrophoresis on cel-

lulose acetate strips; its specific ATCase activity increased by a factor of 5-10 times that of the Ca_3PO_4 eluates. However, such preparations had lost all but traces of their CPSase activity and their ATCase was completely insensitive to FI. Fig. 2 shows an elution profile from DEAE-Sephadex and Fig. 3 a sucrose density gradient run on the combined contents of tubes 6 and 7 of Fig. 2. A single peak of ATCase activity was recovered but this time with a molecular weight of only approx. 140,000. Heat (5 min. at 50°) had exactly the same effect as DEAE-Sephadex treatment.

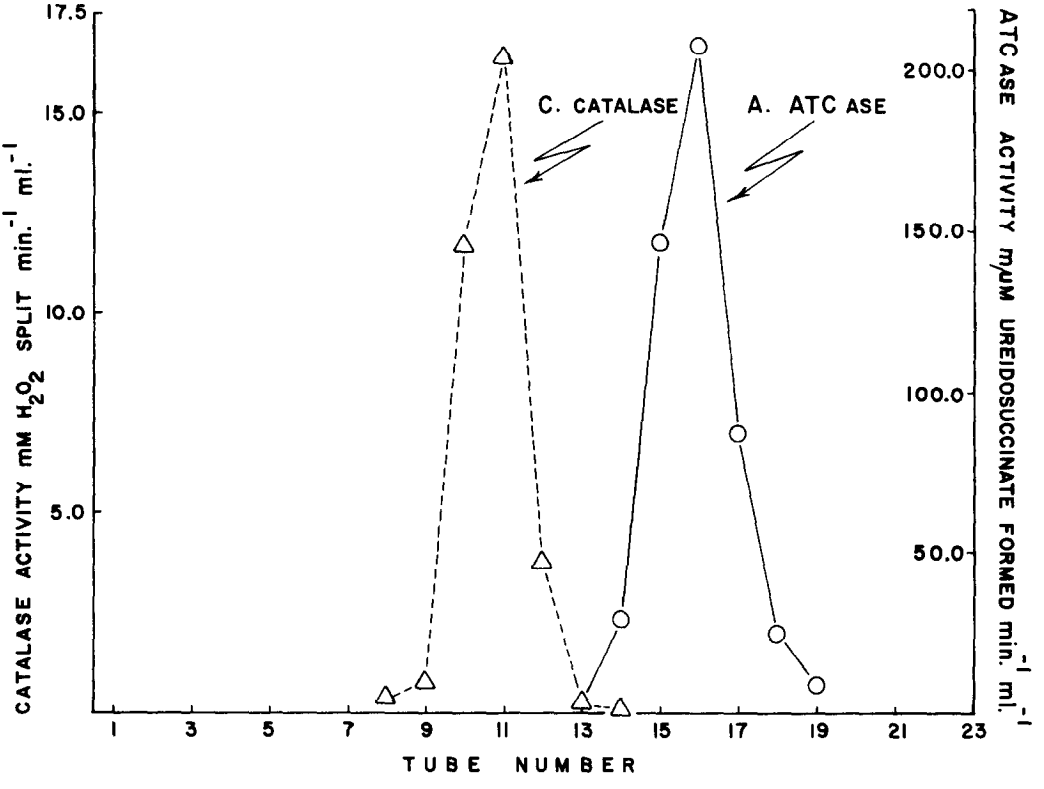

Figure 3. Sucrose density gradient centrifugation of $(NH_4)_2SO_4$ preparation of tubes 6 and 7 (Fig. 2) spun at 60,000 rpm for 5 hours at 5°C. The procedure was the same as in Fig. 1.

DISCUSSION: A method of purification of yeast ATCase resulted in a considerably greater co-purification of CPSase. After centrifugation on sucrose gradients, such preparations showed a single peak for both activities corres-

ponding to a molecular weight for the complex of 600,000, provided that UTP was included in the gradient. We conclude that both activities are associated with a single protein molecule consisting of many individual subunits. Omission of UTP resulted in dissociation of the aggregate into half-molecules of molecular weight 300,000 possessing both activities, but whose ATCase possessed reduced sensitivity to FI. Treatment of purified preparations either on DEAE-Sephadex or by heat caused dissociation into units possessing roughly one-quarter the molecular weight of the fully aggregated material (140,000); such preparations had virtually no CPSase activity and their very active ATCase was completely insensitive to FI by UTP. These data indicate the existence of at least 3 states consisting of 1, 2 and 4 monomers and also confirm a previous conclusion, based on the effects of dilution (4), that individual monomers possess only ATCase activity insensitive to FI.

The reason for the greater increase in specific activity of CPSase during the purification is doubtless due to the presence in the crude extract of some partially dissociated molecules lacking CPSase activity. During purification there is a selection for the larger aggregates resulting in a greater purification of the CPSase than of the ATCase.

In Neurospora, a single gene also controls both CPSase and ATCase activities (11). While these enzyme activities are in many respects different from those of yeast, both are also co-purified, suggesting the presence of a single complex (12). A number of other enzyme aggregates have been reported (15, 16, 17, 18); it is difficult as yet to detect a common regulatory and evolutionary significance. We incline to the view that where a substrate is common to more than one pathway, as in the case of carbamoyl phosphate, aggregation of the CPSase and ATCase may cause a preferential channelling (19) of the common precursor into the pyrimidine pathway. Experiments designed to test this hypothesis are in progress.

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