Biochimica et Biophysica Acta, 309 (1973) 50–57

BBA 66882

CHARACTERIZATION OF THE ASPARTATE CARBAMOYLTRANSFERASE SUBUNIT OBTAINED FROM A MULTIENZYME AGGREGATE IN THE PYRIMIDINE PATHWAY OF YEAST

ACTIVITY AND PHYSICAL PROPERTIES

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(Received November 14th, 1972)

SUMMARY

The carbamoylphosphate synthase (ATP:carbamate phosphotransferase, dephosphorylating, EC 2.7.2.5)–aspartate carbamoyltransferase (ATP:creatine phosphotransferase, EC 2.1.3.2) aggregate of *Saccharomyces cerevisiae* was dissociated by heat and the resulting aspartate carbamoyltransferase subunit purified to homogeneity as determined by polyacrylamide gel electrophoresis and ultracentrifugation. The molecular weight of the subunit was estimated to be 138 000. Treatment with sodium dodecyl sulphate gave a single band on gel electrophoresis corresponding to a molecular weight of 21 000. The pH dependence and Michaelis constants for both substrates were determined and found to be identical with those parameters previously determined for the enzyme aggregate. Antibody to the enzyme aggregate caused the precipitation of aspartate carbamoyltransferase activity when it was reacted with either the aggregate or the subunit. The purified subunit, probably a hexamer, retains many of the properties associated with the intact multienzyme aggregate.

INTRODUCTION

In *Saccharomyces cerevisiae*, the ura-2 locus of Mortimer and Hawthorne[1] was found by Lacroute[2] to control three functions: the pyrimidine-specific carbamoylphosphate synthase (ATP:carbamate phosphotransferase, dephosphorylating, EC 2.7.2.5), the aspartate carbamoyltransferase (ATP:creatine phosphotransferase, EC 2.1.3.2) and the regulatory site at which both activities are inhibited by UTP. Recent work from this laboratory[3] has shown that the ura-2 locus consists of at least two closely linked cistrons, one coding for carbamoylphosphate synthase and one for aspartate carbamoyltransferase, and transcribing into a single molecule of messenger RNA in the direction carbamoylphosphate synthase → aspartate carbamoyltransferase.

Earlier work from this laboratory showed that the gene products of these tightly linked cistrons, the carbamoylphosphate synthase and aspartate carbamoyltransferase activities, were also linked, in the form of a multifunctional enzyme aggregate of mol. wt 800 000 (refs 4, 5). It was possible to dissociate the aggregate under controlled conditions into half-molecules (mol. wt 380 000) with both activities present, but with the aspartate carbamoyltransferase only slightly sensitive to feedback inhibition by UTP[5]. Further dissociation resulted in the appearance of a separate carbamoylphosphate synthase peak in sucrose density gradients (mol. wt 250 000) still fully sensitive to feedback inhibition[5]; indeed, we have been unable by biochemical or physical methods to obtain a UTP-insensitive carbamoylphosphate synthase. On the other hand, early experiments showed that heat caused the aspartate carbamoyltransferase to lose its sensitivity to feedback inhibition[7] and this was found to be due to dissociation of a highly active aspartate carbamoyltransferase subunit of mol. wt 140 000 totally lacking carbamoylphosphate synthase activity and completely insensitive to UTP[6]; the same aspartate carbamoyltransferase subunit with identical kinetic properties was found after passage of the purified aggregate on a column of DEAE-Sephadex, with mol. wt 140 000 (ref. 4).

The present experiments were undertaken to purify the aspartate carbamoyltransferase subunit to homogeneity, to determine its molecular weight and that of its component polypeptide chains and to compare its kinetics with that of the aspartate carbamoyltransferase of the aggregate and with that of the catalytic subunit of *Escherichia coli*. There are several excellent reviews dealing with comparative aspects of the pyrimidine pathway of microorganisms[8] and of aspartate carbamoyltransferase structure and kinetics[9,10].

MATERIALS AND METHODS

Growth of *Saccharomyces cerevisiae* and purification of the carbamoylphosphate synthase–aspartate carbamoyltransferase aggregate was as previously described[5]. The aspartate carbamoyltransferase subunit was prepared by heating the fraction obtained after calcium phosphate gel absorption, elution and precipitation by $(NH_4)_2SO_4$ at 55 °C for 5 min. The heated fraction was centrifuged and the resulting supernatant eluted from a Sepharose 4B column (1.6 cm × 25 cm) with 0.1 M phosphate buffer (pH 6.8) containing $2 \cdot 10^{-4}$ M $MgSO_4$ and $2 \cdot 10^{-3}$ M β-mercaptoethanol. The fractions active in aspartate carbamoyltransferase and showing no inhibition by UTP were pooled and precipitated with 45–65% $(NH_4)_2SO_4$.

The enzyme was assayed as previously described[11,12]. Marker proteins were purchased as follows: cytochrome *c*, myoglobin, ovalbumin, γ-globulin and albumin from Mann Research Laboratories; catalase from Worthington Biochemical Corporation; thyroglobulin and alcohol dehydrogenase from Sigma Chemical Co. Sepharose 4B was purchased from Pharmacia and substrates and calcium phosphate gel from Calbiochem. UTP was obtained from Raylo Chemicals Ltd, the $(NH_4)_2SO_4$ used was ultra-pure from Schwartz-Mann and the sodium dodecyl sulphate was from Sigma.

Gel electrophoresis was carried out on 5% polyacrylamide gels, containing 0.15% methylenebis-acrylamide. Sodium dodecyl sulphate polyacrylamide gel electrophoresis was carried out using the method of Weber and Osborn[13]. Analytical ultracentrifugation was performed using the Beckman Model E ultracentrifuge.

Antibody to the enzyme aggregate was prepared by injecting subcutaneously in the thigh two 2–3-month-old rabbits with 1.0 ml of an enzyme preparation containing 4.0 ml of pure antigen (approximately 2.0 mg protein) and an equal volume of Freund's (complete) adjuvant (Calbiochem). Three injections were given to each rabbit at one-week intervals and blood collected from the ear at 3–8 days after the last injection. The γ-globulin fraction was precipitated from pooled sera by the method of Keckwick[14], resuspended in phosphate buffer (0.005 M, pH 7.0) and dialysed for 48 h in the cold against the same buffer. The resulting preparation was used as the anti-enzyme aggregate serum or antibody.

RESULTS

Physical properties of the aspartate carbamoyltransferase subunit

Fig. 1 shows the elution profile from Sepharose 4B of a semi-purified preparation of the enzyme aggregate heated at 55 °C for 5 min. Heating invariably caused an increase in the aspartate carbamoyltransferase specific activity, varying from 1.5

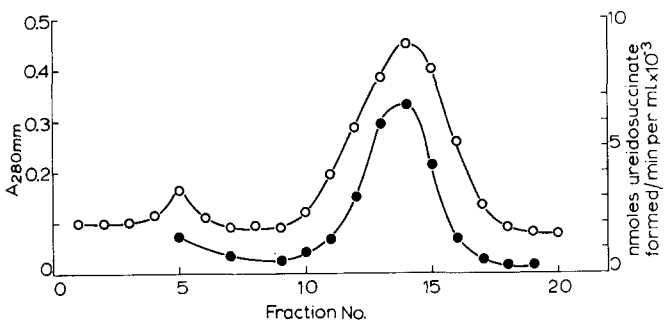

Fig. 1. Elution of heated semipurified enzyme aggregate from Sepharose 4B. ○—○, elution of protein; ●—●, enzyme activity.

to 2.5 times the activity before heat. The major protein peak and the aspartate carbamoyltransferase activity peak were eluted in the same position. The enzyme activity in the most active fractions was not inhibited by $2 \cdot 10^{-3}$ M UTP: this concentration of UTP caused 70% inhibition of the aspartate carbamoyltransferase activity of the original aggregate. A smaller protein peak with aspartate carbamoyltransferase activity which was sensitive to UTP was sometimes eluted before the major peak; this was presumably due to incompletely dissociated aggregate.

A plot of elution volume against the log of molecular weight of several marker proteins was linear and the elution volume of the aspartate carbamoyltransferase subunit corresponded to a molecular weight of 138 000. The purified aspartate carbamoyltransferase subunit migrated as a single band on polyacrylamide gel electrophoresis (Fig. 2) and migrated as a single homogeneous component in the analytical ultracentrifuge. Fig. 2 also shows the electrophoretic pattern on polyacrylamide gel after the subunit had been incubated with sodium dodecyl sulphate. The resulting polypeptide migrated as a single band. The electrophoretic mobilities of six marker proteins subjected to electrophoresis in the presence of sodium dodecyl sulphate gave

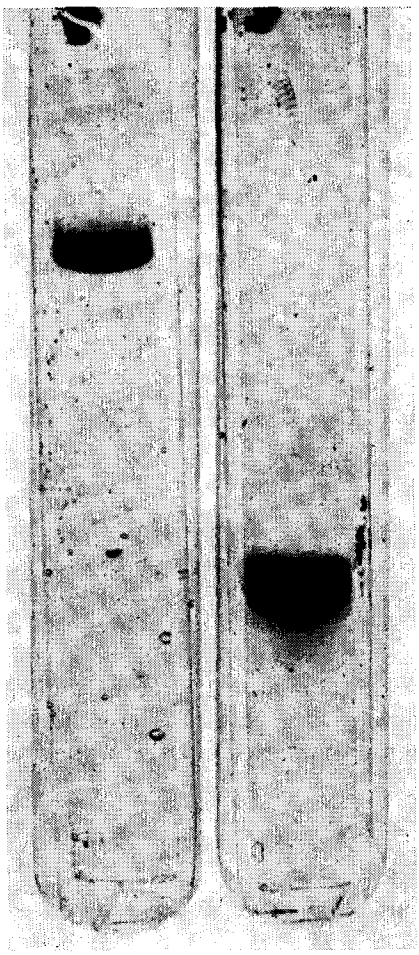

Fig. 2. Migration of purified subunit on polyacrylamide gel electrophoresis. Left-hand tube, untreated subunit; right-hand tube, subunit preincubated with sodium dodecyl sulphate.

a linear plot; from this plot, the molecular weight of the single polypeptide chain obtained after dissociation of the aspartate carbamoyltransferase subunit with sodium dodecyl sulphate was shown to be 21 000.

Kinetic properties of the aspartate carbamoyltransferase subunit

Fig. 3 shows the pH curve for the subunit in the pH range 6.0–9.0. Maximum activity was obtained at a pH value of 8–9. Double reciprocal plots with either aspartate or carbamyl phosphate as variable substrate in the presence of a saturating concentration of the other were linear. The K_m for aspartate was $4.30 \pm 0.12 \cdot 10^{-2}$ M while the K_m for carbamyl phosphate was $3.40 \pm 0.15 \cdot 10^{-3}$ M. Fig. 4 compares the Arrhenius plots of the aspartate carbamoyltransferase activity of the enzyme aggregate and subunit. Activation energies of the subunit, the enzyme aggregate and the enzyme aggregate assayed in the presence of $2 \cdot 10^{-3}$ M UTP were 12.4, 12.0 and 16.1 kcal/mole, respectively.

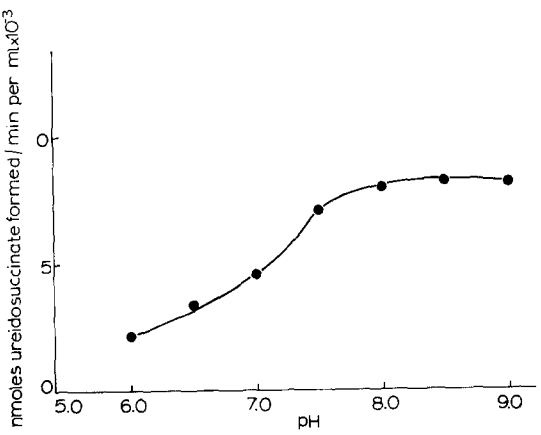

Fig. 3. pH dependence of the subunit under standard conditions of assay.

Reaction of the enzyme aggregate and subunit to anti-enzyme aggregate antibody

Fig. 5 shows the reaction of the antibody, formed in response to intact aggregate, with the original antigen and with the subunit. Under the conditions of the experiment, 80 μl of antibody precipitated all of the aspartate carbamoyltransferase activity of the aggregate from the supernatant. The same volume of antibody precipitated 85% of the subunit activity from the supernatant. From 10–30% of the original enzyme activity could be recovered in the precipitates after resuspension in buffer. The presence of UTP did not modify the degree of precipitation of either aggregate or subunit by the antibody preparation.

DISCUSSION

The aspartate carbamoyltransferase subunit obtained by heating the yeast

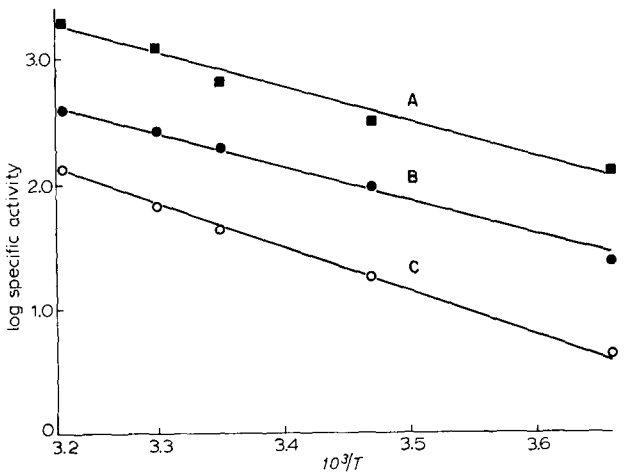

Fig. 4. Arrhenius plots of the aspartate carbamoyltransferase activity of: (A) subunit; (B) enzyme aggregate and (C) enzyme aggregate assayed in the presence of $2 \cdot 10^{-3}$ M UTP.

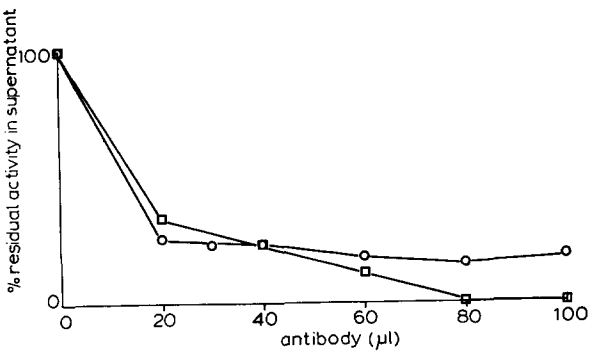

Fig. 5. Reaction of the antibody prepared against the enzyme aggregate with the aggregate (□—□) and subunit (○—○), showing the percentage residual activity remaining in the supernatant after 2 h reaction of antibody with aggregate or subunit. The amount of antigen present was 0.12 mg of enzyme aggregate and 0.02 mg of subunit.

enzyme aggregate has a mol. wt of 138 000 which compares favorably with previous estimates from this laboratory[4,6]. One effect of heating the enzyme aggregate is to abolish sensitivity to feedback control by UTP. A similar situation occurs in *E. coli* in which the aspartate carbamoyltransferase is inhibited by CTP. Heat, urea or mercurials abolish this CTP effect as well as the sigmoidal binding of L-aspartate[11]. Thus the action of heat on the aspartate carbamoyltransferases from *E. coli* and *S. cerevisiae* causes the dissociation of the enzyme into a smaller, catalytically active form, no longer sensitive to feedback control. The molecular weights of the native enzymes from the two sources are quite different (310 000 for *E. coli*[15] and 800 000 for yeast[5]) although the aggregate from yeast contains a carbamoylphosphate synthase subunit and possibly a regulatory subunit. The carbamoylphosphate synthase and aspartate carbamoyltransferase activities in *E. coli* are not linked in the same enzyme aggregate.

In yeast, the enzyme aggregate dissociates, on omission of UTP from a sucrose density gradient, to a half-molecule of mol. wt 380 000 posessing both carbamoylphosphate synthase activity (which is fully sensitive to feedback inhibition by UTP) and aspartate carbamoyltransferase activity (which is only poorly inhibited by UTP)[5]. Further dissociation occurs when glutamine and Mg^{2+} are omitted from the gradient, with the carbamoylphosphate synthase peak, still fully sensitive to feedback control, trailing behind the aspartate carbamoyltransferase in a new peak corresponding approximately to 250 000 daltons. These data are consistent with a model in which the native enzyme contains two aspartate carbamoyltransferase subunits (each of mol. wt 138 000) and two carbamoylphosphate synthase subunits possessing regulatory properties (each of mol. wt 250 000) per enzyme aggregate of mol. wt 800 000. If this is the case, the molecular weight of protein contributing to aspartate carbamoyltransferase activity in the native enzyme from yeast and *E. coli* would be approximately the same. The molecular weights of the catalytically active aspartate carbamoyltransferase subunits are quite similar (100 000 for the C-subunit of *E. coli*[15] and 138 000 for the yeast subunit). Similarly, Coleman and Jones[16] showed that two aspartate carbamoyltransferases exist in *Citrobacter freundii*, one with a mol. wt of 250 000 and a smaller enzyme with a mol. wt of 93 000. The larger enzyme could

be converted to the smaller form by repeated gel filtration. Unlike the larger enzyme, the smaller enzyme is not under feedback control by CTP. Thus in each case cited above, feedback inhibition is associated only with the larger molecular weight form of the enzyme.

The aspartate carbamoyltransferase subunit of yeast was shown to migrate as a single band in sodium dodecyl sulphate electrophoresis. The molecular weight of this polypeptide chain was estimated at 21 000 and thus the subunit may exist as a hexamer. Our colleague, Dr M. Denis-Duphil presently in the laboratory of Lacroute, estimated the molecular weight of the presumed asparate carbamoyltransferase polypeptide chain by the genetic method of Manney and Mortimer[17], assuming that one genetic map unit codes for a stretch of polypeptide 43 amino acids long, as reported by Sherman and Stewart[18] for yeast cytochrome *c*. She found that the aspartate carbamoyltransferase (A) cistron was 4.5 map units in length, corresponding thus to 194 amino acids or to a molecular weight of approximately 20 000 (personal communication). The calibration of Sherman and Stewart[18] would thus seem to apply to aspartate carbamoyltransferase as well as to cytochrome *c*. In comparison, the C-subunit of *E. coli* is made up of polypeptide chains of mol. wt 33 000 and thus exists as a trimer[19]. Little is known about the structure of the subunit from other sources although the aspartate carbamoyltransferase from *Streptococcus faecalis*, with a mol. wt of 120 000–140 000, has been suggested as existing as a dimer[20].

The pH maximum of the subunit was shown above to be in the range 8–9. Kaplan *et al.*[21] previously showed the pH maximum for aspartate carbamoyltransferase activity in the aggregate to be in the range 8.0–8.7. Similarly *E. coli*[22] and *C. freundii*[23] have pH maxima of 8.5. The K_m for aspartate in the yeast enzyme was shown to be $4.3 \cdot 10^{-2}$ M and this compares with the values of $2.8 \cdot 10^{-2}$ M reported for *C. freundii*[16]. The K_m for carbamyl phosphate however was different for the two organisms; the value in yeast was shown to be $3.4 \cdot 10^{-3}$ M while that of *C. freundii* was $3.9 \cdot 10^{-5}$ M[16]. The K_m for carbamyl phosphate in the enzyme aggregate is $4 \cdot 10^{-3}$ M[20]. The activation energy for the subunit was very similar to that reported by Lue and Kaplan[6] for the enzyme aggregate. It thus appears that the increase in specific activity following heat treatment of the aggregate is not due to any change in either K_m or activation energy but is more probably due to an increase in the number of exposed active sites; alternatively it may be due to an increase in velocity of a rate limiting step. In *E. coli* the isolated C-subunit has a maximum specific activity about 2-fold higher than that of the native enzyme[15], but this increase in specific activity is associated with changes in substrate affinity and pH dependence[11].

The antibody prepared against the enzyme aggregate was shown to react with both the original antigen and the subunit derived from it. As some aspartate carbamoyltransferase activity was recovered in the precipitates, presumably some active sites remain available after the antigen–antibody complex is formed. The antibody reacted somewhat more effectively with the enzyme aggregate then with the subunit. It is thus possible that the antigenic determinants of the complex have a different conformation in the whole enzyme than in the subunit. On the other hand, it is likely that some antigenic determinants of the aggregate are due to carbamoylphosphate synthase and regulatory chain(s) or may be created at the points of contact of the subunit with the remainder of the enzyme complex and these are of course lost upon dissociation. Similarly antisera to the native enzyme in *E. coli* react more effectively

with native aspartate carbamoyltransferase than with the isolated catalytic subunit[24]. Preincubation of the aggregate with UTP did not protect the enzyme from precipitation and it is thus unlikely that many antigenic determinants are closely contiguous to a UTP binding site.

Thus, the principal parameters of the aspartate carbamoyltransferase activity of the isolated subunit are not significantly modified by its aggregation into the multifunctional complex previously described[4]. On the other hand, it is only in the aggregated state that the aspartate carbamoyltransferase acquires sensitivity to feedback inhibition, which is of obvious metabolic importance due to spillover into the pyrimidine pathway of carbamyl phosphate formed by the arginine-specific carbamoylphosphate synthase[25,26]. This regulatory control, as well as the channeling of carbamyl phosphate formed by the pyrimidine-specific carbamoylphosphate synthase into the pyrimidine pathway[26], are probably the main evolutionary advantages of the aggregation of the first two enzymes of the pyrimidine pathway into a multi-functional complex.

ACKNOWLEDGEMENTS

The authors are grateful for the technical assistance of Maureen McIntosh and Nicole Folléa and for grants from the National and Medical Research Councils of Canada.

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