ASPARTATE CARAMOYLTRANSFERASE FROM WILD TYPE AND RUDIMENTARY *DROSOPHILA MELANOGASTER*

G. SÖDERHOLM and M. SCHWARTZ

Enzyme Division, University Institute of Biological Chemistry B, Sølvgade 83, DK-1307 Copenhagen K, Denmark

and

S. NØRBY

Institute of Biochemical Genetics, University of Copenhagen, Ø. Farimagsgade 2A, DK-1353 Copenhagen K, Denmark

Received 24 February 1975

1. Introduction

The *rudimentary* locus (*r*; 1-54.5) in *Drosophila melanogaster* [1] is involved in the genetic regulation of pyrimidine biosynthesis [2–5]. Based on genetic and biochemical data it is assumed [3,4,6] that this locus comprises the structural genes for the first two enzymes of the pyrimidine pathway, i.e. carbamoyl phosphate synthetase (CPS) and aspartate carbamoyltransferase (ACT), and that these enzymes form a bifunctional complex [4,7] like in some other eukaryotes [8]. Recent experiments [9] indicate that the *r* locus also determines the activity of the third enzyme in the pathway, i.e. dihydroorotase (DHO), with the possibility that this enzyme is included in the postulated complex, as it is known to be in mammalian systems [8,10].

As part of an effort to further clarify the function of the *r* locus, the present paper reports a partial purification and characterization of ACT from wild type *D. melanogaster* and from the *rudimentary* strain r^{39k}.

* Present address: Institute of Genetics, University of Copenhagen, Ø. Farimagsgade 2A, DK-1353 Copenhagen K, Denmark.

2. Materials and methods

2.1. *Drosophila cultures*

The *Drosophila* strains used were Oregon-R wild type and the *rudimentary* strain r^{39k}, cultured at 25°C on standard yeast-sucrose media [2]. In case of the mutant strain RNA was added to the medium to a final concentration of 1%.

2.2. *Preparation of extract*

2-day old larvae were harvested and homogenized as described earlier [3], except that the homogenization buffer used in the present investigation was as follows: 0.1 M potassium phosphate pH 8.0–8.3, 10% (w/v) in glycerol, and 1 mM in phenylthiourea (PTU), mercaptoethanol, and EDTA.

The enzyme solution obtained after filtration of the centrifuged homogenate is referred to as the crude extract.

2.3. *Enzyme assay and protein determination*

Aspartate carbamoyltransferase (EC. 2.1.3.2.) was assayed by a modification of the colorimetric method described by Gerhart and Pardee [11]. Except when otherwise indicated, the incubation mixture contained: 0.02 ml Tris–Cl pH 9.5, 0.07 ml 0.1 M potassium aspartate pH 7.0, 0.36 ml enzyme solution, and 0.05 ml dilithium carbamoyl phosphate (8 mg/ml) the addition of which started the reaction.

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The assay was carried out for 30 min at 30°C and stopped by transfer of 0.15 ml incubation mixture to 1.0 ml acid monoxime reagent [11].

One unit of activity is defined as the amount of enzyme which produces 1 nmole of carbamoyl aspartate per min. Protein concentrations were determined according to Lowry et al. [12] using bovine serum albumin as standard.

2.4. *Purification procedures*

All operations were carried out at 0–4°C.

2.4.1. *The wild type enzyme*

A summary of the purification is given in table 1.

Approx. 400 ml of crude extract was first subjected to streptomycin sulfate treatment. 10% streptomycin sulfate was added to a final concentration of 1%. After centrifugation the precipitate was discarded, and solid ammonium sulfate was added to the supernatant. Precipitation was allowed to occur for two hours. The enzymatic activity was found in the precipitate between 40 and 60% saturation, which was collected by centrifugation at 12 000 *g* for 10 min, and kept for further purification steps.

The pellet was dissolved in minimal amount of homogenizing buffer and applied to a Sephadex G-200 column (2.5 X 93 cm), equilibrated and eluted with homogenizing buffer without PTU. The active fractions were pooled and concentrated by ammonium sulfate precipitation (70% saturation), and the gel filtration was repeated. The pooled active fractions were applied to a DEAE-Sephadex A-50 column (2.5 X 27 cm) equilibrated with 0.01 M potassium phosphate pH 8.2, 10% in glycerol and 1 mM in mercaptoethanol and EDTA. Elution was carried out with the same buffer utilizing a KCl gradient from 0 to 200 mM. The hardly retarded enzyme was concentrated as above, and was then found to be stable for at least 14 days when kept at 0–4°C.

B. The r^{39k} enzyme

ACT from the *rudimentary* strain was partially purified by streptomycin sulfate and ammonium sulfate precipitation as described above, with the noticeable exception that this enzyme precipitated below 40% saturation with ammonium sulfate.

2.5. *Molecular weight determination*

The mol. wts of the enzymes were estimated by gel filtration on Sephadex G-200 using the following marker proteins, with molecular weights in parentheses. β-galactosidase (515 000), ACT from *E. coli* (310 000), purine nucleoside phosphorylase (140 000), bovine serum albumin (68 000) and cytochrome *c* (12 400). The column (1.5 X 90 cm) was equilibrated and eluted with the homogenizing buffer without PTU as above.

Table 1
Purification scheme for wild type ACT

Source	Activity units/ml	Total act. units	Spec. act. units/mg prot.	Yield %	Fold purified
Crude extract	8.1	2200	0.74	100	1.0
Streptomycin supernatant	7.0	2200	0.59	100	0.8
Ammonium sulfate precip., dissolved	17.1	358	0.82	16.3	1.1
1st G-200 eluate, concentrated	48.6	165	3.22	7.5	4.4
2nd G-200 eluate, concentrated	18.5	37	4.48	1.7	6.1
DEAE A-50 eluate, concentrated	35.5	7	35.50	0.3	48.0

2.6. *Kinetics of the wild type enzyme*

The apparent K_m values for the substrates potassium aspartate and carbamoyl phosphate were determined on purified enzyme and for aspartate on crude extract as well. Furthermore, the possible effect of 2×10^{-4} M ATP, UTP or CTP on the activity of the purified enzyme was investigated within a range of 0–20 mM potassium aspartate.

2.7. *Chemicals*

Sephadex G-200 and DEAE-Sephadex A-50 from Pharmacia Fine Chemicals. Bovine serum albumin, cytochrome *c*, carbamoyl phosphate, carbamoyl aspartate, CTP, streptomycin sulfate, and Tris from Sigma Chemical Company. ATP, UTP and β-galactosidase from Boehringer. All other chemicals from Merck. Purine nucleoside phosphorylase was a gift from Dr Per Nygaard. *E. coli* ACT was prepared according to Gerhart and Holoubek [13].

3. Results

Purification of the wild type enzyme resulted in a 48-fold increase in specific activity relative to that of the crude larval extract (table 1). The enzyme was found to have a mol. wt of about 390 000, whereas the ACT from the mutant strain had an elution profile from the Sephadex G-200 column corresponding to a protein with a molecular weight of only about 175 000 (fig.1). A difference between the two enzymes was also found in their behaviour towards ammonium sulfate precipitation, where the r^{39k} enzyme precipitated below 40% saturation, whereas wild type ACT was recovered in the fraction that precipitated between 40 and 60% saturation.

Kinetic investigations demonstrated that wild type ACT is subject to substrate inhibition at aspartate concentrations above 14 mM (fig.2). The apparent K_m values for aspartate and carbamoyl phosphate were estimated to be 5.4×10^{-3} M and 4.4×10^{-4} M respectively (figs. 2 and 3).

The nucleotides ATP, UTP and CTP were tested for their possible activating and inhibitory influence on the activity of wild type ACT. Neither was found to have any effect at the concentrations used.

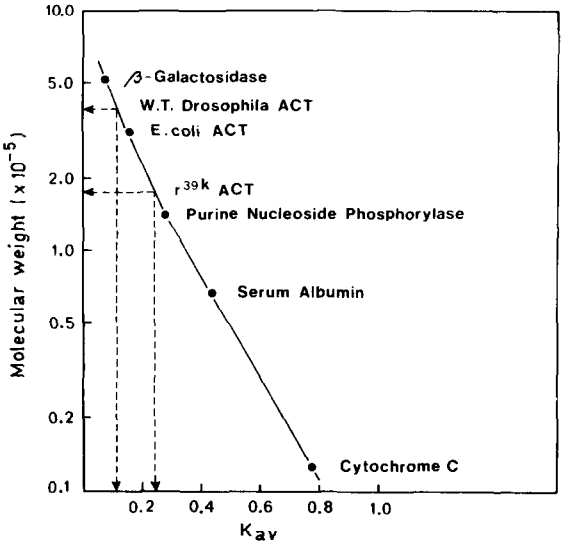

Fig.1. Mol. wt estimates of aspartate carbamoyltransferase (ACT) from wild type (w.t.) *Drosophila melanogaster* and from the rudimentary mutant strain r^{39k} by gel filtration on Sephadex G-200. Marker proteins used were: β-galactosidase, ACT, and purine nucleoside phosphorylase from *E. coli*, bovine serum albumin, and cytochrome *c* from horse heart. $K_{av} = (V_e - V_o)/(V_t - V_o)$. (Sephadex® gel filtration manual).

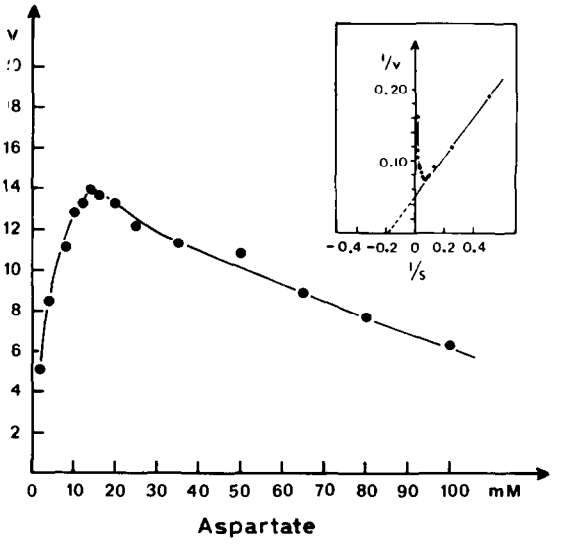

Fig.2. Aspartate saturation curve for wild type *Drosophila* ACT. The reaction mixture (0.500 ml) contained 20 μmol Tris–Cl pH 9.5, 2 μmol dilithium carbamoyl phosphate, 0.200 ml crude extract and the indicated concentrations of L-aspartate. Insert: Lineweaver-Burk plot of the data. *V* = units per ml enzyme.

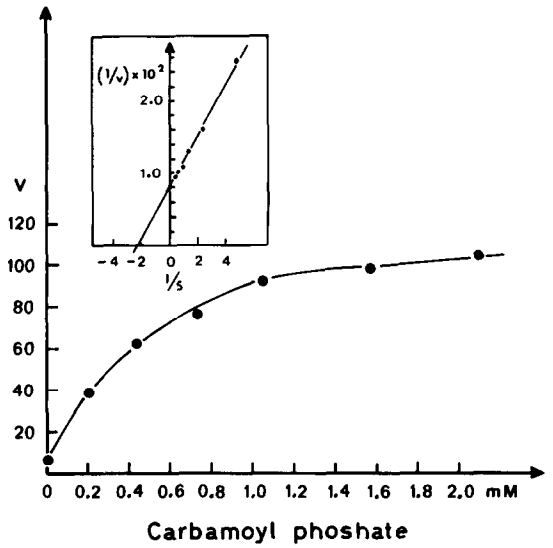

Fig. 3. Carbamoyl phosphate saturation curve for wild type *Drosophila* ACT. The reaction mixture (0.500 ml) contained 20 μmol Tris–Cl pH 9.5, 7 μmol potassium aspartate pH 7.0, 0.025 ml purified enzyme and the indicated concentrations of carbamoyl phosphate. Insert: Lineweaver-Burk plot of the data. V = units per ml enzyme.

4. Discussion

The findings that *rudimentary* mutants of *D. melanogaster* have a nutritional requirement for pyrimidines that can be satisfied by the early intermediate carbamoyl aspartate [2], and that some *rudimentary* strains are deficient in ACT while others are not [3], suggested that the *r* locus determines the activities of the two first enzymes in pyrimidine biosynthesis, CPS and ACT, [3], the latter being coded for by the proximal part of the locus [4]. This has recently been confirmed [6] and extended to include the third enzyme in the pathway, DHO [9].

The complex pattern of complementation within the *r* locus [14–16] indicates that the wild type locus determines a multimeric protein [14–16], possibly an enzyme complex [14], and it has therefore been suggested, as an extension of the above hypothesis, that CPS and ACT in *D. melanogaster* form a bifunctional enzyme complex [4,7]. In that case the situation will be similar to the one found in yeast and *Neurospora*, the two eukaryotes in which the genetic regulation of pyrimidine biosynthesis has so far been studied in greatest detail [8]. Also in mammals, where the genetics of the system is unknown, has ACT been found to exist in a high molecular weight complex, possibly including both CPS and DHO [8,10].

In the present study wild type ACT from *Drosophila* has been found to have a molecular weight of about 390 000. Although much smaller than the CPS-ACT complexes found in yeast (800 000) [17] and *Neurospora* (650 000) [18], this does suggest a multimeric protein big enough to accomodate two enzymes. In fact, in the absence of UTP the enzyme aggregate from yeast dissociates into 'half-molecules' of mol. wt 380 000, which retain both CPS and ACT activities [17]. In preparations from *Neurospora* a CPS–ACT complex of similar size, as determined by sucrose gradient centrifugation, has also been observed, but only in the *presence* of UTP [18].

The hypothesis that ACT in wild type *Drosophila* is part of a CPS–ACT complex has been strengthened through the observation reported here, that ACT from the r^{39k} strain has a mol. wt of only about 175 000. This mutant has wild type levels of ACT and is probably deficient in CPS [3,6]. The simplest explanation for the observed differences in molecular sizes of ACT is, therefore, that in the r^{39k} mutants the CPS moiety of the complex is lacking, either because it is not formed at all, or because it, although present in the cells, is changed in such a way that it is no longer able to form a complex with ACT. The latter property may be essential for the function of the pathway and thereby for the complementation properties of various alleles, regardless of the enzyme activity patterns measured *in vitro*.

It is thus suggested that the 175 000 mol. wt ACT of the r^{39k} mutant represents the ACT subunit in the 390 000 mol. wt wild type complex. The possibility that this complex is only a part of the functional in vivo complex, and that it may include DHO [9], has to be left open at the moment.

According to the proposed model, the 175 000 mol. wt ACT corresponds to the 138 000 mol. wt ACT subunit in yeast, which in itself is probably a hexamer of a single polypeptide chain of a mol. wt of about 21 000 [19]. The *Drosophila* ACT molecule is most probably also a multimeric protein. In fact, preliminary experiments, involving precipitation of

the wild type enzyme with 1.6 potassium phosphate instead of 60% ammonium sulfate, have produced an enzymatically active ACT with a mol. wt of only about 68 000 [20].

Since the discovery in *E. coli* of the regulatory function of ACT in pyrimidine biosynthesis [11,21], the kinetic properties of this enzyme from various other sources have been extensively investigated [8,22]. Substrate inhibition by aspartate, as reported for *Drosophila* ACT in this paper, has also been found for bacterial [23,24], plant [25], and mammalian ACT [10,26].

In vivo regulation of the enzymatic activity through feedback inhibition exerted by pyrimidine nucleotides has been suggested for ACT from bacteria [11,21,28], yeast [29], and higher plants [25,30,31]. Some bacterial enzymes, like the so-called C-class ACT from *B. subtilis,* are not inhibited by nucleotides [28], neither is ACT from *Neurospora* [32] and mammalian tissues [8]. Before the nature of the in vivo complex has been established it is impossible to draw any conclusions from in vitro effector studies about a regulatory function of *Drosophila* ACT. Thus, ACT from yeast is strongly inhibited by UTP when part of the 800 000 mol. wt complex, but only poorly so in the 'half-molecule' of 380 000 mol. wt [17].

The various patterns of regulation of pyrimidine biosynthesis in different organisms reflect the metabolic complexity caused by the dual role of carbamoyl phosphate as precursor for pyrimidines as well as for arginine (urea) [8,22,33]. Pyrimidine-mediated repression of enzyme synthesis does probably not take place in *Drosophila* [3]. As for regulation on the enzyme (complex) level, the *Drosophila* system is of particular interest, also from an evolutionary point of view, since insects apparently do not have (retained?) the ability to synthesize arginine [34,35,36]. If feedback inhibition is involved in the regulation of pyrimidine biosynthesis in *Drosophila,* it seems likely that the target will be the first enzyme in the pathway, i.e. carbamoyl phosphate synthetase.

Acknowledgements

The gift of purine nucleoside phosphorylase from Dr P. Nygaard is gratefully acknowledged.

This work has been supported by the Danish Natural Science Reseach Council (grant no. 511-1675).

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