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Role of Aldolase in Photosynthesis. II Demonstration of Aldolase Types in Photosynthetic Organisms[1]

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Abstract. Spinach leaves and photoautotrophically grown *Euglena* and *Chlorella* possess fructose 1,6-diphosphate aldolases inhibited by *p*-chloromercuribenzoate but insensitive to K^+ or ethylenediamine tetraacetate (Type I). Dark grown *Euglena* and *Chlorella* have aldolases inhibited by *p*-chloromercuribenzoate and ethylenediamine tetraacetate but stimulated by K^+ (Type II). The red alga, *Chondrus,* and the golden-brown alga, *Ochromonas,* appear to possess both types. Bean, pea, and spinach seeds and the leaves and cotyledons of etiolated bean seedlings contain a *p*-chloromercuribenzoate insensitive, apparently non-sulfhydryl variant of Type I. Sensitivity of leaf aldolase to *p*-chloromercuribenzoate occurs in etiolated bean seedlings only after an extended period of illumination. Type II aldolase activity in cell-free extracts of 4 blue-green algae has been demonstrated.

Evidence from many laboratories has led to the recognition that 2 or perhaps 3 fructose 1,6-diP aldolase types may be identified in crude extracts of animals and plants (11). Aldolases which do not require a divalent metal such as Fe^{2+} or Zn^{2+} for activity, are strongly inhibited by mercurials, and yet unaffected by chelating agent and high concentrations of K^+, have been designated as Type I. In contrast, aldolases which use a divalent metal as cofactor, are slightly inhibited by mercurials but are inhibited by chelating agents and stimulated by K^+ have been categorized as Type II. The aldolase of pea seed as reported by Stumpf (15) is not sensitive to mercurials and chelating agents and may be thought of as a third type or as a variant to Type I.

In the previous communication of this series (2), aldolase among other enzymes involved in carbohydrate metabolism was demonstrated in extracts of green, red, and golden-brown algae, a photosynthetic bacterium and spinach leaves. In that report (2), aldolase was not characterized with respect to Type. Three groups have demonstrated an apparent Type II enzyme in crude extracts of the blue-green alga, *Anacystis nidulans* (12, 17, 19). The aldolase from *A. nidulans* has now been purified and characterized (20). Included in this report is the detection of this enzyme in 4 additional blue-green organisms.

While our investigation was in progress, Rutter and coworkers (12) presented evidence that animals and higher plants, including most green algae, possess Type I aldolases while bacteria and fungi have Type II enzymes. Most interesting was their observation that *Euglena gracilis* grown in the presence of light and CO_2, possessed aldolase of Type II, while cells cultured in the dark with acetate as carbon source contained primarily a Type I enzyme. They recorded similar data for *Chlamydomonas reinhardii.* Russell and Gibbs (10) have reported a Type I aldolase in photosynthetically grown *Chlamydomonas mundana* and a Type II enzyme in cells grown on acetate. In an attempt to resolve this difference we have characterized the aldolase in streptomycin—and UV bleached mutants of *Euglena gracilis* var. *bacillaris,* strains Z and B, in addition to normal cells cultured in the light and dark.

Part of this communication is concerned with the classification of the aldolases of ungerminated plant seeds and in determining the aldolase types in the germinating bean plant.

Materials and Methods

Organisms. The blue-green algae, *Anabaena variabilis* (Kutz.) *Nostoc muscorum* G (Kutz.), and *Anabaenopsis* sp., were cultured photoautotrophically in medium C of Kratz and Myers (6). *Plectonema* sp., was grown photoautotrophically on 1 % CO_2 or photoheterotrophically on 1 % CO_2 and 1 % glucose in the same medium C. Cells were harvested after 5 days of growth.

Gram quantities of the red alga, *Chondrus crispus,* and the brown alga, *Fucus vesiculosus,* were rou-

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tinely obtained from the Marine Biological Laboratory, Woods Hole, Massachusetts. *Ochromonas danica,* was grown photoheterotrophically with glucose as a carbon source. One liter cultures were aerated with a mixture of 5 % CO_2 and 95 % air and harvested after 7 days of growth.

Chlorella pyrenoidosa (Emerson Strain) was grown photoautotrophically on a mixture of 5 % CO_2 and 95 % air (2). Cells were grown heterotrophically in the dark on the same mineral media with the inclusion of either 1 % glucose or 0.2 % sodium acetate. Autotrophic cultures were harvested after 4 days of growth; glucose-cells, after 7 days, and acetate-cells, after 2 weeks.

Dark-grown, streptomycin and UV-bleached cells of *Euglena gracilis* var. *bacillaris,* strains Z and B, were cultured with L-glutamate and DL-malate as carbon sources (8). Photoautotrophic wild-type cells were grown on the same media in the absence of all organic carbon sources and aerated with a mixture of 5 % CO_2 and 95 % air. Cells were harvested after 5 days of growth.

Spinach (*Spinacia oleracea*), pea (*Pisum sativum* var. Alaska) and bean (*Phaseolus vulgaris* var. Black Valentine) seeds were routinely obtained from Breck's of Boston.

Spinach leaves were obtained from local markets. Seeds of *Phaseolus vulgaris* var. Valentine were grown in the dark at 22° according to the procedure of Margulies (9). For development in light, the 6 day etiolated seedlings were exposed to a mixture of white and red fluorescent lamps. This corresponded to an intensity of illumination of about 500 ft-c. According to Margulies (9), leaves irradiated in this manner for 24 hours assimilated 100 to 150 μmoles CO_2 per mg chlorophyll per hour. In contrast to the leaves, the cotyledons had little photosynthetic ability.

Preparation of Cell-free Extracts. Cells of the blue-green, golden-brown, and green algae were harvested by centrifugation for 10 minutes at room temperature and were washed once with distilled water. One to 2 grams (wet wt) of cells were suspended in 50 mM tris-HCl (pH 7.6) at 4° to yield a final volume of 15 ml, then sonicated in a 25-ml Rosette cell with the 1.25 cm probe of a Branson Model S-75 Sonifier at full power for about 1 minute. By immersing the Rosette cell in an ice bath, temperatures were maintained below 8°. Sonicates were centrifuged 10 minutes at 10,000 × *g* at 0° and the resultant pellets discarded.

To prepare extracts of *Chondrus* and *Fucus* 10 grams of fresh material were soaked 5 minutes in 50 mM tris-HCl (pH 7.6) at 4°. The washed samples were chopped into small pieces and ground in a chilled mortar with 25 ml 50 mM tris-HCl (pH 7.6) and a small amount of sand until an homogenate of relatively smooth consistency was obtained. The homogenate was filtered through 4 layers of cheesecloth, and the filtrate was centrifuged.

Cell-free extracts of the following organisms in the indicated concentrations of grams wet weight per ml of 50 mM tris-HCl (pH 7.6) were prepared in the same manner: Ten g each of ungerminated bean and pea seeds per 30 ml; 6 g spinach seed per 20 ml; 0.3 g of embryo per 8 ml and 6 g of cotyledons per 15 ml from 36-hour germinated bean seeds: greened and etiolated leaves, 2 g per 10 ml, and cotyledons, 6 g per 10 ml, from bean seedlings of various ages; and 10 g per 30 ml of fresh spinach leaves.

Washed spinach chloroplasts were prepared as previously described (16). Cell-free extracts were obtained by suspending the intact chloroplasts 5 minutes in 50 mM tris-HCl (pH 7.6) and centrifuging the mixture for 10 minutes at 10,000 × *g*. The supernatant solution constituted the chloroplast cell-free extract.

Soluble protein was determined both by the phenol method (7) and spectrophotometrically (18).

Aldolase Assay. The colorimetric assay method of Sibley and Lehninger (13) was routinely employed for the inhibition studies, while the spectrophotometric assay method of Wu and Racker (21) was used for detection of Type II aldolase in the blue-green algae.

Colorimetric assays were conducted at 37° in 2.5 ml reaction mixtures consisting of 40 mM tris-HCl (pH 7.6), 56 mM hydrazine-SO_4, (pH 7.5), 4 mM fructose 1,6-diP, aldolase and other additions as described. In all cases fructose 1,6-diP was added last after a 10 minute preincubation of the aldolase. Reactions were terminated after 20 minutes by the addition of 2.0 ml cold 10 % (w/v) trichloroacetic acid. Blanks consisted of adding fructose 1,6-diP after trichloroacetic acid. Controls without aldolase were run when cofactors or inhibitors were employed. After development of the triose chromagens (13), the resultant absorption at 560 mμ was determined in 1-cm cuvettes.

Spectrophotometric assays of blue-green aldolases were performed at 37° by employing the coupling system of triose-P isomerase and α-glycerol-P dehydrogenase. These enzymes were free of aldolase. In a final volume of 1.0 ml the reaction mixture consisted of 40 mM tris-HCl (pH 7.6), 6 μg crystalline coupling enzymes (Boehringer and Soehne), 0.2 mM DPNH, 5 mM fructose 1,6-diP, 8 mM cysteine, 0.5 mM ferrous ammonium sulfate (Mallinckrodt) and aldolase. Fructose 1,6-diP was added following a 10 minute preincubation at 37°. Reference cuvettes lacked DPNH. Controls lacked fructose 1,6-diP or aldolase. DPNH oxidation at 340 mμ was followed with a Beckmann DU spectrophotometer equipped with a Gilford Model 2000 multiple absorbance recorder.

All unspecified reagents were obtained commercially. One unit of aldolase is defined as the amount of enzyme catalyzing the cleavage of 1 μmole fructose 1,6-diP per hour at 37°. The specific activity is given as units/mg protein.

Results

The aldolase activity of a freshly prepared spinach homogenate was unaffected by cysteine, EDTA, and K^+, but was almost completely inhibited by 0.1 mM *p*-CMB (table I). Similar data were obtained with chloroplast preparations (data not presented). When the extracts were frozen 7 days and then examined, the behavior of the aldolases remained unchanged. The aldolase content of the frozen homogenates appeared greater than that initially present, suggesting that freezing may effect a further solubilization of aldolase.

Aldolase activity in freshly prepared extracts of *Chondrus* was activated by cysteine and EDTA, inhibited by *p*-CMB and stimulated 2-fold by K^+. When the extract was examined after being frozen 24 hours, the activity was now inhibited by cysteine, EDTA and *p*-CMB but stimulated by K^+. In contrast to spinach and *Chondrus* the activity of the *Ochromonas* enzyme was activated equally by both cysteine and EDTA, inhibited by *p*-CMB, but unaffected by K^+. Freezing and thawing did not change the behavior of the enzyme towards these reagents. The activation by cysteine and the insensitivity to K^+ suggest the presence of a Type II, metal-requiring, aldolase of the blue-green type (20). To conclude unequivocally that the aldolase is a Type II enzyme would be premature since EDTA did not inhibit the activity. Aldolase activity could not be detected in extracts of *Fucus*.

Culture conditions had a striking effect on the occurrence of aldolase in *Euglena* (table II). Cells grown in the light with CO_2 as carbon source contained aldolase activity which was insensitive to cysteine, EDTA and K^+, but inhibited by *p*-CMB, suggesting the presence of a Type I enzyme. On the other hand, a Type II enzyme was indicated for non-chlorophyll containing cells. Similar data were recorded for photoautotrophic *Chlorella* when con-

Table I. *Effect of Various Reagents on Aldolases of Spinach,* Chondrus crispus *and* Ochromonas danica

Aldolase was determined by the colorimetric method. Each extract was incubated 10 minutes with various reagents before fructose 1,6-diP was added. The data are expressed as percentage of the control value.

	Spinach		*C. crispus*		*O. danica*	
	Initial	7 Day frozen	Initial	24 Hr frozen	Initial	24 Hr frozen
Protein mg/assay	0.25	0.25	0.47	0.47	0.62	0.62
Control value[1]	17.7	25.8	3.6	4.4	2.5	1.8
$+K^+/-K^+$[2]	1.0	1.0	2.0	1.7	1.0	1.0
Effect of:	%	%	%	%	%	%
Cysteine, 8 mM	111	105	157	67	390	246
EDTA, 5 mM	113	107	120	24	396	240
p-CMB, 1 mM	10	...[3]	11	99	27	34
0.1 mM	9	6	...	...	...	...
0.01 mM	65	35	...	...	...	...

[1] μmoles of fructose 1,6-diP cleaved per mg protein per hour.
[2] Ratio of rates determined in the presence and absence of K^+.
[3] Not determined.

Table II. *Effect of Various Reagents on Aldolases of* Euglena gracilis *Cultured under Different Growth Conditions*

Aldolase was determined by the colorimetric method. Each extract was incubated 10 minutes with various reagents before fructose 1,6-diP was added. The data are expressed as percentage of the control value.

	Strain Z		Strain B			
	Photoautotroph	Dark heterotroph	Photoautotroph	Dark heterotroph	Ultraviolet bleached	Streptomycin-bleached
Protein mg/assay	0.54	0.55	0.61	0.62	0.45	0.51
Control value[1]	9.6	19.2	9.7	22.5	16.8	20.0
$+K^+/-K^+$[2]	0.9	1.8	1.3	1.4	1.6	1.8
Effect of:	%	%	%	%	%	%
Cysteine, 8 mM	100	82	100	75	91	90
EDTA, 5 mM	82	...[3]	74	...	...	...
1 mM	96	25	90	30	27	24
0.1 mM	92	40	90	55	35	38
p-CMB, 0.1 mM	8	42	20	71	26	41
0.01 mM	35	92	66	93	57	95
0.001 mM	87	...	82	...	...	...

[1] μmoles of fructose 1,6-diP cleaved per mg protein per hour.
[2] Ratio of rates determined in the presence and absence of K^+.
[3] Not determined.

trasted with cells cultured in the dark on glucose or acetate.

Aldolase of bean seeds was found to be relatively insensitive to cysteine, EDTA, *p*-CMB and K^+ (table III). Similar data were obtained with spinach and pea seeds. To eliminate the objection that *p*-CMB sensitivity may be due to extraneous protein in the crude extracts, pea seed aldolase, purified roughly 125-fold, was prepared. Using 6 μg of crystalline rabbit muscle or purified pea seed aldolase, the former was completely inhibited by 10 μM *p*-CMB while the latter was unaffected by 100 μM *p*-CMB.

The effects of cysteine, EDTA and K^+ on aldolase activity during the greening of an etiolated bean seedling are also recorded in table III. Aldolases in etiolated leaves and cotyledons were inhibited about 50% by 0.1 mM *p*-CMB. When the seedlings were exposed to continuous light, aldolase activity became progressively more sensitive to *p*-CMB. After 2.5 days of illumination, the inhibition of leaf aldolase by *p*-CMB approached that recorded in table I for aldolase extracted from fully expanded spinach leaves. During this period of greening, the sensitivity of the cotyledon aldolase to *p*-CMB remained unchanged. Aldolase in both leaves and cotyledons were always insensitive to cysteine, EDTA and K^+.

To illustrate the ubiquity of a Type II, metal requiring, aldolase in the blue-green algae, aldolase has been detected in extracts of the blue-greens, *Anabaena, Plectonema, Anabaenopsis,* and *Nostoc* employing assay conditions found optimal for the purified *Anacystis* aldolase (20) (table IV). In all cases there was an absolute requirement for cysteine, and Fe^{2+} stimulated the rate with cysteine alone. When glucose was provided as a carbon source for *Plectonema,* there was no apparent effect on the observed rates or the behavior of its aldolase.

Table III. *Effect of Various Reagents on Aldolase of the Developing Bean Seedling*

For experimental conditions of assay, see table I. The data are expressed as percentage of the control value.

		7 Day etiolated 1 Day light		7 Day etiolated 2.5 Days light		7 Day etiolated 6 Days light		8 Day etiolated		12 Day etiolated
	Seed	Leaves	Cotyledons	Leaves	Cotyledons	Leaves	Cotyledons	Leaves	Cotyledons	Leaves
Protein mg/assay	0.77	0.23	1.05	0.16	0.55	0.29	0.43	0.32	0.93	0.41
Control value[1]	2.4	12.6	0.9	13.9	2.0	16.7	1.6	6.7	1.2	5.9
$+K^+/-K^+$ [2]	1.0	0.93	0.95	1.0	0.99	0.9	0.98	0.9	0.83	1.0
Effect of:	%	%	%	%	%	%	%	%	%	%
Cysteine, 8 mM	100	107	112	100	113	118	100	107	100	100
EDTA, 1 mM	108	100	120	113	110	100	100	112	117	100
0.1 mM	106	100	120	111	112	100	100	112	108	100
p-CMB, 0.1 mM	79	40	60	18	43	9	55	50	59	47
0.01 mM	79	65	100	26	46	14	55	78	71	53

[1] μmoles of fructose 1,6-diP cleaved per mg protein per hour.

[2] Ratio of rates determined in the presence and absence of K^+.

Table IV. *Demonstration of Aldolase in Cell-free Extracts of Some Blue-green Algae*

Aldolase was determined by the spectrophotometric method at 37° with a 10 minute preincubation with 8 mM cysteine and/or 0.5 mM Fe^{2+}.

Aldolase source		Assay condition	Specific activity[1]
A. variabilis	- light, autotrophic	No cofactor	0
		Cysteine	3.0
		Cysteine + Fe^{2+}	3.6
Plectonema sp.	- light, autotrophic	No cofactor	0
		Cysteine	2.7
		Cysteine + Fe^{2+}	4.7
	- light, 1 % glucose	No cofactor	0
		Cysteine	2.7
		Cysteine + Fe^{2+}	6.0
Anabaenopsis sp.	- light, autotrophic	No cofactor	0
		Cysteine	1.1
		Cysteine + Fe^{2+}	5.7
N. muscorum	- light, autotrophic	No cofactor	0
		Cysteine	2.3
		Cysteine + Fe^{2+}	4.0

[1] μmoles of fructose 1,6-diP cleaved per mg protein per hour.

Discussion

Aldolases extracted from chlorophyllous of spinach and bean leaves and from photoautotrophically grown *Euglena* and *Chlorella* are similar in behavior towards *p*-CMB, EDTA, cysteine, and K^+ and may be labeled as Type I (tables I, II). In contrast, aldolase extracted from *Chlorella* cultured in the dark or from bleached mutants of *Euglena* clearly possess the Type II enzyme (table II). Purification of the crude homogenates will be required to determine whether some amount of the Type I remains. These results are in agreement with the report of Russell and Gibbs (10) on *Chlamydomonas* but are in conflict with those of Rutter (12). The latter reported that light-cultured cells of *Euglena* and *Chlamydomonas* possessed a Type II enzyme while cells grown under heterotrophic conditions have Type I enzymes. We envisage that in leaves of higher plants and in the green algae capable of photosynthesizing that a Type I enzyme participates both in photosynthesis and cellular respiration. On the other hand, in those cells which have lost the ability to photosynthesize, we would suggest that a Type II enzyme functions in cellular respiration.

Chondrus and *Ochromonas* appear to possess 2 aldolase types (table I). Enzymic purification is required to resolve the actual amount of each. It is perhaps important to note that *Chrysomonad* was grown in the light but in a medium including glucose, a condition in which the organism might be expected to derive energy from both photosynthesis as well as respiratory pathways (14).

In confirmation of Stumpf (15), seed aldolases appear to be distinct from Type I or Type II. Presently we consider the enzyme obtained from the storage tissue as a variant of Type I. This designation is preferred rather than Type III since the only difference reported here between the leaf and seed enzyme is a difference in behavior to mercurials. We envisage that a fundamental difference between the enzymes may be in their tertiary structure and that during development of the young plant, the protein unfolds and becomes sensitive to *p*-CMB. Treatment with urea and quanidine might be a means of testing this point of view.

Finally it seems appropriate to compare the distribution of aldolase types with the glyceraldehyde 3-P dehydrogenases of plants. It has been demonstrated that chlorophyllous tissues including spinach leaves (2), pea leaves (4, 5) and autotrophically grown *Euglena* (3) *Chlorella* (3) possess both DPN- and TPN-linked dehydrogenases. Evidence has accumulated establishing that the TPN-enzyme is located exclusively in the chloroplast while the DPN-enzyme is divided equally between chloroplast and other components of the cell. These photosynthetic organisms appear to contain only a Type I aldolase. In plant material devoid of significant chlorophyll such as pea seed (M. D. Schulman, personal communication) etiolated pea leaves (4, 5), roots (4), mutants of *Euglena* (1, 3) or where the culturing conditions were of such a nature that respiration either wholly or partially supplants energy derived from photosynthesis for growth including dark grown *Euglena* and *Chlorella* (3), the DPN-linked dehydrogenase occurs solely or has a higher specific activity with respect to protein or chlorophyll over the TPN-enzyme. In general, these materials possess Type I and Type II aldolases but the latter appear to be prevalent. In those organism of this group like dark grown *Chlorella* or *Ochromonas* which contain a functional chloroplast, separation of the organelles is needed to determine whether the aldolases are localized.

Two plant materials which are exceptions to these generalizations are seeds and blue-green algae. Seeds contain only the DPN-linked glyceraldehyde 3-P dehydrogenase (4) and an undetermined aldolase type. Blue-green algae including *Anacystis nidulans, Anabaena variabilis, Plectonema sp., Anabaenopsis* sp., and *Nostoc muscorum,* which grow solely by means of photosynthesis contain both glyceraldehyde 3-P dehydrogenases (2) and a Type II aldolase. Perhaps this exception may be related to the suggestion that the blue-green algae represent a common link between the higher plants and the bacteria; the latter possessing only Type II aldolases (20). The function of the Type II enzyme in the blue-green cell must await an elucidation of their carbohydrate metabolism.

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