DOE/ER/03326-100

PHOTOCHEMISTRY AND ENZYMOLOGY OF PHOTOSYNTHESIS

PROGRESS REPORT

For Period November 1, 1980 through March 31, 1981

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April 1981

Prepared for

The U. S. Department of Energy, Chicago Operations Office

Under Contract
DE-AC01-76ER03326

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I. MASS SPECTROMETER STUDIES OF PHOTOSYSTEM II

During the past reporting period, we have continued our studies of system II donor reactions, particularly the photooxidation of hydroxylamine and hydrazine. Both of these components are close analogs of H_2O (two molecules), and studies of their system II-mediated photooxidations should provide insight into the mechanisms of the O_2-evolving process.

Earlier [Biochim. Biophys. Acta (1979) 546, 418-425], we described the results of experiments in which we used our mass spectrometric techniques to directly monitor the products generated during the photooxidation of hydroxylamine by isolated chloroplasts. We found that molecular dinitrogen, formed by the combination of two 1-electron oxidation products of hydroxylamine, was the only stable reaction product.

Our more recent studies have focused on the processes involved during the photooxidation of hydrazine. We found that, as in the case of hydroxylamine, molecular dinitrogen is the only stable product of hydrazine photooxidation. However, unlike hydroxylamine (NH_2OH), this photooxidation can also involve a series of secondary reactions of hydrazine (NH_2NH_2) with superoxide (O_2^-); these secondary reactions can be eliminated by the addition of superoxide dismutase.

A manuscript describing this work was recently accepted for publication in Biochimica Biophysica Acta (copy attached). The summary is as follows:

"Mass spectrometric techniques were used to directly monitor the products evolved during the course of hydrazine (NH_2NH_2) photooxidation by chloroplasts exposed to short saturating flashes or continuous high light. We found that:

(1) Molecular N_2 was the sole volatile product of hydrazine photooxidation. Isotopic studies showed that the N-N bond remained intact during the $NH_2NH_2 \rightarrow N_2$ transformation. Under conditions in which spurious side reactions were minimized (see item 3 below), the N_2 yield was equal to the O_2 yield during H_2O photooxidation.

(2) In the presence of 3-(3,4-dichlorophenyl)-1,1-dimethylurea, N_2 was evolved, but only on the first flash, suggesting that N_2 was formed by the combination of single electron oxidation products of hydrazine.

(3) In addition to its production by Photosystem II, N_2 can also be generated by a series of secondary reactions mediated by superoxide. This "extra" N_2 evolution can be eliminated by the addition of superoxide dismutase.

Our results indicate that hydrazine can be used as a reliable probe of Photosystem II provided that a) N_2 evolution (rather than O_2 uptake) is monitored, and b) precautions are taken to minimize spurious side reactions. Under conditions in which the participation of superoxide is minimized, N_2 evolution accurately reflects the photooxidation of hydrazine by Photosystem II."

We are currently studying the photooxidation of low concentrations of hydroxylamine by O_2-evolving chloroplasts*. Earlier studies showed that dark adaptation of chloroplasts in the presence of low concentrations of

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*In the earlier studies described above, the chloroplasts used had lost their O_2-evolving capability due to the high concentrations of donor or prior Tris extraction.

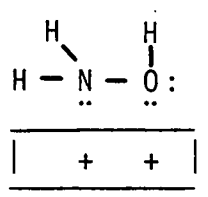
hydroxylamine delayed the evolution of O_2 during a series of flashes (the maximum occurring on the fifth rather than the third flash). These results suggested that two molecules of hydroxylamine were bound to the system II site [B. Bouges-Bocquet, Biochim. Biophys. Acta (1973) 292, 772-785], or that the O_2 system was overreduced to "S_{-1}" [B. Velthuys and B. Kok, Proc. Fourth Int. Cong. Photosynthesis (1977) 397-407].

Recent experiments, in which we directly monitored hydroxylamine photooxidation (as N_2 evolution) in O_2-evolving chloroplasts, showed that hydroxylamine was photooxidized only on the first flash. This finding suggests that one molecule of tightly bound hydroxylamine is oxidized on the first flash, after which O_2 evolution proceeds normally starting from S_0 generated in the dark, i.e.,

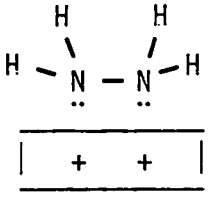
$$S_1 + NH_2OH \longrightarrow S_0 + \tfrac{1}{2} N_2 \text{ (dark reaction)}$$

$$S_0[NH_2OH]_{bound} \xrightarrow{flash} S_0 + \tfrac{1}{2} N_2.$$

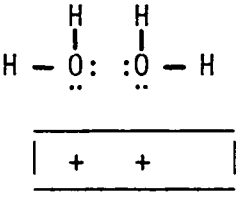
We can draw an intriguing conclusion from this observation: hydroxylamine must bind to the H_2O-oxidation site much more strongly than does H_2O itself, since NH_2OH oxidation occurs at a concentration of 25 μM in the presence of 55 M H_2O. If this inference is correct, it suggests that the H_2O-oxidation site of the chloroplasts has two H_2O binding sites about 1.45 Å apart (the length of the O-N and N-N bonds), i.e.,



(hydroxylamine)



(hydrazine)



(two waters)

The higher binding constant of these artificial donors would then reflect a decreased dissociation probability*; if the dissociation probability for H_2O is p, then that of the artificial donors would be $\sim p^2$. (This hypothesis assumes that the N and O binding constants are roughly equivalent, a supposition supported by the approximately equal degree of binding of NH_2OH and NH_2NH_2 by system II.) We are currently working to clarify other results related to the above experiments.

We have also obtained interesting preliminary data using some substituted NH_2OH and NH_2NH_2 compounds. In these experiments, we determined: 1) the "electrochemical activities" of various substituted donors (by monitoring fluorescence and electron transport in Tris-extracted chloroplasts), and 2) the ability of these same donors to interact with the O_2-evolving system (by measuring the delay in the maximum O_2 flash yield as described above). Some striking differences for a given donor

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*A similar argument has been used to rationalize the stability of chelates.

were observed in the two experiments. For example, the O_2-evolving site of the chloroplasts is virtually inaccessible to disubstituted methylhydrazines [$(CH_3)_2$ NNH_2 and $CH_3HNNHCH_3$] despite their superiority over NH_2NH_2 as electrochemical donors. Similar variations were observed with the hydroxylamines. These results, in conjunction with studies of molecular models, suggest that the O_2-evolving site of the chloroplast is located in a cleft about 4 Å wide.

II. SPECTROPHOTOMETRIC STUDIES: TETRAPHENYLBORON OXIDATION BY PHOTOSYSTEM II

We previously observed that, in the presence of the artificial donor tetraphenylboron (TPB^- *), the enigmatic system II component cyt b 559 underwent extremely rapid redox changes in the light (considerably more rapid than ever seen before). In addition, we found that the reduction of cyt b 559 under the same conditions was accompanied by a new absorbance change, namely a transient bleaching of a carotenoid. Although we did not fully understand our findings, we could draw a few tentative conclusions with reasonable confidence. These experiments were described in a paper, entitled "Carotenoid and Cytochrome b 559 Reactions in Photosystem II in the Presence of Tetraphenylboron," which is in press with FEBS Letters. Our conclusions were: 1) the primary step in the TPB^--induced events is the anionic interaction of TPB^- with an oxidized, positively charged redox carrier at the donor side of system II; and 2) the reduction of cyt b 559 is associated with the

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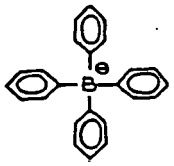
* TPB⁻



2-electron oxidation of TPB^-. However, several features of our measurements, particularly the transient bleaching of carotenoid, were unexplained.

In additional experiments, performed recently, we used another highly lipophilic anion, C-phenyl-1,2-dicarba-undecarborate (PCB^- *), donated by Dr. P. Homann of Florida State University. The lipid solubility of this compound is as large as that of TPB^- (Grinius et al., Biochim. Biophys. Acta (1970) 216, 1-12), and, as shown by Dr. Homann, it affects system II operation strongly without itself acting as a donor [Homann, P. H., in Photosynthetic Oxygen Evolution (Metzner, H., ed.), pp. 195-212, Academic, New York (1978)].

As expected, we found PCB^- to be a very potent catalyst of cyt b 559 oxidation by system II. In fact, it is even more effective than TPB^-. In addition, as with TPB^-, we found a transient carotenoid bleaching in the presence of PCB^- when cyt b 559 was preoxidized. In this case also, lower concentrations of PCB^- were necessary than of TPB^-.

These observations support our earlier conclusion that cyt b

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* PCB^-

and carotenoid oxidation are caused by a coulombic interaction between the added compound and a system II donor, and, unlike cyt b reduction, do not depend on the capacity of the added compound to undergo redox changes. However, some features of the results required clarification. We had speculated that the carotenoid re-reduction, the cytochrome b reduction, and the TPB^- oxidation were caused by one and the same process. If so, a strict, though perhaps complex, kinetic relationship between these reactions should exist. However, we found no support for this theory. Although less cyt b was reduced with PCB^- than with TPB^-, the amount of caroetenoid bleaching was larger with PCB^-. Moreover, this bleaching decayed with similar kinetics under both conditions.

We have concluded that the transiently oxidized carotenoid is not directly involved in cyt b reduction but rather is due to a separate reaction of the system II reaction center. According to this interpretation, system II acts in three competing, alternate ways in the presence of a lipophilic anion: 1) it oxidizes cyt b 559 when this cytochrome is prereduced; 2) when cyt b is preoxidized, it oxidizes the anion (if possible) and also reduces cyt b 559 in the process; or 3) if reactions 1) and 2) are not possible, then it oxidizes a carotenoid.

An unsatisfactory aspect of our current interpretation is that the mechanistic role of the anion in reactions 1) and 2) remains obscure. Understanding this role remains one of the primary objectives of our present studies.

The mechanism of reduction of the carotenoid is also unclear, but the elucidation of this pathway may be less important. If the carotenoid could diffuse away from the photocenter, which is conceivable, it would then be able to react with many different compounds, i.e., it would not be constrained to react with system II components.

Fluorescence measurements by Homann have indicated that PCB^-, like other catalysts of system II deactivation, prevents the reduction of the plastoquinone pool in the light. We have measured plastoquinone directly, and indeed observed a very dramatic inhibitory effect of PCB^- on plastoquinol accumulation. It seems, however, that plastoquinol is not the direct reductant of the carotenoid, because the reoxidation of plastoquinol between flashes is slower than the carotenoid re-reduction. Parallel measurements of P700 during a flash series show a decreased amplitude of re-reduction of P700 between flashes. However, even at saturating concentrations of PCB^-, the supply of electrons, i.e., of plastoquinol, available to system I is still about half that of the control. These data do not confirm the observation of Renger et al. [Biochim. Biophys. Acta (1973) 292, 796-807] that electron flow through system I is (almost) completely suppressed by system II deactivating agents. Our results do support their proposal that the deactivating agents induce an electron transfer shortcut from the system II donor side to the plastoquinone pool, and point to carotenoid as the system II component at which this shortcut starts.

III. BIOCHEMICAL STUDIES: CHLOROPLAST COPPER PROTEINS

In the last Progress Report and Renewal Proposal (August 1980), we reported the isolation and purification of thylakoid-bound polyphenol oxidase from spinach chloroplasts. A paper, entitled "Spinach Thylakoid Polyphenol Oxidase: Activation, Isolation and Properties of the Native Chloroplast Enzyme," has been accepted for publication in Plant Physiology and should appear in the March or April issue.

Because our data now indicate that polyphenol oxidase does not function as an electron transport intermediate in photosynthesis, we are investigating other metal-containing proteins in the electron transport chain. In the last several months, we have been studying the ESR properties of the primary and secondary electron acceptors in system I in collaboration with Dr. Joseph Warden at Rensselaer Polytechnic Institute. In these studies, we have been examining the system I charge separation after the bound iron-sulfur proteins have been oxidatively denatured according to the method of Golbeck et al. [Arch. Biochem. Biophys. (1977) 178, 140-150]. We found the following correlations in various denatured preparations:

1) ESR Centers A and B tend to decline in parallel, although a small amount of ESR Center A is still present even when ESR Center B is completely missing.
2) ESR Signal "X" is absent in the completely denatured particles, lending support to the hypothesis that it contains an iron-sulfur center. Signal "X" also appears less sensitive to

denaturation than do Centers A and B, a phenomenon that is probably due to its location in the particle rather than an inherent stability.

3) A spin-polarized triplet [^{3}P700] which can be observed in the iron-sulfur-depleted particles probably arises from a charge recombination reaction via a radical pair mechanism. The characteristic polarization pattern expected from the recombination reaction between the photoreduced electron acceptor, A_1^-, and the photooxidized primary donor, $P700^+$, was seen. Our investigations indicate that the triplet formulation occurs only after the reduction or destruction of ESR Signal "X" * :

$$\begin{array}{ccccc} A_1^-//\text{"X"}B_A & & A_1//\text{"X"}B_A & & A_1//\text{"X"}B_A \\ \uparrow & \xrightarrow{t_{1/2}\,1ms} & \downarrow & \xrightarrow{t_{1/2}\,30ms} & \\ {}^1P700 & & {}^3P700 & & {}^1P700 \end{array}$$

4) The shape and linewidth of component A_1 in our inactivated preparation is similar to that observed by Heathcote and Evans

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* // indicates a functional separation between A_1 and the secondary electron acceptors, "X," A, and B. Because the order of electron flow between these acceptors is still unresolved, we have not connected them by arrows but rather have shown them in order of increasing redox potential.

[Fifth International Congress on Photosynthesis, Halkidiki, Greece, Abstracts, p. 251 (1980)] but not at all consistent with that published by Baltimore and Malkin [Photochem. Photobiol. (1980) 31, 485-490]. The former authors rendered "X" nonfunctional by chemical reduction while the latter destroyed "X" with SDS; we feel that the detergent technique may cause irreparable damage to the A_1 acceptor system. Due to the difficulty of chemically reducing Signal "X," our "inactivated" particle (devoid of Fe-S centers) will become an excellent starting material for further study of the P700 $\rightleftarrows$ A_1 charge separation/recombination process.

These studies are expected to continue through the summer of 1981. We will continue to investigate A_1 and the process of triplet formation, and will shortly prepare a manuscript for publication detailing these findings.