SEPARATION OF THE CHLORELLA ATP:UREA AMIDO-LYASE INTO TWO COMPONENTS

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SUMMARY

A Chlorella extract capable of cleaving urea to CO_2 and NH_3 in the presence of ATP has been separated into two enzymes neither of which demonstrates this activity alone. The first enzyme forms allophanate in the presence of urea, CO_2, biotin, ATP and Mg^{++}. The second enzyme decomposes allophanate to CO_2 and NH_3 without added co-factors and in the presence of avidin.

Chlorella species readily utilize urea as a source of nitrogen (1) but apparently lack a hydrolytic urease (2,3). In further studies on the utilization of urea by Chlorella, Hodson and Thompson (4) obtained evidence that ATP is required. This evidence is consistent with the finding of Roon and Levenberg (5) that Candida utilis and Chlorella ellipsoidea contain an ATP:urea amido-lyase. With yeast, the latter investigators (6) obtained evidence for two reactions carried out by one enzyme with allophanate as an intermediate. They also showed that CO_2 is required for allophanate formation, as did Whitney and Cooper (7).

This paper shows that a crude extract of Chlorella vulgaris with ATP:urea amido-lyase activity can be separated into two enzymatic fractions; one which synthesizes allophanate and one which hydrolyzes allophanate.

MATERIALS AND METHODS

Chlorella vulgaris Beijerinck var. viridis (Chodat) was grown photoorganotrophically at 20°C for 6 days in 4 liter Erlenmeyer flasks containing 1 liter of media and shaken at one oscillation per second. The composition of the medium was 0.008 M K_2HPO_4, 0.26 M KH_2PO_4, 0.15 M urea, 0.050 M $MgSO_4$, 10^{-4} M NaFeEDTA, and 0.055 M glucose. Trace

elements were added as per Reisner et al. (8). The inoculum for each flask contained 250 µl of packed cells and the yield was 23-25 ml of packed cells per liter.

Cells were harvested by centrifugation at 2500 g for 15 min. and resuspended so that each 15 ml of suspension contained 6 ml of packed cells, 1 millimole of Na Bicine (pH = 8.25) and 80 µmoles of DTE*. Cells were broken by sonication while the temperature was maintained at 10-15°C. The cell debris was removed by centrifugation at 10^5 g for 1 hr. The supernatant was adjusted to pH 8.0 ± 0.1 and fractionated with $(NH_4)_2SO_4$ to yield fractions that precipitated between 0-25%, 25-35%, 35-45% and 45-70% saturation.

Enzyme 1 was purified of enzyme 2 by using the 45-70% saturation $(NH_4)_2SO_4$ fraction. This fraction was diluted so that 50 ml contained 50 mg of protein, 5 mM potassium phosphate buffer (pH = 7.0) and 1 mM DTE. This solution was placed on a 2.8 x 17 cm column of brushite (9) and the brushite was washed with 450 ml of the same buffer-DTE solution. Enzyme 1 was eluted with 0.1 M potassium phosphate buffer containing 1 mM DTE.

Enzyme 2 was separated from enzyme 1 by chromatography on brushite. A portion of the 25-35% saturation $(NH_4)_2SO_4$ fraction was diluted until 50 ml contained 50 mg of protein, 5 mM potassium phosphate buffer (pH 7.0) and 1 mM DTE. This solution was placed on a 2.8 x 17 cm column of brushite and washed with the same buffer-DTE solution. After 300 ml of effluent solution had been collected, the proteins were precipitated with $(NH_4)_2SO_4$ (70% saturation), spun at 10^5 g for 15 min. and dissolved in 0.01 M Na Bicine (pH = 8.25) containing 4 mM DTE.

Enzyme 1 was determined by measuring the allophanate produced from ^{14}C-urea. The incubation mixture (see Table 1) was applied to a 0.9 x 3.5 cm column of tertiary amine resin in the bicarbonate form at 2-4°C. After washing the urea through the resin with 50 ml of water, the allophanate was eluted with 30 ml of 0.05 M NH_4HCO_3. A 5-ml aliquot was counted by liquid scintillation spectroscopy. Allophanate was freed of NH_4HCO_3 by evaporation to dryness at 2°C in vacuo in the presence of 100 µmoles of Na Bicine buffer.

Labeled and unlabeled allophanate was prepared from urea by the method of Dains and Wertheim (10). Allophanate salts were separated from urea and salts as above.

Protein was determined by the method of Lowry et al. (11) using bovine serum albumin as standard. Inorganic phosphate was measured by the method of Ames and Dubin (12) after extraction as the phosphomolybdate complex into isobutanol (13). AMP, ADP and ATP were separated on DEAE-cellulose (14) after deproteinization with phenol and quantitatively measured by absorption at 260 nm. Ammonia was determined by reaction with ninhydrin (15) after distillation in Conway vessels (16).

RESULTS AND DISCUSSION

The results presented in Table 1 show that a cell-free extract of Chlorella vulgaris was separated into two fractions which individually produced negligible $^{14}CO_2$ from ^{14}C-urea as compared with the $^{14}CO_2$ produced

*Abbreviations used: DTE = dithioerythritol; Bicine = N,N-bis(2-hydroxyethyl)glycine.

TABLE 1. THE EFFECT OF TWO PROTEIN FRACTIONS ON THE PRODUCTION OF CO_2 FROM UREA.

PROTEIN COMPONENT OF INCUBATION MIXTURE	$^{14}CO_2$ FORMED
	nmoles
Enzyme 1	0.35
Enzyme 2	0.11
Enzyme 1 + Enzyme 2	69.3
Enzyme 1 (heated) + Enzyme 2	0.12
Enzyme 1 + Enzyme 2 (heated)	0.30

The incubation mixture contained in 0.9 ml, Chlorella protein plus the following components in μmoles: Na Bicine-100 (pH 8.25); DTE-4; KCl-20; $MgSO_4$-4; $NaHCO_3$-4; ATP-4; ^{14}C-urea-0.1 (2 μc/μmole). Heating and incubations were carried out in 10 x 75 mm stoppered test tubes. After 1 hr. incubation at 30°C, 0.1 ml of 1 N KOH was added. An aliquot (0.80 ml) was recovered from each tube and transferred to a Warburg flask with 0.30 ml of 1 N KOH in the center well and 0.5 ml of 6 N H_2SO_4 in the side arm. After sealing the flask, the acid was tipped in and flasks allowed to stand at room temperature for 3 hrs. A portion (0.20 ml) of the KOH was removed and counted by liquid scintillation spectroscopy. Heat treatment was 2 min. in boiling water. Protein content of enzyme 1 solution was 505 μg and of enzyme 2 solution was 500 μg.

when the two fractions are mixed. These activities are destroyed by heating in boiling water for 2 min.

The need for two enzymes shows that there must be an intermediate compound; the latter might be a small molecule or enzyme bound. Table 2 shows that while the enzymes are inactivated in 2 min. at 60°C, the intermediate was still largely intact. The relatively high stability of the intermediate as compared to enzymes indicated that the intermediate was not enzyme bound. This indication was strengthened by the observation that the intermediate was dialyzable.

It was then found that the intermediate compound could be separated from urea by retaining the intermediate on a tertiary amine resin in the bicarbonate form (see methods). The intermediate could be eluted with salt or acid (as shown by radioactivity). Elution of the intermediate compound with ammonium bicarbonate resulted in a product that was mostly

TABLE 2. THE EFFECT OF HEATING ON THE STABILITY OF ENZYMES 1 AND 2 AND ON STABILITY OF INTERMEDIATE.

ENZYME ADDED AT START OF FIRST INCUBATION PERIOD	HEAT TREATMENT	ENZYME ADDED AT START OF SECOND INCUBATION PERIOD	$^{14}CO_2$ PRODUCED
			nmoles
none	none	Enzymes 1 & 2	55.5
none	2 min. at 60°C (with enzyme 1)	Enzyme 2	0.10
none	2 min. at 60°C (with enzyme 2)	Enzyme 1	0.54
Enzyme 1	2 min. at 60°C	Enzyme 2	59.8
Enzyme 1	10 min. at 60°C	Enzyme 2	46.7
Enzyme 1	40 min. at 60°C	Enzyme 2	7.77
Enzyme 2	2 min. at 60°C	Enzyme 1	2.54
Enzyme 1	none	Enzyme 2	94.2
Enzyme 2	none	Enzyme 1	50.3

Enzyme 1 or enzyme 2 was incubated for 1 hr. at 30°C with complete incubation mixture as given in Table 1. Then the incubation mixture was heated as indicated and cooled quickly to 0°C. Enzyme 1 or enzyme 2 was added and the mixture reincubated for 1 hr. at 30°C. Radioactive CO_2 was measured as described in Table 1.

intact as evidenced by its susceptibility to attack by enzyme 2 and its inertness to urease. Acid elution resulted in decomposition of the intermediate as shown by its inability to serve as a substrate for enzyme 2. The decomposition product was urea since it was hydrolyzed by urease to $^{14}CO_2$.

As the latter results were obtained, Roon and Levenberg (6) reported that allophanate is an intermediate compound for the yeast ATP:urea amido-lyase. The instability of the Chlorella intermediate to heat and acid indicated that it was allophanate, and efforts were made to test this possibility. An incubation was carried out as in Table 1 with only enzyme 1 present and the intermediate formed was separated from urea. The purified intermediate was demonstrated to be allophanate by the following criteria: 1) The intermediate cochromatographed at 2-4°C with synthetic allophanate on paper in ethanol:H_2O (7:3), lutidine:H_2O (7:3); and

methanol:pyridine:H_2O (85:4:15); 2) the intermediate and allophanate are effective substrates for enzyme 2; 3) the intermediate and allophanate are not substrates of urease; 4) the intermediate and allophanate are degraded by heat at the same rate to urea (as shown by susceptibility to urease hydrolysis); 5) the intermediate and allophanate are decomposed by acids to urea.

The data in Table 3 (col.2) show that there is an absolute requirement for Mg^{++} and ATP in the formation of allophanate by enzyme 1.

TABLE 3. THE EFFECT OF VARIOUS SUBSTANCES ON THE FORMATION AND DEGRADATION OF ALLOPHANATE.

INCUBATION MIXTURE	^{14}C-ALLOPHANATE FORMED FROM ^{14}C-UREA	FROM $H^{14}CO_3$	$^{14}CO_2$ FORMED FROM ^{14}C-ALLOPHANATE
[1]	[2]	[3]	[4]
	nmoles	nmoles	nmoles
Complete	100.0	20.1	37.9
Complete minus K^+	62.5	13.7	44.6
Complete minus HCO_3^-	76.0	--	37.5
Complete minus ATP	0.56	3.3	37.6
Complete minus Mg^{++}	0.52	2.9	37.2
Complete minus urea	--	3.3	--
Complete + avidin	2.82	4.7	37.4
Complete + avidin + biotin	81.2	10.8	37.5
Complete (heated enzyme)	0.52	3.9	1.7

The results in column 2 were obtained from incubations carried out essentially as in Table 1 using only enzyme 1 (1.31 mg protein). ^{14}C-allophanate was assayed after heating incubation mixtures 2 min. at 60°C. The results in column 3 were obtained from incubations carried out as in incubations of column 2 except that more enzyme 1 was used (3.27 mg protein), unlabeled urea (5 µmoles) was substituted for labeled urea and 2.5 µc of $Na_2{}^{14}CO_3$ (38.4 µc/µmole) was added. After incubation, the mixture was heated 2 min. at 60°C and quickly cooled to 0°C. The water and $H^{14}CO_3^-$ were removed by evaporation to dryness in vacuo at 2-4°C before determination of allophanate. The data in column 4 were obtained from incubations run as for column 1 except that 0.025 µc of ^{14}C-allophanate (2 µc/µmole) replaced radioactive urea and enzyme 2 (208 µg of protein) was used in place of enzyme 1. $^{14}CO_2$ was determined as in Table 1.

In avidin treatments, enzyme was incubated with 200 µg avidin and buffer 1 hr. at 30°C prior to adding other components. A comparable 1 hr. pretreatment with avidin had no effect on enzyme activity. The quantity of biotin added was 100 µg. Heat treatments were as in Table 1.

Preincubation of the enzyme with avidin reduced the production of allophanate to 3% of the activity in complete incubation and the activity was largely restored by the addition of biotin. The omission of K^+ and HCO_3^- resulted in a partial loss of activity.

The biotin requirement and the production of allophanate indicated that HCO_3^- must be a substrate. Purification of reagents of CO_2 permitted a consistent but small (30 to 80%) increase in allophanate formation due to the addition of bicarbonate. Reducing the specific activity of the ^{14}C-urea and increasing the enzyme level 40-fold resulted in a 3.3-fold increase upon adding HCO_3^-. Apparently the Km for HCO_3^- is lower for the Chlorella enzyme than it is for the yeast enzyme (7).

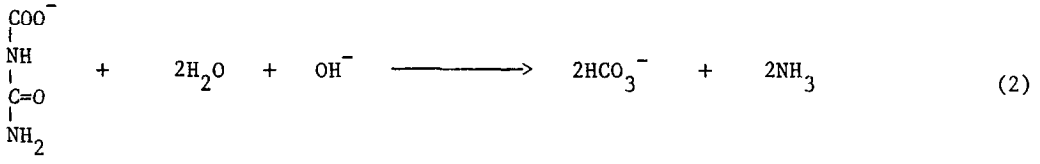
More convincing evidence for a bicarbonate requirement was obtained by forming labeled allophanate from $H^{14}CO_3^-$ and unlabeled urea in the presence of enzyme 1 (Table 3, col.3), The requirements for the formation of allophanate are the same as when labeled urea is the substrate (Table 3, col.2). The identification of the product as allophanate was carried out as before.

The above results show that enzyme 1 is urea:CO_2 ligase (ADP) or urea carboxylase. Although enzyme 1 was not pure enough to give definitive stoichiometry, the amounts of P_i and ADP produced and ATP consumed indicated that the reaction promoted by enzyme 1 can be formulated as in equation (1).

$$\begin{array}{l}NH_2\\|\\C{=}O\\|\\NH_2\end{array} + HCO_3^- + ATP \xrightarrow[\text{Biotin}]{Mg^{++}} \begin{array}{l}COO^-\\|\\NH\\|\\C{=}O\\|\\NH_2\end{array} + ADP + P_i \qquad (1)$$

As indicated by $^{14}CO_2$ production, ^{14}C-allophanate is a substrate for enzyme 2 (Table 3, col.4). None of the cofactors required for allophanate formation are necessary for allophanate hydrolysis. The demonstration of the production of 1.95 molecules of ammonia for each molecule of $^{14}CO_2$ produced from carbonyl-labeled allophanate shows that enzyme 2 is

allophanate amidohydrolase promoting the reaction in equation (2).



$$\begin{matrix} COO^- \\ | \\ NH \\ | \\ C{=}O \\ | \\ NH_2 \end{matrix} + 2H_2O + OH^- \longrightarrow 2HCO_3^- + 2NH_3 \qquad (2)$$

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