MASTER

PHOTOCHEMISTRY AND ENZYMOLOGY OF PHOTOSYNTHESIS

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I. LIGHT HARVESTING AND ELECTRON TRANSPORT IN C₄ PLANTS

During the past decade a large number of studies have been devoted to the interesting features of so-called C₄ plants. This group comprises crops such as sugar cane and corn that are among the most productive in terms of dry weight per unit area. Compared to the more common, so-called C₃ plants, the C₄ types have a different leaf structure. An inner "bundle sheath" tissue surrounds the vascular tissue and is itself surrounded by loosely packed "mesophyll" cells. Presumably CO₂ is pre-fixed in the mesophyll into C₄ acids; these acids diffuse to the bundle sheath, where the CO₂ is released again and permanently fixed into carbohydrate in the normal Calvin-Benson path ("C₃").

One of the difficulties in our understanding of these processes is the source of the reducing power needed by the bundle sheath cells for the final reduction of CO₂. Most of the literature reports that photosystem II (which evolves O₂ from water) is low or absent in these cells so that the source of electrons could not be water. Hardt and Kok have addressed this problem; their paper, "Comparison of Photosynthetic Electron Transport Activities of Spinach Chloroplasts with Those of Corn Mesophyll and Corn Bundle Sheath Tissue," recently appeared in *Plant Physiology*⁽¹⁾. A major point brought up in this report is that bundle sheath chloroplasts of corn are capable of high rates of O₂ evolution.

We succeeded earlier⁽²⁾ in obtaining good rates of O₂ evolution in glutaraldehyde-treated spinach chloroplasts (in which the reaction chain between the photoacts is inhibited) by using lipophilic electron acceptors such as p-phenylenediamine, which presumably penetrate membranes and react directly and rapidly with the primary and/or secondary acceptors of photosystem II.⁽³⁾

We were indeed able to observe reasonably high oxygen rates in bundle sheath chloroplasts using lipophilic acceptors (600 eq./Chl·hr).⁽¹⁾ These

rates, about half those observed with spinach, were of course minimum values. Relatively low rates were observed for electron transport from water to viologen. We believe that the intermediate reaction chain is operative in vivo but is damaged during the harsh preparative procedure used to isolate these bundle sheath chloroplasts.

In these experiments, we mechanically disrupted the tissue with razor blades, a procedure which was adapted from Anderson et al.⁽⁴⁾ The degree of separation of the tissues was judged mainly by microscopic examination. Chlorophyll a/b ratios in bundle sheath chloroplasts varied between 4.5 and 6. Since the lower value might indicate considerable mesophyll contamination, this left an uncertainty whether sufficient purity of the bundle sheath samples had been obtained consistently.

Quantitative interpretation of other measured parameters was also hampered by these uncertainties and by the variability of the bundle sheath preparations. Our results, therefore, did not fully resolve the mode and mechanisms of energy distribution in C_4 photosynthesis -- to the contrary, they raised additional questions.

In view of the above, we have recently begun additional studies, initially of preparative procedures and photosystem II assays. We are comparing bundle sheath strands or chloroplasts prepared in various ways, e.g., by mechanical disruption, by a "polytron" instead of by vibrating razor blades, by enzymatic digestion, and by fractionation using ludox gradients.⁽⁵⁾

Pure preparations of bundle sheath strands can be obtained with relative ease by using the enzyme digestion methods introduced by Kanai and Edwards.⁽⁶⁾ By applying this enzymatic procedure to corn leaves, we have quantitatively confirmed the results of Ku et al.⁽⁷⁾ and Mayne et al.⁽⁸⁾ in terms of the chlorophyll distribution between the mesophyll and bundle sheath tissues, the

respective chlorophyll a/b ratios, and the negligible O_2 -evolution rates in the bundle sheath fragments. We therefore suspect that enzyme digestion, while of great help in separating the tissues, can lead to low O_2 -evolution rates. We have exposed chloroplasts prepared from corn mesophyll or spinach to the same enzymes. We observed very little effect on photosystem I, but considerable damage to the interconnecting chain and loss of photosystem II (~40% in spinach and 60% in mesophyll). Presently, we are trying to develop a method to strip the epidermis and outer (mesophyll) layers of the leaf away before chopping (or digesting) the remaining tissue, which should be mostly bundle sheath.

It appears that the preparative difficulties are less severe with C_4 plants other than corn, sorghum, and sugarcane. (However, these plants might also lack some of the fascinating peculiarities of corn.) Rathnam and Edwards⁽⁹⁾ used enzyme digestion to isolate the bundle sheath from crabgrass (*Digitaria sanguinalis*, another NADP-malic enzyme species). They obtained quite reasonable rates of O_2 evolution (600 eq./Chl·hr) even with PGA as the electron acceptor. Electron transport in this material was previously studied in some detail by Mayne et al.⁽¹⁰⁾ who used differential grinding to separate the two tissues. The bundle sheath fraction showed an average Chl. a/b ratio of 4.5 and an O_2 -evolution rate of ~75 O_2 /Chl·hr with benzoquinone under anaerobic conditions. In preliminary experiments, we have not, however, found this material much more suitable than corn as a source of fully active bundle sheath chloroplasts.

Since we did not have a satisfactory method to prepare fully active chloroplasts from the bundle sheath of corn and since O_2 evolution appears to be most readily lost, we have looked for a more stable, quantitative assay for photosystem II. One candidate for this assay is the oxidation of diphenylcarbazide

(coupled to the photoreduction of indophenol dye).⁽¹¹⁾ This oxidation does not seem to replace the oxidation of water, but occurs only in O_2 centers that are already inactivated. As a result, by monitoring the reduction of DCIP, one views the sum of the two activities. However, the donor reaction is inefficient (30-50%) so that the assay is not quantitative.

We have also used the flash-induced absorption changes at 550 and 320 nm ("C550" and "X320," respectively) as a measure of photosystem II. At both wavelengths (but most severely at 320 nm), there is interference by other absorption changes (mainly due to P700 which occurs in a high concentration in bundle sheath). In one set of experiments (which included the observation of a complete spectrum in spinach), we estimated that, per unit chlorophyll, the C550 content is equal in mesophyll and spinach, but is smaller (~60%) in bundle sheath.

Presently, we are testing yet another assay for photosystem II, which is based on the flash-induced pH changes in the presence of tetraphenylboron, a very effective and very specific photosystem II electron donor.⁽¹²⁾ This assay appears to be reliable, but so far has given low values for the system II concentration in corn bundle sheath chloroplasts.

II. MASS SPECTROMETER STUDIES: MEASUREMENTS IN FLASHING LIGHT

For the past three years we have been studying gas exchange, primarily in algae, using a mass spectrometer (MS) with a special inlet system.⁽¹³⁻¹⁵⁾ We recently developed a measuring technique, derived from this method, by which we can mass spectrometrically monitor the gas exchange elicited by single, short (~5- μ sec), saturating light flashes. As in our previous instrument, gases are admitted to the MS through a silicone rubber membrane (supported by a metal screen). The chloroplast- or algal-suspension is carefully layered on the membrane and

illuminated from above; these aspects of the technique are closely akin to the O_2 -electrode method developed by Joliot.⁽¹⁶⁾ A compensating "bucking" voltage is applied to the resultant MS signal to null out most of the undesired background signal. A mass-stepper-bucking system, built in-house, allows us to independently tune the MS and buck out the background signal of up to eight different m/e values.

We recently ran a series of experiments in which we used this apparatus to monitor the photooxidation of hydroxylamine (NH_2OH) by isolated chloroplasts. (It has been recognized for some time that NH_2OH can serve as a donor to photosystem II,⁽¹⁷⁾ although the precise reactions involved are not clearly understood.) We found that molecular N_2 was formed exclusively when chloroplasts were subjected to a train of saturating flashes (3-sec spacing) in the presence of 1 mM NH_2OH . There was no apparent evolution of nitrogen oxides, which, under many conditions, are the major products of NH_2OH oxidation. When $^{15}NH_2OH$ (97 atom % ^{15}N) was used, we observed the evolution of $^{15}N_2$ (m/e = 30) and an amount of $^{14,15}N_2$ (m/e = 29), which was in accord with the isotopic composition of the labeled NH_2OH .

We were initially worried that we might be indirectly monitoring a photosystem I reaction; e.g.,

However, we are now quite convinced that the observed light-driven N_2 evolution from NH_2OH does not involve photosystem I-generated superoxide or peroxide. We have found that it occurs 1) in the absence of a well-defined stoichiometry between N_2 evolution and O_2 uptake; 2) in the presence of ferricyanide, which abolishes measurable O_2 uptake; 3) in the absence of O_2 (using a glucose-glucose oxidase scrubber); and 4) in particles made from *Scenedesmus* Mutant #8, which lacks photosystem I.

Our data show that equal amounts of N_2 are evolved on each flash. Since there is no binary oscillation of N_2 yields, these results suggest a mechanism involving the abstraction of a single electron from a single NH_2OH molecule at each photosystem II center, and the subsequent combination of these oxidized products to form molecular dinitrogen (a "two-electron" product), e.g.,

This oxidation product ($NHOH\cdot$) is probably the same entity as that detected polarographically by Bennoun and Joliot.⁽¹⁸⁾

It has been recognized for some time that, in the presence of DCMU, NH_2OH inhibits the backreaction between P^+_{690} , the primary PS II donor, and Q^- , the primary acceptor⁽¹⁹⁾. This suggests that the reduction of P^+_{690} by NH_2OH is irreversible, in contrast to the reduction of P^+_{690} by the O_2 system⁽²⁰⁾. We recently observed that, in the presence of NH_2OH and DCMU, N_2 was evolved on the first flash (provided that no light, other than the actinic flashes, reached the sample after the addition of DCMU) but not on subsequent flashes. The irreversibility of the NH_2OH -DCMU system, compared to the normal DCMU-inhibited system, therefore can be attributed to ability of the NH_2OH system to immediately discharge its oxidizing equivalents as N_2 .

In the normal DCMU-inhibited system the oxidizing equivalents are retained -- mainly as S_2 -- and the system can subsequently backreact (deactivate) to restore the initial conditions.

III. SPECTROPHOTOMETRIC STUDIES

We earlier reported the occurrence of a slow phase in the field-indicating 515-nm change in isolated chloroplasts. During the past reporting period, we have supplemented this observation by spectroscopic measurements of proton exchange, and completed a manuscript that describes our findings. A copy of this manuscript is enclosed; it's abstract reads:

"1) Pre-reduction of the intersystem pool in isolated spinach chloroplasts leads to the appearance of a slow phase in the field-indicating 515-nm change induced by a flash. 2) Measurements with the pH-indicator dye cresol red show proton uptake associated with this slow 515-change. 3) With water as the electron donor to photosystem II, electron transfer to ferricyanide is associated with the uptake of more than one proton per electron. 4) Tetraphenylboron, upon oxidation by system II, releases a proton directly into the medium. Yet, flash induced electron transport from tetraphenylboron to ferricyanide is accompanied by a net uptake of protons from the medium. All four results demonstrate the presence of an additional site of proton translocation; observations (1) and (2) localize this site at the oxidising side of the plastoquinone pool."

In some respects, in particular in observation no. 3 of the above summary, these results are merely confirming Fowler's previous measurements of the H^+/e ratio. However, the results also extend our knowledge by demonstrating that the second site of proton-translocation in the intersystem chain is localized at the oxidising side of the plastoquinone pool. This conclusion has considerable consequences for the mechanism of electron transport between the photosystems. The current scheme of electron transport, or modest extensions of this scheme, cannot accommodate the extra "loop" implied by our results. In plastohydroquinone oxidation, already known to be the rate limiting step for electron transfer from

water to the Calvin cycle, more components than previously believed must be involved. It seems more worthwhile than ever to look for a role of cytochrome b in this reaction. Indeed, in preliminary investigations we found that, contrary to common belief, cytochrome b_6 is reduced at the oxidizing side of system I and not at the reducing side. This result shows that cytochrome b_6 is involved in linear electron flow. However, the exact role of this cytochrome and the identity of its immediate redox partners are still obscure. In our enclosed paper we proposed that plastoquinone oxidation by cytochrome f generates the semiquinone anion, and this species seems to be a good candidate for the reductant of cytochrome b_6 .

IV. REDOX TITRATION OF THE PLASTOQUINONE POOL

An important aspect of photosynthetic electron transport that is currently under active investigation concerns events surrounding the plastoquinone pool separating the two photosystems. One property not yet adequately studied is the midpoint potential of the plastoquinone pool. We have approached this problem in a straightforward manner by equilibrating the pool with external redox mediators of known potential and relating this to properties of the rise curve during illumination. Our approach requires that no other pathway be available for the emptying or filling of the pool, such as the presence of oxygen (which accepts electrons from the pool) and the unavoidable action of PS I in draining the pool via plastocyanin. Consequently, we are titrating the plastoquinone pool under strictly anaerobic conditions in an organism lacking photosystem I (*Scenedesmus* Mutant #8). By choosing derivatives of naphthoquinone and benzoquinone redox dyes of the proper potentials, we determined a concentration where the pool will equilibrate with the dyes only very slowly during a dark incubation period of 5 minutes. Such slow equilibration is necessary; the redox dyes must not serve as efficient acceptors during the 5-second illumination period or the rise curve will be severely distorted.

Our preliminary results show the titration of the plastoquinone pool follows an $n = 2$ theoretical curve, indicating two electrons are involved in plastoquinone reduction (as expected). At $\text{pH} = 7$, in the presence of 5 mM Mg^{++} , the midpoint potential is 110 mV and shows a pH -sensitivity that indicates 2 protons are involved during reduction (at least in the $\text{pH} = 6-7$ region). We have observed several interesting anomalies in the titration: a) the redox potential may be sensitive to ionic conditions -- the absence of Mg^{++} shifts the midpoint to 160 mV , and b) under strictly anaerobic conditions, only a fraction of the pool recovers and equilibrates with the external redox buffers. This last finding is particularly interesting: the plastoquinone pool can be oxidized and reduced at will by varying the potential of the external redox buffers provided the chloroplast has not seen light. After illumination, only a small part of the pool equilibrates with the external medium. The reasons for these and several other interesting anomalies in the titration of the plastoquinone pool are under active investigation.

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