INHIBITION OF FERRICYANIDE REDUCTION IN CHLOROPLAST PARTICLES BY ANAEROBICITY[1]

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SUMMARY: The reduction of ferricyanide by spinach and pea chloroplast particles was the same under aerobic or anaerobic conditions. The rate of ferricyanide reduction by these particles uncoupled by ammonium chloride or carboxycyanide p-trifluoromethoxyhydrazone, or Chlamydomonas reinhardi or Euglena gracilis particles uncoupled by sonic oscillation, was inhibited by anaerobic conditions. This inhibition by anaerobicity may explain the cessation of the photolysis of water as measured by H_2 production in reconstituted preparations consisting of chloroplast particles, ferredoxin and fully active hydrogenase.

Recent reports on H_2 photoevolution utilizing reconstituted preparations consisting of chloroplast particles from higher plants, ferredoxin, and hydrogenase functioning under essentially anaerobic conditions have appeared. Bennemann et al (1) have reported a system that produced H_2 by the biophotolysis of water but the system was unstable, losing about 50% of activity after 15 min, apparently due to the presence of evolved O_2. This inhibition could not be accounted for by O_2 inactivation of hydrogenase activity. Improvements were noted in a report by Rao et al (2) with respect to both rates and stability. Maximum lifetime for their system was six and one-half hr at rates of 10 μmol H_2/mg Chl/hr[3]. Further improvements in rate and stability were observed by Fry et al (3). Their photosystem (PS) II-dependent system supported H_2 photoevolution at rates of 15 to 20 μmol/mg Chl/hr for up to 2 hr with much reduced rates up to 20 to 24 hr. Employment of O_2 traps, while increasing the efficiency and rate of H_2 photoproduction, did not extend the lifetime of the system. Since

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[3] Chl: chlorophyll

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TABLE I
The effects of atmosphere on the reduction of ferricyanide

Organism	Air	N_2	Air
	μMol $K_3Fe(CN)_6$ reduced/mg Chl/hr		
Ch. reinhardi, Wt	368	26	262
Ch. reinhardi, F60	594	269	241
E. gracilis, Wt	1143	532	443
Spinach	182	136	121
Peas	454	426	413
Peas + 1mM NH_4Cl	1231	480	52
Peas + 1μM F_3CCP	1376	660	60

Suspensions of chloroplast particles were prepared as in Materials and Methods and the capacity to reduce ferricyanide measured at saturating light intensities. Treatment with N_2 involved evacuation and flushing with N_2 six times; reexposure to air was accomplished by bubbling the suspension in the cuvette with filtered air for 3 to 5 min.

to N_2. When Euglena or F60 were examined for anaerobic sensitivity, inhibition was noted, but only 50 to 55% of the capacity of aerobic ferricyanide reduction was lost. Reexposure to air did not result in a recovery of the original activity. Loss of activity to aerobic ageing was only 3 to 5%.

Spinach and peas demonstrated no significant inhibition by anaerobic conditions (Table I). Spinach particles were quite unstable; activity declined 15 to 20% per hr even in the absence of gas exchanges. Pea particles were relatively stable, losing 3 to 5% of activity per hr due to ageing.

Effect of Uncouplers. Algal particles, sensitive to anaerobic conditions, were found to be uncoupled by preparation as shown by the addition of 1mM NH_4Cl of 1 μM F_3CCP[4] having no stimulatory effect on either O_2 evolution or ferricyanide reduction (data not shown). On the other hand, the rate of ferricyanide reduction by pea particles was enhanced by these uncouplers (Table I) and inhibited 50 to 60% by anaerobiosis following uncoupling. The uncoupled higher plant particles not only did not recover significant activity upon

[4] F_3CCP: carboxycyanide p-trifluoromethoxyhydrazone

hydrogenase remained active, loss of system activity was attributed to inactivation of PS II and a loss of the coupling between low potential electrons and hydrogenase.

We have investigated the effects of anaerobic conditions on the stability of higher plant and algal chloroplast particles. In this report we present data on the effects of anaerobic conditions on the capacity of chloroplast particles to carry out PS-II dependent ferricyanide reduction.

MATERIALS AND METHODS: Chlamydomonas reinhardi wild type and mutant F60 (4; F60 is missing phosphoribulokinase) were grown on the acetate-supplemented medium of Gorman and Levine (5), harvested during late logarithimic growth and resuspended in 50mM HEPES, 2.5 mM $MgCl_2$, pH 7.4. Euglena gracilis was grown on the medium of Greenblatt and Schiff (6), harvested in late logarithimic growth, and resuspended in HEPES-Mg buffer. Algal suspensions were sonicated at full power with a Branson Sonifier 200, 75 w, equipped with a microtip. Sonication time was 8 to 10 sec at 0 C. Following sonication, algal suspensions were centrifuged at 300 x g for 5 min and the supernatant used for ferricyanide reduction.

Spinach and peas were greenhouse grown. Chloroplasts were prepared by the method of Jensen and Bassham (7) utilizing 0.25% bovine serum albumin in the grinding medium. Chloroplasts were harvested by centrifugation at 5000 x g for 1 min and resuspended in 50mM HEPES, 2.5 mM $MgCl_2$, pH 7.4. Particles were used for $K_3Fe(CN)_6$ reduction after 30 min of osmotic shock. The particle preparations from both algal and higher plant chloroplasts were tested for ferricyanide-dependent O_2 evolution with a Clark-type oxygen electrode.

Ferricyanide reduction by these preparations was monitored at 420 nm in a Gilford recording spectrophotometer. Cuvettes were employed which allowed evacuation through rubber septum stoppers. The reaction mixture was: 2.8 ml buffer (50mM HEPES, 2.5mM $MgCl_2$, pH 7.4), 20 µl of 50 mM $K_3Fe(CN)_6$, and 0.2 ml of chloroplast particles containing 40 to 50 µg Chl. Illumination was from a 500 w projection lamp and intensities were saturating for ferricyanide reduction. The treatment sequence was generally air, N_2, air but a N_2, air, N_2 sequence yielded similar results. Anaerobicity was imposed by vacuum pump evacuation followed by pure N_2 (less than 5 µl/l O_2). Evacuation and flushing was repeated six times. A sequence of air to N_2, including measurements and illuminations required about one hr. Nitrogen back to air with determinations, required another 30 min. Re-exposure to air involved gentle bubbling in the cuvette for 3 to 5 min.

RESULTS: Ferricyanide Reduction. Preparations of algal and higher plant particles were subjected to air (21% O_2) or N_2. The results are summarized in Table I. Algal particles demonstrated a sensitivity to imposed anaerobicity. In wild type (Wt) Chlamydomonas, the rate of ferricyanide reduction was reduced over 90% under N_2 but recovered 70% of the original rate when re-exposed to air. In control experiments, particles from Chlamydomonas Wt lost from 12-15% of original ferricyanide reducing capacity per hr when not exposed

reexposure to air, but lost much of their remaining ferricyanide reducing capacity.

DISCUSSION: The effect of anaerobicity on the ability of chloroplast particles to reduce ferricyanide is related to the coupling of electron transport to the photophosphorylation mechanism. Algal systems, uncoupled by sonic oscillation, were sensitive to inhibition by the imposition of anaerobic conditions by 50%, but retained that level of activity even when reexposed to air. Chlamydomonas Wt, although uncoupled in preparation, appeared to be more sensitive to anaerobicity with respect to ferricyanide reduction. On the other hand, this particle recovered most of it's capacity upon reexposure to air. The lack of recovery in F60 and Euglena may be an indication of mechanical breakdown within PS II. Chemical uncoupling of the higher plant preparations rendered them as, if not more, sensitive to inhibition by anaerobiosis as the algal particles.

Where hydrogenase is fully active, the loss of activity in the reported reconstituted systems for H_2 photoproduction may be related to the inactivation of PS II due to the loss of the photophosphorylation coupling mechanism. One report demonstrating short term activity included the addition of NH_4Cl to the system (1). Where the activity reported was eventually lost due to the apparent ageing of particles (2, 3) the chloroplast particles were initially prepared by a procedure that is known to promote stability and retain the coupling of photophosphorylation. Anaerobiosis is required for the protection of hydrogenase from O_2 inactivation; however, extended anaerobiosis may account for the rapid loss of PS II activity in particles which become uncoupled with time. Maximum rates and stability in reconstituted systems may be realized in those systems which are most carefully protected against both uncoupling of photophosphorylation and O_2 inactivation of hydrogenase.

REFERENCES:

1. Benneman, J.R., Berenson, J.A., Kaplan, N.O., and Kamen, M.D. (1973) Proc. Nat. Acad. Sci. 70, 2317-2320.

2. Rao, K.K., Rosa, R., and Hall, D.O. (1976) Biochem. Biophys. Res. Commun. 68(1), 21-28.
3. Fry, I., Papageorgiou, G., Tel-Or, E., and Packer, L. (1977) Z. Naturforsch. 32C, 110-117.
4. Moll, B., and Levine, R.P. (1965) Plant Physiol. 46, 576-580.
5. Gorman, D.S., and Levine, R.P. (1965) Proc. Nat. Acad. Sci. 54, 1665-1669.
6. Greenblatt, C.L., and Schiff, J.A. (1959) J. Protozool. 6, 23-28.
7. Jensen, R.G., and Bassham, J.A. (1966) Proc. Nat. Acad. Sci. 56, 1095-1101.