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Partial Purification and Properties of Ornithine Transcarbamoylase from *Nostoc muscorum* Kützing[1]

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SAMUEL F. BOGGESS[2] AND AUBREY W. NAYLOR
Department of Botany, Duke University, Durham, North Carolina 27706

ABSTRACT

Ornithine transcarbamoylase (carbamoyl phosphate:L-ornithine carbamoyltransferase, EC 2.1.3.3) has been partially purified from the blue-green alga *Nostoc muscorum* Kützing, an organism in which the enzyme seems to be involved in a bicarbonate-fixing pathway leading to citrulline. Pertinent to possible regulation of this pathway, the enzyme shows hyperbolic substrate kinetics, has a molecular weight estimated at 75,000 daltons, and its catalytic capability is little influenced by a selection of metabolites that might conceivably act as regulators *in vivo*. Thus it seems unlikely that this enzyme is the control point for bicarbonate fixation. In terms of energy of activation (12.3 kcal/mole), size and Km for carbamoylphosphate, the *Nostoc* enzyme resembled preparations from liver and higher plants more than preparations from *Streptococcus* and *Mycoplasma*. The enzymes from *Streptococcus* and *Mycoplasma* are probably specialized for citrulline breakdown rather than citrulline synthesis. The Km for ornithine was 2.5 mM at a saturating concentration of carbamoylphosphate and the Km for carbamoylphosphate was 0.7 mM at an ornithine concentration of 2 mM. Ornithine was inhibitory at concentrations greater than 2 mM. Phosphate was a competitive inhibitor with respect to carbamoylphosphate. The pH optimum for citrulline synthesis was 9.5.

The blue-green alga *Nostoc muscorum* Kützing, when exposed to ^{14}C-bicarbonate in either dark or light conditions, shows a marked tendency to accumulate radioactivity in the amino acid citrulline (10, 15, 17). Carbamoylphosphate synthetase and ornithine transcarbamoylase (EC 2.1.3.3) activities have been measured in crude extracts of *Nostoc*, and it has been suggested that this novel pathway of carbon fixation probably occurs via the activity of these two enzymes (10).

We attempted to study the citrulline-synthesizing system in more detail, but in our hands the citrulline-labeling capacity disappeared when the alga was kept in culture (authors' unpublished observations). In seeking an explanation for the disappearance of this synthetic capacity, we have considered the possibility that carbamoylphosphate utilization via OTC[3] could be subject to allosteric control, as in the case of CAP metabolism via aspartate transcarbamoylase (9, 18). If so, the critical change that occurred during culture could have been the result of a buildup of a metabolic effector to concentrations sufficient to inhibit the ornithine transcarbamoylase-catalyzed synthesis of citrulline. In this connection, Burnett and Cohen (5) have pointed out that arginine has an inhibitory effect on beef-liver OTC and Tam and Patil (24) have discovered that the enzyme from *Phaseolus vulgaris* can be strongly inhibited by an as yet unidentified toxin, apparently by an allosteric mechanism. As the investigations on OTC so far have not been directed to this aspect of its nature, we report here some general properties of the enzyme from *Nostoc muscorum* and results pertinent to its possible metabolic control.

MATERIALS AND METHODS

Experimental Organisms. *N. muscorum* Kützing, Indiana culture collection number 486, was grown in unialgal culture in nutrient medium D of Kratz and Myers (14). The cultures were bubbled with 5% CO_2 in air, resulting in a pH of about 6.6; lighting was by banks of fluorescent tubes providing 200 to 250 ft-c of continuous light at the surface of the culture vessels, which were mechanically agitated. Axenic cultures were not routinely used, inasmuch as algal growth and enzyme activity were comparable in sterile and unsterile cultures. Cells used for enzyme preparations were largely freed of bacteria by repeated washing in sterile culture medium, so that *Nostoc* cells (determined by haemocytometer count) outnumbered bacterial cells (determined by plating on nutrient agar after serial dilution) by 100-fold. Cells were freed of growth medium by centrifugation and stored frozen at −20 C until required.

Chemicals. L-Ornithine-HCl was obtained from Aldrich Chemical Co., Milwaukee, Wisc., streptomycin sulfate from CalBiochem, agarose and alumina gel Cγ from Bio-Rad Laboratories, and diacetyl monoxime from Eastman. Other chemicals were obtained from Sigma unless otherwise noted.

Extraction of Enzyme. The green-black paste of pelleted cells (20- to 30-ml lots) was subjected to intermittent sonic disintegration (Bronwill Biosonik apparatus, 20 Kc) over a period of 10 to 15 min at 2 C, until microscopic examination showed an absence of intact cells. The sonicator was operated in 30-sec bursts, with intervals of 1 to 2 min to allow cooling. Total sonication time was 2 to 5 min. The undiluted crude sonicate was dialyzed overnight at 2 C against 3 liters of 1 mM-L-ornithine, pH 7.

Streptomycin Precipitation. One-fifth volume of cold 5% streptomycin sulfate was added to the dialyzed extract. The mix-

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[2] Present address: Department of Agronomy, University of Illinois, Urbana, Illinois 61801.

[3] Abbreviations: OTC: ornithine transcarbamoylase; CAP: carbamoylphosphate.

ture was stirred and allowed to stand 5 min before centrifuging at 10,000*g* for 10 min at 5 C. These conditions were used in all centrifugations unless specified otherwise. The heavy pellet was discarded.

Heat Treatment. L-Ornithine-HCl was added to the supernatant from the streptomycin treatment to give a concentration of 2 mM, and the solution was heated to 61 C in a water bath. The solution was maintained at 61 to 62 C for 5 min, then cooled in ice, and centrifuged. The precipitate was washed in a small amount of deionized water, recentrifuged, and the supernatant was combined with the first. The pellet was discarded.

Ammonium Sulfate Fractionation I. To the cold supernatant solution from the heat treatment, solid ammonium sulfate was added gradually, with mechanical stirring, to 40% of saturation (8), and the precipitate was centrifuged off and discarded. Ammonium sulfate was added to the supernatant to 55% saturation, the solution was stirred for 10 min and centrifuged. The precipitate contained most of the ornithine transcarbamoylase activity, and was disssolved in twice the volume of the original cells of 1 mM L-ornithine, pH 7.

Alumina Gel C$_\gamma$ Fractionation. The dissolved ammonium sulfate pellet was stirred in the cold for 5 min with an equal volume of alumina gel C$_\gamma$ slurry as supplied by Bio-Rad Laboratories, and then centrifuged. The supernatant was discarded, and the enzyme was washed from the gel with four washes (each equal to the original slurry volume) of 10 mM Na-phosphate buffer, pH 7.5. Activity was usually contained in the second and third wash solutions. Proteins were precipitated by the addition of solid ammonium sulfate to 60% saturation, and were collected by centrifugation after the solutions stood overnight in the cold room (2 C). The precipitate was dissolved in 10 mM Na-phosphate buffer, pH 7.5.

DEAE-Cellulose Chromatography. Short columns (10 cm of cellulose in 2.7-cm diameter glass columns) of Whatman DE 52 microcrystalline DEAE-cellulose were equilibrated with the starting buffer, 10 mM Na-phosphate, pH 7.5, containing 1 mM L-ornithine. The sample was equilibrated with starting buffer by passing it through a column of Sephadex G-25 that had been previously equilibrated with this buffer. The column was eluted with a stepwise gradient of NaCl in the starting buffer. Thirty-milliliter steps of 0.1, 0.2, and 0.4 M NaCl were used; elution was carried out at room temperature after it was found that elution at a lower temperature did not increase enzyme yield. Five-milliliter fractions were collected and assayed for OTC activity.

Ammonium Sulfate Fractionation II. The OTC-containing fractions from the DEAE-cellulose column were pooled, and solid ammonium sulfate was added at room temperature to 45% saturation. The precipitate was removed by centrifugation and discarded. Additional ammonium sulfate was added to make the supernatant 65% saturated. After 30 min, the precipitate was centrifuged down and dissolved in a small amount of 0.1 M Na-glycylglycine buffer, pH 9.5, containing 1 mM L-ornithine.

Gel Filtration. The sample was loaded at room temperature on a 50 × 2.5 cm column packed with agarose A-0.5m, and eluted with 50 mM Na-phosphate buffer, pH 7.5. The absorbance at 280 nm of the effluent was recorded continuously with a Gilford recording spectrophotometer, and fractions of 7.5 ml were collected automatically. Peak OTC activity was in fraction 17. Fractions containing enzyme activity were pooled, and ammonium sulfate added to 60% saturation. After 4 hr, the protein was collected by centrifugation and dissolved in Na-glycylglycine buffer, pH 9.5.

Estimation of Molecular Weight by Gel Filtration. The agarose column was calibrated by noting the elution volumes of BSA, urease, lysozyme, and trypsin. The ratio of the elution volume to void volume (determined with blue dextran) for each was plotted against log of the mol wt. This gave a roughly linear plot (Fig. 7) from which the observed elution volume of the enzyme could be correlated with an approximate mol wt (1).

Enzyme Assay. The OTC assay was based on the colorimetric determination, modified from Archibald (2), of citrulline formed from ornithine and carbamoylphosphate. An enzyme unit was taken as that amount that would catalyze the formation of 0.1 μmole of citrulline in 1 min at 37 C under standard assay conditions. Each tube contained 1 μmole of L-ornithine, 50 μmoles of glycylglycine buffer, pH 9.5, 5 μmoles of dilithium carbamoylphosphate, and appropriate amounts of enzyme (usually 0.1–0.2 unit) in a total volume of 500 μl. The reaction was started by the addition of CAP and terminated after 10 min by the addition of 2 ml of an acid mixture consisting of 10% perchloric acid-85% phosphoric acid-water-concentrated sulfuric acid (20:3:2:1 by volume). This was followed with 0.5 ml of 0.75% (w/v) diacetylmonoxime (2,3-butanedionemonoxime). The tubes were mixed well, capped with a marble, and placed in a boiling water bath for 30 min. The tubes were cooled and the absorbance at 490 nm was read. Absorbance readings were converted to amounts of citrulline formed by use of a standard curve constructed by assaying known amounts of citrulline in the presence of the enzyme assay reagents. Enzyme catalyzed citrulline formation proceeded linearly for at least 20 min (Fig. 5) and was linearly dependent on the amount of enzyme added, at least over the range of protein concentrations used in the studies reported here.

Determination of Protein. Protein was determined either by the method of Lowry *et al.* (16) or by measurement of the absorbance at 280 nm; BSA was used as a standard in both cases.

RESULTS

Enzyme Purification. Table I summarizes a representative preparation of *Nostoc* ornithine transcarbamoylase. The streptomycin and heat treatments resulted in slight increases in activity, but we cannot distinguish between the possibilities that enzymes competing for substrates were removed, that OTC inhibitors were removed or, that inhibitor(s) of the citrulline assay itself were removed. L-Ornithine was essential to protect the enzyme against heat. The enzyme was stable through the ammonium sulfate I stage, but all subsequent steps resulted in significant losses in activity not counteracted by low temperature,

Table I. *Purification of Ornithine Transcarbamoylase from Nostoc muscorum*

The starting material was 20 g of wet-packed cells.

Material	OTC	Protein	Specific Activity	Yield
	units	*mg*	*units/mg protein*	%
Sonicated cells	810	3940	0.2	100
Streptomycin supernatant	880	3200	0.27	109
Heat treatment supernatant (61 C, 5 min)	910	1660	0.55	112
Ammonium sulfate I, 40–55% pellet	710	400	1.8	88
Alumina gel C-γ (60% ammonium sulfate precipitate of eluate)	414	73	5.7	51
DEAE-cellulose active fractions	200[a]	8	25	24
Ammonium sulfate II, 45–60% pellet	165	4	45	20
Agarose gel filtration (65% ammonium sulfate pellet of active fractions)	80	0.69	116	9

[a] Corrected for inhibition due to phosphate buffer.

or by the presence of substrates or reducing agents. Dialysis at the ammonium sulfate I stage caused a loss in activity (about 50% on overnight dialysis) that could not be restored by addition of concentrated diffusate. The purest preparations were unstable in all conditions tested. Therefore enzyme at the ammonium sulfate II stage of purification (about 200- to 250-fold purified), which lost little activity when stored frozen for 2 months, was used for the determination of properties.

Effect of Substrate Concentration on Reaction Velocity. Figure 1 (inset) shows the hyperbolic response of reaction velocity to increasing carbamoylphosphate concentrations and a double reciprocal plot of the data, from which a *Km* of 0.67 mM may be determined. Although this determination was done at a nonsaturating concentration of ornithine (2 mM, which was chosen to avoid ornithine inhibition shown in Fig. 2), it has been determined that for ox liver OTC the concentration of neither ornithine nor CAP changes the *Km* value with respect to the other (12). Thus our *Km* value is probably valid. When ornithine concentration was varied (Fig. 2), the response of reaction velocity is hyperbolic only at low ornithine concentrations, with higher concentrations being inhibitory. Figure 3 shows that the double reciprocal plot diverges upward. If the linear portion of the double reciprocal plot is extended, a *Km* of 2.5 mM may be determined. Figure 2 also shows that the inhibitory effect of high ornithine concentrations decreased with pH, and was barely noticeable at pH 8.

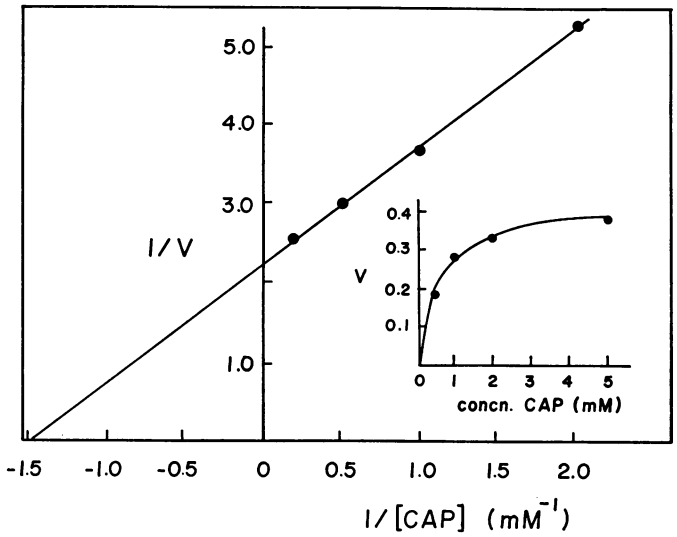

FIG. 1. Reaction velocity as influenced by concentration of carbamoylphosphate. The inset figure shows the hyperbolic response of reaction velocity to substrate concentration. An apparent *Km* of 0.67 mM (L-ornithine concentration at 2 mM) can be determined from the double reciprocal plot. The standard assay conditions were used with the exception that the concentration of carbamoylphosphate was varied.

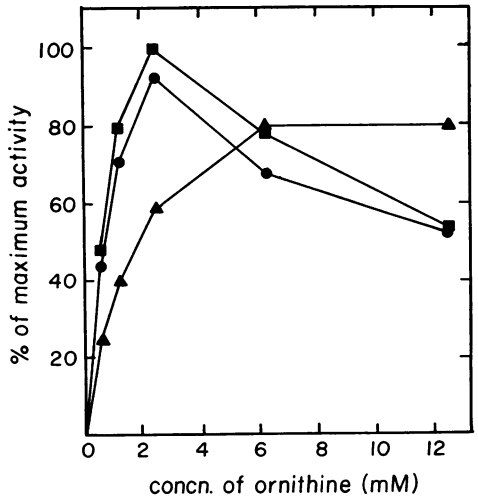

FIG. 2. Reaction velocity as influenced by the concentration of ornithine at different pH values. Standard assay conditions were used except that the concentration of L-ornithine was varied, and the pH of the reaction mixture was adjusted with NaOH or HCl to pH 8 (▲), pH 9 (●) or pH 9.5 (■).

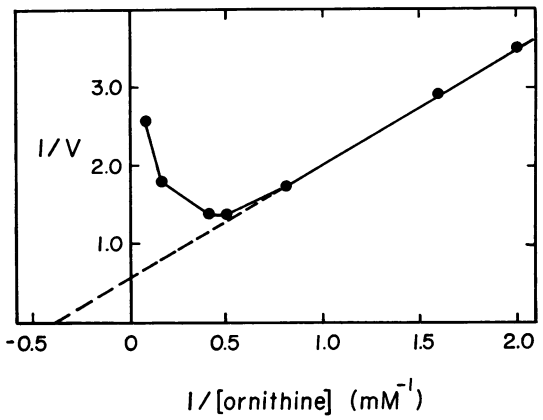

FIG. 3. Double reciprocal plot of reaction velocity as influenced by ornithine concentration. The plot is linear only when the concentration of L-ornithine is below 2 mM. Assay conditions were standard except for the variation in L-ornithine.

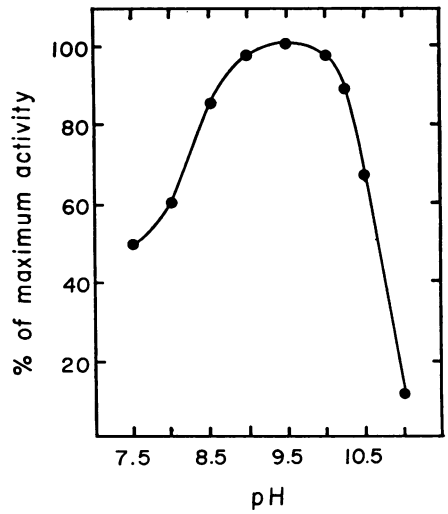

FIG. 4. Dependence of enzyme activity on pH. The standard reaction mixture was buffered with 50 μmoles diethanolamine-HCl.

pH Optimum. Figure 4 shows the result of assays done with the standard reaction mixture buffered over a range of pH values with diethanolamine-HCl. The enzyme showed almost full activity from pH 9 to 10, with the optimum value at pH 9.5.

Activation Energy. Figure 5 shows the time course of citrulline formation at 37, 33, and 29 C. When the slopes were used as rate constants, an activation energy of 12.3 kcal/mole was calculated from an Arrhenius plot (inset).

Inhibitors. The enzyme was slightly inhibited by divalent zinc and copper. Cyanide, fluoride, maleic hydrazide, lead, calcium, and ferric ions had no effect (Table II). Phosphate buffers were observed to be inhibitory and Figure 6 shows that phosphate is a competitive inhibitor of carbamoylphosphate. The inhibitor con-

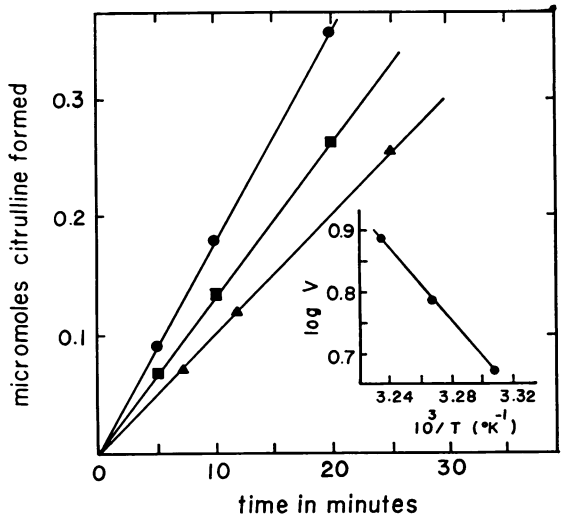

FIG. 5. Progress of citrulline synthesis at different temperatures. Standard assay conditions were observed except for different reaction times and temperatures. Lines represent experiments done at 29 C (▲), 33 C (■), and 37 C (●). Inset is an Arrhenius plot of the data. The activation energy calculated from the slope of the line is 12.3 kcal/mol.

Table II. *Response of Nostoc OTC to Some Common Ions and Inhibitors*

Standard assay conditions were used except for inclusion of test substances at concentrations indicated. Enzyme was purified to the ammonium sulfate II stage (22-fold purification). Data are the average of three determinations.

Substance	Concn	Percentage of Control Activity
	mM	
p-Hydroxymercuribenzoate	0.1	15
Maleic hydrazide	20	102
Cyanide	0.1	99
Fluoride	0.1	99
Zn^{2+}	1	80
Pb^{2+} [a]	1	101
Ca^{2+}	1	104
Fe^{3+}	1	100
Cu^{2+}	1	79

[a] Precipitate formed at assay pH.

stant (K*i*) for phosphate was calculated (8) as 0.55 mM, close to the K*m* for carbamoylphosphate.

Estimated Molecular Weight. Figure 7 shows the ratio of elution volume to void volume of the agarose A-0.5m column plotted against log mol wt for lysozyme, trypsin, BSA, and urease. The elution volume-void volume ratio for ornithine transcarbamoylase allows an estimate of about 75,000 for its mol wt.

Effect of Possible Regulators. The following compounds were tested for effect on enzyme activity: adenosine, AMP, ADP, ATP, guanosine, GMP, GDP, GTP, cytidine, CMP, CDP, CTP, uridine, UMP, UDP, UTP, ITP, TMP, orotic acid, aspartic acid, arginine, arginino-succinic acid, uriedosuccinic acid, proline, glutamic acid, urea, glutathione, and cysteic acid. Compounds were included in the standard assay mixture at 0.2 to 20 mM. The tests were first done with enzyme purified by the method described and stored frozen, but when none of the metabolites showed appreciable influence on enzyme activity, it seemed desirable to eliminate the possibility that heating and freezing might have destroyed the enzyme's regulatory properties. Therefore, another preparation was made omitting the heat treatment, and used without refrigeration. Activity of this preparation was also only slightly influenced by the presence of the metabolites tested.

DISCUSSION

In comparing various preparations of OTC in terms of activation energy of citrulline synthesis, K*m* for CAP, and estimated mol wt, the *Nostoc* enzyme is typical of a group of preparations from mammals, *Neurospora*, and *E. coli*, but the data in Table III show that it differs from the *Streptococcus* and *Mycoplasma* enzyme. Our value for energy of activation (12.3 kcal/mole) is in good agreement with 13.1 kcal/mole, the value found for beef and frog liver; *S. lactis* OTC, on the other hand, yielded a figure of 20.5 kcal/mole. Likewise, while *S. lactis*, *S. faecalis*, and *Myco-*

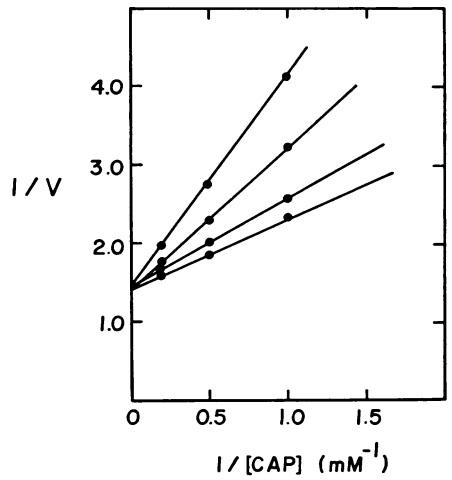

FIG. 6. Phosphate as an inhibitor competitive with carbamoylphosphate. Assay conditions were standard except for variable amounts of carbamoylphosphate and Na_2HPO_4, adjusted to pH 9.5 with NaOH. The four lines represent experiments done at phosphate concentrations of 0, 0.2, 0.5, and 1 mM, from bottom to top of the figure.

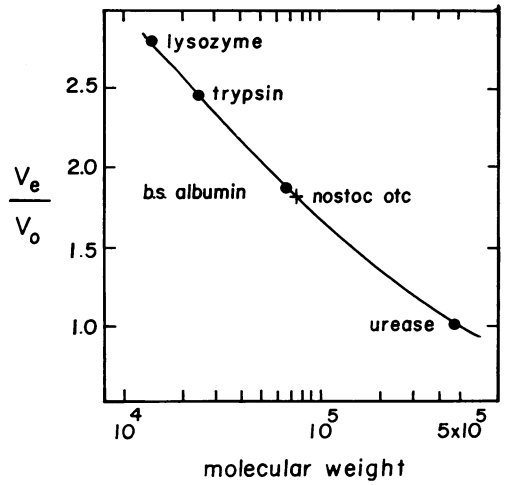

FIG. 7. Estimation of molecular weight by gel filtration. Standard proteins and a *Nostoc* preparation were eluted from a 2.5 × 35 cm column of agarose A-0.5m with 0.05 M Na phosphate, pH 8. Fractions of 7 ml were collected and the elution volume (V_e) for each protein was determined by observing the absorbance at 280 nm, or by pertinent enzyme assay. The void volume (V_0) of the column was taken as the elution volume of blue dextran.

Table III. *Some Collected Data on OTC from Various Sources*

	Km for Ornithine	Km for CAP	Activation Energy	Reference
	mM	mM	Kcal/mole	
Mycoplasma hominis 07	3.6	2.3		22
Streptococcus lactis	2	3.7	20.5	19
Streptococcus faecalis	2	3.7		11
E. coli	1.5	0.2		21
Rat liver	1.4	0.4		20
Beef liver	3	1.2	13.1	5, 12
Frog liver			13.1	4
Neurospora crassa	1.9	0.3		7
Nostic muscorum	2.5	0.7	12.3	This paper

plasma OTCs have a K*m* for CAP greater than 2 mM, the values for other OTCs are about 1 mM or less. K*m* values for ornithine vary from 1.4 to 3.6 mM. Molecular weight comparisons are more tenuous, because rigorous determinations are lacking; however, several sedimentation coefficients are available which allow a rough comparison of mol wt provided the various enzymes are similar in shape. For the green pea (13) and rat liver enzymes (20) $S_{20,w}$, values of 5.5S have been reported; on the other hand, S_{25} of 16.6S and S_{20} of 8.95S have been reported for the enzyme from *Mycoplasma hominis 07* (22) and *Streptococcus* D_{10} (3). The compilation of sedimentation coefficients and mol wt given by Tanford (25) shows that for globular proteins there is a reasonable correlation between sedimentation coefficients and mol wt, and that a protein of the approximate size of *Nostoc* OTC (about 75,000 daltons) might reasonably have a sedimentation coefficient of 4S to 5S. Thus it seems that the *Nostoc* enzyme is one of the smaller specimens studied, approximating in size the pea and rat liver enzymes rather than the bacterial form.

These differences in physical and catalytic properties may be related to metabolic specialization, as in the *Streptococcus* and *Mycoplasma* work referred to above. The organisms were grown on arginine, thus inducing the arginine dihydrolase pathway (22), a group of three enzymes (arginine deiminase, OTC, and carbamate kinase) that facilitates catabolism of arginine with a gain in ATP. Thus the OTC from these organisms is catabolic in function. In other organisms, notably in the livers of ureotelic animals, OTC-catalyzed conversion of ornithine to citrulline is basic to arginine synthesis, but arginine breakdown is initiated by arginase, which catalyzes formation of ornithine and urea, thus bypassing the OTC reaction; OTC in these organisms serves in arginine synthesis but not directly in its breakdown. That catabolic and anabolic forms of OTC can have different properties has been shown by Stalon *et al.* (23) who separated an arginine-inducible (catabolic) from an arginine-repressible (anabolic) OTC by ammonium sulfate fractionation of extracts of *Pseudomonas*.

The similarity of properties exhibited by OTC from *Nostoc* and liver suggests that *Nostoc* OTC is biosynthetic in function. This is consistent with the optimum pH of 9.5 (citrulline synthesis releases a proton, hence alkaline conditions would favor the reaction) and our unpublished observation that *Nostoc* OTC preparations would convert citrulline to ornithine very slowly if at all.

Our results do not support the proposition that *Nostoc* enzyme might be regulated by metabolites. Cohen (6) has summarized the data on several enzymes which are known to be so regulated, and emphasized three common characteristics of these enzymes: (*a*) they are polymeric proteins of high mol wt; (*b*) the subunits interact so that the binding of one substrate molecule facilitates the binding of another, with the result that enzyme activity is a sigmoid function of substrate concentration rather than a hyperbolic function as is the case with enzymes following classical Michaelis-Menten kinetics; and (*c*) treatment with heat, cold, or other denaturing agents can often cause a dissociation of subunits resulting in a change in mol wt and a loss of both sensitivity to regulators and sigmoid kinetics. That the *Nostoc* enzyme does not fit this pattern is illustrated by its lack of response to a variety of conceivable regulators; this lack of response was not attributable to heating or freezing the preparations. The enzyme is small compared to most allosteric enzymes, and the substrate kinetics, with the exception of ornithine inhibition, are hyperbolic. Although these criteria are probably not definitive taken individually, together they cast strong doubt on the possibility that ornithine transcarbamolyase is a regulatory enzyme in *Nostoc*.

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