THE PYRUVATE PHOSPHOROCLASTIC REACTION IN *CHROMATIUM* A PROBABLE ROLE FOR FERREDOXIN IN A PHOTOSYNTHETIC BACTERIUM

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Mortenson, *et al.* originally reported the presence and involvement of ferredoxin, the non-heme iron containing protein, in the phosphoroclastic split of pyruvate in *Clostridium pasteurianum* (Mortenson, *et al.*, 1962). The reaction mechanism of this anaerobic cleavage of pyruvate forms acetylphosphate plus molecular H_2 and CO_2 as follows:

$$CH_3CO\text{-}COOH + H_3PO_4 \xrightarrow[\text{enzymes}]{\text{ferredoxin}} CH_3C\text{-}OPO_3H_2 + CO_2 + H_2$$

The intervention of a reduced C_1 intermediate such as formate was not indicated in this phosphoroclastic mechanism. Since the initial report, it has been shown that there is a coupling of the H_2 from the phosphoroclastic reaction with ferredoxin and the concomitant fixation and reduction of nitrogen in *Clostridium* (Mortenson, 1964; Eustachio and Hardy, 1964). Tagawa has shown that photosynthetic pyridine nucleotide reductase discovered by San Pietro in higher plant chloroplasts is a non-heme iron and has designated this as "chloroplast ferredoxin" (Tagawa, *et al.*, 1963). This compound certainly functions in the electron transfer coupled with pyridine nucleotide reduction as originally proposed by San Pietro in green plants (San Pietro, *et al.*, 1958). Tagawa and Arnon (1962) have also isolated ferredoxin from the photosynthetic bacterium *Chromatium*. However, evidence of the existence of ferredoxin in other photosynthetic bacteria is tenuous and limited to showing that

purified extracts from the bacteria can activate the light dependent photosynthetic pyridine nucleotide reductase in chloroplasts. Chromatium is the only obligate anaerobe among the purple bacteria and is missing several key enzymes for the citric acid cycle (Fuller, et al., 1961). Our investigations of the oxidative enzymes of this organism have shown that Chromatium, strain D carried out the anaerobic phosphoroclastic split of pyruvate to acetylphosphate, CO_2 and H_2 in a manner identical to Clostridium pasteurianum. We suggest the function of ferredoxin in Chromatium is as a co-factor in this reaction and is coupled with the light independent evolution of hydrogen from pyruvate or nitrogen fixation in this photosynthetic anaerobe in a manner similar to the mechanism proposed for Clostridium.

Materials and Methods - Chromatium, strain D was grown in one liter glass stoppered bottles at 35° C with an instant light intensity of 2,000 ft. candles. The majority of experiments were performed with cells grown with pyruvate as the sole source of carbon in a medium lacking $Na_2S_2O_3$. Rhodospirillum rubrum, strain S1 was grown photosynthetically in the medium described by Ormerod, et al., (1961). Cells were harvested at the desired stage of growth, washed in a potassium phosphate buffer at pH 7.5 containing 0.01% thiamine pyrophosphate and 0.01% glutathione. Washed cells were suspended in the same buffer solution and treated for 3 minutes in a Mullard 20 kc sonic oscillator. These particular experiments were carried out on the broken cell preparation directly without further cell fractionation. All respiration experiments were carried out in a Warburg vessel at 30° C under helium gas atmosphere. CO_2 and H_2 evolution was measured by using duplicate vessels with and without KOH in the center well. Radioactive measurements were made in a liquid scintillation counter or after chromatographic purification directly on paper chromatograms with an end window hand counter as described by Fuller (1956).

Results - The stoichiometric evolution of CO_2 and H_2 from pyruvate under anaerobic conditions was measured in Clostridium and compared to activities in Chromatium, strain D and Rhodospirillum rubrum. The data presented in Table I show an equal stoichiometry of H_2 and CO_2 from Clostridium pasteurianum, and Chromatium.

TABLE I Stoichiometry of CO_2 and H_2 Evolution

Organism	Substrate (10 μmoles)	μmoles H_2	μmoles CO_2
Clostridium	Pyruvate	7.1	8.7
Chromatium	Pyruvate	7.4	6.6
Chromatium	DPNH	0	-
Chromatium	Formate	0	0
R. rubrum	Pyruvate	0	Trace
	Formate	10	10

Clostridium experiments were run with cell-free preparations containing 10 mg protein per ml and the above figures report an average of 4 experiments. The cells were grown as described by Mortenson, et al. Chromatium extracts from cells grown on pyruvate and the experiments were carried out under the same conditions as Clostridium. The formic hydrogen lyase reaction was active only in whole cells of R. rubrum.

When a cell-free extract from Chromatium grown on pyruvate as a sole source of carbon was incubated under helium with 10 μmoles of pyruvate as a substrate in a Warburg vessel, approximately equimolar amounts of H_2 and CO_2 were evolved. There is no anaerobic evolution of hydrogen gas from added DPNH. If formate is used for a substrate under the same conditions with whole cells or with cell-free extracts of Chromatium, there is neither evolution of H_2 or CO_2 indicating the absence of a formic hydrogen lyase system. However, in the facultative phototroph Rhodospirillum rubrum using pyruvate as a substrate under these same conditions, no hydrogen and only a trace amount of CO_2 evolved. This

organism, however, has an active formic hydrogenlyase system as indicated by stoichiometry of H_2 and CO_2 evolution. The reaction of Chromatium with pyruvate never goes to completion with the production of 10 µmoles of H_2 and CO_2 from 10 µmoles of pyruvate. However, this may be a factor of growth conditions as cells grown on malate produce only 5 umoles each of H_2 and CO_2 from 10 µmoles of pyruvate. The fate of the remaining pyruvate is being investigated.

It has been clearly demonstrated that Rhodospirillum rubrum has a normal pyruvic oxidase system and although cell-free extracts of Chromatium can aerobically oxidase pyruvate, the mechanism of this reaction is not clear at the moment. However, under anaerobic conditions, either in whole cells or cell-free extracts, Chromatium can evolve equimolar amounts of CO_2 and H_2 from pyruvate, indicating the presence of the anaerobic phosphoroclastic reaction.

Either ferredoxin or low potential dyes such as methyl viologen will stimulate the phosphoroclastic split in Clostridium (Mortenson, et al., 1962). The phosphoroclastic enzyme system in Chromatium is an extremely labile one and any attempt to "de-ferredoxinize" the cells, as has been done with Clostridium, resulted in inactivation of the enzyme. Since Chromatium contains a large amount of ferredoxin it was difficult to obtain convincing stimulation of the reaction with added ferredoxin. However, if methyl viologen, which can substitute for ferredoxin as a co-factor in Clostridium was added to the solution, a striking stimulation could be obtained as shown in Table II.

With pyruvate as a substrate, the evolution of H_2 and CO_2 in the presence of methyl viologen remains stoichiometric but the rate of gas evolution triples. The use of dithionite as a substrate for the measurement of hydrogenase gives similar results. Again, this is in complete agreement and substantiates that the low potential dye methyl viologen, which can

TABLE II Effect of Methyl Viologen on Rate of Anaerobic Gas Evolution in Chromatium

Substrate	Rate - M. V.		Rate + M. V.	
Pyruvate	H_2	CO_2	H_2	CO_2
	2	2.4	6.4	6
Dithionite	8	-	29	-

100 µg of Methyl Viologen per vessel after the method of Peck and Gest (1956). Rate of gas evolution equals µl gas/10 mg protein/10 minutes.

substitute for ferredoxin, can stimulate the rate of the phosphoroclastic reaction.

Since the phosphoroclastic reaction involves the formation of acetyl phosphate attempts were made to isolate acetylphosphate from the reaction in Chromatium. Repeated attempts using several assay methods for the formation of acetylphosphate met with negative results. However, upon addition of acetyl phosphate to the reaction mechanism, a very rapid acetylphosphatase was detected, accounting for the rapid breakdown of this compound to acetate and inorganic phosphate. About 1.5 umoles of acetyl phosphate were destroyed in 30 minutes in the reaction mixture per 10 mg protein. Therefore, attempts were made to isolate acetate from the reaction mixture. Pyruvate 2-C^{14} was incubated with the reaction mixture and the results shown on Table III. It is apparent that hydrogen gas and C^{14} acetate appear in stoichiometric amounts from the reaction mixture. In the presence of an acetylphosphatase, this gives clear indication that three stoichiometric products, CO_2, H_2, and acetylphosphate are formed under anaerobic conditions in the dark from the photosynthetic bacterium Chromatium.

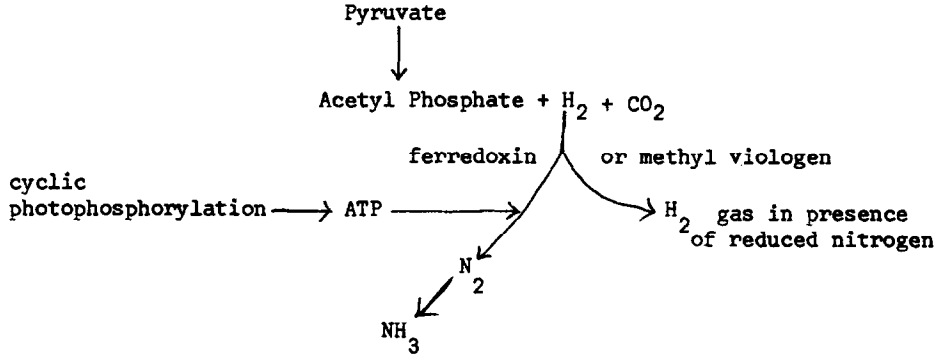
TABLE III Stoichiometry of H_2 Evolution and Acetate Formation in Chromatium.

Exp. #	cpm in Reaction Mixture (10 um Pyruvate)	Volatile cpm (Acetate)	Residual cpm (unknown)	μm C^{14} Acetate	μm H_2
#1	11.9×10^6	7.6×10^6	3.26×10^6	6.4	7.8
#2	10.26×10^6	7.76×10^6	1.54×10^6	7.5	7.3

All reactions were carried out under helium in Warburg vessels as described for Table I and II.

Discussion - It is clear that the anaerobic photosynthetic bacterium, Chromatium, strain D can carry out the phosphoroclastic split of pyruvate to form acetylphosphate, CO_2 and H_2. Ferredoxin or methyl viologen can act as electron transfer co-factor in the same reaction in Clostridium. Methyl viologen stimulates the reaction in Chromatium. The phosphoroclastic reaction has also been demonstrated in the facultative enterobacteriaceae and the anaerobic desulfovibrios. However, in these systems formate is an intermediate in the breakdown of pyruvate to acetate, CO_2 and H_2. Although ferredoxin does not exist in these cells, a cytochrome of low potential has been demonstrated and is thought to be involved in the formic hydrogenlyase reaction (Gray, 1964; Williams, et al., 1964). Tagawa, et al., (1964) have suggested the function of ferredoxin as the primary electron acceptor from chlorophyll in all photosynthesis, using as their argument, the presence of ferredoxin in all photosynthetic bacteria. The only clear cut evidence that ferredoxin is present in photosynthetic bacteria is in Chromatium, where it has been isolated, crystallized and chemically identified. We feel we have shown a function for ferredoxin in the anaerobic photosynthetic bacterium, Chromatium, that involves anaerobic electron transport in the dark phosphoroclastic reaction of pyruvate in this organism.

All the purple photosynthetic bacteria are able to fix atmospheric nitrogen and it is tempting to speculate that the role of such a low potential cofactor as ferredoxin coupled with the phosphoroclastic reaction, would be to serve as the hydrogen donor in nitrogen fixation in a mechanism similar to that proposed by Mortenson (1964) and D'Eustachio and Hardy (1964). A diagrammatic representation of that process adapted for a photosynthetic bacterium is given below.



Since Rhodospirillum rubrum, Rhodopseudomonas, and other species of the facultative photosynthetic bacteria can also fix nitrogen, it might be that a low potential compound, other than ferredoxin, could serve a role in nitrogen fixation in these cells. Mortenson (1964) has shown that the reduction of nitrogen by the phosphoroclastic mechanism is an energy requiring step, requiring the presence of ATP. It seems logical that the cyclic phosphorylation mechanism for the production of ATP in the light by Chromatium could be used to drive the reaction when N_2 is being fixed. In the presence of reduced nitrogen, the dark evolution of H_2 we have demonstrated would occur.

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