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PROGRESS REPORT

Kinetic Analysis of the Oxidizing and Reducing Sides of the O_2 Evolving System

During the reporting period, we analyzed a number of kinetic observations made during the last few years, concerning the loss of the oxygen precursors under conditions where the System II acceptor was not in its optimal (oxidized) state. Without an added electron acceptor, illumination with 650 nm light reduces the electron acceptor pool of System II and, probably due to a back reaction, the O_2 precursors are rapidly lost. In subsequent dark, this acceptor pool becomes reoxidized and System II again operative. To unravel the measurements of two concurrent phenomena, loss of S_3 and S_2 states and oxidation of the acceptor in the dark, we used computer simulation and were able to obtain a coherent interpretation of both phenomena. A novel approach in these measurements was the use of flashing light to drive the reactions. Since brief, saturating light flashes drive the system synchronously, all traps moving one step at a time, the analysis was relatively simple. Our analysis yielded several interesting conclusions. Of these, we might mention that the primary acceptor of System II reacts as a homogeneous pool of about 5 equivalents per trapping center. We surmise that this pool represents two molecules of plastoquinone. These and other conclusions are reported in a paper by Radmer and Kok which is accepted for publication in BBA.

Rapid Events in Photosystem II

Kinetic measurements of fluorescence emission (a) during a brief saturating light flash and (b) subsequent to the flash in the time range $\sim 80 \mu\text{sec}$, which were made by Zankel during the last two years, have been collected in

a manuscript. The results indicated that the fluorescence changes $\gg 200 \mu\text{sec}$ after a flash are governed primarily by the degree of oxidation of acceptor Q: Q^- made in a light flash equilibrates with a neighboring molecule of similar redox potential in about $200 \mu\text{sec}$, leaving part of Q still reduced. The slower components of fluorescence decay - reflecting further oxidation of Q^- - occur when the neighbor interacts with other intermediates in the electron transport chain. The fluorescence during this time range is also slightly perturbed by positive charges on the donor side of System II.

At shorter times, the fluorescence kinetics after the light flash appear related to movement of quenching positive charges on the donor side of System II. We observed a $35 \mu\text{sec}$ rise of emission in the dark after a flash which appears to be related to recovery of the electron donor. Slower changes ($\sim 1 \text{ msec}$) appear to be related to + charges moving to the S-states from some intermediate.

The fluorescence kinetics during a flash indicate that carotenoid radicals produced by excess quanta in System II quench fluorescence. The disappearance of the radicals and the unquenching of fluorescence occur in a few μsecs . Both are hastened by the addition of the paramagnetic quencher, oxygen. A copy of the manuscript submitted for publication is attached.

Charge Cooperation in Oxygen Evolution

The linear mechanism of charge accumulation in oxygen evolution proposed by Kok et al⁽¹⁾ was reviewed in a paper by Joliot and Kok, proposed during this reporting period for publication in "Bioenergetics of Photosynthesis." The paper gives a detailed account of the observations supporting this mechanism as well as the remaining difficulties.

Last year's proposal discussed our attempts to improve the time response of a membrane covered polarograph electrode. We recently have developed a

workable though not yet ideal apparatus. The membrane, while passing O_2 , protects the platinum electrode from reacting with chemical additions in the chloroplast suspension medium. The apparatus allows the measurement of flash yield sequences provided the spacing between the flashes is a second or longer. We have applied the apparatus quite extensively in attempts to chemically influence the state of the O_2 precursor enzyme. Bouges⁽²⁾ reported last year that a mixture of ferricyanide and indophenol dye (DPIP) converted the S_0 state into the S_1 state. She used a bare electrode and minimized artifacts using the criterion that the first two flash yields after a dark period should be zero. With the covered electrode, we could readily corroborate this result. In addition we have surveyed the effect of a number of oxidizing reagents including several with a midpoint potential much higher than that of ferricyanide. However, we have had no success in oxidizing the system chemically beyond the S_1 state. Moreover with all oxidants tried, the reaction was extremely sluggish. Several chemical oxidants proved deleterious to all chloroplast activity; others, including potassium iridium chloride, were withstood very well. Thus far, the result of this somewhat frustrating series of experiments is that the reaction $S_1 + e^- \rightleftharpoons S_0$ indeed has a relatively low midpoint potential. So far, sluggish equilibration and interference of deactivation have hindered a precise determination of this midpoint potential. Since S_2 could not be produced by chemical oxidation, at least by our unsophisticated approach, the reaction $S_2 + e^- \rightleftharpoons S_1$ must have a high oxidizing potential.

In addition we have noticed some other features of the system. One of these is that, even in the presence of high concentrations of oxidizing agents, deactivation (i.e. reduction of the S_2 and S_3 states) still occurred albeit with rather long halftimes. The question now arises what type of intermediate

serves as electron donor in this case. Furthermore, we observed that the addition of small amounts of benzoquinone or phenazin metho sulfate (PMS) to the ferricyanide solution accelerated this deactivation considerably. Again we have no explanation except maybe that penetration through the chloroplast membrane and equilibration with various intermediates is more rapid. Some of these aspects will be pursued further. Other interesting phenomena have been noticed when we added reducing rather than an oxidizing agents to the suspension. Such agents invariably cause loss of S_1 states in darkness, i.e., accelerate deactivation of the system. PMS proved the most effective, causing a rapid loss of precursor states at higher concentrations. Such a rapid deactivation readily expands earlier observations⁽³⁾ which showed that PMS can interfere with the electron transport chain at the level of Z and inhibit O_2 evolution.

Proton Evolution in Synchrony with O₂ Evolution

Four protons per O₂ evolved are released during the photooxidation of water. In order to understand the mechanism(s) underlying this process, it is important to know how these protons are released; one in each S-state transition, in pairs, or all 4 in a terminal "concerted reaction" within each unit as seen for O₂ evolution. Using our sensitive pH electrode technique, we have obtained evidence supporting the latter alternative. Normally, the detection of protons liberated inside the thylakoid membrane is masked by the classical alkaline pool formed during illumination and the slow re-equilibration of the protons in the dark period following. These problems were overcome to a large extent by the use of suitable uncouplers. Methylamine and ammonium chloride at uncoupling concentrations suppressed the classical proton uptake and allowed those protons being made on the inside of the membrane to be observed. During a sequence of short saturating flashes, chloroplast suspensions in the presence of methylamine or ammonium chloride released protons in synchrony with O₂. Pre-flashing, DCMU, and hydroxylamine all modified the oscillatory behavior in much the same manner that they affect O₂ evolution. Other types of uncouplers, for example, gramicidin allowed the oscillatory behavior to be observed, however, the release was superimposed upon the classical uptake which was not suppressed. The data strongly support the concepts that (1) water is split in "one concerted reaction" following the accumulation of 4 + charges and (2) the O₂ evolving process is compartmentalized inside the thylakoid membrane. These data have been reported at two meetings and a paper reporting the measurements is in preparation.

Photoinduced Electric Field in Chloroplasts

Using a very simple set-up consisting of two identical Ag-AgCl electrodes connected to a rapid amplifier, it proved possible to observe a photo-induced electric field in suspensions of isolated chloroplasts. If the two electrode tips are placed asymmetrically in the light beam, a brief flash induces a potential transient, the electrode closest to the light source going negative. The rise time of the pulse was as fast as the time resolution of our instrument. The decay time of the pulse ranged between 10 μ sec to milliseconds and was inversely proportional to the conductivity of the suspension medium in which the chloroplast were suspended. Evidently, light induces a separation of charges which are not randomly distributed in the suspension. Redistribution of these charges occurs faster, the greater the conductivity of the medium. We ascribed this phenomenon to an intensity gradient across the chloroplast vesicle membranes due to the high pigment concentration. so that relatively more charges are released on the "brighter side".

Experiments with specific poisons showed that this charge transient reflected the photoevents directly, each of the two photoacts contributing equally to the signal. In addition, if the experiment was made with chloroplasts which had been in dark for a long time, the very first flash induced an abnormally high electrical transient. This "extra" signal equalled the ones induced by each of the photoacts in subsequent flashes. Therefore, we have tentatively ascribed it to an additional photoact in System II, occuring only once after long dark. Evidence for such an "extra" photoact was brought forth earlier by Doschek and Kok⁽⁴⁾ on the basis of fluorescence observations.

Photoinduced Electric Field in Chloroplasts (cont'd)

The literature contains considerable speculation about anisotropic properties of the chloroplast membrane. These presumably underly the chemiosmotic theory of phosphorylation⁽⁵⁾ while Witt et al.⁽⁶⁾ have interpreted the 515 nm absorption change as being caused by an electric field across the thylakoid membrane. The measurements discussed above appear to constitute the first direct observation of this long surmised light driven electrical field. A preliminary report of this effect is given⁽⁷⁾ and a paper is being prepared for publication.

Photoactivation of O₂ Evolution in Vivo and in Vitro

During this year's reporting period, we summarized our studies on the "Absence of Oxygen Evolving Capacity in Dark Grown *Chlorella*: The Photoactivation of Oxygen-Evolving Center." This paper will appear in the June issue of Photochemistry and Photobiology.

These studies were an out-growth of some of our previous studies on photoactivation of O₂ evolving centers of Mn deficient^(8, 9) and NH₂OH extracted cells.⁽¹⁰⁾ Based on these previous studies we had postulated, in contradiction to results of the older literature^(11, 12) that photoactivation of the O₂ evolving centers might be required in cells such as *Chlorella* which are capable of synthesis in darkness of the photosynthetic apparatus. Briefly, the following conclusions were made in the above manuscript:

1. Growth under a variety of heterotrophic conditions yielded cells containing functional System II and I trapping centers and interconnecting electron transport chains but without O₂ evolving centers;
2. The Mn pool which we have correlated with O₂ evolving centers was conspicuously absent;
3. Brief illumination ($t_{1/2}=2-3$ min) of such dark grown cells resulted in incorporation of Mn into the O₂ evolving centers and the capacity to evolve O₂ (photoactivation);
4. The kinetic data from flashing or continuous light proved entirely similar to those deduced from studies with cells depleted of Mn by autotrophic growth or by NH₂OH extraction -- i.e., evidence for a multi-quantum process was obtained.
5. Measurements of O₂ yield oscillations indicated that throughout the photoactivation process each newly formed O₂ yielding center was independent of its neighbors.

6. It was concluded that photoactivation is a fundamental process in all O_2 evolving tissue and that in the development of the photosynthetic apparatus the formation of O_2 yielding sites must be preceded by formation of System II trapping centers.

The oscillations of fluorescence in a series of flashes first reported by Delosme⁽¹³⁾ and later by Zankel⁽¹⁴⁾ to be typical for O_2 evolving material were notable absent in the dark grown cells or chloroplast particles isolated therefrom. We observed however that photoactivation resulted in appearance of the typical fluorescence oscillations pattern which Delosme has attributed to difference in fluorescence quenching by S states, notably the S_2+S_3 states. If we accept the current general interpretations offered by Delosme⁽¹³⁾, Joliot⁽¹⁵⁾ and Zankel⁽¹⁴⁾ for the oscillatory behavior of fluorescence observed within flashes, we might conclude these observations offer another line of evidence that photoactivation builds the S states within the System II complex. Since this conclusion only corroborated conclusions previously reached by us from O_2 measurements, we did not pursue these studies in great kinetic detail.

In this reporting period, we also began systematic rather than our previous sporadic attempts to obtain procedures allowing photoactivation with "cell-free material" such as chloroplasts and/or permeaplasts^(16, 17) of blue-green algae. Such studies are aimed toward supplementing out kinetic picture of photoactivation by gaining insight into the actual chemical events occurring in the process. Most of these studies have been made with material in which the concentration of O_2 evolving centers has been diminished or eliminated by extraction with NH_2OH (≈ 2 mM/ 10 min). We believe that this procedure for destroying the O_2 evolving mechanism is the mildest and most specific of all such procedures. Moreover, we know⁽¹⁰⁾ that at least in whole cells the destroyed O_2 evolving centers can be entirely remade via photoactivation.

Thus far these studies have been frustrating, yielding only occasional success (5-10% of the attempts). We briefly qualitatively summarize the results thus far from such studies:

1. Occasional successes with isolated chloroplasts have been obtained only with chloroplasts prepared in ways to preserve their outer membranes (chloroplasts capable of CO_2 fixation). Incubation of the initial homogenate (Mn^{+2} of the medium omitted to avoid oxidation of the NH_2OH) with NH_2OH (1 m M) for 10 min before centrifugation sufficed to destroy 90% of O_2 evolution, measured after osmotic shock to allow penetration of Hill reagents (FeCN or viologen) through the outer membrane. Resuspension and incubation of the pelleted chloroplasts in medium containing Mn^{+2} (to oxidize remaining traces of NH_2OH and to serve as substrate for photoactivation) has proven to yield the only material with which we can occasionally obtain significant (3-4 fold increase) cell-free photoactivation. Osmotic shock or repeated washings of such chloroplasts in isolation medium completely abolished their capacity to photoactivate. Presently, we tend to think these results suggest that possibly some required water soluble, low molecular weight substance other than Mn^{+2} is lost during such manipulations.

2. Possibly some support for this tentative conclusion is found in the following observations:

- a) Brief sonication of dark grown *Chlorella* for times (60 sec), insufficient to disintegrate the cells or to affect the benzoquinone Hill reaction of such photo-activated cells, but sufficient to essentially abolish photosynthesis and permit support of Hill reaction by dichlorophenolindophenol, led to loss of capacity to photoactivate.

b) Preincubation of cells with glutaraldehyde⁽¹⁸⁾ or benzoquinone⁽¹⁹⁾, which increase the permeability of algal membranes but do not inhibit the Hill reaction of the cells, caused loss of capacity to photoactivate. This inhibition persisted even after washing of cells to remove these chemicals. Curiously, the glutaraldehyde concentration vs inhibition of photosynthesis and photoactivation capacity were almost identical. We know, however, that photoactivation is independent of CO₂ fixation as judged by the independence of photoactivation on KCN concentrations sufficient to abolish photosynthesis.

Such results, while offering a possible general explanation for our previous use of benzoquinone to quantitate photoactivation, do not give us any critical insight. Both of these chemicals in addition to making the cell membrane leaky also are known to cross-link proteins. Thus, we question whether the data imply that photoactivation requires an unknown freely diffusable substrate in addition to Mn⁺² or whether subtle configurational changes of membrane structures are involved. Presently, our data do not permit discrimination between these or other alternatives.

Charge Recombination in System II

During this reporting period we have broadened an effort aimed towards gaining a better insight into the mechanisms of recombination of charges occurring within the System II traps of DCMU poisoned tissue. Current dogma concerning such events is as follows: 1) illumination of DCMU poisoned System II results in a separation of charge producing an oxidant (Z⁺) of unknown potential and a reductant (Q⁻), presumably of ~0.0 V redox potential; 2) in darkness the separated charges recombine leading to trap recovery; 3) hydroxylamine⁽²⁰⁾ and CCCP⁽²¹⁾ have been reported to inhibit this recombination; 4) the inhibition by NH₂OH has been postulated to result from the reduction of Z⁺ leaving Q⁻ without a charge to recombine with.

A number of observations have led us to doubt this simplistic explanation and have led us to study this "simple" reaction by measuring the dark decay kinetics of the light induced variable fluorescence. These kinetics have been studied as a function of the concentration of O_2 evolving centers, artificial electron donors, and inhibitors of the oxidant side of System II. We varied the concentration of O_2 evolving centers by: 1) heterotrophic growth of *Chlorella* in darkness followed by photoactivation to various extents; 2) extraction with NH_2OH to various extents (algae or chloroplasts) or extraction of algae with NH_2OH to eliminate O_2 centers followed by photoactivation to various extents; 3) autotrophic growth on a Mn deficient medium; and 4) concentrations of certain amines which have proven to be effective reversible inhibitors of O_2 .

Thus far we have obtained the following results:

1. Irrespective of the manner of decreasing the concentration of O_2 evolving centers, we observe that the rate of decay of the light induced fluorescent state (Q^- oxidation) decreases as the concentration of O_2 evolving centers is diminished. In the absence of O_2 evolving centers, the light induced high fluorescent state (trap closed) remains "locked-in" even after ~30 min darkness, but is restored immediately with addition of oxidants of redox potential ≥ 100 mv. In contrast, in normal O_2 evolving tissue, the half-time for return to the open trap state is reached in ~0.3 sec without addition of oxidants.

2. In all instances, the second-order (with respect to Q^-) rate constant for fluorescence decay appears to be linear with respect to the quantum yield for O_2 ; this relationship is maintained as the O_2 center concentration is decreased or increased via photoactivation; or by different concentrations of certain amines

3. The behavior of certain amines such as NH_3 on the decay of Q^- , prompted us to confirm and extend the observations of Izawa et al. ⁽²²⁾ who reported that in strong light NH_3 inhibited H_2O photolysis but not the photooxidation of NH_2OH , an artificial electron donor to System II. We found that the inhibition of O_2

evolution by a series of amines to be independent of intensity. Inhibition of flash yields of O_2 was independent of dark times between flashes, thus the effect of amines is notably different than the effects of CCCP and the anilinothiophenes which apparently inhibit O_2 by increasing rates of deactivation of the S states. ⁽²³⁾

Though the amines abolish O_2 evolution they scarcely (<10%) alter the kinetics of the fluorescence rise associated with either Q or Q+A reduction. Such amines, in contrast to NH_2OH , do not apparently serve as artificial electron donors to System II as judged by their inability to support steady-state photoreduction of DCIP via System II.

Since the Q-A pool represents about 10 equivs per System II trap ⁽²⁴⁾, the results seem to imply that amine inhibited O_2 evolving centers are capable of supplying these 10 equivs without turnover from H_2O photooxidation. Apparently, the concentration of endogenous catalysts on the oxidant side of System II may be as great as 10 per trapping center. If we assume one turnover each of the two cyt_{559} and the 6 Mn per System II trap such a tentative conclusion might not be absurd.

Presently, we have no coherent explanation for the totality of these results on the effect of concentration of O_2 evolving centers on the reoxidation of Q^- of DCMU poisoned System II. We can only speculate that in the presence of DCMU the reoxidation of Q^- occurs via an S state ($S_2?$, $S_3?$) and either not at all or extremely slowly with Z^+ , the primary donor to the System II trap. This tentative conclusion is surprising, especially since the redox potential gap between Z^+ and Q^- is thought to be at least 0.8 V; thus the results might imply a very specific route for recombination of Q^- with the hole(s) on the oxidant side of System II.

Publications During Last Reporting Period

- Doschek, W., and Kok, B., "Photon Trapping in Photosystem II of Photosynthesis, The Fluorescence Rise Curve in the Presence of DCMU," *Biophysical J.* 12, 832-838 (1972).
- Kok, B., "Photoacts and Electron Transport," presented at the VI International Congress on Photobiology, Bochum, Germany, (1972), in press.
- Joliot, P., and Kok, B., "Oxygen Evolution in Photosynthesis," in "Bioenergetics of Photosynthesis," Govindjee, Ed., Academic Press, N.Y., in press.
- Kok, B., "Photosynthesis: The Path of Energy" in: *Plant Biochemistry* (Second Ed.) J. Bonner and J. Varner, Eds, Academic Press, N. Y., in press.
- Radmer, R., and Kok, B., "A Kinetic Analysis of the Oxidizing and Reducing Sides of the O_2 -Evolving System of Photosynthesis," *Biochim. Biophys. Acta.*, in press.
- Cheniae, G., and I. F. Martin, "Absence of Oxygen-Evolving Capacity in Dark-Grown *Chlorella*: The Photoactivation of Oxygen-Evolving Centers", *Biochim. Biophys. Acta*, in press.
- Cheniac, G., and I. F. Martin, "Effects of Hydroxylamine on Photosystem II," *Plant Physiol.* 50, 87-94 (1972).
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