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Communication

Localization of the Enzymes Involved in the Photoevolution of H_2 from Acetate in *Chlamydomonas reinhardtii*[1]

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ABSTRACT

The localization of a series of enzymes involved in the anaerobic photodissimilation of acetate in *Chlamydomonas reinhardtii* F-60 adapted to a hydrogen metabolism was determined through the enzymic analyses of the chloroplastic, cytoplasmic, and mitochondrial fractions obtained with a cellular fractionation procedure that incorporated cell wall removal by treatment with autolysine, digestion of the plasmalemma with the detergent digitonin, and fractionation by differential centrifugation on a Percoll step gradient. The sequence of events leading to the photoevolution of H_2 from acetate includes the conversion of acetate into succinate via the extraplastidic glyoxylate cycle, the oxidation of succinate to fumarate by chloroplastic succinate dehydrogenase, and the oxidation of malate to oxaloacetate in the chloroplast by NAD dependent malate dehydrogenase. The level of potential activity for the enzymes assayed were sufficient to accommodate the observed rate of the photoanaerobic dissimilation of acetate and the photoevolution of H_2.

Studies following the photodissimilation of acetate in *Chlamydomonas reinhardtii* F-60 adapted to a fermentative hydrogen metabolism suggested the functioning of anaerobic and light-driven citric acid and glyoxylate cycles (9). It was of interest to us to elucidate further the profile of enzymic activity in *Chlamydomonas* so that the carbon metabolism necessary to support the anaerobic photodissimilation of acetate into H_2 and CO_2 could be more clearly defined.

By modifying the procedure of Klein *et al.* (13) to include differential centrifugation on a Percoll step gradient, we were able to obtain cytoplasmic and mitochondrial fractions as well as intact *Chlamydomonas* chloroplasts. The isolated chloroplasts were judged to be about 90% intact by the ferricyanide assay and essentially free of mitochondrial and cytoplasmic contamination, as determined by enzymically assaying for the presence of their respective markers (Cyt *c* oxidase and NADP isocitrate dehydrogenase). These samples were assayed for enzymic activity critical for the required carbon metabolism of acetate photodissimilation, whereupon a scheme was developed to account for enzymic compartmentation.

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MATERIALS AND METHODS

Algae and Growth Conditions

Chlamydomonas reinhardtii Wt[2] 137 c (+), 137 c (−), and the mutant strain F-60 (obtained from R. K. Togasaki, Indiana University) were grown under fluorescent light on an acetate-supplemented medium as described previously (13). Gametogenesis was induced in the Wt (±) strains by excluding nitrogen from the growth media so that autolysine could be isolated through the mating of these strains (2).

Cellular Fractionation

The procedure for isolating chloroplasts followed the method of Klein *et al.* (13) except that the two centrifugations through Percoll cushions were replaced by fractionation with a three-phase Percoll step gradient. The step gradients, composed of 4 mL each of 60, 40, and 30% Percoll in 20 mM Tricine-NaOH (pH 7.7), 150 mM mannitol, 5 mM $MgCl_2$, 5 mM $MnCl_2$, and 2 mM EDTA, were prepared in 15 mL Corex tubes. The sample material was brought to 0.025 mg Chl/mL with the above buffer whereupon 2.0 mL aliquots were layered onto the step gradients and immediately centrifuged at 10,400*g* (4°C) for 20 min on a swinging bucket rotor. The chloroplastic material banding at the 40/60% Percoll interface was harvested, washed, and brought to approximately 1 mg Chl/mL in 50 mM Hepes-NaOH (pH 7.5), 120 mM mannitol, 1 mM $MgCl_2$, 1 mM $MnCl_2$, and 2 mM EDTA.

Enzyme Assays

Enzyme activities were measured with a Gilford recording spectrophotometer (model 250) according to the following published procedures: Cyt *c* oxidase (23) NADP glyceraldehyde-3P dehydrogenase (14), isocitrate dehydrogenase (4), succinate dehydrogenase (E_{DCPIP} = 21,900 L/mol·cm [19]) (7), malate dehydrogenase (5), lactate dehydrogenase (6), isocitrate lyase (8), citrate synthase (21), adenylate kinase (20), aceto-CoA kinase—using hydroxyamine as the trapping agent (11), pyrophosphatase (15), and fumarase (18). The reaction mixtures included Triton X-100 at a final concentration of 0.1% v/v to lyse completely the protoplasts and organelles and make the enzymes more accessible to added cofactors and substrates. The specific activities of the enzymes were

[2] Abbreviations: Wt, wild type; PEP, phospho*enol*pyruvate; E, molar extinction coefficient; DCPIP, 2,6-dichlorophenolindophenol.

Table I. *Comparison of Enzymic Activities in the Chloroplast and Protoplast of Chlamydomonas reinhardtii*

Enzyme	Specific Activity		Enzymic Activity in the Chloroplast
	Protoplast	Chloroplast	
	μmol/mg Chl·min		%
Succinate dehydrogenase	1.6	0.4	25
Isocitrate dehydrogenase			
NADP	0.8	0	0[a]
NAD	ND[b]	ND	[c]
Isocitrate lyase	1.5	0	0[b]
Lactate dehydrogenase			
NADP	ND	ND	
NAD	1.1	1.1	100
Citrate synthase	1.8	0	0[d]
Malate dehydrogenase			
NADP	ND	ND	
NAD	590	180	30
Aceto CoA kinase	680	160	23
Adenylate kinase	5.3	2.3	43
Pyrophosphatase	71	49	69
Glyceraldehyde-3P dehydrogenase			
NADP	3.2	3.2	100
Cytochrome c oxidase	5.4	0.1	2

[a] Activity was found exclusively in the cytoplasmic fraction. [b] Activity was not detected by the methods used. [c] Activity was observed in the mitochondrial fraction. [d] Activity was observed in both the mitochondrial and cytoplasmic fractions.

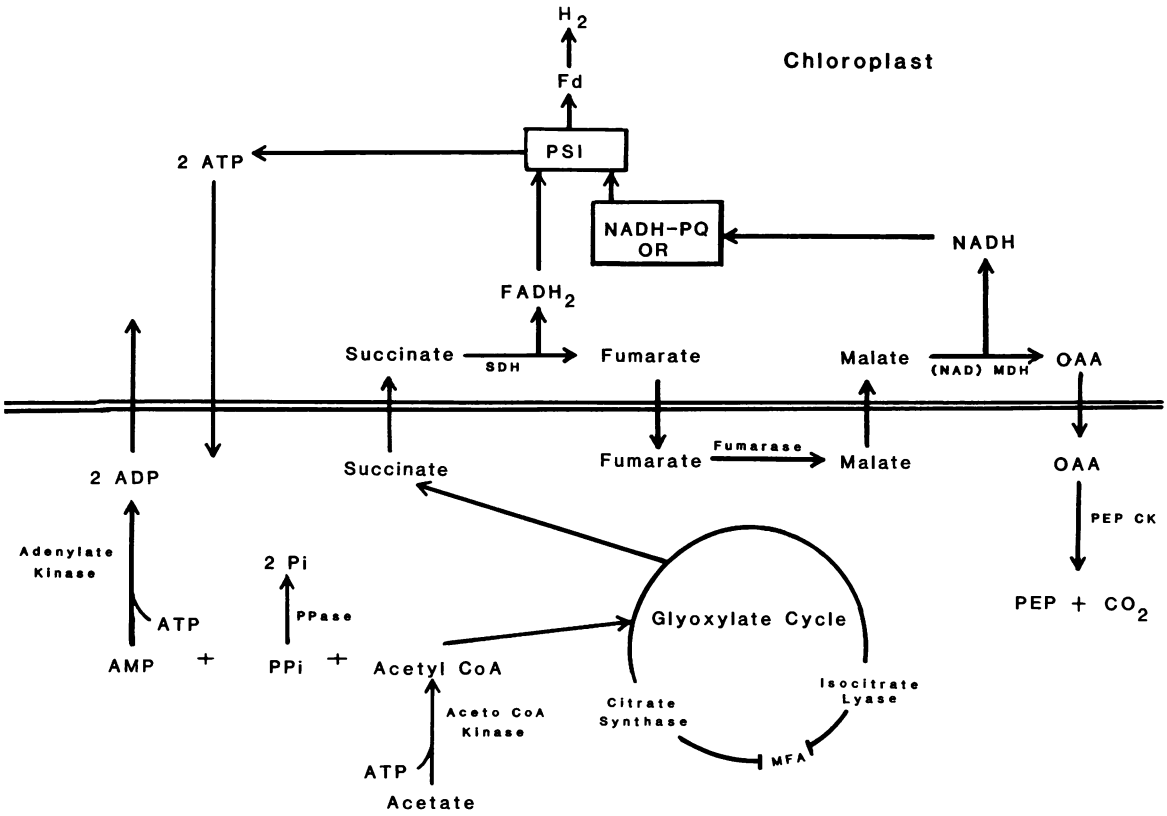

Figure 1. Proposed scheme for the evolution of H_2 and CO_2 resulting from the anaerobic photodissimilation of acetate in *Chlamydomonas*. SDH, succinate dehydrogenase; (NAD) MDH, NAD dependent malate dehydrogenase; NADH-PQ OR, NADH-plastoquinone oxidoreductase; MFA, monofluoroacetate; PEP CK, phospho*enol*pyruvate carboxykinase; PPase, pyrophosphatase.

determined on a per mg Chl basis so that, if necessary, a correction could be made for extraplastidic contamination (12). The marker enzymes were Cyt *c* oxidase for the mitochondria, NADP isocitrate dehydrogenase for the cytoplasm (3), and NADP glyceraldehyde-3P dehydrogenase for the chloroplast (13).

Chloroplast Intactness

This was measured by the ferricyanide assay developed by Lilley *et al.* (17) using 5 mM NH_4Cl as the uncoupler.

Chl Determination

This was assayed according to the method of Arnon (1).

RESULTS AND DISCUSSION

Earlier work (9) examining the photodissimilation of acetate in *Chlamydomonas* adapted to a hydrogen metabolism revealed that carbohydrate and lipid production, coupled to the evolution of CO_2 and H_2 occurred in part through the reactions of the glyoxylate and citric acid cycles. It was postulated that the enzymic oxidation of succinate to fumarate proceeded within the chloroplast. An investigation was undertaken by this laboratory to examine critically this postulate. In so doing, a method for obtaining intact *Chlamydomonas* chloroplasts was developed. The isolated chloroplasts were assessed to be approximately 90% intact (as determined by the ferricyanide assay), free of cytoplasmic contamination (as judged by NADP isocitrate dehydrogenase activity, Table I), and from 2 to 5% in mitochondrial contamination (as evaluated by Cyt *c* oxidase activity). The chloroplastic, cytoplasmic, and mitochondrial fractions were assayed for enzymic activity believed to be important to the fermentative carbon metabolism for the photodissimilation of acetate. A composite of the chloroplastic enzyme profile is presented in Table I.

Acetate can be a precursor to succinate via the actions of aceto-CoA kinase and the glyoxylate cycle. Aceto-CoA kinase activity was detected in all three fractions. Citrate synthase, an essential enzyme to both the glyoxylate and citric acid cycles, was observed to be in only the cytoplasmic and mitochondrial fractions, whereas isocitrate lyase (a marker enzyme characteristic for the glyoxylate cycle) was found to be only in the cytoplasmic fraction. Therefore, the entire enzymic sequence necessary to take acetate to succinate is present in the *Chlamydomonas* cytosol.

Monofluoroacetate inhibits the photodissimilation of acetate in *Chlamydomonas* (9), but since aconitase (an enzyme common to both the glyoxylate and citric acid cycles) was the enzyme site ultimately blocked through the action of monofluoroacetate, it was unclear whether the glyoxylate or citric acid cycle, or both, were involved in acetate's anaerobic photodissimilation. Of the cell's overall succinate dehydrogenase activity, 23% was localized within the chloroplast (Table I). Thylakoidal material, derived from the intact chloroplasts, express PSI activity when supplied with succinate and stimulated by light (this laboratory, unpublished data). The chloroplast's ability to use the electrons generated from the chloroplastic oxidation of succinate eliminates the need for participation by the mitochondrial citric acid cycle and supports the notion that the glyoxylate cycle is directly involved in the anaerobic metabolism of acetate. These features require that succinate be able to enter the chloroplast.

Fumarase was not detected in the chloroplastic samples obtained (data not shown) and has been observed to be extraplastidic in the unicellular green alga *Dunaliella tertiolecta* (10). This offers the cell the potential for invoking a succinate/fumarate exchange via the dicarboxylate transport system, thereby supplying the chloroplast with easy access to the succinate pool. Malate, generated from the transported fumarate, could reenter the chloroplast to be acted upon by the abundant supply of NAD dependent malate dehydrogenase (Table I). This would again provide the chloroplastic electron transport chain with an electron source and metabolite balance could be maintained by exporting the generated oxaloacetate in exchange for malate. The exported oxaloacetate could be acted upon by PEP carboxykinase to liberate CO_2. PEP carboxykinase was not assayed in this study but has been placed in the cytosol of other photosynthetic organisms (16).

To facilitate the process described in Figure 1, the chloroplast must provide the cytosolic aceto-CoA kinase with ATP. The AMP formed in this reaction, along with an additional ATP, may be further processed to two ADPs through the action of adenylate kinase (Table I). This creates a potentially unstable system for the organism, as it calls for the depletion of the plastidic adenine nucleotide pool, and an increasing cytosolic adenine nucleotide concentration. The most convenient means to affect the proper balance would be to enroll a rapid ATP/ADP exchange system. Adenine nucleotides, complexed to Mg^{2+}, were shown to undergo an exchange with [U-^{14}C]ADP in *Sedum* chloroplasts (22).

The photoanaerobic rate of acetate uptake, 20 μmol/mg Chl·h (9), would yield at most 10 μmol succinate/mg Chl·h. It is well within the potential of the available chloroplastic succinate dehydrogenase, which can oxidize succinate at a rate of 21.6 μmol/mg Chl·h, to metabolize any succinate generated from fermentative acetate incorporation and to remain consistent to the observed rate for the accompanying photoevolution of H_2 (9).

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