INDUCTION OF CARBAMYL-P SPECIFIC OXAMATE TRANSCARBAMYLASE BY PARABANIC ACID IN A STREPTOCOCCUS

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Oxamate transcarbamylase found in Streptococcus allantoicus and in Arthrobacter allantoicus (Valentine and Wolfe, 1960; Vogels, 1961), catalyzes reaction 1:

1) Oxamate + Carbamyl-P $\xrightleftharpoons{Mg^{++}}$ Carbamyl Oxamate + Pi

This reaction is of much interest because in contrast to all other known transcarbamylases, oxamate transcarbamylase requires magnesium for activity and catalyzes an easily reversible reaction. This paper outlines the conditions for induction of oxamate transcarbamylase in Strain D10 group D Streptococcus, the partial purification of the induced enzyme and some of its properties. Induction of the enzyme with this microorganism to higher levels than hitherto recorded in other microorganisms does not require anaerobic conditions or allantoin (Bojanowsky et al., 1964; Vogels, 1961), but parabanic acid. As recently shown in this laboratory with several enzymes, all transcarbamylases thus far tested can use acetyl-P in place of carbamyl-P (Grisolia and Raijman, 1964). It was therefore of interest to check if oxamate transcarbamylase could use acetyl-P; as noted in this paper oxamate transcarbamylase is inactive under the conditions tested with acetyl-P.

ATP, carbamyl-P, acetyl-P were from Sigma Chemical Company. Carbamyl oxamate, oxamic and parabanic acids were obtained from Nutritional Biochemicals Corporation. Tripticase and Bacto Yeast Extract were Baltimore Biological Laboratory and Difco products, respectively. Carbamyl oxamate was determined by the method of Archibald (1944), Pi by the method of Gomori (1941), oxamate by the Vogels technique (1961). Protein was determined by the procedure of Mockrasch and McGilvery (1956).

Carbamylation of oxamate was assayed routinely with the following components in a volume of 2 ml:200 μmoles of Tris-HCl buffer (pH 8.0); 20 μmoles of carbamyl-P; 20 μmoles of Na oxamate; 10 μmoles of $MgSO_4$ and the enzyme. 10 min incubation at 38°. The reaction was terminated by the addition of 2 ml of 1 M $HClO_4$. One enzyme unit is defined as that required

for the formation of 1 μmole carbamyl oxamate under these conditions.

As illustrated in Table I there is marked appearance of activity with addition of parabanic acid. Addition of Mg^{++} increases slightly the yield and the specific activity; also, glucose addition is necessary. While not illustrated both yeast extract and tripticase are essential. The final pH was 6.5. Increasing the pH to 8 did not increase yield or specific activity. It is of interest that upon the addition of parabanic and magnesium the specific activity of ornithine transcarbamylase in the crude extract decreases from about 600 to 170 and that of carbamate kinase decreases from 600 to 4.

Table I. THE INFLUENCE OF SEVERAL COMPONENTS ON INDUCTION OF OXAMATE TRANSCARBAMYLASE ACTIVITY IN STRAIN D10 GROUP D STREPTOCOCCUS

In all cases 6 g of $Na_2HPO_4 \cdot 7H_2O$, 3 g of yeast extract and 1.2 g of tripticase were combined with the indicated components (in g and made up to 300 ml). 30 ml were inoculated. After 2 hours (or 12 hours for stationary conditions) at 37°, 270 ml of fresh medium were added and the mixtures were incubated 2 more hours (12 more hours for stationary conditions). Good oxygenation was insured by shaking the cultures in partially filled flasks (120 strokes per min). The cells were harvested by centrifugation, washed, taken up in 10 ml of water and sonicated as described in the text.

Additions	Stationary Conditions					Aerobic Conditions (Shaking)					
Arginine	3	--	--	--	--	--	--	--	--	--	--
Allantoin	--	2	--	--	--	--	2	--	--	--	--
Parabanic	--	--	2	1	1	--	--	0.5	1	2	1
Glucose	1.8	2	1	--	1	1	1	1	1	1	1
$MgSO_4 \cdot 7H_2O$	--	0.8	0.8	0.8	0.8	--	--	--	--	--	0.8
Protein	60	23	21	21	69	180	210	188	180	40	172
Units	--	1	32	--	227	--	--	300	360	45	380
Specific Activity	--	--	1.5	--	3.3	--	--	1.6	2	1.1	2.2

Enzyme Purification: All operations were at 0° and all centrifugations at 14,000 x g for 30 min, unless otherwise indicated. The ammonium sulfate solutions were at pH 5.5 and saturated at 25°. The following solutions were made and to contain per liter: A) 20 g parabanic acid; B) 20 g glucose (tap water); C) 60 g $Na_2HPO_4 \cdot 7H_2O$; D) 7.5 g $MgSO_4 \cdot 7H_2O$, 12 g tripticase and 30 g yeast extract (tap water). All were autoclaved

separately; 1 liter each of A, B and 2 liters each of C and D were mixed before inoculation. 60 ml were inoculated. After 12 hours at 37°, 540 ml of fresh medium were added and after 12 more hours made up to 6 liters with fresh medium and incubated for 12 more hours. The cells were harvested by centrifugation (4,000 x g), washed with distilled water, made up to 180 ml and sonicated (10 ml portions) 1.5 ml for four periods (cooling for 1 min in between) in a MSE ultrasonic disintegrator. 90 ml of ammonium sulfate were added, the preparation was centrifuged, and the precipitate discarded. 250 ml of supernatant fluid (crude fraction) were mixed with 750 ml of ammonium sulfate, the supernatant discarded and the precipitate taken up in 70 ml of water. It was then heated at 60° for 5 min, cooled and centrifuged. The supernatant fluid (Fraction I) was diluted to 240 ml with water and mixed with 40 ml of a bentonite suspension, (30 mg/ml). After 15 min contact, with occasional stirring the bentonite (containing much absorbed inactive protein) was centrifuged off. The supernatant fluid was mixed with 160 g of ammonium sulfate and then centrifuged. The precipitate was dissolved in water (Fraction II, see Table II). Further purification by acetone fractionation, precipitation with heavy metals, phosphate gel or gradient centrifugation was unsuccessful. However, the specific activity of the purified enzyme is about twice greater than previously recorded by others.

Table II. PURIFICATION OF OXAMATE TRANSCARBAMYLASE

Fraction	Volume (ml)	Units	Protein (mg)	Specific Activity	Yield %
Crude	250	5,695	1,722	3.3	--
I	70	5,250	720	7.2	92
II	15	3,200	159	20.1	56

Properties: There was linearity with time for 20 min when carbamyl oxamate syntheses was kept below 1 μmole. Table III illustrates the effect of protein concentration and of temperature on rate.

The formation of oxamate from carbamyl oxamate was measured by incubating the enzyme for 10 min at 38° with 200 μmoles of Tris-HCl buffer (pH 8.0), 10 μmoles of sodium phosphate buffer pH 8.0, 10 μmoles of $MgSO_4$ and 10 μmoles ADP in 2 ml. The reaction was terminated by the addition of $HClO_4$ or of hydroxylamine. The disappearance of Pi, of carbamyl oxamate, or the appearance of oxamate were measured. 335, 670 and 1340 μg of the enzyme formed, respectively, 0.65, 1.50 and 3.10 μmoles of oxamate. Similar

Table III. EFFECT OF PROTEIN CONCENTRATION AND TEMPERATURE ON RATE

Fraction II was assayed under the standard conditions except as indicated.

μg Enzyme Added	Temperature				
	21°	30°	37°	37°*	50°
	μmoles carbamyl oxamate found				
30	0.15	0.32	0.40	(0.37)	0.35
45	--	--	--	(0.56)	--
60	0.37	0.63	0.72	(0.70)	0.70
75	--	--	--	(0.90)	--
90	0.56	0.82	1.08	(1.08)	0.94
105	--	--	--	(1.17)	--
120	0.69	1.08	1.32	--	1.20

*The figures between parentheses are from another experiment.

results were obtained by measuring carbamyl oxamate and Pi disappearance. The apparent initial velocity of reaction 1 from right to left is roughly half that from left to right.

The enzyme preparations can be kept frozen, after precipitation with ammonium sulfate, for at least two weeks with little decrease of activity. A concentrated enzyme solution (9 mg/ml) was stable for at least 24 hours at 0°; a dilute solution (0.22 mg/ml) retained at 6 and 24 hours, 45 and 27%, respectively, of its original activity. Addition of 2.5×10^{-4} M oxamate, carbamyl-P or $MgSO_4$ did not increase the stability of diluted solutions. Dilution with 1% albumin increased the activity slightly, but did not protect against inactivation. Dialysis inactivated the enzyme except when dialyzed against a dilute ammonium sulfate solution. Fraction II (containing 8.6 mg protein per ml) when held for 1 hour at pH values 3.0, 4.0 and 5.2 retained 0, 65, and 85% activity respectively; from pH 5.8 to 9.0 it was stable. Samples of Fraction II were held for 5 min at 60°, 65° and 70°. The enzyme retained 100%, 62% and none, respectively, of the initial activity; in the presence of 10% Na_2SO_4 it was stable up to 70°.

Effect of pH on rate and Mg^{++} requirement. These are illustrated in Tables IV and V respectively.

Table IV. EFFECT OF pH ON RATE

35 μg Fraction II were used under the standard conditions, except that the pH was changed as indicated.

pH	7.1	7.4	7.6	7.8	8.0	8.3	8.4	8.6	9.0
μmoles carbamyl oxamate found	0.23	0.30	0.32	0.39	0.46	0.47	0.49	0.45	0.39

Table V. Mg^{++} REQUIREMENT

2 ml containing 12 mg protein (Fraction II) were absorbed in a G 25 Medium Sephadex column (2 and 2.5 ml external and internal vol respectively) and eluted with 0.5 M tris buffer pH 8.0. Once the protein started to appear 1.5 ml were collected, and portions containing 300 μg were assayed under the standard conditions except that Mg^{++} were changed as indicated.

Mg^{++} added (μmoles)	--	1	2	3	5	8	10
μmoles carbamyl oxamate found	0.12	0.40	0.58	0.78	1.02	1.38	1.50

The effect of other cations on activity was tested. There was 90% activity when Mn^{++} replaced Mg^{++} and 50% with Co^{++}; with 2.5×10^{-3} M Mg^{++} in the incubation mixture, equimolar Hg^{++} and Zn^{++} inhibited completely while Cu^{++} and Pb^{++} inhibited 90 and 60% respectively.

When carbamyl-P was replaced by acetyl-P under the assay conditions outlined above, no evidence for acetyl-P utilization was found even when adding 250 units of enzyme. Thus it appears that either oxamate transcarbamylase has no action on acetyl-P, or if it does the activity is below 1/500 that shown with carbamyl-P, since we should have been able to detect 0.5 μmole change. When carbamyl alanine, carbamyl-β-alanine, carbamyl aspartate, carbamyl glutamate or carbamyl-β-amino iso butyrate replaced carbamyl oxamate in a standard assay, no activity was found.

Oxamate Transcarbamylase from Other Sources: The standard assay for carbamyl oxamate formation was used except that the samples were incubated for 20 min. The following water extracts containing the indicated mg protein were tested: yeast (7.5), corn germ (1.5), wheat germ (2.5), rice germ (1.7), chick peas (9.5), split peas (2.3), rat liver (7), rat brain (7), rat muscle (5.5). No evidence for oxamate transcarbamylase activity was found. If there had been 0.5 μmoles of synthesis, we should have been able to detect it.

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