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# CHARACTERIZATION OF ORNITHINE CARBAMOYLTRANSFERASE FROM CULTURED CARROT CELLS OF LOW EMBRYOGENIC POTENTIAL

STEVEN R. BAKER and ROBERT J. YON

School of Biological Sciences and Environmental Health, Thames Polytechnic, Wellington Street, London SE18 6PF, U.K.

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**Key Word Index**—*Daucus carota*; Umbelliferae; carrot; suspension culture; embryogenesis; ornithine carbamoyltransferase; MW; enzyme kinetics.

**Abstract**—Ornithine carbamoyltransferase was partially purified from carrot cell suspension cultures of low embryogenic potential. The enzyme had a MW of $1.58 \times 10^5$ and a pH optimum at 7.5. It was not inhibited by L-lysine or by α-methyl-L-ornithine, but was inhibited weakly by putrescine and more strongly by agmatine, L-arginine and L-norvaline. The enzyme catalyses an ordered-sequential mechanism in which carbamoylphosphate binds first, followed by L-ornithine, and in which L-citrulline leaves first, followed by phosphate. Support for this mechanism comes from product inhibition, substrate-analogue inhibition and evidence of abortive ternary complex formation. Kinetic analysis gave Michaelis constants of 3.3 mM for L-ornithine and 9 μM for carbamoylphosphate, and a dissociation constant of 2.5 μM for the enzyme–carbamoylphosphate complex.

## INTRODUCTION

Ornithine carbamoyltransferase (carbamoylphosphate: L-ornithine carbamoyltransferase, EC 2.1.3.3)* catalyses an essential step in the biosynthesis of the amino acid arginine. In addition to its roles in protein biosynthesis and urea production, arginine appears to be the major source of the polyamines, putrescine, spermidine and spermine, in several higher plants [1–3] and may, thereby, be implicated in the regulation of genome expression during differentiation processes [4]. We have shown [5] that embryonic development in cultured carrot cells appears to be preceded at an early stage by a transient rise in the activity of OCTase, against a normal background level of activity that is also seen in non-embryogenic cells, and we speculated that the 'burst' of OCTase activity might be related to transient peaks of polyamine synthesis that had been reported previously [6]. A role for OCTase in polyamine production has been established in seedlings of *Lathyrus sativus*, where a unique OCTase is a component activity in a multifunctional enzyme complex dedicated to putrescine synthesis [7]. It would be of interest, therefore, to compare the properties of the 'normal' OCTase, present in both embryogenic and non-embryogenic carrot cells, with the OCTase produced during the transient, embryo-related rise in activity. As the first phase of this comparison, we describe here some properties of the 'normal' OCTase produced in cultures of low embryogenic potential maintained in medium containing the auxin 2,4-dichlorophenoxyacetate.

## RESULTS

### *Molecular weight*

The MW of the enzyme was determined by gel filtration chromatography on a column of Sephacryl G200. The eluted enzyme peak was symmetrical and indicative of a single size species. The enzyme eluted close to lactate dehydrogenase and was assigned a MW of $1.58 \times 10^5 \pm 0.20 \times 10^5$ (mean and range of three runs). For comparison, the MW of the enzyme from wheat germ was also determined and found to be $1.21 \times 10^5$ (one run).

### pH *optimum*

Using imidazole, Tris and glycine buffers, the pH optimum was determined over the range 7–10.5, using substrate concentrations of 10 mM ornithine and 1 mM carbamoyl phosphate. Maximum activity was at pH 7.5, with half-maximal activity at pH 9.0. The acidic half-maximal activity was not determined.

### *Inhibition by ornithine analogues*

Inhibitory effects were tested in an assay system containing 10 mM L-ornithine and 1 mM carbamoyl phosphate in 0.1 M Tris–acetate, pH 7.5. At a concentration of 30 mM, neither L-lysine nor α-methyl-L-ornithine inhibited the enzyme. Putrescine (1,4-diaminobutane) inhibited the enzyme weakly, 50% inhibition occurring at a concentration ($I_{50}$) of 100 mM. More inhibitory were agmatine ($I_{50}$ = 13 mM) and L-arginine ($I_{50}$ = 8 mM). L-Norvaline ($I_{50}$ = 2 mM) was the strongest inhibitor tested.

### *Steady-state kinetic mechanism*

*General features of initial rate plots.* The kinetics of the bisubstrate–biproduct reaction were studied in the forward (citrulline synthesis) direction, at a pH of 7.5 and temperature of 25°. The results were analysed according to Cleland [8–10] whose terminology is used here. Except at high concentrations of ornithine, plots of reciprocal

*Abbreviation: OCTase, ornithine carbamoyltransferase.

velocity vs reciprocal substrate concentration were linear. When the concentration of either substrate was varied at a series of fixed concentrations of the other, reciprocal plots displayed a pattern of lines intersecting to the left of the vertical axis (Fig. 1) indicating a sequential mechanism, i.e. both substrates must bind before either product leaves. Slope–intercept analysis of the two patterns (Fig. 1, insets) gave two sets of kinetic constants that were in close agreement; the mean values of the two sets are given in Table 1.

*Product inhibition by phosphate.* Distinguishing between kinetic mechanisms, in which the two substrates bind randomly or in a compulsory order, is achieved by studying the inhibition of the reaction by its products. A diagnostic feature of the compulsory-order mechanism is that inhibition by the last-leaving product is competitive with respect to the first-binding substrate [10]. Figure 2 shows that the inhibition by phosphate is competitive when carbamoyl phosphate is the variable substrate, indicating a compulsory-order mechanism in which carbamoyl phosphate binds first, followed by ornithine, then the catalysed reaction follows after which citrulline leaves first, followed by phosphate. A consequence of this conclusion is that $K_{ia}$ in Table 1 is the dissociation constant of the enzyme–carbamoyl phosphate complex. From the horizontal intercept of the slope replot in Fig. 2, the dissociation constant of the enzyme–phosphate complex is estimated to be 2.5 mM [11].

Table 1. Kinetic constants for the bi-substrate reaction catalysed by OCTase from non-embryogenic carrot cells

| | |
|---|---|
| $V_{max}$ | $1.82 \pm 0.09$ nmol/min |
| $K_a$ | $9 \pm 4$ $\mu$M |
| $K_b$ | $3.3 \pm 0.1$ mM |
| $K_{ia}$ | 2.5 $\mu$M |

Carbamoyl phosphate is denoted by *a* and L-ornithine by *b*. $K_a$ and $K_b$ are Michaelis constants, i.e. the concentration of *a* or *b* giving half-maximal velocity when the other substrate is saturating. $K_{ia}$ is the dissociation constant for the enzyme–carbamoyl phosphate complex. Values of $V_{max}$, $K_a$ and $K_b$ are each the mean ± range of the two values obtained from the slope–intercept analysis of Fig. 1(a and b). The value of $K_{ia}$ was obtained from the slope–intercept analysis of Fig. 1(a).

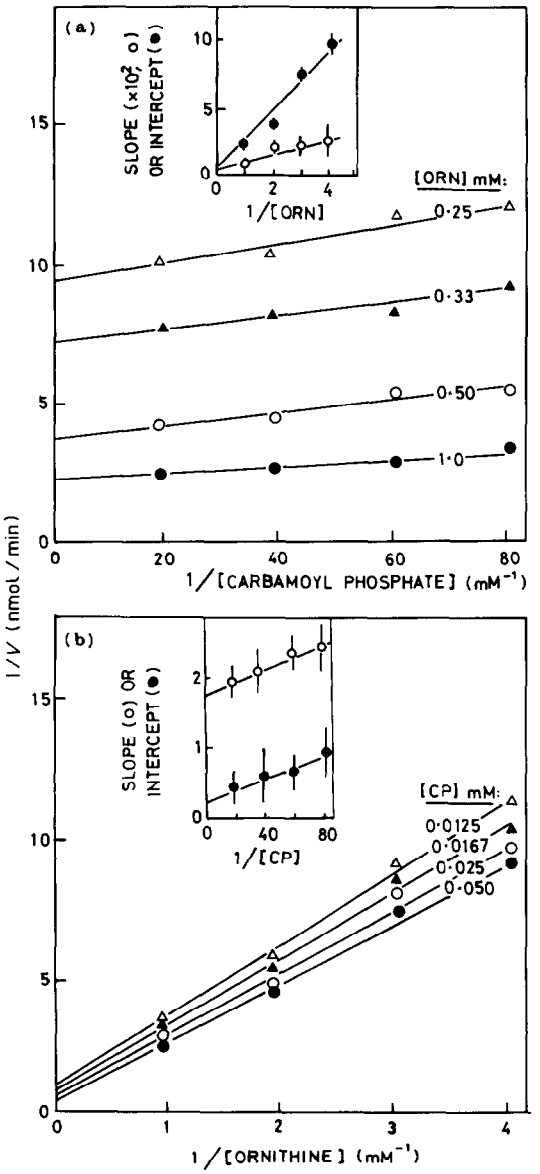


Fig. 1. Double-reciprocal plots of initial velocity as a function of the concentration of each substrate. Slopes, intercepts and their s.e.m. values were generated by a computer programme based on Cleland's [9].

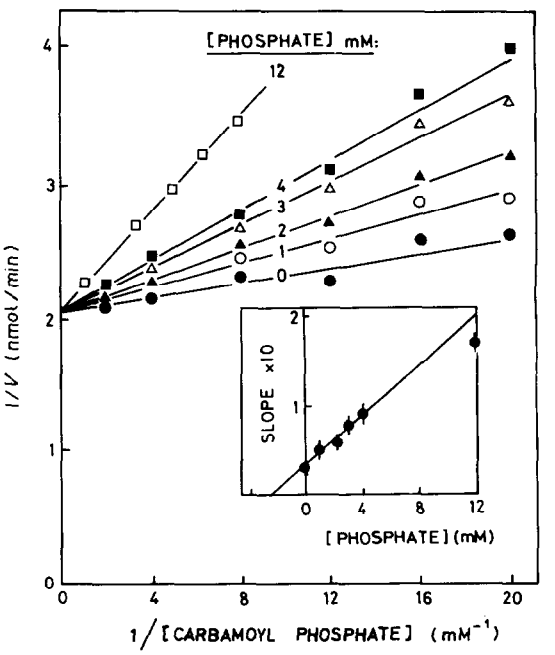


Fig. 2. Product inhibition by phosphate with carbamoyl phosphate as the variable substrate.

*Inhibition by substrate analogues.* Further confirmation of the binding order may be obtained by a study of the inhibition by substrate analogues. In preliminary experiments (not shown), inhibition by norvaline was shown to be competitive with respect to ornithine, and inhibition by pyrophosphate competitive with respect to carbamoyl phosphate, confirming that these were substrate analogues for kinetic purposes. A diagnostic feature of the ordered mechanism proposed above would be the inhibition by norvaline with respect to carbamoyl phosphate. This is predicted to be uncompetitive, i.e. characterized by a set of parallel lines in double reciprocal plots [10]. Within the limits of experimental error this is shown by the result in Fig. 3. The alternative set of lines, for pyrophosphate inhibition with respect to ornithine, are predicted to be non-competitive; however, analysis is complicated by severe substrate inhibition by ornithine in the presence of pyrophosphate (see next paragraph and Fig. 4b).

*Inhibitory ternary complexes.* In a group-transfer reaction, a further characteristic of the ordered mechanism is the ease of formation of the unproductive ternary complex involving the substrate and product lacking the transferred group, in this case ornithine and phosphate [10, 12]. Since the concentration of this complex increases with ornithine concentration, the reaction should be inhibited by excess of ornithine, this inhibition being intensified by the addition of extraneous phosphate or any other analogue of carbamoyl phosphate. Figure 4 shows the predicted substrate inhibition by high concentrations of ornithine and its intensification by phosphate (Fig. 4a) and by pyrophosphate (Fig. 4b).

## DISCUSSION

The MW of the OCTase from non-embryogenic carrot cells (158 000) is significantly different from that of the cytoplasmic (224 000) and mitochondrial (79 000) forms of the sugar-cane enzyme [13], but was quite close to that of another monocot, wheat (121 000), determined in the present study. Since the enzymes from a number of sources [14–16] have been shown to exist in multiple forms, it may be that the various plant enzymes are different multimers of a similar subunit. The OCTase from non-embryogenic carrot is substantially larger than the multifunctional enzyme putrescine synthase (MW 55 000) from *L. sativus* which includes OCTase as a component function [7]. It will be of interest to see which enzyme most resembles the OCTase produced early in embryogenesis [5] which we have speculated may be related to polyamine biosynthesis.

With one or two notable exceptions, the majority of carbamoyltransferases appear to catalyse a compulsory-order sequential reaction with carbamoylphosphate as the leading substrate. The data presented in this paper show

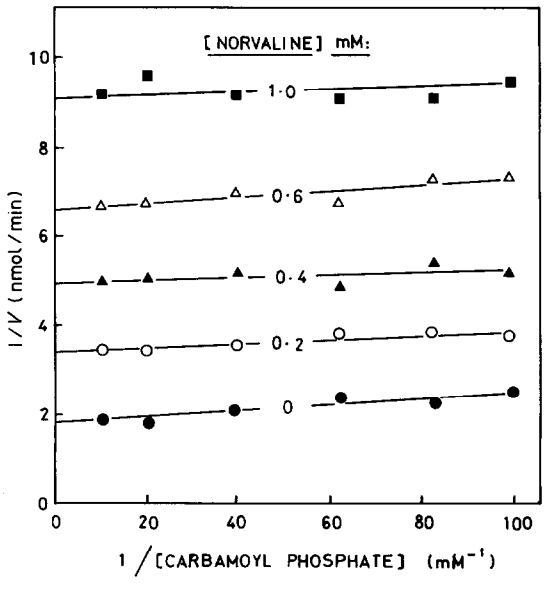


Fig. 3. Inhibition by L-norvaline, an analogue of the substrate L-ornithine, with carbamoyl phosphate as the variable substrate.

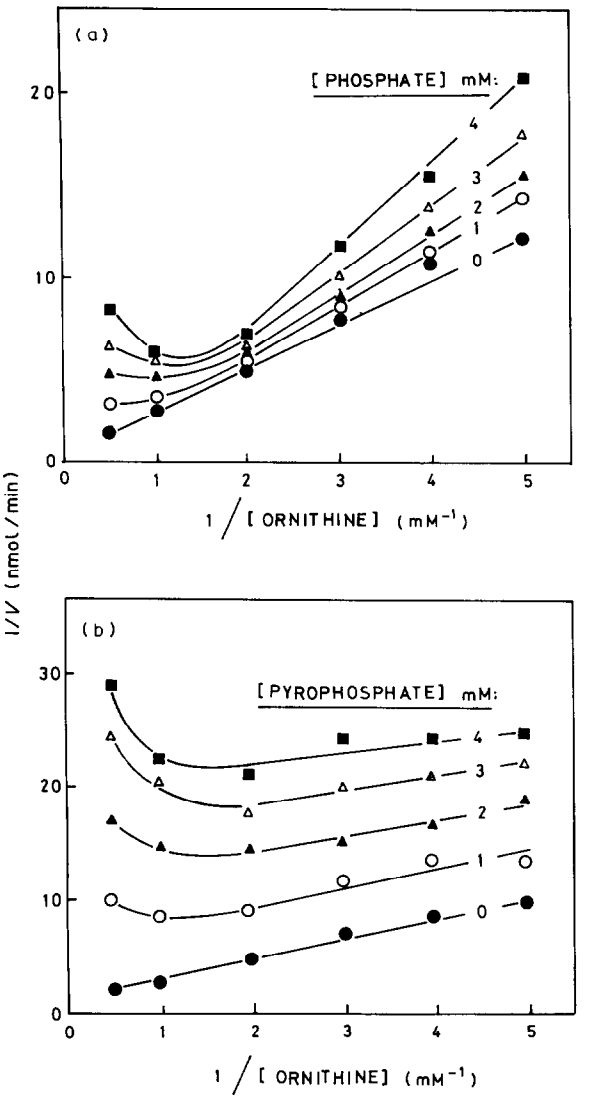


Fig. 4. Inhibition by phosphate (a) and by pyrophosphate (b) with L-ornithine as the variable substrate. Inhibition by high concentrations of ornithine is seen to become increasingly severe as the concentration of phosphate or pyrophosphate is increased.

that the OCTase from non-embryogenic carrot cells in culture is no exception. The $K_m$ for L-ornithine (3.3 mM) is of the same order of magnitude as in 15 bacterial, fungal, plant and mammalian OCTases for which data are available, all in the range 1.5–5.5 mM, with the exception of the sugar-cane mitochondrial enzyme at 0.5 mM (data listed in ref. [17]). In contrast, the $K_m$ for carbamoylphosphate is much more variable, values ranging from 0.11 to 6.0 mM for these 15 enzymes. It is noteworthy that the $K_m$ for carbamoylphosphate in the case of OCTase from non-embryogenic carrot cells, at 9 $\mu$m (2.5 $\mu$M for the corresponding dissociation constant) is an order of magnitude smaller than the smallest value previously recorded, indicating an exceptionally high affinity for carbamoylphosphate.

## EXPERIMENTAL

*Partial purification of the enzyme.* Carrot cell cultures were initiated and propagated as described previously [5]. After *ca* 1 year in culture, in the presence of 0.1 mg/l. 2,4-dichlorophenoxyacetate (2,4-D), embryogenic potential had fallen to a low value. An inoculum (5% v/v packed) of these cells was allowed to grow in culture medium containing 2,4-D for another 21 days and harvested (yield 26 g wet wt). All subsequent operations were at 4°. The cells were ground, filtered, homogenized and centrifuged as described previously [5]. The clear extract (vol. 45 ml) was loaded onto a column (2 cm diameter × 4 cm) of Whatman DE52 anion-exchanger equilibrated with 0.1 M Tris–acetate, pH 7.5. The column was washed with 60 ml of the same buffer, and then developed with a gradient (0.01–0.20 M) of NaOAc (total vol. 240 ml) also in the same buffer. The active enzyme was usually between 0.06 and 0.12 M NaOAc; these fractions were pooled and concd by dialysis against Carbowax (high MW polyethylene glycol). The concd enzyme (4 ml), purified *ca* nine-fold, was stored at 4° and used within the next 24–48 hr. The residual phosphatase activity (with carbamoyl phosphate as substrate) was found to be negligible in this preparation.

*Gel filtration chromatography.* Gel filtration was performed by using a column (2 × 50 cm) of Sephacryl G200 (Pharmacia, G.B.) equilibrated with 0.1 M Tris–acetate, pH 7.5. The sample consisted of 1.0 ml enzyme preparation containing MW markers (Blue Dextran, $NH_4Cl$, ferritin, catalase, lactate dehydrogenase, haemoglobin and myoglobin, the proteins added three at a time). For assays see ref. [18]. The MW was obtained from a plot of $K_d$ vs log (MW) [19].

*Enzyme kinetics.* By using the highest and lowest combination of substrate concns likely to be encountered, initial trials showed that the catalysed reaction was linear with time for up to 30 min, and that after 20 min the extent of the reaction was less than 15% of the theoretical yield (the reaction is very nearly irreversible) confirming that 'initial rate' conditions pertained. Thereafter, initial rates were measured by single time-point assays (in duplicate) after 20 min. The reaction mixture (1.5 ml) contained 0.1 M Tris–acetate, pH 7.5, varying concns of substrates and inhibitors, and enough enzyme to ensure 'initial rate' conditions. The reaction was started by the addition of carbamoyl phosphate from a frozen stock soln. After 20 min at 25° (water bath), the reaction was stopped by the addition of 1.0 ml phenazone–$H_2SO_4$ reagent followed by 0.5 ml of the diacetyl monoxime–HOAc reagent of Prescott and Jones [20], and the concn of the product citrulline determined according to method one of these authors. Citrulline standards were run for each set of expts, as well as controls to compensate for citrulline produced non-enzymically.

*Analysis of kinetic data.* Preliminary double-reciprocal plots were made to check linearity. Exptal data were then fitted directly to the Michaelis–Menten equation by a computer programme adapted from that of Cleland [9], which provided the slopes and intercepts of double-reciprocal plots together with their s.e.m. values. Data for each line of the double-reciprocal plot were fitted separately and without reference to the intersection with other lines. No attempt was made to fit non-linear double-reciprocal plots (see Fig. 4) to theoretical equations.

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