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Collaborative Research Programme

TERMINAL EVALUATION REPORT

UNIDO contract #: 91/066/CW **ICGEB ref. #:** (CRP/GRE90-01)

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TERMINAL EVALUATION REPORT

Title of Project: Photosynthetic water cleavage and inhibitory effects of herbicides

Keywords: Oxygen evolution; Photosystem II; herbicides.

UNIDO contract #: 91/066/CW **ICGEB ref. #:** (CRP/GRE90-01)
Project initiation: June 1991 **Project termination:** June 1994

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Abstract

The properties of the water photolyzing enzyme and the reducing side of Photosystem II were investigated by using a series of biochemical and biophysical techniques. The location of calcium and its role in the photosynthetic water oxidation was studied by a selective substitution of the cation with various lanthanide ions. Selective substitution of calcium by lanthanides in Photosystem II has shown that there is at least one calcium binding site which is close to $TyrZ^+$. When this site is occupied by a paramagnetic lanthanide ion there is a magnetic interaction between the ion and $TyrZ^+$ (1*). The herbicide binding site of Photosystem II was investigated by using a highly resolved core preparation and it was found that the 22, 10 kDa, CP 26 and CP 29 proteins are not involved in the binding of the extrinsic proteins (33, 23 and 17 kDa) or the sensitivity of Photosystem II to herbicides (2, 3). Finally, the effects of environmental stress on the photosynthetic activity were investigated and it was found that singlet oxygen is involved in the destructive mechanism of photoinhibition (4, 5).

*see publication section for references

OBJECTIVES (proposed at the time of submission of the proposal)

The aim of the project was to investigate the mechanism of photosynthetic water oxidation. More specifically, the elucidation of the properties of the oxidizing and reducing sides of Photosystem II through a series of biochemical and spectroscopic experiments was the main interest of the proposal.
(see also appendix I)

RESULTS

The main results of the project are summarized below:

Calcium binding sites of Photosystem II as probed by lanthanides

The calcium is a cofactor required for oxygen evolution activity in Photosystem II (PSII), the oxygen evolving enzyme of plants. Although various proposals have been made regarding its role, its binding site(s) has(have) not been characterized. Lanthanides and calcium compete for binding site(s) at the oxidized side of PSII. When the trivalent ions of lanthanides (Ln^{3+}) replace calcium, the oxygen evolving complex is unable to proceed to the higher oxidation states. In addition to blocking electron transport from the Manganese-complex to Tyrosine Z^{+}($TyrZ^{+}$), the presence of the lanthanides also affects the electron transport from TyrZ to Chlorophyll P_{680}^{+}. This latter effect depends on the ionic radius of the trivalent lanthanide ions; lanthanide ions with an ionic radius smaller than that of calcium ion (Ca^{2+}), exert a more pronounced inhibitory effect. A low temperture EPR study has demonstrated that the lanthanide ions which occupy the calcium site(s) in PSII interact magnetically with the Tyrosine radical D^{+}. The paramagnetic lanthanide ions also interact with the $TyrZ^{+}$. The properties of the oxygen evolving complex in lanthanide-treated PSII membranes were also studied in collaboration with Dr. Vass at B.R.C.-Hungary, by using the Thermoluminescence technique.

Effect of copper at the oxidizing side of Photosystem II

It has been known that Copper inhibits electron transport in Photosystem II, but the site of inhibition was debated. We have developed a procedure which allows the preparation of PSII which selectively bind copper. Characterization of these samples, which was carried out both in Crete and at B.R.C.-Szeged, demonstrated that one action site of copper is located at the donor side of PSII, most likely at the level of TyrZ (manuscript in preparation).

Characterization of the herbicide binding site of Photosystem II

A highly purified oxygen evolving Photosystem II core complex was isolated from PS II membranes solubilized with the non-ionic detergent *n*-octyl-b-D-thioglucoside. The three extrinsic proteins (33, 23 and 17 kDa) were functionally bound to the PS II core complex. Selective extraction of the 22, 10 kDa, CP 26 and CP 29 proteins demonstrated that these species are not involved in the binding of the extrinsic proteins (33, 23 and 17 kDa) or the DCMU sensitivity of the Photosystem II complex.

Effects of environmental stress on the photosynthetic activity of Photosystem II

Strong illumination of the Photosystem II (PS II) core complex, at 35°C under aerobic conditions, resulted in rapid inactivation of electron transport activity, and pigment photobleaching which was followed by the degradation of the D1 polypeptide. Concomitant with D1 degradation there was a significant disappearance of the 43 and 29 kDa Chl binding proteins and an appearance of high molecular weight species originating from the cross-linking of other PS II proteins with the D1 polypeptide and/or D1 fragments. Strict anaerobic conditions during illumination almost completely prevented pigment photobleaching and protein degradation and subsequent cross-linking. The presence of singlet oxygen scavengers, histidine and rutin, during illumination, significantly protected against photoinduced damages to the PS II complex, suggesting an involvement of singlet oxygen in the destructive process. Singlet oxygen-generating chemicals led to similar pigment bleaching and protein cross-linking. We propose that singlet oxygen, which is generated during aerobic illumination of the PSII complex, is responsible for the photobleaching of photosynthetic pigments, D1 protein degradation and protein cross-linking.

Exposure of a PS II core complex to singlet oxygen, generated either chemically in the dark or through the photodynamic action of rose bengal, caused a rapid degradation of D1 protein. Photoinhibitory illumination of the PS II complex, either at 25°C or at 4°C, resulted in D1 protein fragments similar to those observed upon exposure of the PS II complex to singlet oxygen. Histidine, a singlet oxygen scavenger, provided a significant protection of D1 protein against degradation. We therefore suggest that singlet oxygen, which is generated during acceptor side induced photoinhibition, is responsible for the *in vitro* fragmentation of D1 protein.

WORK PLAN AND TIME SCHEDULE

originally envisaged

(see Appendix I)

actual

The three different tasks and the time schedule which was followed are shown below:

i) Role of inorganic cofactors in Oxygen evolution: 1991-1993
ii) Effect of stress conditions on electron transport: 1992-1994
iii) Role of various proteins in herbicide binding: 1992-1994

The three significant constributions of this particular project are the following (see list of publications):
A. It has provided new information regarding the binding of calcium in Photosystem II.
B. It has demonstrated the involvement of singlet oxygen in the destructive mechanism of photoinhibition.
C. It has provided new information regarding the role of certain proteins at the herbicide binding site of Photosystem II.

This project gave the opportunity to young scientists to be trained in various biochemical and spectroscopic techniques. Dr. Ranjit Mishra spent two years in Crete, working as a postdoctoral fellow. In addition, Nikos Lydakis, a graduate student at the University of Crete spent a month at B.R.C.-Hungary where he carried out thermoluminescence experiments, and Laszlo Sass, a Ph.D. student in Szeged, spent one month in Iraklion where he was trained in various isolation techniques. During the third year of the project, Dr. Imre Vass, the coprincipal investigator from the Szegedd group, spent two weeks in Iraklion performing EPR experiments on photoinhibited samples.

NETWORKING

Throughout this research project the Greek team worked closely with the the group at B.R.C. in Hungary. The exchange of investigators has established an effective collaboration of the two groups, which will continue in the future. The results of common experiments are now under evaluation and will be submitted for publication.

PUBLICATIONS (UNIDO support acknowledged)

1. "Substitution of calcium by lanthanides affects electron transport from Tyrosine *Z* to P680+ in Photosystem II"
Bakou, A. and Ghanotakis, D.F.
Biochim. Biophys. Acta **1141**, 303-308 (1993).

2. "Selective extraction of 22 kDa and 10 kDa polypeptides from Photosystem II without removal of 23 kDa and 17 kDa extrinsic proteins"
Mishra, R. and Ghanotakis, D.F.
Photosynthesis Research **36**, 11-16 (1993)

3. "Selective extraction of CP 26 and CP 29 proteins without affecting the binding of the extrinsic proteins (33, 23 and 17 kDa) and the DCMU sensitivity of a Photosystem II core complex".
Ranjit K. Mishra & Demetrios F. Ghanotakis
Photosynthesis Research, in press (1994).

4. "Destructive role of singlet oxygen during aerobic illumination of the Photosystem II core complex".
Neelam P. Mishra, Christof Francke, Hans J. van Gorkom and Demetrios F. Ghanotakis
Biochim. Biophys. Acta, in press (1994).

5. "Exposure of a Photosystem II complex to chemically generated singlet oxygen results in D1 fragments similar to the ones observed during aerobic photoinhibition"
Neelam P. Mishra and Demetrios F. Ghanotakis
Biochim. Biophys. Acta, in press (1994)

STATEMENT OF EXPENDITURES

Budgets as per original proposal

1) Capital equipment	US$	.....
2) consumables	US$	.....
3) training	US$	.....
4) literature	US$	.....
5) miscellaneous	US$	.....
TOTAL GRANT	US$	.....

Summary of expenditures

1) Capital equipment	US$	15,000
2) consumables	US$	23,000
3) training	US$	*
4) literature	US$	-
5) miscellaneous	US$	14,000
TOTAL GRANT	US$	52,000

*Dr. Mishra, who spent two years in our lab as a postdoctoral fellow, received his salary directly from UNIDO.

APPENDIX I

(proposed work at the time of submission of the proposal)

INTERNATIONAL CENTRE OF GENETIC ENGINEERING AND BIOTECHNOLOGY

COLLABORATIVE RESEARCH PROJECT APPLICATION FORM

Follow instructions carefully

100 PROJECT STATEMENT

101 TITLE : Photosynthetic water cleavage and inhibitory effect of herbicides.

102 SUMMARY:

Using a series of refined Photosystem II preparations we will investigate the mechanism of the photosynthetic cleavage of water and characterize the herbicide binding site. Through a series of biochemical and biophysical experiments we will try to give answers to the following questions:

1. What are the properties of the oxygen evolving complex and which is the role of the inorganic cofactors (Mn, Ca, Cl)?
2. What are the properties of the herbicide binding site in Photosystem II?

The above questions are relevant to two major biotechnological applications: i) Since the end of the petroleum era is at hand, an understanding of the mechanism by which photosynthetic organisms convert solar energy into chemical energy will facilitate our search for alternative sources of energy. ii) A characterization of the herbicide binding site will lead to the design of more effective herbicides and will also help the molecular biologists in their attempt, through genetic engineering, to modify the herbicide binding proteins.

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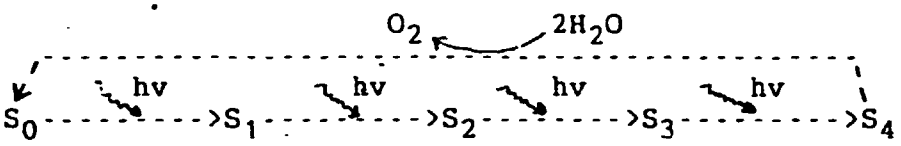
200 PROJECT STATUS

201 Review of Status of Research and Development in the Subject Field

The oxygen evolving center in Photosystem II catalyzes the four electron oxidation of water, forming oxygen and releasing protons in the inner side of the thylakoid membrane. As shown in the scheme below, photochemistry at Photosystem II (PSII) sequentially advances the oxygen evolving center through each state until the S_4 state is formed; the S_4 state decays rapidly to the S_0 state releasing O_2 (Joliot, P. and Kok, B. (1975) In Bioenergetics of Photosynthesis (Govindjee, ed.) Academic Press.)



Competent functioning of this sequence requires manganese, calcium and chloride ligated to an ensemble of polypeptides. Although substantial progress has been made in defining the polypeptide and inorganic cofactor requirements for the photosynthetic oxygen evolution, the mechanism for the sequential reaction and the organization of its constituents still presents a formidable challenge to the investigators working in the field of photosynthesis (for a review see Babcock, G.T. (1987) In New Comprehensive Biochemistry, Vol. 15, Photosynthesis. Amsterdam. Elsevier)

The most highly purified PSII preparations capable of oxygen evolution consist only of the 47, 43, 34 (D_2), 33, 32 (D_1) and the 9+4.5 kDa ($Cytb_{559}$) species; of these polypeptides a complex consisting of the D_1-D_2-$Cytb_{559}$ is believed to contain the binding sites for the photochemically relevant Chls, the Pheos and the primary quinone (Q_A), as well as the herbicide binding site (Ghanotakis et al (1989) Biochim. Biophys. Acta 974, 44-53). This particular model is very attractive because there is a sequence homology between D1 and D2 and between D1 and D2 and the L and M subunits of the bacterial reaction center, and the reported crystal structure of *Viridis* shows that the L and M subunits are involved in binding the primary donor and acceptor (Deisenhofer, J. et al. (1984) J. Mol. Biol. 180, 385-398).

202 Importance of the Project in the Context of Current Status

The proposed research is intended to investigate the mechanism of photosynthetic water oxidation. Manganese has long been known to have an important role in the oxygen evolving center of PSII, but despite intensive research over many years, the structure and function of Mn in PSII, and the involvement of cofactors such as Calcium and Chloride in this structure, remain poorly characterized. The use of highly resolved PSII preparations will allow us to carry out a series of biochemical and spectroscopic studies in order to characterize both the oxidizing (water splitting) and the reducing (herbicide binding) sides of Photosystem II. Such a characterization would help us understand the mechanism by which photosynthetic oxygenic organisms transfer electrons singly and store oxidizing equivalents that can be accumulated to oxidize water.

For the study of the catalytic properties of Photosystem II we are planning to use:
i) Membrane preparations enriched in PSII (Berthold, D. et al. (1981) FEBS Lett. 134, 231-234; Ghanotakis, D. and Babcock, G. (1983) FEBS Lett. 153, 231-234).
ii) A highly active PSII core complex which has been depleted of the main light harvesting complex (LHC) (Ghanotakis, D. et al. (1987) Biochim. Biophys. Acta 891, 15-21).
iii) A stable PSII reaction center complex (Fotinou, C. and Ghanotakis, D. (1989) manuscript in preparation).

Of the above preparations, the intact PSII membranes contain all three extrinsic proteins (17, 23 and 33kDa) and thus the Oxygen Evolving Complex (OEC) is in its native state; on the other hand the PSII core complex, which has lost the 17 and 23 kDa polypeptides and is highly active only in the presence of external Ca^{+2}, shows a 3.7-fold enrichment in manganese content, on a chlorophyll basis, and so is more amenable to spectroscopic studies. The PSII reaction center complex, although not capable of splitting water, contains the primary donor P_{680} and the acceptor Pheo a and undergoes primary photochemistry.

The three focal points of the proposed research are:

A. *CHARACTERIZATION OF THE OXYGEN EVOLVING COMPLEX: THE ROLE OF THE INORGANIC COFACTORS*

There is no controversy over the requirement for manganese ion as a critical cofactor in photosynthetic oxygen evolution. Earlier uncertainties over the number of atoms per oxygen evolving PSII reaction center have been resolved in favor of a stoichiometry of 4 metal atoms per reaction center; this ratio is consistent in unresolved thylakoid membranes and in a variety of purified preparations of photosystem II (Ghanotakis, D. and Yocum, C. (1985) Photosynth. Res. 7, 97-114). At the same time, the sites of manganese ligation and the structure of the tetranuclear assembly are not clearly understood.

Proposals as to the role of selected halides, or nitrate, in activating the oxygen evolving reaction are drawn into two camps. Several groups have suggested that the role of these anions is to act as counterions within the environment of the active site of the oxygen evolving reaction, neutralizing positive charges on amino acid side chains, for example lysine (Homan, P. (1987) J. Bioenerg. Biomembr. 19, 105-123). The alternative hypothesis is that activating anions bind to manganese, acting to facilitate electron transfer among the metal atoms (Sandusky, P. and Yocum, C. (1986) Biochim. Biophys. Acta 849, 85-93).

Calcium ion is required for oxygen evolution activity as evidenced by the need to include the ion in assays of PSII preparations deprived of the extrinsic 23 and 17 kDa proteins (Ghanotakis, D. et al. (1984) FEBS Lett. 170, 169-

173). Of the two extrinsic species, it is the 23 kDa protein which most strongly influences calcium binding. The actual number of calcium atoms minimally required for activity has been set at 2 per reaction center in PSII preparations from wheat; a similar number has been obtained in PSII material from cyanobacteria. At this time, results on the Kd for the calcium binding site in PSII vary. In spinach preparations, a low-affinity site is seen, whereas in wheat, a high affinity binding site has been detected. The use of lanthanides led us to suggest a Con A type structure according to which Ca binds close to the Mn complex and effects its structure. Such a stuctural arrangement seems now very possible because of the recent data of EPR experiments with samples in which Ca^{+2} had been replaced by Sr^{+2}, which demonstrated a change in the Mn complex upon replacement of calcium. Although at this point a structural role for calcium seems very attractive, a direct involvement of the ion in the water splitting mechanism can not be excluded.

The properties of the OEC in intact PSII membranes and the refined PSII core complex will be studied by the following techniques:

I. Oxygen evolution measurements in continuous light by a Clark-type oxygen electrode.

II. Flash-induced oxygen evolution measurements by a Joliot-type electrode.

III. Measurements of thermoluminescence (TL), fluorescence (FL) induction and delayed light (DL) emission in order to probe the charge stabilization and recombination in PSII.

The role of Cl^- and Ca^{+2} will be studied by selective depletion of the ions and replacement by other ions (e.g. F^- for Cl^-, La^{+3} for Ca^{+2} etc.,).

B. *CHARACTERIZATION OF ELECTRON TRANSPORT ACTIVITY IN THE VARIOUS PSII PREPARATIONS*

Fluorescence, thermoluminescence and delayed light measurements will yield useful information about the electron transport activity in the various PSII preparations. Such measurements will help us understand the properties of the various redox components which participate in the electron transfer in PSII, and will also provide new information about the role of the various polypeptides (e.g. the 43 and 47 kDa) in the charge separation process.

C. *CHARACTERIZATION OF THE HERBICIDE BINDING SITE OF PSII*

We are planning to use two different approaches for the characterization of the herbicide binding site. First, we will isolate and characterize the PSII core and the PSII reaction center complex from herbicide resistant higher plants (e.g. Solanum nigrum or Erigeron canadensis). Second, we will substitute the Q_B quinone with other quinones/quinone analogs and will study the effects on the

302 WORK PLAN

In the original hypothesis of Kok, the oxygen yield optimum on the third flash, with a lower yield on the fourth flash, has been proposed to result from an unequal distribution of S-states in the resting, dark-adapted system. The most common estimate for the S_1/S_0 distribution in the dark is 75%/25%. It is now clear that imperfections in electron transfer reactions within PSII influence the apparent distribution of S-states, as deduced from O_2 yield measurements. A thorough study of the period-4 oscillation in oxygen yield from PSII membrane preparations and the refined PSII core complex will help us understand the properties of the Oxygen Evolving Complex and the role of calcium and chloride in the various S-state transitions. Oxygen evolution measurements will be accompanied by thermoluminescence, delayed luminescence and fluorescence induction measurements; such measurements are very useful in studying charge stabilization and recombination in PSII (e.g. charge recombination between the S_2, S_3 states and Q_A^-, Q_B^-). The effect of removal and reconstitution of Q_B on the above measurements in combination with biochemical experiments in which the PSII core and the PSII reaction center complex will be isolated from herbicide resistant plants will reveal new information about the herbicide binding site.

Thermoluminescence (TL) components observed below -20 ^oC most probably originate from charge recombination between donor and acceptor components close to the site of primary charge separation in PSII i.e. Z^+, P_{680}^+ and Q_A^-, $Pheo^-$ (Vass, I. and Inoue, Y. (1986) Photosynth. Res. 10, 431-436). From the study of these TL signals we expect to get information about the functioning and redox state of the involved electron transport components in different preparations (PSII membranes-PSII core-PSII R.C. complex).

303 TIME SCHEDULE

The experiments which have been described in the previous sections will be carried out by two collaborating groups, one in the Institute of Molecular Biology and Biotechnology, at Crete, and the other in the Biological Research Center, at Szeged. Most of the biochemical work will be carried out at Crete, whereas the group at Szeged will perform most of the biophysical studies. Although, during the first year, an emphasis will be given on the characterization of the oxygen evolving complex, we plan to start the part with the herbicides early in the begining of this project.

A potential time schedule is shown below: