

Purification and Properties of Ornithine Carbamoyltransferase from Loggerhead Turtle Liver

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Summary

Ornithine carbamoyltransferase has been purified from the liver of the loggerhead turtle *Caretta caretta* by a single-step procedure using chromatography on an affinity column to which the transition-state analogue, δ -N-(phosphonoacetyl)-L-ornithine (δ -PALO), was covalently bound. The procedure employed yielded an enzyme which was purified 373-fold and was judged to be homogeneous by nondenaturing and sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The enzyme showed a specific activity of 224. The molar mass of the *C. caretta* enzyme was approximately 112 kDa, the single band obtained by SDS-PAGE indicated a subunit molar mass of 39.5 kDa; hence, the enzyme is a trimer of identical subunits. It catalyzes an ordered sequential mechanism in which carbamoyl phosphate binds first, followed by L-ornithine. The Michaelis constants were 0.858 mM for L-ornithine and 0.22 mM for carbamoyl phosphate, the dissociation constant of the enzyme-carbamoyl phosphate complex was 0.50 mM.

Key words

Loggerhead turtle • *Caretta caretta* • Ornithine carbamoyltransferase • Enzyme kinetics • Enzyme thermostability

Introduction

Ornithine carbamoyltransferase (OCT, EC 2.1.3.3.) catalyzes the synthesis of citrulline from ornithine and carbamoyl phosphate with the liberation of orthophosphate. OCT is widely distributed throughout the phylogenetic spectrum. In prokaryotes (Legrain *et al.* 1972) and fungi (Eisenstein *et al.* 1984), the enzyme is involved in both synthesis and degradation of citrulline, in plants it serves to synthesize citrulline as a nitrogen reservoir (Schubert *et al.* 1986). In ureotelic organisms the enzyme is an intermediate in the detoxication process of ammonia, leading to urea and arginine biosynthesis.

Marine elasmobranchs (sharks, skates and rays) synthesize and retain in their tissues high concentrations of urea (up to 0.4 M) and certain amines, such as trimethylamine oxide (up to 0.2 M) for the purpose of osmoregulation. Although the pathway of urea synthesis in ureosmotic elasmobranchs is similar to the classical urea cycle in ureotelic species, there are several properties that are unique. Indeed, the primary function of urea synthesis in elasmobranchs is osmoregulation rather than ammonia detoxication. In fact, in these organisms the most significant difference is that the initial step of ammonia assimilation for urea synthesis in hepatic mitochondria of elasmobranchs is the formation of

glutamine which is subsequently utilized as substrate for carbamoyl phosphate synthesis (Anderson *et al.* 1984). This is due to the presence of high levels of glutamine synthetase (GSase) and a unique acetylglutamate and glutamine-dependent carbamoyl phosphate synthetase (CPSaseIII), both localized exclusively in the mitochondrial matrix (Casey and Anderson 1982, 1985). In mammalian and amphibian species, glutamine synthetase is localized in liver cytosol, and the first step of ammonia assimilation for urea synthesis in liver is the direct conversion of ammonia to carbamoyl phosphate, catalyzed by the corresponding mitochondrial acetylglutamate and ammonia-dependent carbamoyl phosphate synthetase that cannot utilize glutamine as the nitrogen-donating substrate. OCT has been purified from a variety of sources: bovine (Marshall *et al.* 1972), rat (Clarke 1976), elasmobranch (Xiong *et al.* 1988), human liver (Kalousek *et al.* 1978), and a variety of microorganisms (Legrain *et al.* 1976, Bates *et al.* 1985, Murata *et al.* 1996, Sanchez *et al.* 1997) and plants (Slocum *et al.* 1991).

In the present communication we report the purification and biochemical characterization of the loggerhead liver OCT.

Methods

Material and reagents

Liver was obtained from *Caretta caretta* specimen with traumatic and mortal wounds but still living, kindly supplied by the Museum of Natural History of Calimera (Lecce). The animal was sacrificed by a single injection of Tanax (embutramide, mebezonium iodide, tetracaine hydrochloride) and immediately dissected.

δ -PALO was synthesized and further purified essentially as described by De Martines *et al.* (1981). Bovine albumin, citrulline, L-ornithine hydrochloride, carbamoyl phosphate dilithium salt, *N*-ethylmorpholine, MES, diethanolamine, diacetylmonoxime, thiosemicarbazide and Tris-(hydroxymethyl)-aminomethane were purchased from SIGMA Co. (St. Louis, MO, USA), epoxy-activated Sepharose 6B was obtained from Pharmacia LKB (Sweden)

Purification of ornithine carbamoyltransferase

All operations were carried out at 0–5 °C unless otherwise stated, except for adsorption and elution from affinity adsorbent.

Step 1: preparation of crude extract of *C. caretta* liver. Defrosted portions of 3 g were homogenized in a Potter homogenizer in 15 ml of 50 mM Tris-HCl (pH 7.8)

containing 1 mM 2-mercaptoethanol. The homogenate was centrifuged at 13 000 *g* for 10 min. The supernatant containing ornithine carbamoyltransferase activity was separated from the upper fat pad by filtration through glass wool.

Step 2: heat treatment. The filtrate obtained in step 1 was heated with stirring to 61 °C in a water bath, the solution was maintained at that temperature for 1 min., then cooled in an ice bath and centrifuged at 13 000 *g* for 10 min.

Step 3: affinity chromatography on δ -PALO Sepharose. The supernatant obtained in step 2 was mixed with the affinity adsorbent (δ -PALO) equilibrated with 50 mM Tris-HCl (pH 7.8), and incubated for 30 min at 27 °C. The mixture was filtered and washed with 80 ml 50 mM Tris-HCl (pH 7.8) and 80 ml of the same buffer containing 100 mM KCl. The enzyme was eluted with 50 ml 2.5 mM carbamoyl phosphate in 50 mM Tris-HCl (pH 7.8). Enzyme obtained from several purifications was dialyzed against 10 volumes of 50 mM Tris-HCl (pH 7.8) containing 1 mM 2-mercaptoethanol and kept frozen at –20°C.

Protein determination

Protein was determined from binding of Coomassie brilliant blue G-250, as described by Bradford (1976) using bovine serum albumin as standard.

Assay of OCT

OCT activity was measured as the rate of citrulline formation from ornithine and carbamoyl phosphate as described by Lusty *et al.* (1979). The reaction mixtures, in a final volume of 1.0 ml, contained the tri-buffer diethanolamine/MES/*N*-ethylmorpholine (0.051 M/0.1 M/0.051 M) at pH 7.8, 10 mM ornithine, 10 mM carbamoyl phosphate and an appropriate amount of ornithine carbamoyl transferase. After incubation for suitable intervals at 37 °C, the reaction was terminated by the addition of 2.0 ml acid reagent (3.7 g antipyrine and 2.5 g ferric ammonium sulfate in 500 ml water, 250 ml concentrated H₂SO₄, and 250 ml 85 % H₃PO₄). One milliliter of 0.4 % diacetylmonoxime was added and the samples were incubated for 15 min in boiling water. Citrulline concentration was determined from the absorbance at 464 nm, using an appropriate standard. We varied the length of the reaction time between 6 and 12 min according to the velocity of the reaction and the degree of optical density produced for accurate data analysis.

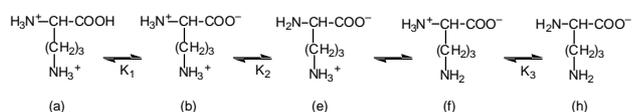
For the determination of kinetic parameters, the amount of enzyme used was kept low in all assays so that a sufficiently high ratio of [S]/[E] was maintained to

ensure steady-state turnover. We varied the length of the reaction time between 6 and 12 min according to the velocity of the reaction and the degree of optical density produced for accurate data analysis. To compensate for the loss of activity and the increase in $K_{m,app}$ at lower values of pH, the concentration of both enzyme and ornithine in the reaction mixture were increased.

Unit of enzyme activity was taken as the amount which catalyzes the formation of 1 μ mol citrulline per min at 37 °C under standard assay conditions.

Ionic species of L-ornithine

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



The total concentration of ornithine is then $[a + b + e + f + h]$. The ratio between the zwitterionic form and the total species is $[e + f]/[a + b + e + f + h]$; it can be calculated from the molecular dissociation constants of L-ornithine determined in water at ambient temperature (8.68 and 10.78) and by using the following relationship

$$K_{a1}=[b][H]/[a]; K_{a2}=[e+f][H]/[b]; K_{a3}=[h][H]/[e+f]$$

The ratio $[e + f]/[a + b + e + f + h]$ is equal to:

$$10^{\text{pH}-\text{p}K_{a,2}} / (1 + 10^{\text{p}K_{a,1}-\text{pH}} + 10^{\text{pH}-\text{p}K_{a,2}} + 10^{2\text{pH}-\text{p}K_{a,2}-\text{p}K_{a,3}})$$

and, considering $\text{p}K_1 \ll \text{pH}$:

$$10^{\text{pH}-\text{p}K_{a,2}} / (1 + 10^{\text{pH}-\text{p}K_{a,2}} + 10^{2\text{pH}-\text{p}K_{a,2}-\text{p}K_{a,3}}) \quad (1)$$

Data analysis

The nomenclature used here is that of Cleland (1967) Reciprocal velocities were plotted against reciprocal substrate concentration.

Assay for heat inactivation

Two mg enzyme per ml were incubated in 50 mM Tris-HCl (pH 7.8), 2 mM β -mercaptoethanol with or without a saturating concentration of ligand in a water bath thermostated at ± 0.1 °C of the selected temperature. The enzyme activity was determined for each set of experimental conditions.

Determination of molecular mass

The molecular mass for native OCT was estimated by gel filtration chromatography using a column (1.6 \times 133 cm) of Sephacryl S-200 HR equilibrated with buffer containing 50 mM K-phosphate, 1 mM 2-mercaptoethanol, 1 mM EDTA (pH 8.5), at a flow rate of 8 ml/h at 4 °C. The column was calibrated with aldolase (158 kDa), BSA (67 kDa), ovalbumin (43 kDa), chymotrypsinogen (25 kDa). The void volume was determined with ferritin (440 kDa).

Electrophoresis (SDS-PAGE)

The degree of homogeneity of the δ -PALO-Sepharose 6B-purified OCT protein sample was determined by SDS-PAGE (Laemmli 1970). OCT was electrophoresed through 6 % stacking and 12 % resolving gels at 20 mA constant current. Size estimation for the OCT monomer was based on the mobility of the enzyme protein relative to molar mass standards: myosin (200 kDa), β -galactosidase (130 kDa), phosphorylase (100 kDa), BSA (65 kDa), ovalbumin (45 kDa), carbonate dehydratase (30 kDa).

Table 1. Summary of OCT purification from *Caretta caretta*.

Purification step	Total volume mL	Total protein mg	Total activity units	Yield %	Specific activity units/mg	Purification -fold
Filtration	290	1876	1100	100	0.6	–
Heat treatment	25	375	930	84	2.48	4.2
δ -PALO-affinity chromatography	5	1.33	299	27	224	373

Results

The OCT from *C. caretta* was purified to homogeneity. The results of the purification procedure are shown in Table 1. The affinity chromatography step has been used for purifying ornithine carbamoyl transferase from other species in a single step (Hoogenraad *et al.* 1980; De Martinis *et al.* 1981), but in our experience with the *C. caretta* enzyme a heat-treatment step preceding the affinity chromatography had to be carried out to obtain highly purified enzyme. The final step yielded an enzyme of high purity as indicated by the presence of a single protein band after gel electrophoresis in the presence of SDS and by the observation that the enzyme activity eluted as a sharp peak that correlated well with the protein in elution profile in the final step (data not shown). The *C. caretta* OCT protein was purified 373-fold with an 27 % yield exhibiting a specific activity of 224 units/mg protein. The specific activity value obtained with *C. caretta* was similar to those observed for human liver (233 U/mg; Kalousek *et al.* 1978), and is higher than that reported for porcine (26.4 U/mg; Koger *et al.* 1994), for dogfish (27 U/mg; Xiong *et al.* 1988), for chicken kidney (77 U/mg; Tsuji 1983), and it is lower than those reported for other organisms, e.g. rat (885 U/mg; Lusty *et al.* 1979), beef (780 U/mg; Marshall *et al.* 1972).

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 38 kDa. The molecular mass of native OCT was about 112 kDa.

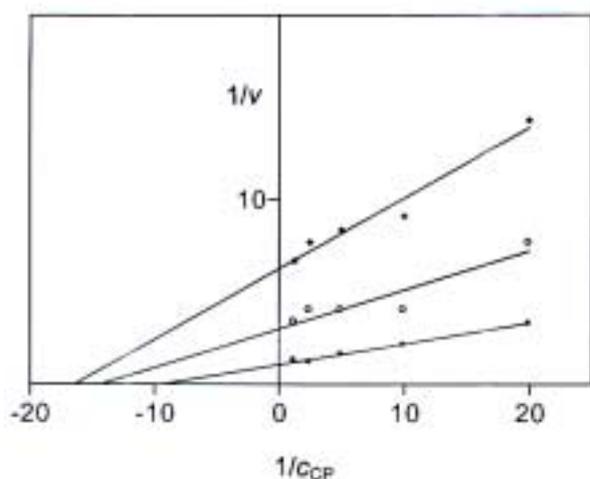


Fig. 1. Lineweaver-Burk plot of the reciprocal rate of reaction, $1/v$ (in $\mu\text{mol}\cdot\text{1ml}^{-1}\cdot\text{min}^{-1}$) against the reciprocal concentration of carbamoyl phosphate $1/c_{\text{CP}}$ (mM^{-1}) at different concentrations of ornithine (\bullet 0.4, \circ 0.1, \blacklozenge 0.05 mM).

Kinetic mechanism and regulatory properties

The tribuffer used (Ellis-Morrison 1982), maintains an essentially constant ionic strength in the pH 5–11 range, the OCT is stable under the conditions of the assay. The kinetics of the bisubstrate-biproduct reaction has been analyzed by varying the concentration of carbamoyl phosphate (CP) between 0.05 and 0.8 mM at different concentrations of ornithine (0.05–0.4 mM). This analysis produced a double-reciprocal plot in which lines converging at the left of vertical axes, indicating a sequential mechanism in which both substrates bind before the release of products (Fig. 1)

A similar behavior was observed when ornithine was varied at a fixed concentration of CP (Fig. 2).

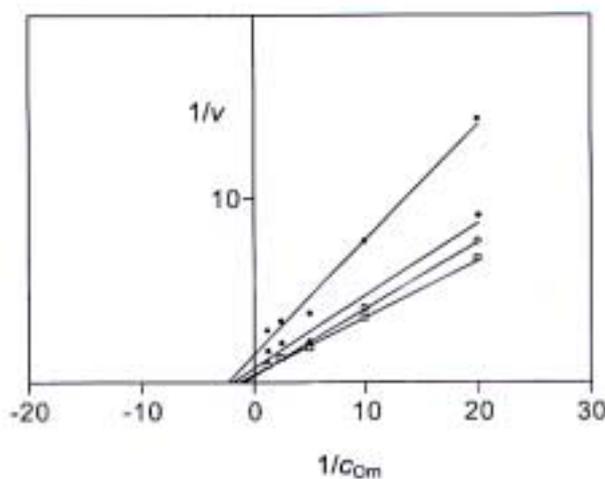


Fig. 2. Lineweaver-Burk plot of the reciprocal rate of reaction $1/v$ ($\mu\text{mol}\cdot\text{1ml}^{-1}\cdot\text{min}^{-1}$) against the reciprocal concentration of ornithine $1/c_{\text{Orn}}$ (mM^{-1}) at different concentrations of carbamoyl phosphate (\circ 0.8, \circ 0.4, \blacklozenge 0.1, \bullet 0.05 mM).

The kinetic constants calculated according to Cleland (1967) were K_{CP} 0.22 mM and K_{Orn} 0.86 mM. The dissociation constant of the complex of OCT with CP was 0.5 mM.

The sequential mechanism is demonstrated by the inhibition exerted by the last product (inorganic phosphate) which is competitive toward carbamoyl phosphate (Fig. 3). From the horizontal intercept of the secondary plot, the inhibition constant of the $E\text{-}P_i$ complex is seen to be 4.25 mM.

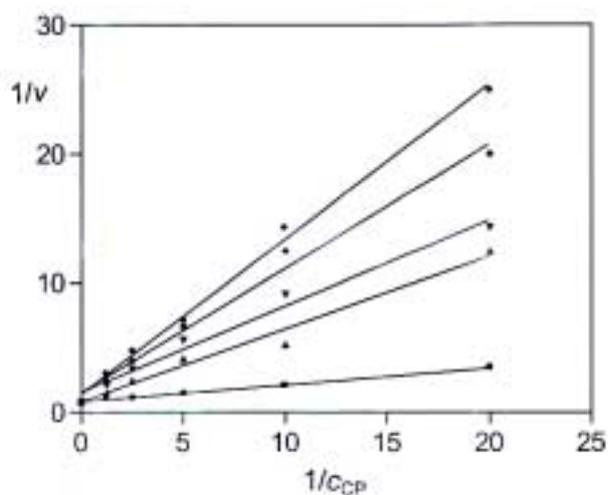


Fig. 3. Product inhibition by phosphate with carbamoyl phosphate as the variable substrate. OCT activities were determined in the presence of \blacksquare 0, \blacktriangle 2, \blacktriangledown 4, \blacklozenge 6, \bullet 10 mM Na_2HPO_4 ; ordinate: $1/v$ ($\mu\text{mol}\cdot\text{mL}^{-1}\cdot\text{min}^{-1}$); abscissa: $1/c_{\text{CP}}$ (mM^{-1}).

Table 2. Values of $K_{m,\text{app}}$ and $K_{m,\text{zwitter}}$ for ornithine.

pH	$c_{\text{Orn,zwitter}}/c_{\text{Orn,tot}}$	$K_{m,\text{app}}$ (mM)	$K_{m,\text{zwitter}}$ (mM)
6.35	0.0046	14.04	0.065
6.66	0.0094	4.73	0.044
6.95	0.018	2.73	0.049
7.25	0.036	2.04	0.073
7.71	0.097	0.94	0.091
8.00	0.173	0.51	0.088
8.38	0.333	0.20	0.065
8.85	0.592	0.21	0.124
9.95	0.839	0.19	0.157

The values of $K_{m,\text{zwitter}}$ were obtained from $K_{m,\text{app}}$ through equation (1). Carbamoyl phosphate was maintained at 10 mM.

Determination of kinetic parameters as a function of pH

The effect of pH on $K_{m,\text{app}}$ of ornithine of *C. caretta* OCT is shown in Table 2. The $K_{m,\text{app}}$ is seen to decrease as pH values increase and the concentration of zwitterionic form increases. For each value of pH obtained from equation (1) the ratio $[e + f]/[a + b + e + f + h]$ is reported in the table, together with the value of K_m for the zwitterionic form ($K_{m,\text{zwitter}}$), referring to the concentration of real substrate available at each value of pH. No substantial variation in the pH range (6.5-10) for zwitterionic ornithine affinity constants was observed. We examined the dependence of activity on pH, by

varying the total ornithine concentration according to equation (1) so to obtain, in every sample, the same concentration of $\text{Orn}_{\text{zwitter}}$ with the situation in which a constant concentration of Orn_{tot} is maintained. Under these conditions the pattern for constant $\text{Orn}_{\text{zwitter}}$ is flatter than that for constant Orn_{tot} (Fig. 4). We also calculated the affinity constants for carbamoyl phosphate to check the influence of the ionization of this substrate on enzyme activity. The kinetic parameters were not influenced by varying pH (data not shown).

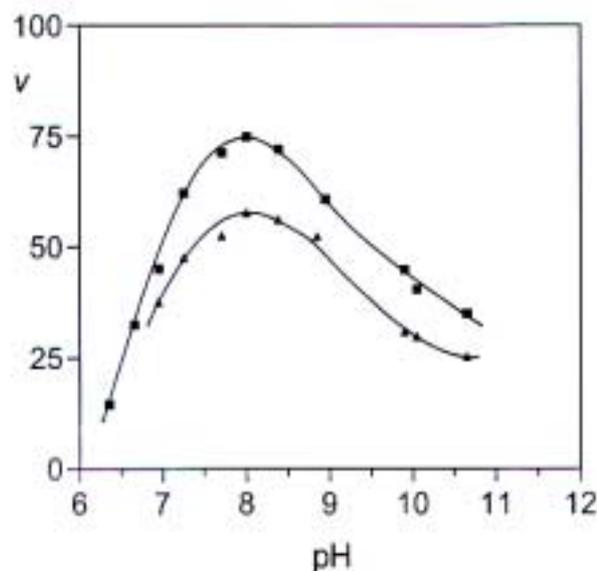


Fig. 4. Rate of OCT reaction (v , in $\mu\text{mol mL}^{-1}\cdot\text{min}^{-1}$) in dependence on pH in the presence of 0.2 mM $\text{Orn}_{\text{zwitter}}$ (\blacktriangle) and of 10 mM Orn_{tot} (\blacksquare).

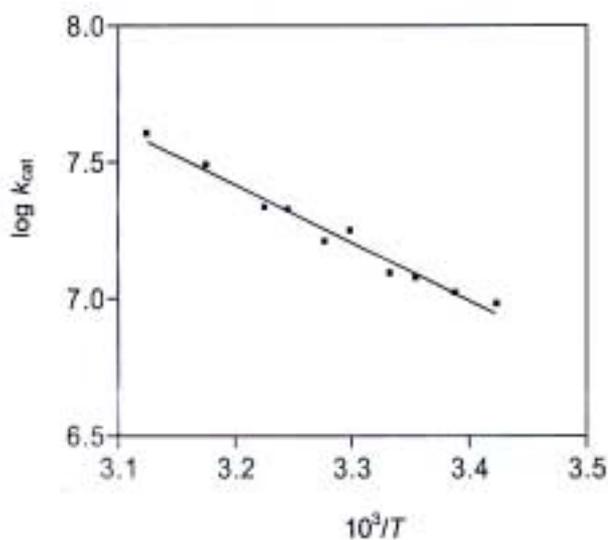


Fig. 5. Arrhenius plot of the turnover number k_{cat} of *Carettta caretta* OCT; T is expressed in K.

Thermal stability

The effect of temperature on the rate of citrulline synthesis was studied between 60 and 70 °C. In samples containing 370 µg/ml of enzyme extract, the half-lives (in min) were found to be 116 at 60 °C, 39 at 64 °C, 10 at 66 °C, 7 at 67 °C and 5 at 70 °C. At 68 °C and a protein concentration 10-fold lower, the $t/2$ was reduced only two-fold, indicating that thermostability was not markedly concentration-dependent. The presence of CP produced total protection only for 3 min; phosphate protected more than ornithine, whereas association of two substrates provides total protection (Table 3). The effect of temperature on the turnover number k_{cat} has been determined for temperatures between 19 and 47 °C. Within this range the data follow the Arrhenius rate law. The activation energy of the enzyme reaction was found to be 41 kJ/mol (Fig. 5).

Table 3. Protection exerted by ligands during thermal inactivation of OCT.

Ligand	Concentration (mM)	Half-life (min)
None	–	7.15
Orn	50	8.89
P _i	25	37
Orn + P _i	50–25	>200
Ethylene glycol	20 %	3.93

The OCT (0.37 mg/ml) was heated at 68 °C for different time intervals in 50 mM Tris-HCl (pH 7.8) and 2 mM β-mercaptoethanol.

Discussion

The *C. caretta* OCT was purified here to homogeneity. The crucial step, also used for purification

by other authors, is the affinity chromatography using as ligand the transition-state analog δ-phosphonoacetyl-L-ornithine (δ-PALO). Kinetic analysis and substrate inhibition confirm that the reaction proceeds by an ordered sequential mechanism, with CP identified as the leading substrate. The effect of 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.

Thermal stability studied in the range 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.

Such protective effects on ligands of OCT were observed previously in *Escherichia coli* and in thermophiles (Legrain *et al.* 1977, Van de Castele *et al.* 1997). The authors indicate that the complex enzyme–Orn–P_i is very stable with a half-life of more than 20 h. They suggest that in order to ensure the stability of CP, a molecule heat-labile with a $t/2$ of 1 min at 70 °C, and potentially toxic owing to its decomposition into cyanate, carbamoyl phosphate synthase and OCT associate to form a complex which channels carbamoyl phosphate toward the synthesis of citrulline, thus preventing exposure to the aqueous phase and hence protecting it against degradation (Legrain *et al.* 1995). Hydrophobic solutions, such as ethylene glycol, destabilize the enzyme drastically, suggesting that, also for *C. caretta* OCT, hydrophobic interaction contributes to the stability.

The activation energy was found to be 41 kJ/mol, in accordance with the values reported for enzymes from mesophilic organisms (Kuo *et al.* 1985). No breakpoints are observed in the Arrhenius plots as are seen in OCT from the thermophilic eubacterium *Thermus* Z05.

Taken together, the loggerhead turtle OCT appears to be similar to OCT's from other sources.

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Reprint requests

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