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29 JUN 1994

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TELEFAX COVER PAGE

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To:

From: I. Vass

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Date:

cc: M. Protas

Mr. V. Koloskov
Contracts Officer
Contracts Section
General Services Division
Department of Administration
UNIDO
A-1400, Vienna
Austria

FAX: 43-230 8272

June 29, 1994

Dear Mr. Koloskov,

Enclosed please find the Terminal Evaluation Report about our project GE/GLO/89/001 contract No. 91/054/ (CRP/HUN).

The original is sent to you by mail.

Yours sincerely,

Imre Vass

Imre Vass

International Centre for Genetic Engineering and Biotechnology
United Nations Industrial Development Organization

Collaborative Research Programme

TERMINAL EVALUATION REPORT

Part 1

Title of Project

Photosynthetic water cleavage and inhibitory effects of herbicides

Keywords: photosynthesis, oxygen evolution, herbicides

UNIDO contract # 91/054 **ICGEB ref. #:** CRP/ GE/GLO/89/001

Project initiation: 1991 **Project termination:** 1994

Principal Investigator's name: Imre Vass

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Abstract:

Photosystem II is a multisubunit pigment-protein complex embedded in the thylakoid membrane of oxygenic photosynthetic organisms. Its main function is the light-induced oxidation of water to protons and molecular oxygen, as well as the reduction of plastoquinone to plastoquinol. The catalytic site for water cleavage is consisted of a cluster of four protein-bound manganese ions, whose redox functioning is regulated by protein components and further inorganic cofactors. This delicately balanced machinery is very sensitive to various environmental factors which include light (both in the visible and ultraviolet spectral range), heat, cold and heavy metals. PSII is also the site of action for a large number of herbicides used in agriculture.

During the project we concentrated on various aspects of PSII functioning, and obtained the following main results. (i) The redox cycling of the water-oxidizing complex is inhibited between the S2 and S3 oxidation states in the absence of Cl^-, and the stability of a redox-active tyrosine (Tyr-Z), which mediates electron transfer from the water-oxidizing complex to the reaction centre, is largely increased. (ii) The 33 kDa extrinsic protein (*psbO* gene product) stabilizes the optimal conformation of the catalytic Mn cluster, but not absolutely required for water splitting. The 9 kDa phosphoprotein is likely involved in the regulation of electron transfer between the Q_A and Q_B quinone acceptors. The 5 kDa intrinsic protein (*psbK* gene product) has no influence on the redox function of PSII. (iii) Copper has an inhibitory action site at the donor side of PSII. (iv) An important step of visible light induced inhibition of PSII electron transfer is the modification of the Q_A acceptor, which leads to triplet chlorophyll formation in PSII. (v) Highly damaging singlet oxygen and free radicals are formed during photoinhibition by visible light, and are involved in the destruction of protein structure of the PSII reaction centre. (vi) Ultraviolet-B light preferentially inhibits the redox cycling of the water-splitting complex, but damages also the quinone electron acceptors and the redox active tyrosines. (vii) Site specific mutations around the Q_B binding site in the D1 protein (Ser264Ala, Ser264Pro, Ala263Pro, Val219Ile, Ala251Val) were characterized and shown to induce changes in herbicide binding and lead to herbicide resistance.

Part 2

OBJECTIVES/METHODOLOGY
(proposed at the time of the submission of the research proposal)

The oxygen evolving complex of Photosystem II catalyzes the four-electron oxidation of water, forming oxygen and releasing protons in the inner side of the thylakoid membrane. This process requires manganese, calcium and chloride ligated to an ensemble of polypeptides. Although there had been a substantial progress in defining the polypeptide and inorganic cofactor requirements for the photosynthetic water splitting by the time of the submission of our research proposal, the mechanism for the water-splitting reaction and the organization of its constituents was not understood in detail. Light-induced electron transfer from water to plastoquinone is blocked by a number of different chemicals at the binding site of the QB quinone electron acceptor. These chemicals can be used as herbicides and some of them, e.g. atrazine or diuron, has a large agricultural importance. The mode of herbicide action and the mechanism of resistance against various herbicides was an other important topic at the time when our proposal was submitted.

The proposed research was intended to investigate the mechanism of photosynthetic water oxidation, and to get a better understanding of the role of inorganic cofactors and protein constituents in the process. An other aim was to study the properties of the herbicide binding site in PSII. A further major objective of the research was to strengthen the collaboration between the two partners of the project (Dr. Imre Vass in Szeged, Hungary and Dr,. Demetrios Ghanotakis, Iraklion, Crete) by introducing new methods and experimental techniques in each others laboratories.

For the experiments we planned to use and characterize various PSII preparations, which are developed or modified by our collaborating partner in the project, at the University of Crete. These preparations included PSII enriched membranes, a highly active PSII core complex (consisting the D1 and D2 reaction centre proteins, the CP43 and CP47 chlorophyll binding antenna proteins, cytochrome b-559 and the 33 kDa extrinsic protein) and reaction centre complexes (consisting only the D1 and D2 proteins, and cytochrome b-559).

In methodology we planned to apply various optical spectroscopy techniques (thermoluminescence, chlorophyll fluorescence) in combination with EPR spectroscopy, flash-oxygen polarography and protein analysis methods.

The three focal points of the proposed research were:

A, Characterization of the oxygen evolving complex: The role of the inorganic cofactors and polypeptide constituents. Oxygen evolution, thermoluminescence and fluorescence induction measurements were planned in PSII preparations from which chloride or calcium was selectively removed or replaced by other ions, or from which different polypeptides were removed by biochemichal methods.

B, Characterization of electron transport activity in the various PSII preparations. Fluorescence, thermoluminescence and oxygen measurements were planned to obtain information about electron transport activity in the various preparations and about the role of various polypeptides in the charge separation process.

C, Characterization of the herbicide binding site of PSII. Isolation and characterization of core- and reaction centre complexes were planned from herbicide resistant plants by using fluorescence, thermoluminescence and oxygen measurements.

RESULTS
(compare against the set objectives)

When the proposal was submitted, our group in Szeged had mainly biophysical background and instrumentation, with limited experience in biochemical isolation techniques and protein analysis methods. During the project we utilized the collaboration with the biochemically oriented Crete group, and established methods for isolation of PSII core- and reaction centre complexes as well as for gel electrophoresis of PSII polypeptides. On the other hand, we helped the members of the Crete group in applying thermoluminescence to characterize various PSII preparations.

Besides the mutual methodological benefits, the project has yielded a number of important results, most of which are already published in high quality international journals (listed on page 6 and cited below). These results are the following, as arranged according to the three focal points of the planned research:

A, Characterization of the oxygen evolving complex: The role of the inorganic cofactors and polypeptide constituents. (i) Our results show that in the absence of chloride the redox cycling of the water-oxidizing complex is inhibited between the S_2 and S_3 oxidation states, and the stability of Tyr-Z is increased in PSII enriched membranes from spinach [11]. For studying the role of polypeptide components, we used genetically modified cyanobacterium mutants. This way, it was possible to study the role of both extrinsic and transmembrane proteins, which can not be removed by the originally planned biochemical methods. We have shown that (ii) the 33 kDa extrinsic protein (*psbO* gene product) stabilizes the optimal conformation of the catalytic Mn cluster, but not absolutely required for water splitting [1]. (iii) the 9 kDa phosphoprotein (*psbH* gene product) is likely involved in the regulation of electron transfer between the Q_A and Q_B quinone acceptors [3], and (iv) the 5 kDa intrinsic protein component (*psbK* gene product) has no influence on the redox function of PSII [8].

B, Characterization of electron transport activity in the various PSII preparations. The original idea for this point was to perform a general characterization of different PSII preparations in order to get background information for further studies. Besides doing that, we studied the mechanisms of inhibition of oxygen evolution by various environmental factors (heavy metals, visible light, ultraviolet-B light). These studies provide information not only about the way of damaging PSII, but also help to understand the functioning of intact PSII centers.

(i) Copper has been known to inhibit PSII electron transport, but its action site within PSII was debated. The group of Dr. Ghanotakis in Crete has developed a method to produce purified PSII membranes which bound externally added copper, that can subsequently be removed. From the characterization of these samples, which was performed partly in Crete and partly in Szeged, we could show that one action site of copper is located at the donor side of PSII, most likely at the level of Tyr-Z [15]. (ii) One of the environmental factors to which PSII is very sensitive is light itself. By using isolated PSII membranes we showed that an important step of photoinhibition is the modification of the Q_A acceptor, which leads to triplet chlorophyll formation in PSII [4,6]. We have also shown that highly damaging singlet oxygen and free radicals are formed during photoinhibition, and involved in damaging the protein structure of the PSII reaction centre [10,12,13]. (iii) Free radical formation was also demonstrated during heat treatment of thylakoids [2,5]. (iv) Ultraviolet light is an other important damaging factor of PSII. We demonstrated that UV-B light preferentially inhibits the redox cycling of the water-splitting complex, but damages also the quinone electron acceptors and the redox active tyrosines [7,14,16]. We have also identified the site of UV-B induced cleavage of the D1 protein in the middle of the second transmembrane helix [9].

C, Characterization of the herbicide binding site of PSII. This problem was originally planned to be approached by measurements in isolated PSII preparations. In this line we have shown that (i) UV-B irradiation modifies the herbicide binding site, and decreases the binding affinity of diuron [7]. During the project, site directed mutants of green algae and cyanobacteria have also became available for us, with modified aminoacid residues around the QB (herbicide binding) site on the D1 protein, which were utilized for further studies on the herbicide binding site. (ii) Thermoluminescence and flash oxygen characterization of Ser264Ala, Val219Ile and Ala251Val mutants of *Chlamydomonas* reinhardtii indicated a decreased redox potential of QB, and decreased affinity of herbicide binding, and a modification of the donor side components of PSII [manuscript in preparation]. Replacement of Ser264 or Ala263 to Pro in *Synechocystis* 6803 induces also herbicide resistance and indicates that not only Ser264 but Ala263 is an important part of the herbicide binding niche [manuscript in preparation].

Part 3

Work plan and time schedule (originally envisaged)

In the original workplan oxygen evolution, thermoluminescence, fluorescence induction measurements were proposed in various, biochemically modified PSII preparations.

The basic share of work between the Szeged and Iraklion groups was planned in a way that most of the biochemical preparations will take place in Iraklion, while most of the biophysical measurements will be performed in Szeged.

The potential time schedule, proposed at the time of submitting the proposal is shown below.

	1st year	2nd year	3rd year
Oxygen evolution :	<--------->		
Electron transport:		<--------->	
Herbicides		<--------->	

Work plan and time schedule (actual)

Project landmarks, duration of individual tasks (use bar charts); evaluation criteria (publications, patents, services, training)

The actual time schedule followed essentially the one originally planned. There was one important difference in methodology as compared to the proposed one. By the time the project started, and also during the project, a number of very interesting genetically modified algal and cyanobacterial mutants became available for us. These mutants provided an alternative, and very efficient way for modifying the structure of the PSII complex. Thus many of the experiments were performed with the mutants to complement the studies with the biochemically modified PSII preparations. This approach was very important in studying the role of the polypeptide constituents of PSII and mapping the herbicide binding site. This way, the actually performed work proceeded far beyond that planned at the submission of the proposal.

There was an important improvement in the subject of the studies as well. In the original workplan, the main emphasis was put on a general characterization of various PSII preparations, in order to obtain information that can be utilized in later experiments. During the actual work these studies were combined with exploring the damaging mechanisms of very important environmental factors (heavy metals, visible and ultraviolet light) that inhibit the function of PSII and the water-splitting activity and destroy its protein structure. Thus, besides reaching the originally planned aims concerning the characterization of PSII functioning, it became possible to get important information about the inhibition of PSII by the above environmental factors.

The time schedule of work performed by the Szeged group is the following:

	1st year	2nd year	3rd year
Development of biochemical techniques:	<------>		
Role of chloride in oxygen evolution:	<------>		
Role of protein consituents:	<---------->		
Copper inhibition of PSII:	<-------->		
Mechanism of visible photoinhibition:	<---------->		
Mechanism of ultraviolet photoInhibition:		<---------->	
Characterization of herbicide binding:		<---------->	

We consider the project very successful. This is clearly demonstrated by the 14 papers, already published in international journals and books form the results, and 3-4 manuscripts being under preparation. The experience gained by our group in Szeged by establishing a number of biochemical techniques through the collaboration is also considerable, and will have a long lasting impact on our scientific potential.

The work during the project was done mainly in the respective laboratories of the Szeged group. In addition, three successful visits were realized to each others laboratories. In the first year Laszlo Sass, a Ph.D. student from Szeged, spent one month in Iraklion learning isolation techniques. In the second year Nikos Lydakis, a Ph.D. student from Iraklion spent one month in Szeged, performing oxygen and thermoluminescence characterization of a number of PSII preparations from the Iraklion group, and studying the inhibitory effect of copper in PSII. In the third year Dr. Imre Vass, the coprincipal investigator from the Szeged group, spent two weeks in Iraklion performing EPR experiments on the mechanism of UV-B photoinhibition.

NETWORKING

Publications

1. Vass, I., Cook, K.M., Deák, Zs., Mayes, S.R. and Barber, J. (1992) "Thermoluminescence characterization of the IC2 deletion mutant of Synechocystis sp. PCC 6803 lacking the photosystem II 33 kDa protein" Biochim. Biophys. Acta 1102, 195-201
2. Hideg, É. and Vass, I. (1992) "The high temperature thermoluminescence band of green tissues originates in the chemiluminescence of chlorophyll promoted by free radicals" in Research in Photosynthesis (Murata, N. ed.) Vol. III, 107-110, Kluwer, Amsterdam
3. Mayes, S.R., Dubbs, J.M., Vass, I., Hideg, É., Nagy, L. and Barber, J. (1993) "Further characterisation of the *psbH* locus of *Synechocystis* sp. PCC 6803: Inactivation of *psbH* impairs Q_A to Q_B electron transport in Photosystem 2." Biochemistry 32, 1454-1465
4. Vass, I. and Styring, S. (1993) "Characterization of chlorophyll triplet promoting states in photosystem II sequentially induced during photoinhibition" Biochemistry 32, 3334-3341
5. Hideg, É. and Vass, I. (1993) "The 75 °C thermoluminescence band of green tissues: Chemiluminescence from membrane-chlorophyll interaction" Photochem. Photobiol., 58, 280-283
6. Vass, I., Garzen, G. and Holzwarth, A.R. (1993) "Picosecond time-resolved fluorescence studies on photoinhibition and double reduction of Q_A in photosystem II" Biochim. Biophys. Acta, 1183, 388-396
7. Hideg, É., Sass, L., Barbato, R. and Vass, I. (1993) "Inactivation of photosynthetic oxygen evolution by UV-B irradiation. A thermoluminescence study" Photosynth. Res. 38, 455-462

PUBLICATIONS

8. Zhang, Z.-H., Mayes, S.R., Vass, I., Nagy, L. and Barber, J. (1993) "Characterization of the *psbK* locus of *Synechocystis* sp PCC 6803 in terms of photosystem two function" Photosynth. Res. 38, 369-377
9. Friso, G., Spetea, C., Giacometti, G.M., Vass, I. and Barbato, R. (1994) "Degradation of the photosystem II reaction center D1 protein induced by UV-B radiation in isolated thylakoids. Identification and characterization of C- and N- terminal breakdown products" Biochim. Biophys. Acta, 1184, 78-84
10. Hideg, É., Spetea, C. and Vass, I. (1994) "Singlet oxygen production in thylakoid membranes during photoinhibition as detected by EPR spectroscopy" Photosynth. Res. 39, 191-199
11. Deák, Zs., Vass, I. and Styring, S. (1994) "Redox interactions of Tyrosine-D with the S-states of the water-oxidizing complex in intact and chloride-depleted Photosystem II" Biochim. Biophys. Acta 1185, 65-74
12. Hideg, É., Spetea, C. and Vass, I. (1994) "Singlet oxygen and free radical production during acceptor and donor side induced photoinhibition. Studies with spin trapping EPR spectroscopy" Biochim. Biophys. Acta, in the press
13. Hideg, É., Spetea, C. and Vass, I. (1994) "Singlet oxygen and free radical production during acceptor and donor side induced photoinhibition. Evidence from EPR spectroscopy" in. Frontiers of Reactive Oxygen Species in Biology and Medicine (Asada, K. and Yoshikawas, T., eds.), Kluwer Academic Publishers (in the press)
14. Vass, I., Spetea, C. and Petrouleas, V. (1994) "Sequential impairement of photosystem II electron transport components by UV-B radiation. An EPR study" Biochemistry, submitted
15. Lydakis, N., Vass, I., Hideg, É. and Gahnotakis, D. (1994) "Inhibition effects of copper at the donor side of photosystem II" manuscript in preparation

Part 4

STATEMENT OF EXPENDITURES

To be filled by ICGEB		To be filled by the Affiliated Centre	
Budgets as per original proposal		**Summary of expenditures** *	
1) Capital equipment	US$	1) Capital equipment	US$ 10,650
2) consumables	US$	2) consumables	US$ 14,784
3) training	US$	3) training	US$ .....
4) literature	US$	4) literature	US$ .....
5) miscellaneous	US$	5) miscellaneous	US$ 15,842
TOTAL GRANT	US$.....	**TOTAL**	US$ 41,276

Please itemize the following budget categories (if applicable)

Capital equipment

-Personal computer	3,030
-Printer	520
- Photon counter	5,400
-Gel ELFO power sup.	1,400
-Recorder	300

Training (provide names, duration of training, host laboratory)

Literature

* Please do not send invoices, receipts etc.: these should be kept by the Affiliated Centre for future reference and sent to ICGEB upon request

co-principal investigator

June 29, 1994

Managing director