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PARTIAL PURIFICATION AND CHARACTERIZATION OF TWO FRUCTOSE DIPHOSPHATE ALDOLASES FROM *CHLAMYDOMONAS MUNDANA*

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SUMMARY

1. Two fructose-1,6-diphosphate aldolases (EC 4.1.2.7) have been detected in extracts of the unicellular green alga *Chlamydomonas mundana*. The two activities can be resolved by gel filtration of crude cell extracts on Sephadex G-200.

2. A metal-requiring enzyme has been purified 30-fold from cells cultured in the light on acetate. The enzyme is markedly stimulated by Fe^{2+} and is strongly inhibited by metal-chelating agents. The Michaelis constant for the enzyme is $3 \cdot 10^{-4}$ M fructose diphosphate.

3. An enzyme not dependent upon a metal for activity has been highly purified from cells cultured with carbon dioxide by elution of the activity from cellulose phosphate columns with fructose diphosphate. The purified enzyme is unaffected by high concentrations of chelating agents and is strongly inhibited by heavy metals. The Michaelis constant is $4 \cdot 10^{-5}$ M fructose diphosphate.

INTRODUCTION

Fructose-1,6-diphosphate aldolase (ketose-1-phosphate aldehyde-lyase, EC 4.1.2.7) is presumed to participate in at least three reactions in the intermediary metabolism of plants; (1) hexose formation during photosynthesis by means of the reductive pentose phosphate cycle[1,2], glycolysis[2] (2), and (3) the conversion of acetate to carbohydrate through the glyoxylate cycle and the reversal of glycolysis[3]. Aldolase activity has been detected in a variety of plants including several groups of algae and higher plants[4–6].

Recently it has been demonstrated that the unicellular green alga *Chlamydomonas mundana* contains two distinct aldolase activities[7]. The enzyme activity in crude extracts of photosynthetically grown *C. mundana* is similar to the enzyme from rabbit muscle, many protozoa, photosynthetic algae and higher plants (Class I aldolase according to the terminology of RUTTER[6]). The activity is insensitive to high

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concentrations of metal-chelating agents and is not stimulated by a variety of divalent cations. In addition, the enzyme has a high affinity for Fru-1,6-P_2.

The aldolase activity of cells cultured with sodium acetate as the sole source of cellular carbon corresponds closely to the Class II aldolase of RUTTER[6] (yeast, all bacteria, many fungi). The activity is completely inhibited by 1 mM *o*-phenanthroline and 1 mM EDTA and is markedly stimulated by Fe^{2+}. In addition, the enzyme from acetate cells is stimulated approx. 2-fold by K^+ and has a lower affinity for Fru-1,6-P_2 than the Class I enzyme.

The present communication describes certain aspects of the metabolic control of enzyme formation and the partial purification and characterization of the two enzymes.

METHODS

Organism

The organism used in this study was the Mojave desert strain of *C. mundana*. A slant of the organism was obtained from Dr. R. W. EPPLEY, Northrop Aviation Corporation, Hawthorne, California. Cells were grown in 10-l batches as described previously[7]. Sodium acetate (0.3%) was added to the medium where required. Cultures were grown in continuous light at 2000-ft candles.

Preparation of cell-free extracts

Cell-free extracts of *C. mundana* were prepared by sonication of whole cells in the MSE (Measuring and Scientific Equipment Company) sonicator (10 kcycles) for 60 sec. The sonicates were clarified by centrifugation at 10 000 × *g* for 10 min. Soluble protein was measured by the Folin reaction[8].

Assay of aldolase activity

Aldolase activity was measured colorimetrically using the procedure of SIBLEY AND LEHNINGER[9]. The spectrophotometric assay of WU AND RACKER[10] was also used. All measurements were linear with time and were linear with respect to enzyme concentration. One unit of activity is defined as the amount of enzyme catalyzing the cleavage of 1 μmole of Fru-1,6-P_2 per h.

The colorimetric assay was performed in test tubes at 38°. The reaction mixture contained Chlamydomonas extract, 20 mM Tris (pH 7.5), 60 mM hydrazine (pH 7.5) in a total volume of 2.5 ml. For determination of Class II aldolase activity, 0.25 μmole Fe^{2+} and 12.5 μmoles cysteine were added to each tube. Fe^{2+} was added as $Fe(NH_4)_2(SO_4)_2$. The reaction was started by adding 10 μmoles Fru-1,6-P_2 to the reaction mixture. At the end of 10 min the reactions were terminated by the addition of 2.0 ml 10% trichloroacetic acid. For each assay a blank was run in which acid was added before the addition of substrate. The killed reaction mixtures were clarified by centrifugation and an aliquot was taken for determination of hydrazone formation[9].

Aldolase was measured spectrophotometrically by coupling the reaction with triosephosphate dehydrogenase and α-glycerophosphate dehydrogenase. The reaction mixture contained Chlamydomonas extract, 50 mM Tris (pH 7.5), 0.15 μM DPNH, excess commercial triosephosphate dehydrogenase and α-glycerophosphate dehydro-

genase (Sigma) in a total volume of 1.0 ml. DPNH was omitted from the blank. The reaction was started by adding 5 μmoles Fru-1,6-P_2 to the test cuvette and was measured at 25° in the Cary recording spectrophotometer as the decrease of absorption at 340 nm. For measurements of Class II aldolase activity, 0.1 μmole Fe^{2+} and 5 μmoles cysteine were added to each cuvette. Fe^{2+} was added as $Fe(NH_4)_2(SO_4)_2$.

Gel filtration with Sephadex

Sephadex G-200 was allowed to swell in 50 mM Tris (pH 7.5) for several days with occasional decanting of the buffer. After 72 h a 2.5 cm × 45 cm column (Pharmacia laboratory column) was poured and equilibrated overnight at 4° by passing 50 mM Tris (pH 7.5) through the column. A 1.5-ml sample was applied and eluted with buffer at a flow rate of 3 ml per h. Fractions of 2.0 ml each were collected and assayed for aldolase activity. The fractions were assayed a second time in the presence of 1 mM EDTA to determine the sensitivity of the various fractions to the metal chelator.

Cellulose phosphate chromatography

0.5–0.6 g cellulose phosphate (Nutritional Biochemical) were washed several times in 5 mM Tris (pH 7.5) and poured into a column 1.0 cm in diameter containing a small layer of washed glass beads at the bottom. The column height was 10 cm. A small piece of glass wool was placed at the top of the column. 1 ml of autotrophic *C. mundana* extract in 5 mM Tris (pH 7.5) containing 15 mg soluble protein and 750 units aldolase activity was applied to the column and eluted with 5 mM Tris buffer (pH 7.5) until no soluble protein could be detected in the effluent. At this point, elution was continued with the same buffer to which 2 mM Fru-1,6-P_2 was added. All operations were performed at 4°. The flow rate of the column was 2 to 3 ml per min. 2-ml fractions were collected manually and assayed for aldolase activity. Control assays were performed on the tubes which had been eluted with Fru-1,6-P_2 to correct for the small amount of triosephosphate formed during elution.

Partial purification of Class II aldolase

10 l of acetate-grown Chlamydomonas were used for the partial purification of the Class II aldolase. Cell-free extracts were centrifuged for 15 min at 10 000 × *g*. The resulting supernatant fraction is referred to as the crude sonicate.

The extracts were frozen overnight at −20°. This treatment regularly gave a 3–5-fold purification of the enzyme without substantially affecting total enzyme activity. The frozen and thawed material was clarified by centrifugation for 15 min at 10 000 × *g*. The extract was dialyzed for 12 h against 4 l 50 mM Tris (pH 7.5).

Protamine sulfate (Sigma) was added to the dialyzed extract to a final concentration of 0.25 mg protamine sulfate per mg soluble protein. After 30 min stirring at 4°, the precipitated nucleic acids were removed by centrifugation.

Fractionation with solid $(NH_4)_2SO_4$ was carried out at 4°. Aldolase activity was found to precipitate between 55–75% saturation.

RESULTS

Resolution of two aldolase activities

Previous work[7] had shown that crude extracts of cells of *C. mundana* cultured

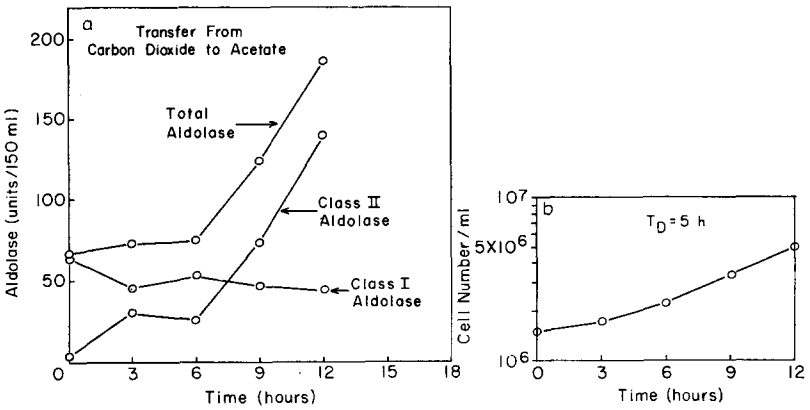

Fig. 1a. Kinetics of aldolase formation during adaptation from carbon dioxide media to media containing acetate. At time zero cells cultured with carbon dioxide as the sole source of cellular carbon were harvested and resuspended in media containing 0.3% sodium acetate. At each time point an aliquot was harvested and assayed for total aldolase activity. The fraction of the total activity which was insensitive to EDTA was taken as a measure of Class I aldolase activity. The difference between the total activity and the Class I activity was taken as a measure of Class II aldolase.

Fig. 1b. The increase in cell number as a function of time during adaptation from carbon dioxide media to acetate media.

with carbon dioxide contain a Class I aldolase activity; extracts of acetate-grown cells contain a Class II aldolase. The kinetics of enzyme formation during adaptation from carbon dioxide media to acetate media are shown in Fig. 1. Total aldolase activity was determined and the fraction of the activity which was insensitive to EDTA was used as a measure of the Class I enzyme. The difference between the total activity and the EDTA-insensitive activity was taken as a measure of the Class II enzyme.

Fig. 1a demonstrates that cells of *C. mundana* which have been cultured autotrophically with carbon dioxide as the sole source of carbon contain an aldolase activity which is totally insensitive to the metal-chelating agent EDTA. After transfer to media containing acetate, the synthesis of autotrophic enzyme ceases and a second enzyme dependent upon Fe^{2+} for maximal activity is formed. Approximately equal amounts of the two enzymes are present after 7 h. Growth, measured as the increase in cell number per ml, shows an initial lag of about 3 h and then proceeds exponentially with a doubling time of 5 h (Fig. 1b). The data in Fig. 1a are plotted as the amount of enzyme per aliquot of cells. Since the cell number is increasing with time, the drop in the amount of autotrophic enzyme per aliquot represents more than a simple dilution of enzyme per cell due to cell division. This may represent protein turnover or inactivation of enzyme activity.

The presence of two distinct aldolase activities in cells undergoing adaptation from carbon dioxide to acetate media is clearly demonstrated in Fig. 2. Two separate peaks of activity were resolved by gel filtration on Sephadex G-200 of soluble extracts of cells undergoing adaptation. The slower-moving peak, corresponding to the enzyme characteristic of acetate-grown cells, was completely inhibited by 1 mM EDTA; the faster-moving peak, corresponding to the enzyme from autotrophic cells, was not inhibited by the same concentration of chelating agent.

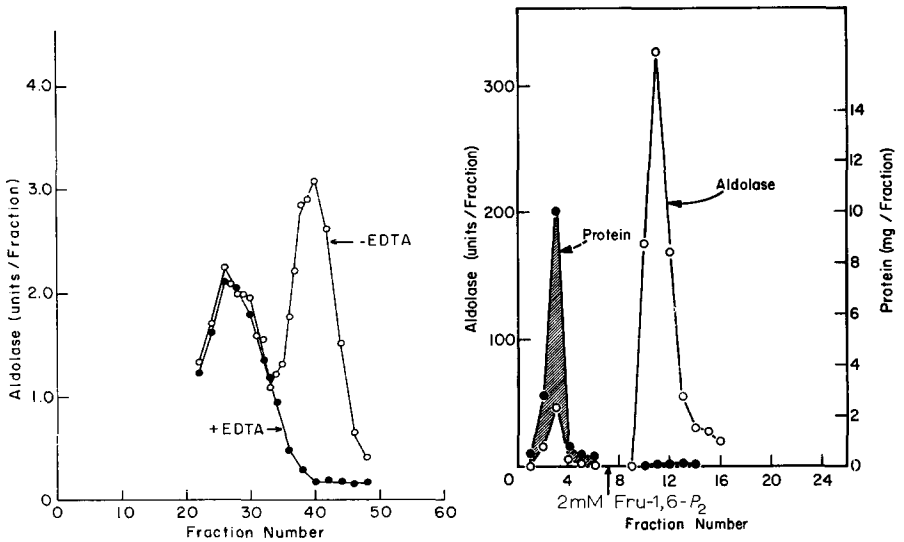

Fig. 2. Resolution of two aldolase activities on Sephadex G-200. An extract of cells which were in the process of adapting from carbon dioxide to acetate media was applied to a 2.5 cm × 45 cm Sephadex G-200 column and eluted with 50 mM Tris (pH 7.5) at a flow rate of 3.0 ml/h. Each tube was assayed for total aldolase activity. The fractions were reassayed in the presence of 1 mM EDTA.

Fig. 3. Purification of Class I aldolase by column chromatography on cellulose phosphate. A soluble extract of cells cultured with carbon dioxide as the carbon source was applied to a 1 cm × 10 cm column of cellulose phosphate and eluted with 5 mM Tris (pH 7.5) until no soluble protein could be detected in the effluent. At this point the elution was continued with 2 mM Fru-1,6-P_2.

Partial purification of Class I aldolase

The aldolase from autotrophic cells was partially purified by chromatography on a 1 cm × 10 cm cellulose phosphate column. A crude extract of cells cultured with carbon dioxide as the source of carbon was applied to the column and specifically eluted with 2 mM Fru-1,6-P_2, the substrate of the enzyme, according to the procedure of POGELL[11]. The elution profile is given in Fig. 3. Over 80% of the soluble protein added to column passed directly through and was collected in the Fractions 1–6; only small amounts of aldolase activity were found in these fractions.

The material eluted from the column with Fru-1,6-P_2 had a specific activity of approx. 4000 units per mg protein and was essentially free of nucleic acids as judged by the ratio of optical densities at 260 and 280 nm. This represented an approximate 200-fold purification since the crude sonicate had a specific activity of 20 units per mg protein. The purified material was used in the characterization of the enzyme described below.

Partial purification of Class II aldolase

Attempts to purify the Class II enzyme from acetate-grown cells by substrate elution under the conditions employed above were unsuccessful; the enzyme passed directly through the column.

The enzyme was partially purified by freezing and thawing the extract, protamine sulfate precipitation of nucleic acids, and $(NH_4)_2SO_4$ fractionation (Table I). The final material had a specific activity of 240 units per mg protein, representing a

TABLE I

PARTIAL PURIFICATION OF CLASS II ALDOLASE FROM *C. mundana*

Treatment	*Protein (mg)*	*Percent recovery of protein*	*Units of enzyme*	*Percent recovery of enzyme*	*Specific activity*	*Purification*
Crude sonicate	1280	100	10 250	100	8	—
Extract frozen and thawed, centrifuged	230	18	9 200	92	40	5.0
Dialysis	206	16	9 450	94	46	5.8
Protamine sulfate	122	9.5	8 600	84	71	8.9
55–75% saturation $(NH_4)_2SO_4$	28	2.2	6 650	65	240	30.0

30-fold purification over the crude sonicate. This material was used to characterize the enzyme.

Characterization of purified enzymes

The Class II enzyme was active in the absence of added metal ions but a 4.5-fold stimulation of activity was noted in the presence of 0.1 mM Fe^{2+} (Table II). Co^{2+} gave a slight stimulation, but only at a concentration 10-fold higher than the optimal Fe^{2+} concentration. Mn^{2+}, Cu^{2+}, and Zn^{2+} were inhibitory at higher concentrations.

0.1 mM EDTA and 1 mM *o*-phenanthroline completely inhibited aldolase

TABLE II

EFFECT OF VARIOUS COMPOUNDS ON CLASS II ALDOLASE ACTIVITY

The effects of various compounds on the Class II aldolase activity were measured using the SIBLEY AND LEHNINGER assay system[9]. The reactions were run for 10 min at 38°. The control rate (100) above represents a specific activity on 35–50 units per mg protein. Fe^{2+} was added as $Fe(NH_4)_2(SO_4)_2$; Co^{2+} was added as $CoCl_2 \cdot 6\,H_2O$; Mn^{2+} was added as $MnCl_2 \cdot 4\,H_2O$; Cu^{2+} was added as $CuSO_4 \cdot 5\,H_2O$, Zn^{2+} was added as $ZnSO_4 \cdot 7\,H_2O$. The reaction mixtures contained 5 mM cysteine in addition to the metals indicated above. The effects of PCMB, iodoacetamide, EDTA and *o*-phenanthroline were measured in the absence of cysteine.

Compound	*Rate*	*Compound*	*Rate*
Control	100	10^{-3} M Zn^{2+}	54
		10^{-4} M Zn^{2+}	64
10^{-3} M Fe^{2+}	460	10^{-5} M Zn^{2+}	67
10^{-4} M Fe^{2+}	450		
10^{-5} M Fe^{2+}	350	10^{-3} M PCMB	22
10^{-6} M Fe^{2+}	104	10^{-4} M PCMB	57
		10^{-5} M PCMB	85
10^{-3} M Co^{2+}	160		
10^{-4} M Co^{2+}	100	10^{-3} M iodoacetamid	100
10^{-5} M Co^{2+}	100		
10^{-3} M Mn^{2+}	24	10^{-3} M EDTA	0
10^{-4} M Mn^{2+}	70	10^{-4} M EDTA	0
10^{-5} M Mn^{2+}	79	10^{-5} M EDTA	25
10^{-3} M Cu^{2+}	48	10^{-3} M *o*-phenanthroline	0
10^{-4} M Cu^{2+}	86		
10^{-5} M Cu^{2+}	90		

TABLE III

EFFECT OF VARIOUS COMPOUNDS ON CLASS I ALDOLASE ACTIVITY

The effects of various compounds on the Class I aldolase activity were measured using the SIBLEY AND LEHNINGER assay system[9]. The reactions were run for 10 min at 38°. The control rate (100) above represents a specific activity of 2000–4000 units per mg protein. Fe^{2+} was added as $Fe(NH_4)_2(SO_4)_2$; Cu^{2+} was added as $CuSO_4 \cdot 5\ H_2O$. The reaction mixture contained 5 mM cysteine in addition to the metals indicated above. The effects of EDTA, *o*-phenanthroline, PCMB, and iodoacetamide were measured in the absence of cysteine.

Compound	*Rate*	*Compound*	*Rate*
Control	100	10^{-3} M *o*-phenanthroline	100
10^{-3} M Fe^{2+}	84		
10^{-4} M Fe^{2+}	92	10^{-4} M PCMB	0
10^{-5} M Fe^{2+}	96	10^{-5} M PCMB	3
		10^{-6} M PCMB	44
10^{-3} M Cu^{2+}	0	10^{-7} M PCMB	97
10^{-4} M Cu^{2+}	0		
10^{-5} M Cu^{2+}	97	10^{-3} M iodoacetamid	105
10^{-3} M EDTA	100	10^{-4} M iodoacetamid	94

activity. The effect of EDTA could be restored approx. 60% by the addition of 1 mM Fe^{2+} subsequent to treatment with 0.1 mM EDTA.

1 mM PCMB inhibited the enzyme 78%, but 1 mM iodoacetamide was without effect.

The purified Class I enzyme was not affected by concentrations of EDTA and *o*-phenanthroline which completely inhibited the enzyme from acetate cells (Table III). Fe^{2+} had a slightly inhibitory effect at high concentrations.

The enzyme from autotrophic cells was extremely sensitive to the sulfhydryl inhibitor PCMB; 10 μM PCMB completely inhibited enzyme activity. 0.1 mM Cu^{2+} virtually abolished enzyme activity. Iodoacetamide had no effect at 1 mM.

The pH optima and Michaelis constants for the two enzymes are listed in Table IV. The Class I enzyme has an approximate 8-fold higher affinity for Fru-1,6-P_2.

TABLE IV

PROPERTIES OF TWO ALDOLASES FROM *C. mundana*

The Michaelis constants for the two partially purified enzymes were determined using the WU AND RACKER[10] spectrophotometric assay. The reaction mixture contained 50 mM Tris (pH 7.5), 0.15 μM DPNH, excess triosephosphate isomerase and α-glycerophosphate dehydrogenase and enzyme in a total volume of 1.0 ml. The Class II aldolase activity was measured in the presence of 5 mM cysteine and 10 μM Fe^{2+} (added as $Fe(NH_4)_2(SO_4)_2$). Higher Fe^{2+} concentrations interfered with the spectrophotometric assay. The effect of 0.1 M K^+ (added as KCl) and the pH optima for the two enzymes were measured using the SIBLEY AND LEHNINGER[9] assay as outlined in the METHODS section. Phosphate buffers were used for the pH range 6.0–7.2; Tris buffers were used for the pH range 7.2–8.8.

	Class I	*Class II*
K_m (Fru-1,6-P_2)	$4 \cdot 10^{-5}$ M	$3 \cdot 10^{-4}$ M
Effect of 0.1 M K^+	none	1.9 × stimulation
pH optimum	6.9–8.5	7.4–7.6

DISCUSSION

The aldolase activity for acetate-grown *C. mundana* is classified as Class II aldolase on the basis of (1) inhibition by metal-chelating agents, (2) stimulation by Fe^{2+}, (3) stimulation by K^+, (4) relatively low affinity for Fru-1,6-P_2, and (5) narrow pH optimum.

Cells of *C. mundana* cultured in the light with sodium acetate carry out a photoassimilation of acetate to carbohydrate through the glyoxylate cycle and the reversal of glycolysis[12]. Under these conditions of growth the cells are unable to carry out typical green plant photosynthesis (light-dependent CO_2 fixation and O_2 evolution); acetate specifically represses the synthesis of several enzymes of the reductive pentose phosphate cycle[7]. Isocitrate lyase and malate synthase, two enzymes of the glyoxylate cycle, have been detected in extracts of acetate-grown *C. mundana*[12]. The Class II aldolase found in cells cultured with acetate presumably functions in the formation of glucose units from acetate.

The enzyme from acetate-grown *C. mundana* is very similar to the aldolase activity recently described in *Anacystis nidulans*[13,14], several other blue-green algae[13,14] the photosynthetic bacterium *Rhodopseudomonas spheroides*[13], and the green flagellate *Euglena gracilis*[6]. The enzyme from Chlamydomonas as well as the above-mentioned organisms is strongly inhibited by chelating agents and is stimulated by Fe^{2+}. The metal activated enzyme seems to function in photosynthetic carbon dioxide fixation in Rhodopseudomonas and Anacystis since it is the only aldolase activity detectable in extracts of these organisms grown photosynthetically[13]. The Class II enzyme performs a glycolytic function in *C. mundana*.

Class II aldolases derived from a variety of bacteria and fungi were shown to have molecular weights of approx. 70 000 (ref. 6). The molecular weight for the Chlamydomonas enzyme has not been determined directly but the behavior of the enzyme on Sephadex G-200 parallels that of a metal-activated enzyme from *Euglena gracilis* described by RUTTER[6] (5.4 S, molecular weight about 70 000), (RUSSELL, unpublished observation).

The aldolase activity of autotrophic *C. mundana* is classified as Class I on the basis of (1) insensitivity to chelating agents, (2) absence of stimulation by divalent cations, (3) high affinity for Fru-1,6-P_2 and (4) broad pH optimum. Molecular weight determinations have not been carried out on this enzyme but the elution profile of the enzyme on Sephadex G-200 is identical to a Class I enzyme from *Euglena gracilis* (7.8 S, molecular weight about 150 000 (ref. 6)) (RUSSELL, unpublished observation).

The Class I aldolase from *C. mundana* is remarkably sensitive to PCMB. A 56% inhibition was obtained at 1 μM; 10 μM PCMB gave virtually complete inhibition. Cu^{2+} was also an effective inhibitor. STUMPF[5] has described a Class I aldolase in pea seeds, but this activity is quite insensitive to heavy metal inhibition, in marked contrast to the Chlamydomonas enzyme.

Since the Class I aldolase is essentially the only aldolase activity in autotrophic cells of *C. mundana*, it presumably functions in carbon dioxide assimilation through the reductive pentose phosphate cycle. It is not certain whether one or both of the aldolase activities can participate in the breakdown of carbohydrate through the glycolytic pathway. Autotrophic cells presumably carry out glycolysis and the Class I enzyme may function in this capacity. However, small amounts of Class II aldolase

in autotrophic cells may be sufficient for glycolysis. *C. mundana* does not utilize exogenously supplied glucose as a carbon source so it has not been possible to determine aldolase activity under conditions where the cell is known to be actively carrying out the glycolytic breakdown of carbohydrate.

The kinetics of enzyme appearance and disappearance during adaptation from carbon dioxide to acetate illustrate the regulatory mechanisms controlling the formation of these proteins. Although definitive evidence for synthesis *de novo* has not been presented, it seems reasonable to assume that the proteins are newly synthesized during adaptation. RUTTER[6] has dismissed the possibility that the Class I enzyme is simply a multiple of the Class II enzyme on the basis that the available evidence (including peptide mapping) reveals no basic similarity between Class I and Class II aldolases. Conversion of one enzyme to the other seems unlikely and is inconsistent with the kinetics of enzyme change during adaptation reported here.

The working hypothesis to explain the metabolic control of aldolase formation in *C. mundana* is that high concentrations of certain metabolic intermediates serve to activate the cytoplasmic products of regulatory genes which in turn control the activity of the respective structural genes of the enzymes. Presumably some intermediate(s) are present in high concentration during growth on carbon dioxide which prevent the functioning of the structural gene for the Class II aldolase; during growth on acetate the concentration of the intermediate is presumed to be low. The same concept can be applied to control of Class I aldolase formation. This model has been used to explain the regulation of isocitrate lyase formation in *Escherichia coli*. KORNBERG[15] has shown that the concentration of a glycolytic intermediate (possibly phosphoenolpyruvate) controls the rate of synthesis of isocitrate lyase, an enzyme of the glyoxylate cycle.

It has not been possible to isolate chloroplasts from *C. mundana* using the nonaqueous extraction procedure of SMILLIE[16]. Determination of the intracellular localization of the two enzymes must await improvements in methodology.

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