# ISOLATION OF A MULTIFUNCTIONAL COMPLEX CONTAINING THE FIRST THREE ENZYMES OF PYRIMIDINE BIOSYNTHESIS IN *DROSOPHILA MELANOGASTER*

B. JARRY

*Centre de Biochimie et de Biologie Moléculaire, Centre National de la Recherche Scientifique, 31 chemin Joseph Aiguier, 13274 Marseille Cédex 2, France*

Received 9 July 1976

## 1. Introduction

In *Drosophila melanogaster*, the rudimentary locus (*r*; 1–54.5) was found recently to control three functions: the pyrimidine specific carbamoyl phosphate synthetase (CPase EC 2.7.2.5), the aspartate transcarbamylase (ATCase EC 2.1.3.2) and the dihydroorotase (DHOase EC 3.5.2.3). [1–3]. These enzymes catalyse the first three reactions of pyrimidine biosynthesis. Based on genetic and biochemical evidence it is assumed that this locus contains the structural genes for these enzymes ([2,3] and B. Jarry, unpublished results).

Recent studies have shown that in at least two mammalian species and also in bull-frog the three enzymes exist as a macromolecular entity [4–6]. In *Drosophila* larvae Söderholm et al. have reported data suggesting the existence of a bifunctional complex between at least two of the enzymes [7]. This paper reports the partial purification of a complex containing the three enzymes from a wild type *D. melanogaster* cultured cell line.

## 2. Materials and methods

### 2.1. *Drosophila cell line culture*

Subline M.1 of the KC *Drosophila* cell line [8] was grown in suspension culture in D 22 medium with 5% calf serum. Cells were collected and kept frozen at −80°C.

Larvae of *Drosophila*, strain wild type Amherst, were cultured at 22°C on standard molasses corn meal media. Second instar larvae were harvested three days after egg deposition and kept frozen at −20°C.

### 2.2. *Preparation of extracts*

All purification steps were performed at 0–4°C. Cells were resuspended in 0.05 M potassium phosphate, pH 7.2, 0.01 M $MgCl_2$, 1 mM dithioerythritol, 1 mM EDTA and 30% (v/v) glycerol and broken in a French press cell. The extract was immediately centrifuged at 30 000 × *g* for 20 min. The supernatant was collected and will be referred as S 30 supernatant.

Larvae were resuspended in the same buffer but containing 1 mM phenylthiourea and homogenized in a Dounce homogenizer. The extract was then centrifuged at 30 000 × *g*, fat and debris discarded and the S 30 supernatant collected.

### 2.3. *Standard enzyme assays*

CPSase activity was assayed with $^{14}$CNa bicarbonate as substrate by following the formation of [$^{14}$C] citrulline in coupled reaction with ornithine transcarbamylase. The reaction mixture contained 30 μmol KCl, 10 μmol ATP, 15 μmol $MgCl_2$, 100 μmol HEPES, pH 7.5, 2 μmol glutamine, 5 μmol ornithine, 5 μmol aspartate, excess of OTCase, 7.5% $Me_2SO$, 2.5% glycerol, 2 μmol $^{14}$CNa $HCO_3$ (0.75 μCi/μmol) and extract in 1 ml. The incubation time was 30 min at 37°C. The reaction was stopped by addition of 0.6 ml of 1 N PCA, 0.3 ml of 0.01 M glutamine and the mixture bubbled with $CO_2$ for 5–10 min. The $^{14}$C-citrulline formed was separated from the other labelled compounds by chromatography on Dowex

A-50 columns and estimated by measuring the radioactivity in a liquid scintillation counter.

ATCase activity was assayed by measuring the carbamoyl aspartate formed from carbamoyl phosphate and aspartate. A 1 ml reaction mixture containing 100 μmol Tris–HCl pH 9.3, 5 μmol K aspartate, 50 μmol carbamoyl phosphate and extracted was incubated for 30 min at 30°C. Carbamoyl aspartate was determined by the method of Prescott and Jones [9].

Dihydro-orotase activity was assayed by measuring the carbamoyl aspartate formed from dihydro-orotate. A 1 ml reaction mixture containing 100 μmol of potassium phosphate buffer pH 8.5, 2 μmol dihydro-orotate and extract was incubated for 1 h at 37°C. Carbamoyl aspartate was determined colorimetrically as for the ATCase assay. One unit of CPSase, ATCase or DHOase activity is defined as that amount of enzyme which produces 10 nmol of product per hour under the above conditions calculated on initial rate. The specific activity is expressed as units of enzyme per milligram of protein determined by the Folin test.

2.4. *Sucrose gradient centrifugation*

Linear 5 to 20% sucrose gradient (12 ml) containing 0.1 M HEPES, pH 7.2, 1 mM dithioerythritol, 30% $Me_2SO$ and 5% glycerol were centrifuged at 1°C at 41 000 rpm for 18 h using a SW41 Spinco rotor. Gradients were collected from the top. Relative sedimentation coefficients were estimated using [$^{32}$P] *Drosophila* 18S ribosomal RNA as an internal standard. Molecular weights were calculated using yeast ATCase–CPSase complex centrifuged on the same rotor as reference [10].

2.5. *Polyacrylamide gel electrophoresis*

Disc-gel electrophoresis under non-denaturing conditions was performed in 0.5 × 10 cm gels (5% w/v cyanogum, 0.19 M Tris–HCl pH 8.9, 10% glycerol). Up to 300 μl of sample in 10% sucrose containing bromophenol blue were applied at the top of the gel. Electrophoresis was usually at 4 mA per tube with 0.1 M Tris–glycine, pH 8.9, as top buffer and 0.1 M Tris–acetate, pH 8.4, as bottom buffer. The apparatus was kept at 1°C during the run.

To locate enzyme activities, 0.5 cm slices were cut with a razor blade and crushed in 0.2 ml of 0.05 M potassium phosphate pH 7.2 containing 10% glycerol. The enzymatic activities were then measured directly in the mixture. 75 – 80% of the activity was recovered by this treatment. Gels were stained with Coomassie blue R–250 after fixing with Ethanol–TCA mixture [11].

2.6. *Enzyme purification*

ATCase activity has been purified from the cell and larvae S 30 supernatants by following exactly the procedure described by Mori et al. [6].

## 3. Results

3.1. *Copurification of ATCase, DHOase and CPSase activities*

KC *Drosophila* cell line and wild type second instar larvae contain the three first enzymes of the pyrimidine pathway (table 1). Indeed, specific activities measured in the S 30 supernatant of cells are 2–3 times higher than specific activities measured

Table 1
Purification of ATCase, CPSase and DHOase from a *Drosophila* cell line

| Fraction | Total activity (units) | | | Total protein (mg) | Specific activity (units/mg of protein) | | |
|---|---|---|---|---|---|---|---|
| | ATCase | CPSase | DHOase | | ATCase | CPSase | DHOase |
| S30 (larvae)[a] | 2452 | 66 | 1082 | 273 | 8.9 | 0.24 | 3.9 |
| S30 (cells)[b] | 7194 | 235 | 2550 | 327 | 22 | 0.72 | 7.8 |
| Ammonium sulfate | 5680 | – | 2016 | 140 | 40.6 | – | 14.4 |
| Second hydroxylapatite | 208 | 1.0 | 56 | 1.6 | 130 | 0.62 | 27.6 |

[a] Starting material: 5 g second instar larvae
[b] Starting material: 5 g wet weight KC cells.

in the S 30 supernatant of second instar larvae.

The purification of ATCase using precipitation with ammonium sulfate and chromatography on hydroxylapatite columns resulted in the concomitant purification of DHOase and CPSase (results in table 1). Unfortunately the CPSase activity recovered at the end of the purification was too low to be reliably measured. It must be noted in addition that during the final chromatography step, purification of ATCase activity was significantly greater than for DHOase. Extreme lability of CPSase activity has been previously reported in other systems [4,6] and could be responsible for the poor recovery of this activity.

When the proteins in the peak fractions of the hydroxylapatite chromatography containing all three activities were analyzed on sucrose gradient after concentration by ultrafiltration, both measurable activities, ATCase and DHOase, sedimented as a single peak with a relative sedimentation coefficient of 20 S.

The pH optimum for both ATCase and DHOase reactions was found to be 9.5 using the purified enzyme preparation. We obtained the same apparent $K_m$ values as those reported by Söderholm et al. [7] for ATCase, i.e., $K_m = 5.2 \times 10^{-3}$ M and $4.4 \times 10^{-4}$ M for aspartate and carbamoyl phosphate respectively. For the DHOase reaction, the apparent $K_m$ value for dihydro-orotate was $6 \times 10^{-4}$ M at pH 9.0.

### 3.2. *Cosedimentation of CPSase, ATCase and DHOase*

The poor recovery of the enzyme activities after partial purification with classical methods prompted us to try a more direct approach to estimate the molecular size of the enzymes in the extracts. When analyzed under the conditions described for the mammalian systems on sucrose gradient [4] the three activities sedimented in two peaks with relative sedimentation coefficients of 13 S and 20 S (fig.1A). Molecular weights determined using the 800 000 mol. wt. ATCase–CPSase complex of yeast [10] as reference were found to be 350 000 and 800 000 respectively.

Analysis of the same extract kept 24 h at −20°C show a drastic change in the sedimentation coefficients and repartition of the three activities (fig.1B). Under these conditions ATCase activity sediments under 2 peaks with sedimentation coefficients of 9 S (mol. wt. 150 000) and 20 S. DHOase and CPSase activities cosediment under the 20 S peak and a 12 S peak. A new peak which also contains the three activities and has a sedimentation coefficient of 28 S also becomes apparent. It must be noted, however, that although the total recovery of both ATCase and DHOase activities is only slightly decreased from one analysis to the other, the CPSase activity dropped considerably. This result has been consistently observed.

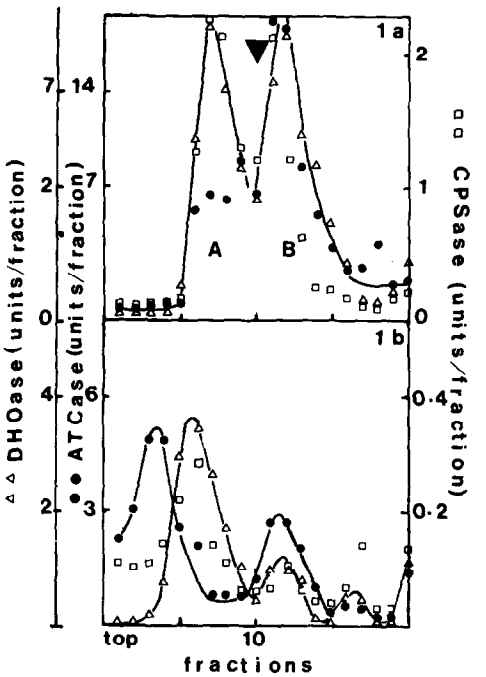


Fig.1. (a) Molecular weight estimates of ATCase–CPSase–DHOase complex from wild type *Drosophila melanogaster* by sucrose gradient centrifugation. S 30 supernatant (13 mg of protein) immediately after extraction was centrifuged at 41 000 rpm for 18 h at 1°C in a SW41 rotor. (b) S 30 supernatant (10 mg of protein) was kept frozen at −20°C for 24 h after extraction and centrifuged in the same conditions as for fig.1a. $^{32}$P-18 S rRNA was added in each tube as internal sedimentation marker. The position of the peak is indicated by an arrow: (● ●), ATCase; (△ △), DHOase; (□ □), CPSase.

### 3.3. *Dissociation of the different activities*

The different activities present in the 20 S peak of the first gradient can be quantitatively recovered and separated by electrophoresis on acrylamide gels at pH 8.9. As shown on fig.2 each enzyme activity is now split into a specific pattern. Exactly the same pattern has been obtained for different preparations.

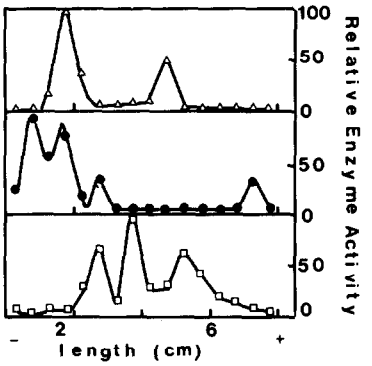


Fig.2. Dissociation by gel electrophoresis at pH 8.9 of the trifunctional '20 S' complex isolated on sucrose gradient. Fractions of the peak B of the fig.1a were pooled and submitted to electrophoresis on 5% polyacrylamide gels. 0.5 cm slices were cut with a razor blade and the presence of the three activities measured in each fraction. The ordinate represents the activity of each enzyme as relative activity to the major peak fraction: (● ●), ATCase; (△ △), DHOase; (□ □), CPSase.

A similar pattern was also obtained when the preparation purified on hydroxylapatite was analyzed with the same technique (not shown). Although the band pattern obtained by staining the gels with Coomassie blue is relatively simple, we did not try to correlate any specific band with the recorded activities, due to the crudity of our enzyme preparations.

## 4. Conclusion and discussion

To explain the complex pattern of complementation presented by the various mutants at the rudimentary locus, we recently hypothetised the existence of a multienzymatic complex containing the enzymes the synthesis of which was controlled by this locus [2]. Copurification of CPSase, ATCase and DHOase had already been described in mammals and frog [4–6]. This paper describes the purification of a large 800 000 molecular weight complex containing the two activities, ATCase and DHOase.

Although no CPSase activity could be found associated with the complex at the final stage of purification, we are confident that the protein was in fact also present in this multienzymatic structure but in a denaturated form. This interpretation is substantiated by the finding of all three activities in a complex of identical sedimentation coefficients obtained after centrifugation of the S30 supernatant on a sucrose gradient. This complex was indeed found unstable and spontaneously dissociable at low temperature into at least two units carrying ATCase and DHOase–CPSase activities respectively. The same high molecular weight complex has also been observed in larval extracts (not shown).

The greater purification of ATCase activity when compared with recovery of DHOase and CPSase, when classical biochemical techniques are used could also be due to partial dissociation of the different units of the complex during the purification.

Similarly the obtention of the 13 S complex from the S30 extract suggest partial dissociation of the complex. Its absence in the purified enzyme preparation could be explained by a lower stability than the 20 S complex.

A direct demonstration of the existence of different units in the 800 000 mol. wt. complex is obtained by analysis on polyacrylamide gels at pH 8.9. Although there is some overlap in the top region of the gel, which could reflect uncomplete dissociation of the complex, each enzyme activity gives a unique reproducible pattern.

We conclude therefore that in Drosophila, the first three activities of the pyrimidine pathway are associated with a single protein complex consisting of many individual subunits, the biosynthesis of which is controlled by the *r* locus. Various combinations of the subunits can occur, still carrying enzyme activities. These results are in perfect agreement with the genetic data [2]. Söderholm et al. [7] have described a 325 000 MW protein carrying ATCase activity in wild type *Drosophila* larvae. This protein could be similar to our 13S protein containing ATCase activity isolated on sucrose gradients.

These authors have postulated that in vivo regulation of the enzymatic activities could be expected at the level of the complex. Taking into account the close relationship of the structural genes of the three enzymes on the genetic map [2,3] other mechanisms could also be invoked. Experiments are now in progress to study the molecular and genetic basis of this regulation.

## Acknowledgements

The author acknowledges helpful discussions with R. Rosset and excellent technical assistance of N. Folléa throughout this investigation.

## References

[1] Nørby, S. (1973) Hereditas 73, 11–16.
[2] Jarry, B. and Falk, D. (1974) Mol. Gen. Genet. 135, 113–120.
[3] Rawls, J. and Fristrom, J. (1975) Nature 255, 738–739.
[4] Shoaf, W. and Jones, M. (1973) Biochemistry 12, 4039–4051.
[5] Kent, R., Lin, R., Sallach, H. and Cohen, P. (1975) Proc. Natl. Acad. Sci. USA 72, 1712–1716.
[6] Mori, M., Ishida, H. and Tatibana, M. (1975) Biochemistry 14, 2622–2630.
[7] Söderholm, G., Schwartz, M. and Nørby, S. (1975) FEBS Lett. 53, 148–153.
[8] Echalier, G. and Ohanessian, A. (1970) In Vitro 6, 162–172.
[9] Prescott, L. and Jones, M. (1969) Anal. Biochem. 32, 408–419.
[10] Lue, P. and Kaplan, J. (1971) Can. J. Biochem. 49, 403–411.
[11] Weber, K. and Osborn, M. (1969) J. Biol. Chem. 244, 4406–4412.
[12] Lue, P. and Kaplan, J. (1969) Biochem. Biophys. Res. Commun. 34, 426–433.