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METABOLIC COMPARTMENTATION AT THE MOLECULAR LEVEL: THE FUNCTION OF A MULTIENZYME AGGREGATE IN THE PYRIMIDINE PATHWAY OF YEAST

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SUMMARY

In yeast, the pyrimidine-specific carbamyl phosphate synthetase and aspartate transcarbamylase exist as an aggregate, together with a regulatory site. The hypothesis, originally proposed by DAVIS, that such complexes serve to channel the product of the first enzyme (*i.e.* carbamyl phosphate which is common to both the pyrimidine pathway and the arginine pathway) into the pathway for which it was produced has been subjected to two experimental tests. In both types of experiment, controls were run in which the two enzymes were separate. When the activities were separate, cold carbamyl phosphate in the assay mixture could easily dilute label from [^{14}C]bicarbonate subsequently recovered in the form of carbamyl aspartate, the product of aspartate transcarbamylase. Where the activities were associated in the aggregate, the label recovered in the product could not be diluted by the presence of moderate concentrations of cold exogenous carbamyl phosphate. When the activities were separate, purified bacterial ornithine transcarbamylase could compete for the carbamyl phosphate formed by the action of carbamyl phosphate synthetase on equal terms with the aspartate transcarbamylase; the proportion recovered as either citrulline or carbamyl aspartate was determined by the ratio of activity ornithine transcarbamylase: aspartate transcarbamylase. Where the carbamyl phosphate synthetase and the aspartate transcarbamylase activities were associated in the complex, most of the product of the first reaction was not free to become converted to citrulline but was channeled preferentially into carbamyl aspartate. Both types of experiment confirm the channeling hypothesis.

INTRODUCTION

We have previously shown that in yeast the carbamyl phosphate synthetase of the pyrimidine pathway and aspartate transcarbamylase, the next enzyme in the

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sequence, exist as a multi-functional enzyme aggregate, in which form they co-purify together with a regulatory site at which UTP binds to inhibit both activities[1]. The molecular weight of the complex was found to be about 800000 (ref. 2). Heat (5 min at 50°) caused total disappearance of both the carbamyl phosphate synthetase activity and of the sensitivity of the aspartate transcarbamylase activity to feedback inhibition; the aspartate transcarbamylase was recovered as a subunit having a mol. wt. of 140000 (ref. 3).

DAVIS[4] first showed that in *Neurospora crassa* there existed two isofunctional carbamyl phosphate synthetases, one under the genetic control of the pyrimidine genes and the other of the genes for arginine biosynthesis[5]. Deficiency of either enzyme caused the organism to become auxotrophic for the end-product of the corresponding pathway (*e.g.* for either uracil or arginine); thus the carbamyl phosphate formed by either carbamyl phosphate synthetase was not free to equilibrate in a common intracellular pool, but was compartmented, or segregated, except in certain mutants where there was overflow of carbamyl phosphate into the competing pathway[6]. Lacroute and his collaborators then showed that in yeast there also existed two carbamyl phosphate synthetases, under the genetic and regulatory control of the pyrimidine and arginine pathways respectively[7]; however, in this case loss of either enzyme did not cause an auxotrophy for the corresponding end-product, such mutants remaining wild-type phenotypically[7]. Thus, in bakers' yeast there is some overflow of carbamyl phosphate formed by either enzyme into a common pool.

What is the physiological and evolutionary significance of such multifunctional complexes? In a much cited paper, DAVIS[6] advanced the hypothesis that carbamyl phosphate, the product of the first enzyme in the sequence, remains bound to the aggregate where it is preferentially coupled with aspartate by aspartate transcarbamylase, the second enzyme of the aggregate, to form carbamyl aspartate; since carbamylaspartate is common to no other pathway, the carbamyl phosphate would thus be irreversibly committed, or channeled, to the pyrimidine pathway for which it was produced. Thus, intracellular compartmentation of intermediates common to more than one pathway need not require membranes or organelles but might rather be a function of multi-enzyme aggregates in which enzymes which catalyze consecutive steps in a sequence are complexed.

At first glance, this molecular model of compartmentation might seem unlikely to apply to the fate of carbamyl phosphate in yeast where, unlike the case of Neurospora, there is overflow from one pathway to the other. On the other hand, together with M. Duphil-Denis of this laboratory, we have noted that the growth of a mutant deficient in the carbamyl phosphate synthetase of the arginine pathway was extremely slow in minimal medium but approached wildtype growth rate when the medium was supplemented with arginine. The reciprocal effect was never noted in the case of mutants lacking the carbamyl phosphate synthetase of the pyrimidine pathway: these mutants grew as well in minimal medium as in uracil-supplemented medium. It is thus not *a priori* unlikely that some of the carbamyl phosphate formed by the pyrimidine-specific carbamyl phosphate synthetase might, in fact, be preferentially channeled into the pyrimidine pathway.

We have therefore adopted the working hypothesis that the carbamyl phosphate synthetase aspartate transcarbamylase aggregate channels the carbamyl phosphate formed by the pyrimidine-specific enzyme into an intermediate (carbamyl

aspartate) specific to the corresponding pathway. The present paper presents two successful tests of this hypothesis.

MATERIALS AND METHODS

The carbamyl phosphate synthetase–aspartate transcarbamylase aggregate was extracted from *Saccharomyces cerevisiae* FL 233-3C and purified as previously described[1]; such preparations were devoid of ornithine transcarbamylase activity. In all experiments to be reported, an aliquot of purified aggregate was heated at 60° for 3 min which completely destroyed the carbamyl phosphate synthetase activity which is highly heat labile[3]; carbamyl phosphate synthetase was then added to such tubes, making use of purified preparations from *Escherichia coli*, generously given us by Dr. P. Anderson of Southern Illinois University and by Dr. S. Kalman, Stanford University. The ornithine transcarbamylase used was prepared from *E. coli* and purified according to the method of ROGERS AND NOVELLI[8]; such preparations were free of carbamyl phosphate synthetase and aspartate transcarbamylase activities.

Aspartate transcarbamylase was assayed by the method of GERHART AND PARDEE[9] as modified[10], with the exception that the reaction was carried out in 0.05 M Tris–sulphate buffer, pH 7.6. Ornithine transcarbamylase activity was estimated by means of the citrulline produced, utilizing the reaction-stopping mixture devised by KORITZ AND COHEN[11]; absorbance at 560 nm was read after 18 min at 30° when it was maximum and found to be linear with citrulline concentration. Citrulline standards were always run with the assays. Ornithine transcarbamylase activity thus measured was directly proportional to enzyme concentration and the color developed was proportional to duration of incubation of the enzyme–substrate system. The ornithine transcarbamylase reaction mixture contained, in 0.5 ml final volume, 25 μmoles Tris–sulphate buffer, pH 7.6; 3 μmoles ornithine and 10 μmoles carbamyl phosphate. Carbamyl phosphate synthetase activity was routinely estimated by a colorimetric procedure previously described[1].

In the tracer experiments to be reported, $NaH^{14}CO_3$ (Nuclear Chicago) was diluted with cold $NaHCO_3$ to give a final specific radioactivity of $2 \cdot 10^4$–$6 \cdot 10^4$ counts/min per μmole. In the dilution experiments, only carbamyl aspartate was isolated and counted as described below. In competition experiments, the complete reaction mixture contained in a final volume of 0.5 ml; $MgSO_4$, 10 μmoles; ATP, 10 μmoles; $NaHCO_3$, 5 μmoles; KCl, 50 μmoles; KCl, 50 μmoles; glutamine, 3 μmoles; aspartate, 25 μmoles; ornithine, 6 μmoles; and either aspartate transcarbamylase (in the form of the aggregate with carbamyl phosphate synthetase or heated plus added bacterial carbamyl phosphate synthetase) or ornithine transcarbamylase, or mixtures which contained constant amounts of the former and variable amounts of the latter. The reaction was incubated at 25° for 20 min. The reaction mixture for the dilution experiments was the same except that ornithine and ornithine transcarbamylase were not included.

After the incubation period, the reaction was stopped by addition of 0.1 ml of 0.4 M HCl. 100 μl of this mixture was pipetted onto a glass planchet, 300 μl of a 1:1 mixture of 4 M HCl and ethanol was added, and the sample was evaporated to dryness. The planchet was then counted by means of a Nuclear-Chicago gas flow counter. This method takes advantage of the fact that both citrulline and carbamyl aspartate are

acid-stable, but is useful only in the dilution experiments where only the latter was being formed due to the presence of aspartate transcarbamylase and aspartate.

In the competition experiments it was necessary to separate the two products of the competing reactions, citrulline and carbamyl aspartate. The reaction was stopped as above and 0.3 ml was passed through Dowex 50 (W-X-12, Baker Co.,) held in columns made from Pasteur pipettes plugged with glass wool; the gel bed was approx. 4 cm in height. The gel was then eluted with 0.01 M HCl. Carbamyl aspartate was not adsorbed and was found in the initial eluate. After 5 ml or more had been collected, citrulline was then eluted by means of 4 M HCl. Aliquots of both were then treated as above for counting. Despite the variation in ratio of ornithine transcarbamylase: aspartate transcarbamylase in such experiments, the total counts recovered (as carbamyl aspartate + citrulline) were fairly constant throughout the series, and there was a good reciprocal relation between amount of carbamyl aspartate and of citrulline formed.

RESULTS

Preliminary experiments

Fig. 1 shows the carbamyl phosphate synthetase activity of the purified aggregate as a function of duration of incubation. The reaction was linear with time, whether the carbamyl phosphate formed was measured subsequently as carbamyl aspartate (Curve A) or as citrulline (Curve C). Curve B shows that ornithine, at the concentration to be used in the competition experiments, did not affect significantly the activity of aspartate transcarbamylase. Aspartate, at the concentration used in these experiments, caused little or no inhibition of ornithine transcarbamylase activity. Curve C demonstrates that, in the absence of aspartate, carbamyl phosphate formed by the aggregated carbamyl phosphate synthetase is perfectly free to take part in the reaction catalyzed by ornithine transcarbamylase.

Other preliminary experiments showed that exogenous carbamyl phosphate

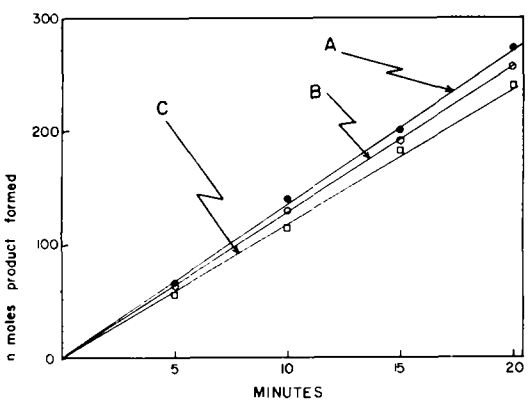

Fig. 1. Formation of carbamyl phosphate as a function of duration of the carbamyl phosphate synthetase reaction. Curve A (● ● ●), product determined as carbamyl aspartate by coupling with aspartate and aspartate transcarbamylase; Curve B (○ ○ ○) same as in A, except that ornithine at the usual concentration was present; Curve C (□ □ □), product determined as citrulline by coupling with ornithine and ornithine transcarbamylase.

did not inhibit carbamyl phosphate synthetase activity[2] and that the aspartate transcarbamylase of the aggregate quantitatively converted exogenous carbamyl phosphate into carbamyl aspartate even while the carbamyl phosphate synthetase of the aggregate continued to function[2].

Dilution experiments

The points shown in Fig. 2 represent two separate dilution experiments. Curve B shows that significant dilution of the label recovered in carbamyl aspartate oc-

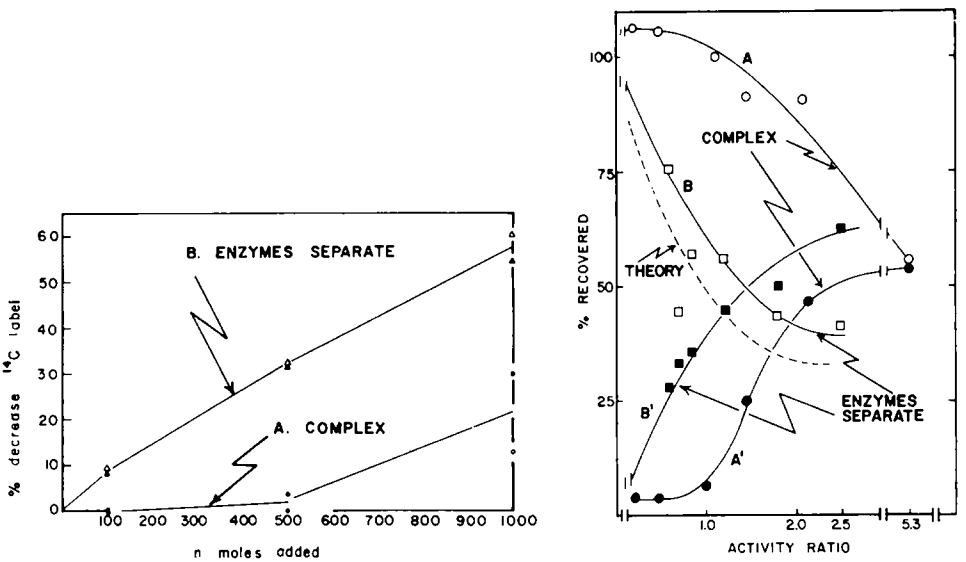

Fig. 2. Decrease of the label in the recovered carbamyl aspartate as a function of the quantity of non-radioactive carbamyl phosphate present during the reaction. A, carbamyl phosphate synthetase and aspartate transcarbamylase present as the aggregate; B, bacterial carbamyl phosphate synthetase and heated yeast aspartate transcarbamylase. The experimental points represent two separate experiments.

Fig. 3. Variation of yield of product as a function of the ratio of activity ornithine transcarbamylase : aspartate transcarbamylase. A (○—○), carbamyl aspartate, yeast enzymes in the aggregate; B (_ — _), carbamyl aspartate, bacterial carbamyl phosphate synthetase and yeast aspartate transcarbamylase; A′ (●—●) citrulline, yeast enzymes in the aggregate; B′ (■—■), citrulline, bacterial carbamyl phosphate synthetase and yeast aspartate transcarbamylase. Dotted line is theoretical curve expected if quantity of carbamyl aspartate formed depended entirely on the ratio of aspartate : ornithine transcarbamylases. For Curves A and B, 100% was taken as amount of carbamyl aspartate produced in presence of aspartate transcarbamylase alone. For Curves A′ and B′, 100% was taken as the amount of citrulline produced in the presence of ornithine transcarbamylase alone.

curred when 100 nmoles of cold carbamyl phosphate was added to the reaction mixture provided that the carbamyl phosphate synthetase and aspartate transcarbamylase were separate. When the formation of carbamyl phosphate was catalyzed by the carbamyl phosphate synthetase of the enzyme aggregate (Curve A), 500 nmoles of unlabeled exogenous carbamyl phosphate caused little or no dilution of label in final product, carbamyl aspartate. At very high concentrations of unlabeled external carbamyl phosphate (1000 nmoles) significant dilution of the label in carbamyl

aspartate was noted. In one of the experiments shown, the dilution was 30 percent; in most of the experiments it was between 10 and 20 percent.

Competition experiments

The K_m for carbamyl phosphate was virtually identical for the native aspartate transcarbamylase of the aggregate, for the heated aspartate transcarbamylase and for the bacterial ornithine transcarbamylase ($3 \cdot 10^{-3}$ M, $2 \cdot 10^{-3}$ M and $3 \cdot 10^{-3}$ M) respectively.

Fig. 3 represents a typical competition experiment, in which the percent of product recovered as either carbamyl aspartate or as citrulline (taking as 100 percent the amount produced by aspartate transcarbamylase alone or by ornithine transcarbamylase alone respectively) is plotted against the ratio of activity of the two transcarbamylases in the reaction mixtures. It will be noted that when the carbamyl phosphate synthetase was separate from the aspartate transcarbamylase, the amount of carbamyl aspartate recovered (Curve B) was quite close to that predicted on the basis of the ratio of activities of the two transcarbamylases (dotted line); an experiment in which the fit of experimental points to this theoretical curve was even closer has been presented elsewhere[12]. On the other hand, where the carbamyl phosphate synthetase was part of the enzyme aggregate, the points fell well above this theoretical curve (Curve A); indeed, at a ratio of 1.0, where ornithine and aspartate transcarbamylase activities were equal, essentially all of the radioactivity was recovered in the form of carbamyl aspartate, in this and in all our other experiments. As the high ratios of ornithine to aspartate transcarbamylases increased to high values, progressively more of the radioactivity was recovered in the form of citrulline (Curve A') and correspondingly less as carbamyl aspartate (Curve A).

DISCUSSION

Two distinct lines of experimentation, utilizing ^{14}C-labelled bicarbonate, a precursor of carbamyl phosphate and substrate of carbamyl phosphate synthetase, have indicated that when this enzyme exists as an aggregate with aspartate transcarbamylase, some of its product, carbamyl phosphate, is preferentially channeled into the pyrimidine precursor carbamyl aspartate. The first argument is based on the finding that exogenous cold carbamyl phosphate cannot dilute the label recovered in the final product, carbamyl aspartate, produced by the aggregate at concentrations effective when carbamyl phosphate synthetase and aspartate transcarbamylase were separate. The second argument utilizes the finding that ornithine transcarbamylase in the reaction mixture cannot compete on even terms with aspartate transcarbamylase for carbamyl phosphate formed by the aggregate, but can do so when the carbamyl phosphate is produced by bacterial carbamyl phosphate synthetase. These competition experiments serve as an *in vitro* model of compartmentation of intermediates common to more than one pathway and thus provide strong support for the hypothesis of DAVIS[6].

Recently, Davis' group[13] has demonstrated channeling of carbamyl phosphate into either the arginine or pyrimidine pathway, by direct measurement of carbamyl phosphate pools in logarithmically growing Neurospora. Since Davis and his collaborators[14,15] have recently shown that the pyrimidine-specific carbamyl phosphate

synthetase exists in an aggregate with aspartate transcarbamylase, it would be interesting to repeat the dilution and competition experiments reported above with the purified aggregate from Neurospora, despite the failure of Davis[6] to observe channeling in preliminary experiments of the latter type.

In wild-type yeast, the spillover of carbamyl phosphate from one pathway to the other[7] makes it doubtful that the physiological significance of the aggregate lies exclusively in the channeling of carbamyl phosphate into the pyrimidine pathway. However, it is worth noting that the carbamyl phosphate synthetase of the arginine pathway of yeast is highly repressible by arginine in the growth medium[7]. Thus, the synthetase transcarbamylase aggregate would offer an important selective advantage to yeast growing in an arginine-rich medium, since under these conditions virtually all the carbamyl phosphate produced would be channeled into the biosynthesis of pyrimidines.

Part of the selective advantage of enzyme aggregates, such as that of the synthetase and transcarbamylase, is the presence of the common regulatory site at which UTP inhibits both activities[2,7]. This is also true of the enzyme aggregate of the threonine pathway of *E. coli* in which the first and third enzyme of the pathway are complexed[16]; channeling is most unlikely to occur under these conditions. There is also the possibility that enzyme aggregates may be in some way a consequence or by-product of an advantageous mechanism of coordinate regulation of the biosynthesis of the enzymes in question.

Isofunctional carbamyl phosphate synthetases have been demonstrated in mammalian systems and there is a suggestion that the arginine-specific enzyme may channel carbamyl phosphate into the arginine pathway by virtue of its localization within the mitochondria[17–20], although evidence to the contrary has been presented[21]. There seems to be no evidence at present as to whether the carbamyl phosphate synthetase and aspartate transcarbamylase of higher eucaryotic plants and animals are joined in an aggregate or whether there is channeling of carbamyl phosphate into pyrimidine biosynthesis in these organisms.

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