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Ornithine carbamoyltransferase from *Spinacea oleracea*: purification and characterization

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Abstract

Ornithine carbamoyltransferase (OCT) from spinach (*Spinacea oleracea* L.) was purified to homogeneity and studied for some kinetic and structural properties. The enzyme showed a specific activity of 436 U mg^{-1}, its molecular mass was approximately 118 kDa as estimated by *Sephacryl S-200* gel filtration chromatography, the purified protein ran as a single band of 38 kDa in sodium dodecyl sulfate-polyacryamide gel electrophoresis. The enzyme catalyses an ordered bi-bi-sequential reaction in which carbamoyl phosphate binds first, followed by L-ornithine; L-citrulline leaves first, followed by phosphate. The Michaelis constant was 0.19 mM for L-ornithine and 13.1 μM for carbamoyl phosphate; the dissociation constant for the enzyme and carbamoyl phosphate complex was of 19 μM. The K_m of the reaction decreases from pH 6.0 to pH 10.4. The enzyme is heat-labile, but it was protected from thermal inactivation by substrates; more by ornithine alone than by two substrates acting together.

Additional key words: enzyme kinetics, enzyme purification, δ-*N*-(phosphonoacetyl)-L-ornithine, spinach.

Introduction

Ornithine carbamoyltransferase: (OCT, EC 2.1.3.3 carbamoyl-phosphate: L-ornithinecarbamoyltransferase) catalyzes the formation of citrulline from L-ornithine (Orn) and carbamoyl phosphate (CP). 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 (Thompson 1980). In bacteria, the enzyme is involved in both synthesis and degradation of citrulline. In general, the anabolic OCTs are characterized by a low molar mass and a trimeric structure, whereas the catabolic OCTs display much higher molar mass and a more elaborate quaternary structure. Contradictory reports exist on the presence of OCT isoenzymes in higher plants (Glenn and Maretzki 1977). Two papers (De Ruiter and Kolloffel 1985, Acaster *et al.* 1989) provide evidence for the presence of only one form of OCT in higher plants.

In the present communication, we report some kinetic and structural properties of ornithine carbamoyltransferase purified from leaves of spinach. These properties are compared with those of OCT from other higher plants.

Materials and methods

Plants: Leaves of *Spinacea oleracea* L. cv. Fortune and cv. Virofly (height about 30 - 50 cm) were used. The first had curly leaves, the second had smooth leaves. Leaves were kept on crushed ice until transfer to the laboratory, then washed with distilled water and frozen at -20 °C.

Chemicals: Epoxy-activated Sepharose 6B was obtained from *Pharmacia LKB*, Uppsala, Sweden, L-ornithine hydrochloride, carbamoyl phosphate dilithium salt, citrulline, diacetylmonoxime, thiosemicarbazide, tris-(hydroxymethyl)aminomethane and phosphonoacetic acid

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were purchased from *Sigma*, St. Louis, USA, δ-*N*-(phosphonoacetyl)-L-ornithine, δ-PALO), a transition-state analog inhibitor of OCT, was synthesized in our laboratory essentially as described by De Martines *et al.* (1981).

Purification of OCT from *Spinacea oleracea*: All steps of enzyme purification were carried out at 4 °C (the purification steps are summarized in Table 1).

For protein extraction portions of 2 000 to 2 500 *g* of frozen *S. oleracea* leaves were homogenized in the same amount of 50 mM Tris-HCl (pH 7.8) and 1 mM 2-mercaptoethanol in a Waring blendor. The extract was filtered through muslin and centrifuged for 10 min at 20 000 *g*. The supernatant fluid was brought to 40 % saturation with solid $(NH_4)_2SO_4$. The supernatant obtained after centrifugation (20 min at 22 000 *g*) was brought to 65 % $(NH_4)_2SO_4$ saturation and centrifuged again, the precipitate was dissolved in about 300 cm^3 buffer containing 50 mM Tris-HCl (pH 7.8), 50 mM L-ornithine and 1 mM 2-mercaptoethanol.

The extract was heated under stirring to 61 °C in a water bath. The solution was maintained at that temperature for 3 min, then cooled in ice and centrifuged (7 min at 20 000 *g*) to sediment the denatured proteins. Solid charcoal was added to the supernatant at 20 mg cm^{-3}. The supernatant obtained after centrifugation (20 min at 22 000 *g*) was brought to 75 % $(NH_4)_2SO_4$ saturation and centrifuged again, the precipitate was resuspended in 20 cm^3 buffer containing 50 mM Tris-HCl (pH 8.5) and 1 mM mercaptoethanol.

A column (2 × 8 cm) of δ-PALO-Sepharose 6B was equilibrated with buffer containing 50 mM Tris-HCl (pH 8.5) and 1 mM 2-mercaptoethanol. The precipitate obtained after centrifugation from the previous step was added to the column and was incubated for 30 min at 27 °C. The mixture was filtered and washed with 80 cm^3 of 50 mM Tris-HCl (pH 8.5), and with 80 cm^3 of the same buffer containing 50 mM KCl. The enzyme was eluted by using a 80 cm^3 (total volume) linear gradient of 0 - 10 mM carbamoyl phosphate in 50 mM Tris-HCl, (pH 8.5) and 1 mM 2-mercaptoethanol.

Fractions containing most of the enzyme activity were pooled, concentrated and dialyzed. The enzyme was routinely stored at -20 °C. Before reuse, the δ-PALO-Sepharose 6B resin was washed with 50 cm^3 of 300 mM sodium acetate buffer (pH 5.2) and then washed exhaustively with distilled water. The resin was stored a 4 °C in 0.02 % sodium azide.

Table 1. Summary of OCT purification from *S. oleracea*.

Purification step	Total volume [cm^3]	Total protein [mg]	Total activity [a.u.]	Yield [%]	Specific activity [U mg^{-1}]	Purification fold
Filtration	2500	5925	1010	100	0.15	-
Ammonium sulfate treatment	297	1941	247	74	0.38	2.5
Heat treatment	210	980	610	60	0.62	4.1
Activated charcoal treatment. 70 % $(NH_4)_2SO_4$ precipitate of the supernatant	20	525	439	43	0.84	5.6
δ-PALO-affinity chromatography	2.9	0.39	170	17	436	2907

OCT assay: OCT activity was measured according to the method of Lusty *et al.* (1979) based on the colorimetric detection of citrulline with diacetylmonoxime. The standard assay mixture contained the tri-buffer, diethanolamine/MES/*N*-ethylenemorpholine (0.051 M/ 0.1 M/0.051 M) at pH 8.0, 10 mM L-ornithine, 10 mM carbamoyl phosphate and an appropriate amount of OCT in a final volume of 1 cm^3. The reaction was started by the addition of freshly prepared carbamoyl phosphate. After incubation for suitable time intervals at 37 °C, the reaction was stopped by the addition of 2 cm^3 of acid reagent (3.7 g antipyrine and 2.5 g ferric ammonium sulfate in 250 cm^3 concentrated H_2SO_4 and 250 cm^3 85 % H_3PO_4). After mixing, 1 cm^3 0.4 % diacetylmonoxime was added. The samples were then incubated for 15 min in a boiling-water bath.

We varied the length of the reaction time between 6 and 12 min according to the velocity of the reaction and the degree of absorbance produced for accurate data analysis. The quantity of enzyme used was kept low in all assays so that a sufficiently high ratio of [S]/[E] was maintained to ensure steady-state turnover. To compensate for the loss of activity and increase in $K_{m,app}$ at lower values of pH, the concentrations of both enzyme and ornithine in the reaction mixture were increased. A unit of enzyme activity was taken as the amount which catalyzes the formation of 1 μmol of citrulline per min at 37 °C under standard assay conditions.

Data analysis: The nomenclature used here is that of Cleland (1967). Reciprocal velocities were plotted versus reciprocal substrate concentration. Analysis of secondary plots yielded the following parameters: K_a as the Michaelis constant for CP, K_b the Michaelis constant for ornithine,

K_{ia} the dissociation constant for the enzyme-carbamoyl phosphate complex and v_{lim} as the limiting velocity.

Protein determination: Protein was determined by measurement of A_{280} or by the method of Bradford (1976) using bovine serum albumin as standard .

Electrophoresis: SDS/polyacrylamide-gel electrophoresis was performed as described by Laemmli (1970). OCT was electrophoresed through 6 % stacking and 12 % resolving gels at 20 mA costant current. Size estimation for the OCT monomer was based on the mobility of the enzyme protein relative to standards (myosin 200 kDa; β-galactosidase 130 kDa; phosphorylase 100 kDa; BSA 65 kDa; ovalbumin 45 kDa; carbonate dehydratase 30 kDa). Non-denaturing gel electrophoresis was performed on a 6 % stacking and 12 % resolving slab gel. Proteins were stained with Coomassie Brilliant Blue R-250.

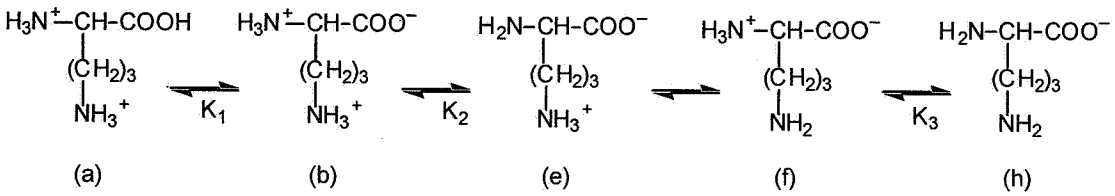
Molecular mass determination: The molar mass for native OCT was determined by gel-filtration chromatography using a column (1.6 × 133 cm) of *Sephacryl S-200 HR* equilibrated with buffer containing 50 mM K-phosphate (pH 8.5) and 1 mM 2-mercaptoethanol. The column was calibrated by noting the elution volumes (V_e) of aldolase, BSA, ovalbumin, chymotrypsinogen. The void volume (V_o) was determined with ferritin. The partition coefficient, K_{av}, was calculated according to the formula $K_{av} = (V_e - V_o)/(V_t - V_o)$ in which V_t represents the total bed volume.

Heat inactivation: An appropriate amount of enzyme (200 μg cm^{-3}) was incubated in 50 mM Tris-HCl (pH 7.8), 2 mM β-mercaptoethanol with or without a saturating concentration of ligands in a water bath thermostated at ± 0.1 °C of the selected temperature. The enzyme activities were determined for each set of experimental conditions.

Results

The spinach OCT protein was purified 2907-fold with a 17 % yield exhibiting a specific activity of 436 units per mg protein (Table 1). Gel-filtration chromatography on a *Sephacryl S-200* column and SDS-PAGE indicated that the enzyme has a trimeric structure made up of identical or similar subunits of apparent molecular mass of 40 kDa. The mass of the native OCT was about 118 kDa.

Earlier studies (Kuo *et al.* 1985) showed that between the ionic forms of ornithine in the pH 6 - 10 range, only the minor zwitterionic species with uncharged δ-amino group binds the enzyme productively. The dissociation steps of ornithine are shown as follows:



The total concentration of ornithine is then [a + b + e + f + h].

The ratio between the zwitterionic form and the total species [e + f]/[a + b + e +f + h] can be calculated from the molecular dissociation constants (8.68 and 10.78).

$K_{a,1}=[b]\,[H]/[a]$; $K_{a,2}=[e + f]\,[H]/[b]$; $K_{a,3}=[h]\,[H]/[e + f]$

Thus:

$[e + f] = K_{a,2}\,10^{pH}\,[b] \rightarrow [e + f] = 10^{pH-pK_{a,2}}[b]$

$[a] = [H]\,1/K_{a,1}\,[b] \rightarrow [a] = 10^{pK_{a,1}-pH}\,[b]$

$[h] = 10^{2pH-pK_{a,3}}\,10^{pH-pK_{a,2}}\,[b] \rightarrow [h] = 10^{2pH-pK_{a,3}-pK_{a,2}}\,[b]$

Taking $pK_i \ll$ pH the ratio is then

$$10^{pH-pK_{a,2}}/(1 + 10^{pH-pK_{a.2}} + 10^{2pH-pK_{a,2}-pK_{a,3}}) \qquad (1)$$

The kinetics of the bisubstrate-biproduct reaction were studied in the tribuffer diethanolamine/MES/N-ethylenemorpholine at pH 8.5 and 37 °C. Plots of reciprocal velocity *vs.* reciprocal substrate concentration were linear when the concentration of either substrate was varied (CP 0.05 - 0.1 - 0.2 - 1.0) in a series of fixed concentrations of the other (ornithine 0.25 - 0.5 - 1 - 2). Moreover, in both plots the lines intersect to the left of the vertical axis, indicating a sequential mechanism. A similarly converging initial velocity pattern was also observed when ornithine concentration was varied at fixed levels of CP. The results were analysed according to Cleland (1967) whose terminology is used here.

The following kinetic constants were obtained for the bisubstrate reaction catalyzed by OCT: v_{lim} (the limiting rate) 7.0 ± 1.5 pmol s^{-1}, K_a (Michaelis constant for carbamoyl phosphate) 3.5 μM, K_b (Michaelis constant for L-ornithine) 0.19 mM, K_{ia} (dissociation constant of the enzyme-carbamoyl phosphate complex) 19 μM.

In order to distinguish between kinetic mechanisms,

in which the two substrates bind either randomly or in a sequential order, the effect of the product on the reaction was studied. During the determination of the initial velocity pattern in the presence of phosphate (0 - 2 - 4 - 8 - 12 mM) when carbamoyl phosphate is the variable substrate (0.05 - 0.1 - 0.2 - 1.0 mM), was observed that a higher ornithine concentration (10 mM) causes substrate inhibition of the reaction. Ornithine, in the presence of P_i, caused competitive inhibition with respect to CP. All these observations indicated that the mechanism is ordered, with CP being the obligatory first substrate. Further ornithine inhibition induced by P_i indicated that the mechanism involves the formation of dead-end enzyme-P_i-ornithine complex.

The value of the dissociation constant of the enzyme-carbamoyl phosphate complex was 19 μM.

The effect of pH on $K_{m,app}$ of ornithine in spinach OCT is shown in Table 2. It was observed that $K_{m,app}$ decreases as pH values increase and the concentration of zwitterionic form increases. For each value of pH the ratio [e + f]/[a + b + e + f + h] was obtained using equation (1) and the value of K_m for the zwitterionic form ($K_{m,zwitt}$), referring to the concentration of real substrate available at each value of pH. In this way no appreciable variation in the pH range of 6.5 - 10 of the zwitterionic ornithine affinity constants was observed.

Table 2. Values of $K_{m,app}$ and $K_{m,zwitt}$ for ornithine in OCT. $K_{m,zwitt}$ were obtained from $K_{m,app}$ using equation (1). Carbamoyl phosphate was mantained at 10 mM; ornithine concentration was varied at every value of pH, to respect the hyperbolic distribution of points.

pH	$c_{Orn,zwitt}/c_{Orn,tot}$	$K_{m,app}$ [mM]	$K_{m,zwitt}$ [mM]
6.0	0.0029	166.8	0.34
6.5	0.0066	91.6	0.60
6.8	0.0130	49.1	0.64
7.0	0.0205	23.6	0.48
7.8	0.1165	1.45	0.17
8.0	0.1728	1.31	0.22
8.4	0.3437	0.57	0.19
9.0	0.6688	0.36	0.24
10.0	0.8238	0.32	0.26

As zwitterionic ornithine concentration depends both on total ornithine in the mixture reaction and on the pH value of each assay, any set of points gave a different value of Orn_{zwitt}. For this reason we combined in a single graph the points obtained for each zwitterionic ornithine at different pH values (Fig. 1).

It was observed that for values higher than 7.8 the results are fairly consistent. The same situation is

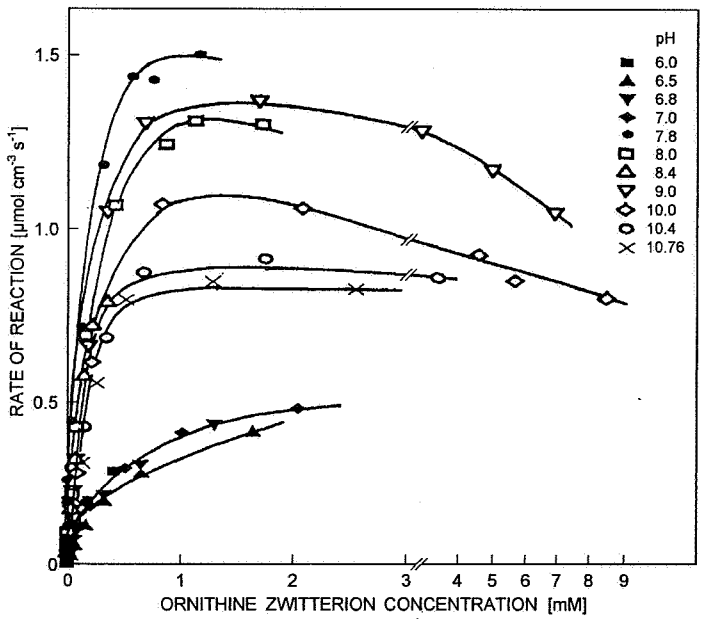


Fig. 1. Rate of reaction (v) of spinach OCT vs. ornithine zwitterion concentration (in mM) at different values of pH.

observed for values lower than pH 7 when considering only the zwitterionic form of ornithine. The complete graph gives evidence of a sharp change at pH 7 but all the differences observed are exclusively due to the fact that all ionic forms of ornithine have enzymic activity.

To confirm this hypothesis we have again analysed the activity *vs.* pH, varying the total ornithine concentration to obtain, in every sample, the same concentration of Orn_{zwitt}. Under these conditions the pattern for pH > 7.8 can be expressed as a straight line (Fig. 2).

In order to verify the influence of the ionization of carbamoyl phosphate on the enzyme activity, we calculated the affinity constants for this substrate. When

keeping in every assay the same concentration of Orn_{zwitt}, the kinetic parameters were not influenced by varying pH (results not shown). As reported by Baker and Yon (1983) OCT shows the typical pattern of excess substrate

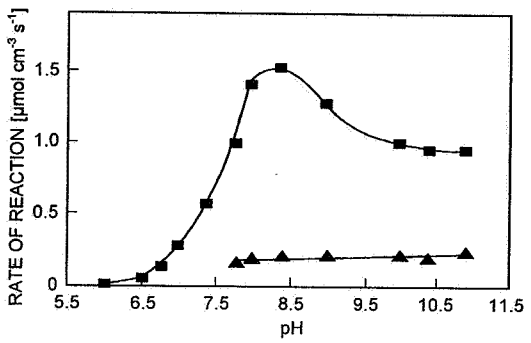


Fig. 2. Rate of reaction (v) of spinach OCT in the presence of 10 mM Orn_{tot} (*squares*) and of 0.2 mM Orn_{zwitt} (*triangles*) at different pH values.

mechanism in which dead-end complexes E-P_i-ORN occur. Substrate inhibition was observed (Fig. 1) for values of Orn_{zwitt} higher than 1.8 mM.

The effect of temperature on the velocity of citrulline synthesis was also studied. In samples containing 200 µg cm^{-3} enzyme extract, the half-lives obtained ranged from 99 min at 56 °C to 60 min at 58 °C to 11 min at 64 °C.

By adding ligands, the thermal inactivation of spinach OCT was modified. At 68 °C, the half-life of spinach OCT in the control was 10.5 min, in the presence of 50 mM ornithine it was greater than 45 min, with 25 mM orthophosphate it was 11.8 min, and in the presence of both Orn and P_i it was greater than 40 min.

The effect of temperature on the turnover number k_{cat} was determined for temperatures between 19 and 47 °C. Within this range the data lie on a straight line, following Arrhenius law. The activation energy of the reaction was calculated to be 39.6 kJ mol^{-1}.

Discussion

Within a broader study of ornithine carbamoyltransferases including both animal and plant sources, OCT from spinach leaves was purified to homogeneity in 5 steps. A single step of affinity chromatography was used to purify ornithine carbamoyltransferase from other species (Hoogenraad *et al.* 1980, De Martines *et al.* 1981, Slocum and Richardson 1991, Koger *et al.* 1994) but in our experience with spinach 4 steps were necessary preceding the affinity chromatography in order to obtain a highly purified enzyme.

De Martines *et al.* (1981) also reported that the pea enzyme bound PALO-Sepharose 6B better at pH 6.5 than at pH 7.5, the pH used in the purification animal OCTs. In contrast, we observed in accordance with Slocum and Richardson (1991) that binding of *S. oleracea* OCT is maximum near pH 8.5 and we estimate that the enzyme binds to the inhibitor with a considerably lower affinity than does its mammalian counterpart (Mori *et al.* 1977, Hoogenraad 1978). Indeed, Acaster *et al.* (1989) reported K_i values for PALO between 1.7 and 3.5 × 10^{-4} M for OCTs from eight different plant species.

The value of specific activity (436 U mg^{-1}) is higher than that reported for *Pisum sativum* and for the human enzyme by previous workers (139 U mg^{-1}, Slocum and Richardson 1991; 158 U mg^{-1}, De Ruiter and Kolloffel 1985; 340 U mg^{-1}, De Martines 1981; 233 U/mg, Kalousek *et al.* 1978) and it is lower than those reported for other organisms, *e.g.* rat (885 U mg^{-1}, Lusty *et al.* 1979) and bovine (780 U mg^{-1}, Marshall *et al.* 1972).

The same trimeric structure has been reported for OCT from *Pisum sativum* (Slocum and Richardson 1991) and other species. It is interesting to note that nontrimeric structures have been reported for other plant OCTs. De Ruiter and Kolloffel (1985) concluded that partially purified pea OCT was a dimer (M_r = 77 600), Acaster *et al.* (1989) reported that the mean M_r value for OCTs from several plant species was 158 kDa and the same value was obtained for the carrot enzyme (Baker and Yon 1983), suggesting that the enzyme was most likely a tetramer.

Other molar masses for plant OCT's have been reported and it is not clear to what extent the differences may be due to different methods for their preparation and analysis.

In agreement with the majority of carbamoyltransferases, the spinach OCT appears to catalyse an obligatory sequential reaction with carbamoyl phosphate as the leading substrate.

The effect on pH on the apparent Michaelis constant of L-ornithine suggests that this diamino acid in its cationic form is not the substrate, and that only the minor zwitterionic form binds enzyme productively.

Consequently, we decided to keep constant the value of Orn_{zwitt}, the real substrate of reaction, so that the activity registered at any single value of pH can be described by a straight line in contrast with the bell-shaped pattern obtained when maintaining constant Orn_{tot}. This provides evidence that for pH >7.8 the enzyme can be considered to be pH-independent. The sharp change registered in the pattern at pH 7 could be ascribed to an enzyme group ionization.

The thermal stability studied in the range of 56 - 60 °C did not appear to be markedly concentration-dependent. The presence of ligands during the heat treatment had a striking protective effect on the activity of the enzyme. In particular the presence of ornithine

alone protects from inactivation more than Orn and P_i together, while P_i alone has no effect.

Such protective effects on ligands on OCT were observed previously in *Escherichia. coli* and in thermophiles (Legrain *et al.* 1977, Van de Casteele *et al.* 1997). In *Pyrococcus furiosus* OCT, isotopic competition experiments strongly suggest that carbamoyl phosphate synthase and OCT associate to form a complex channeling carbamoyl phosphate towards the synthesis of citrulline, thus preventing the exposure to the aqueous phase and hence protecting it against degradation (Legrain *et al.* 1995).

Statistical analysis revealed that *Thermus thermophilus* OCT has 55 % more arginine and 35 % more proline than the average for all other OCT's. Similarly in a number of proteins an increase of these residues appears to be a feature for thermal stability. On the other hand, heat-labile residues, such as asparagine and glutamine, are as expected, less abundant (Murata and Shachman 1996). It was also reported that hydrophobic interactions are important for enzyme stability; indeed, proteins from hyperthermophiles were found to have an increased content of hydrophobic amino acids (Van de Casteele *et al.* 1997).

The activation energy found here (39.6 kJ mol^{-1}) was in accordance with values reported for enzymes from mesophilic organisms (Kuo *et al.* 1985).

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