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HISTORICAL MINIREVIEW

Photosynthesis research in Italy: a review

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Abstract This *historical review* was compiled and edited by Giorgio Forti, whereas the other authors of the different sections are listed alphabetically after his name, below the title of the paper; they are also listed in the individual sections. This *review* deals with the research on photosynthesis performed in several Italian laboratories during the last 50 years; it includes research done, in collaboration, at several international laboratories, particularly USA, UK, Switzerland, Hungary, Germany, France, Finland, Denmark, and Austria. Wherever pertinent, references are provided, especially to other historical papers in Govindjee et al. [Govindjee, Beatty JT, Gest H, Allen JF (eds) (2005) Discoveries in Photosynthesis. Springer, Dordrecht]. This paper covers the physical and chemical events starting with the absorption of a quantum of light by a pigment molecule to the conversion of the radiation energy into the stable chemical forms of the reducing power and of ATP. It describes the work done on the structure, function and regulation of the photosynthetic apparatus in higher plants, unicellular algae and in photosynthetic bacteria. Phenomena such as photoinhibition and the protection from it are also included. Research in biophysics of photosynthesis in Padova (Italy) is discussed by G.M. Giacometti and G. Giacometti (2006).

Keywords Antenna structure and function · ATP synthase · Bacterial photosynthesis · Biophysics of photosynthesis · Chemiosmosis ·

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Excitation energy transfer · FNR · International congress on photosynthesis in Stresa · Photoinhibition · Photophosphorylation · Photosynthesis research in Italy · Photosynthetic electron transport · Photosynthetic membranes · Q-cycle · Reaction center structure and function · Respiration · State changes

Abbreviations

AFR	ascorbate free radical
Chl	chlorophyll
DBMIB	2,5-Dibromo-3-methyl-6-isopropyl benzoquinone
DCCD	Dicyclohexyl carbodiimide
DCMU	3-(3,4-Dichlorophenyl)-1,1-dimethylurea
DNP-INT	2,4-(Dinitrophenol)-dinitrophenylether of iodonitrothymol
Fd	ferredoxin
FNR	ferredoxin-NADP$^+$ reductase
HiPIP	high-Potential Iron-sulfur Proteins
LHC	light harvesting complex
NADP	nicotinamide adenine dinucleotide phosphate
NPQ	non-photochemical quenching of the excited state of Chl
PS I	photosystem I
PS II	photosystem II
THC-RC	tetra-heme RC-bound cytochrome

Introduction (by Giorgio Forti)

History is the orderly narration of events, with the aim that ''*the memory of the enterprises of humans are not erased by time*'' (Herodotus, V century B.C.). Herodotus insisted on the necessity of thorough investigation before accepting any ''fact'' as truly documented. In modern times, *Science* requires that the ''facts'' are, or should be, documented ''objectively,'' i.e., by reproducible experiments reported and discussed in papers accepted in peer-reviewed scientific journals. But what can be defined as an ''objective'' description, and, even more difficult, an ''acceptable'' interpretation of experiments? We quote an outstanding ancient historian (Thucidides, V century B.C.): ''*...it has been an arduous undertaking, because the persons present at every event did not report on it in the same way, but everyone according to what he remembered or his personal preferences for one or the other of the conflicting parts''.*

Keeping in mind the ancient historians, we will adopt here the criteria of the critical description and discussion of experiments, documented by the published papers: this is the reason for including a long list of references, which were prepared by the authors of the nine sections of this chapter of history.

The scientists involved in photosynthesis research in Italy are many, and most of their names can be found in the list of references. Extensive collaboration with scientists from many countries is documented in the text and in the references. The authors of the nine sections, except for the first author (GF), are listed in alphabetical order in the authors' list; they are fully responsible for their contributions: their opinions and personal views on historical events may differ on some points, as observed by Thucidides long time ago.

For time-lines of discoveries in anoxygenic photosynthesis and oxygenic photosynthesis, see, in Govindjee et al. (eds) (2005), Gest and Blankenship (pp 51–62), and Govindjee and Krogmann (pp 63–105), respectively. Readers are requested to consult other chapters in Govindjee et al. (2005), which are related to the topics presented here. A limited number of examples of some of these chapters are included in this paper.

Photosynthetic electron transport and photophosphorylation in oxygenic photosynthesis (by Giorgio Forti and Giovanni Finazzi)

The studies on photosynthesis after World War II in Italy were initiated, in the Department of Botany at the University of Milano, by Erasmo Marrè and one of us (Giorgio Forti); the late Sergio Tonzig led the department in those years. Marrè was mostly interested, at that time, in the mechanism of action of auxins at the metabolic level; most of the research in the field of photosynthesis was pursued by Forti, who made it his life-time scientific interest. He spent 2 years (1958–1960) in the laboratory of André T. Jagendorf, at the Johns Hopkins University in Baltimore, MD. There he investigated the effect of ascorbate on the so-called ''endogenous'' photophosphorylation (endogenous because it was observed without the addition of any electron transport carrier or acceptor to the washed chloroplast fragments): the large stimulation observed was connected with the Mehler reaction by the observation that O_2 was required by the electron transport coupled to ATP synthesis. This was the first demonstration that the Mehler reaction is coupled to photophosphorylation (Forti and Jagendorf 1961). [For a historical perspective on 'photophosphorylation', see Jagendorf (pp 561–569) in Govindjee et al. (2005).]

Many years later, after the discovery of ascorbate peroxidase in plants by Asada (1992), Forti and Ehrenheim (1993) showed that the ascorbate free radical (AFR), generated either via oxidation of ascorbate by O_2^- formed at the reducing side of Photosystem I (PS I), or by peroxidation of ascorbate, catalyzed by the peroxidase, is photoreduced by PS I in competition with NADP. The rate of this reaction is about 50% of the maximal rate of NADP reduction and the process shows an Emerson enhancement effect of the same magnitude as NADP reduction. [For the discovery of the Emerson enhancement effect in NADP reduction, see R. Govindjee et al. (1962, 1964).] The overall balance of O_2 changes is zero (Forti and Elli 1995), as it is well known to be the case for the Mehler reaction followed by the combined action of superoxide dismutase and catalase. Forti and Elli (1995) showed that this pathway of electron transport (also called the ascorbate-Mehler reaction) is triggered by the slow electron transport to O_2 of the Mehler reaction, and is coupled with ATP synthesis with the same ATP/2 electron ratio of 1 as for the NADP reduction.

Forti and Gerola (1977) showed the involvement of the water to O_2 electron transport (the Mehler reaction) in steady-state photosynthesis by isolated intact chloroplasts, performing CO_2 assimilation at high rates, to be an essential part of higher plant photosynthesis, in agreement with the earlier results of Egneus et al. (1975). The two laboratories used different methods to estimate O_2 photoreduction: Forti and Gerola measured the initial rate of H_2O_2 formation upon the addition of KCN (an inhibitor of both catalase and ascorbate peroxidase) to the illuminated chloroplasts, while Egneus et al. measured the initial rate of incorporation of $^{18}O_2$ into H_2O during steady-state photosynthesis. The ratios of the rates of O_2 reduction to CO_2 reduction were found to be of 0.25–0.30 in both the laboratories. In the Mehler reaction, the ratio O_2 uptake/electron transported by the linear system through the two Photosystems in series is 0.25, when the ascorbate/AFR turnover, superoxide dismutase, and catalase are inhibited (see line 1 in the scheme in Table 1, calculated from Forti and Elli 1995).

Forti (1996) proposed an overall picture of the continuous alternation of electron transport at the reducing side of PS I to NADP *or* to O_2−AFR (see Table 1) for higher plant photosynthesis. The regulation is affected by the redox state of NADP, because NADPH cannot be reoxidized, if ATP is not produced in the linear electron transport to NADP in the ratio of not less than 1.5/1, required for the operation of the Calvin–Benson cycle. Thus, the electron transport pathways of higher plant photosynthesis, using alternatively NADP or O_2−AFR as the final acceptor at the reducing side of PS I, involves the two Photosystems operating in series; this allows a more substantial utilization of light energy absorbed than would be possible in alternating linear electron transport to NADP and cyclic electron transport around PS I to produce the needed ATP. Indeed, in the latter process the energy absorbed by PS II is totally wasted. This of course does not minimize the importance of Daniel Arnon's discovery of cyclic electron flow and photophosphorylation in isolated chloroplasts (Arnon et al. 1954).

Though cyclic photophosphorylation has been shown to occur in vivo in higher plants, it seems to be limited to particular conditions of stress, in the presence of the PS II inhibitor DCMU (3-(3,4-Dichlorophenyl)-1,1-dimethylurea) (Forti and Parisi 1963), and it has been shown to occur extensively during the dark–light transition, at variance with steady-state photosynthesis (Joliot and Joliot 2005). In cyanobacteria, Myers (1987) found no evidence for cyclic electron transport, and Xu et al. (2005) discovered that a thylakoid protein was putatively required for $P700^+$ reduction in the presence of DCMU and for photoheterotrophic growth in the presence of diuron and glycerol, but not for normal photoautotrophic growth and photosynthetic O_2 evolution, indicating that cyclic electron transport around PS I is not required for steady-state photosynthesis.

On the other hand, a new research line started in the 1990 by Giovanni Finazzi indicated that cyclic electron transport coupled to ATP synthesis might represent a major process for the utilization of light energy in the unicellular green alga *Chlamydomonas reinhardtii.* In

Table 1 Scheme of electron transport in the Mehler-ascorbate reaction

	ΔO_2	O_2/e^-	ATP	ATP/e^-
$2H_2O + 4O_2 \xrightarrow{PSII+PSI} O_2 + 4O_2^- + 4H^+$	−3	−0.75	2	0.5
$4O_2^- + 4AsA + 4H^+ \xrightarrow{spontaneous} 4H_2O_2 + 4AFR$	0.0	0.0	0.0	0.0
$4H_2O_2 + 8AsA \xrightarrow{ApX} 8AFR + 8H_2O$	0.0	0.0	0.0	0.0
$6H_2O + 12AFR \xrightarrow{PSII+PSI} 3O_2 + 12AsA$	+3.0	0.5	6	0.0
Overall balance	0.0	−0.25	8	0.5

(Abbreviations: ApX: ascorbate peroxidase; AFR: ascorbate free radical; AsA: ascorbate)

this alga, onset of cyclic electron flow was suggested by Wollman and co-workers (Vallon et al. 1991) to be regulated by the State 1–State 2 transition. While in plants the occurrence of State transitions involves a limited fraction of the major light-harvesting complex (LHC) of Photosystem II (LHC II), and is supposed to represent a physiological device to allow optimization of light redistribution between Photosystem I and Photosystem II (Allen 1992, see also a later section, by Jennings), this process apparently involves a larger fraction of LHC II in *Chlamydomonas*, up to 85% of the LHC II, according to Delosme et al. (1996). Due to this large redistribution of the LHC II complexes, State transitions in *Chlamydomonas* would hardly be able to fulfill the role of balancing light absorption between the two Photosystems. (For identification of the mobile LHC II polypeptides in *Chlamydomonas*, see Takahashi et al. 2006.) Instead, they would tend to increase PS I performance at the expense of PS II, therefore representing a mechanism to allow the switching on and off of cyclic electron flow around PS I (see e.g., Vallon et al. 1991). This hypothesis was experimentally tested by the application of pump and probe spectroscopy to intact cells of *Chlamydomonas* (Finazzi et al. 1999). When light induced electron injection into cytochrome b_6f complex was probed in cells that were adapted to either State 1 or State 2 in the dark (Wollman and Delepelaire 1984), a differential sensitivity of electron injection into the cytochrome b_6f complex to the addition of the PS II inhibitor DCMU was observed. Consistent with this hypothesis, we found that this inhibitor blocked electron flow to Cytochrome b_6f in State 1 only, suggesting that PS II activity was not required to reduce the plastoquinone (PQ) pool in State 2 (Finazzi et al. 1999). On the other hand, an identical sensitivity to the addition of the cytochrome *b6f* inhibitor DBMIB (2,5-dibromo-3-methyl-6-isopropyl benzoquinone) was observed in both State 1 and State 2 conditions (Finazzi et al. 1999). This result is consistent with the occurrence of a switch between linear and cyclic flow upon State 2 transition.

The strict relationship that exists between State transitions and DCMU sensitivity of electron flow through Cytochrome b_6f complex was confirmed by the analysis of the stt7 mutant of *Chlamydomonas*, which is locked in State 1 because the LHC II kinase is knocked out in this mutant (Fleischmann et al. 1999). In this mutant, electron flow remained sensitive to DCMU inhibition under both State 1 and State 2 promoting conditions (Finazzi et al. 2002). The study of the relationship between State transitions and the occurrence of cyclic electron flow has been extended to conditions approaching the physiological ones (i.e., phototrophic growth under moderate light intensity). Under these conditions, cells appear to be in an intermediate state between State 1 and State 2, and both linear and cyclic flows seem to take place at the same time (Forti et al. 2003). Measurement of thylakoid swelling upon illumination of *Chlamydomonas* mutants devoid of the ATP-synthase CF_0-F_1 (Majeran et al. 2001) indicated that the building of a transthylakoid ΔpH can be inhibited by DCMU addition in State 1, but not in State 2 conditions, consistent with the notion that the ΔpH is built by linear flow in State 1, and by cyclic flow around PS I in State 2 in this alga. Cardol et al. (2003) showed that a reduced metabolic interaction between mitochondria and chloroplasts, which is observed in respiratory mutants of *Chlamydomonas*, promotes a systematic transition to State 2; this results in a reduced oxygen evolution capacity and in an enhanced cyclic flow activity around PS I, as indicated by photoacoustic measurements.

The above observations indicated that, from an energetic point of view, State transitions in *Chlamydomonas* seem to represent a shift from an oxygenic type of photosynthesis (that generates both reducing power and ATP, '*State 1*') to an anoxygenic bacterial one, where only ATP is synthesized ('*State 2*'). This switch may represent an advantage in terms of the capacity of adaptation to environmental changes. By maintaining a high quantum yield of ATP synthesis in State 2, cells might be able to maintain vital processes and therefore face successfully stress conditions, where photosynthetic CO_2 assimilation and respiration are inhibited. Consistently, it had been observed that a systematic transition to State 2 is induced in *Chlamydomonas* under nutrient deprivation conditions (reviewed in Davies and Grossman (1998)), which often lead to a systematic decrease of PS II activity, and therefore of the linear electron flow. Under more physiological conditions, i.e., intermediate conditions between State 1 and State 2, cyclic flow might provide the extra ATP required by the Calvin–Benson–Bassham cycle in excess of that produced by the linear electron transport (Forti et al. 2003). It seems therefore that *Chlamydomonas* is able to match the extent of cyclic flow to the energetic cellular needs by modulating the amplitude of State transitions. This would occur, it seems, due to the sensing of the redox state of the plastoquinone pool, which is expected to become more reduced upon decrease in the CO_2 fixation performance of the chloroplasts. [For a historical perspective on '*state changes*', see Allen (pp 177–186) in Govindjee et al. (2005).]

On the other hand illumination, when electron flow is mainly occurring with cycling, is expected to pro-

mote back electron flow to PS II from reduced quinones. This is expected to increase photoinhibition of PS II (see e.g., Keren and Ohad 1998, for discussion). In this sense, the ''removal'' of the antennae from PS II might represent a useful way to protect it.

Binding of PQH_2 to the quinol-binding site of the cytochrome b_6f complex (Vener et al. 1995; Zito et al. 1999) leads to the activation of the stt7/stn7 kinase responsible for the phosphorylation of LHC II (Allen 1992, Depege et al. 2003, Bellafiore et al. 2005). This is likely to stem from conformational changes occurring in the lumenal portion of the Rieske Iron–sulfur subunit of the Cytochrome b_6f complex upon binding of PQH_2 to its quinol binding pocket (reviewed in Breyton 2000, see also Kurisu et al. (2003) for a discussion). A role of the conformational changes in the activation of the kinase has been proposed in *Chlamydomonas.* This has been done in Milano, by comparing the effect of three well known competitive inhibitors of the Cytochrome b_6f complex: stigmatellin, DBMIB, and DNP-INT (2,4 Dinitrophenol)-dinitrophenylether of iodonitrothymol) (see e.g., Frank and Trebst 1995) on both the catalytic mechanism of the Cytochrome b_6f complex (Barbagallo et al. 2000) and the induction of the State 1–State 2 transition (see e.g., Finazzi et al. 2001). Thus, a number of relevant phenomenological information has emerged that will lead to final understanding of the relationship between reduction of the plastoquinone pool to activation of the kinase.

Figure 1A illustrates a model for the relationship between the State transitions and its relationship with the linear and cyclic electron flow in the green alga *Chlamydomonas reinhardtii*. Fig. 1B shows a 2005 photograph of Giovanni Finazzi. Finazzi, who was associated with Giorgio Forti in the Department of Plant Physiology at the University of Milano from 1989 to 2003, is now a researcher at the Institut de Biologie Physico-Chimique in Paris, France.

Detailed information is not available on the mechanism leading to the State 2–State 1 transition. We all recognize that the oxidation of the plastoquinone pool is an essential step in the recovery of State 1 in State 2-adapted plants or algae (Allen 1992). However, we also recognize that this transition is under the control of the size of the cellular ATP pool. When the ADP/ATP ratio exceeds a certain critical level, cells are locked in State 2 (Bulté et al. 1990). Only when both conditions are fulfilled (i.e., re-oxidation of the PQ pool and enhancement of the ATP/ADP ratio), State 1 can be attained (Bulté et al. 1991). In *Chlamydomonas*, cyclic flow is responsible for the regeneration of a high ATP/ADP ratio upon

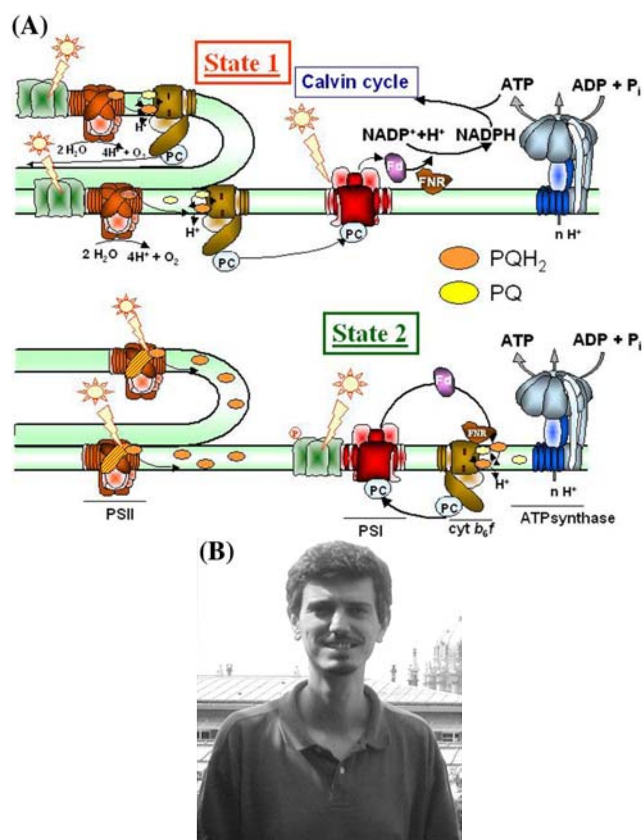


Fig. 1 (**A**) State transitions in *Chlamydomonas reinhardtii*. The physical movement of the light-harvesting complex II (LHC II) and the cytochrome *b6f* complex (Cyt *b6f*) complex, that follows the transition to State 2, modify the diffusion properties of plastoquinone (PQ), thus preventing the functional connection between photosystem II (PS II) and Cyt *b6f*. This promotes cyclic electron flow around photosystem I (PS I), and the over-reduction of the PQ pool connected to PS II. This enhances acceptor side photoinhibition while protecting PS II degradation (dashed subunit) through PQH_2 binding to its quinone-binding site. Calvin cycle should be read as Calvin–Benson–Bassham Cycle (Source: Giorgio Forti and Giovanni Finazzi). (**B**) A 2005 photograph of Giovanni Finazzi

illumination of State 2 cells (Forti et al. 2003). However, this process cannot provide any means to oxidize the plastoquinone pool, since it is also kinetically limited by the rate of plastoquinol oxidation. We have shown that this latter function might be performed by the Mehler reaction in *Chlamydomonas*. By reducing molecular oxygen with electrons coming from PS I photochemistry, this reaction might allow sustained oxidation of the plastoquinone pool, and therefore inactivation of the stt/stn 7 kinase, followed by the transition to State 1 (Forti and Caldiroli 2005).

We acknowledge the contribution of Alberto Vianelli, who measured the changes of PS I activity, at different wavelengths, in isolated spinach thylakoids in States 1 and 2, helping to establish the difference

between higher plants and *Chlamydomonas*' state transitions (Forti and Vianelli 1988). (Dr. Vianelli is now on the Staff of the University of Insubria, Varese, Italy.)

Studies on ferredoxin-NADP$^+$ reductase (FNR) and ferredoxin (Fd) (by Giuliana Zanetti)

In the 1960s, it was shown in the photosynthesis laboratory in Milano that the thylakoid flavoprotein (ferredoxin-NADP$^+$ reductase, FNR), which has the main function of reducing NADP$^+$ in photosynthesis (Shin and Arnon 1965), is also active in the reduction of solubilized cytochrome *f* (Zanetti and Forti 1966), and forms a complex with it during the catalysis (Forti and Sturani 1968); this observation preceded the discovery that FNR is able to associate with the Cytochrome b_6f complex (Zhang et al. 2001). The discovery that this flavoprotein is required for photophosphorylation coupled to cyclic electron transport around PS I was based on the observation that the activity could be titrated with an antibody against pure FNR (Forti and Zanetti 1969; Forti and Rosa 1971).

Zanetti and co-workers have extensively studied the properties of this important electron carrier. A thorough characterization of the spinach leaf enzyme was achieved through kinetic studies with various electron acceptors (Zanetti and Curti 1980), and preparation of the apoprotein and reconstitution with flavin adenine dinucleotide (FAD) or its analogues (Zanetti et al. 1983). Using chemical modification, it was suggested that different residues of the enzyme are involved in the catalytic mechanism: a cysteine (Zanetti and Forti 1969) and a lysine important for NADP$^+$/NADPH binding (Zanetti 1976), later on shown to be Lys 116 (Cidaria et al. 1985). Further, many studies were concerned with the interaction of FNR with the protein substrate ferredoxin (Fd). By using a cross-linking agent, a soluble carbodiimide, a covalent complex between the two proteins was obtained which behaved the same as the natural dissociable complex. Peptide mapping of the cross-linked complex allowed us to define the polypeptide regions of the two proteins which interact in the complex: the polypeptide segment 72–91, containing Lys 85 and 88, was found to be covalently linked to the Fd region 76–97 containing the acidic cluster Glu 92–94 (Zanetti et al. 1988). Studies of limited proteolysis of FNR in the presence and absence of its substrates further identified the 25–35 N-terminal region as part of the binding site for Fd and the 235–250 polypeptide segment for interaction with NADP$^+$/NADPH (Gadda et al. 1990). Thus, the N-terminal moiety of the reductase, where the flavin-binding domain is localized, was shown to be that mainly involved in the binding of Fd.

The powerful technique of chemical cross-linking was used also to study the interaction of purified Fd with PS I particles. A covalent, active complex PS I–Fd was successfully isolated and the subunit PsaD was identified as the main partner for Fd docking to PS I (Zanetti and Merati 1987). Later on, the interaction of Fd in solution with a recombinant form of PsaD was demonstrated (Pandini et al. 1999). All these studies supported the hyphothesis that Fd acts as a shuttle between PS I and the FNR.

A real breakthrough in the structure–function relationship of FNR was the achievement of the recombinant form of the enzyme by A. Aliverti and the fruitful collaboration with the crystallographer P.A. Karplus who had solved the three-dimensional structure of the spinach leaf FNR (see Fig. 2A). A series of studies of selected FNR mutants, designed on the basis of the previous chemical modification results, and of the three-dimensional structure, allowed us to define the roles for specific residues in the reaction mechanism of this essential catalyst of photosynthesis. Thus, Lys116 and Lys 244 (Aliverti et al. 1991), Glu 312 (Aliverti et al. 1998) were demonstrated to be required for NADP$^+$/NADPH binding and Lys 88 (Aliverti et al. 1994) for interaction with Fd. A direct involvement in the catalytic mechanism was then shown for Cys 272 (Aliverti et al. 1993) and Ser 96 (Aliverti et al. 1995). Mutants of the C-terminal tyrosine allowed us to finally have a picture of how the nicotinamide group of NADP$^+$/NADPH could come close to the isoalloxazine ring of FAD in the enzyme's active center for electron transfer to occur (Deng et al. 1999). Furthermore, these mutant proteins showed an impressive increase (up to 1700-fold) in catalytic efficiencies with NADH as compared with wild-type FNR. Thus, the C-terminal tyrosine plays a key role in the discrimination of the pyridine nucleotides by destabilizing the interaction with the nicotinamide. A bipartite binding mode for NADP$^+$/NADPH interaction with FNR was proposed (Piubelli et al. 2000).

Figure 2A shows a three dimensional structure of ferredoxin-NADP reductase (FNR) showing the NADP and the ferredoxin sites, and Fig. 2B shows a 2005 photograph of the photosynthesis research group in the Laboratory of Biochemistry, at the University of Milano.

Spinach leaf ferredoxin I was also produced in a recombinant form and its plasmid has been distributed to many laboratories over the world. Mutants of the

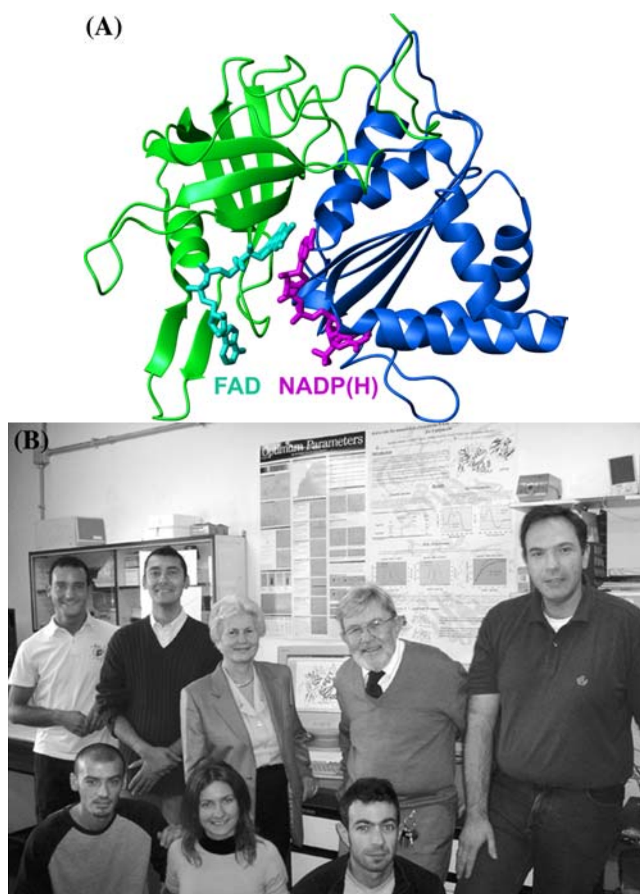


Fig. 2 (A) A three dimensional structure of Ferredoxin-NADP reductase (FNR) showing the NADP and the ferredoxin sites (Source: A Aliverti et al. 2001). (B) A 2005 photograph of members of the Laboratory of Biochemistry at the University of Milano. Back row: from left to right: Andrea Pennati, Vittoria Pandini, Giuliana Zanetti, Bruno Curti and Alessandro Aliverti. Front row: from left to right: Gianluca Caprini, Sara Baroni and Matteo De Rosa

conserved Glu 92 allowed us to define its role in the modulation of the iron-sulfur cluster redox potential as well as in the interaction with FNR (Piubelli et al. 1996) and to obtain the three-dimensional structure of the spinach protein (Binda et al. 1998). By protein engineering, the genes of Fd and the reductase were fused to yield a non-dissociable protein complex, which possesses many of the properties of the physiological one (Aliverti and Zanetti 1997). [For historical perspectives on FNR and ferredoxin, respectively, see Buchanan et al. (pp 859–866) and M. Shin (pp 867–873) in Govindjee et al. (2005).]

Recently, new forms of FNR and Fd have come to the fore: the so-called non-photosynthetic ones. The physiological reactions catalyzed by these FNRs run in the opposite direction with respect to that occurring in photoysnthesis, i.e., electrons are transferred from NADPH to Fd. The structure–function relationship of corn root FNR (Aliverti et al. 2001) was thoroughly evaluated and more recently, involvement in the characterization of the FNR/Fd system present in the apicoplast of the apicomplexan, protozoan parasites (*Toxoplasma gondii* and *Plasmodium falciparum*) (Pandini et al. 2002) is keeping the laboratory busy to find how these redox systems (photosynthetic and non-photosynthetic), that are highly similar to each other, are modulated in the redox potential of their prosthetic groups to accomplish their different metabolic roles.

The Second International Congress on Photosynthesis in Stresa, Italy (by Giorgio Forti)

In June 1971, Giorgio Forti and his associates organized the Second International Congress on Photosynthesis in Stresa, on the Lago Maggiore. (For a list of International Congressses on Photosynthesis, see Appendix F in Govindjee et al. 2005, pp 96–97.) A total of 438 scientists, from all over the world, participated in this Congress. The opening lecture was delivered by Robin Hill (1972), who commemorated Joseph Priestley in the second centenary of his ''Discovery of Photosynthesis''; he discussed Priestley's experiments and their interpretations. The Proceedings of that Congress, edited by Forti, Avron and Melandri, were published in 1972 by Dr W. Junk N.V. Publishers, The Hague.

Mechanisms and regulation of energy transduction and electron transport pathways in bacterial photosynthesis (by Bruno Andrea Melandri, Giovanni Venturoli, and Davide Zannoni)

Studies on the ATP synthase of purple photosynthetic bacteria

The research activities in this field began in 1970 when the existence of F_1 in *Rhodobacter (Rb) capsulatus* was firmly established (Baccarini-Melandri et al. 1970; Melandri et al. 1970). This discovery, made when the authors were working at the University of Indiana, Bloomington, represents the first demonstration that photosynthetic phosphorylation in bacteria is catalyzed by an enzyme homologous to that previously demonstrated in chloroplasts, and was also the first demonstration of the presence of F-type ATP synthases in prokaryotes (Baccarini-Melandri and Melandri 1972). Subsequently, the presence of this enzyme was demonstrated also in *Rhodospirillum rubrum* (Johansson et al. 1973). More importantly the same enzyme was shown to be active in the respiratory ATP synthesis of

Rb. capsulatus, thereby unifying the enzymology of ATP synthesis in the dual functional membrane of facultative photosynthetic prokaryotes (Melandri et al. 1971).

The functional properties of the ATP synthase were further characterized with the demonstration that the ATPase activity was strongly stimulated by illumination (Melandri et al. 1972) and by inorganic phosphate (Baccarini-Melandri et al. 1975), pioneering the work on the activation of the enzyme by $\Delta\mu_{\mathrm{H}}^{+}$ in prokaryotic ATP synthases (Turina et al. 1992). Further, Turina et al. (2004) have discovered subtle regulatory mechanisms on the proton translocation efficiency operating on the enzyme by ADP at submicromolar concentrations and by phosphate at submillimolar concentrations.

The ATP synthase of *Rb. capsulatus* has been chosen as a model for functional studies on prokaryotic ATP synthases, since its association with a photosynthetic cyclic electron transport system allows experiments with excellent time resolution, unattainable in respiratory systems. The complete sequence of the two operons coding for the enzyme in *Rb. capsulatus* B100 are available (Borghese et al. 1998a, b) and genetic techniques for site-specific mutagenesis have been set up (Turina and Melandri, 2002). These studies have lead to the production of a number of site- specific mutants and, for some of these, to the functional characterization of their phenotypes (Turina and Melandri 2002). [For a historical perspective on ATP Synthase, see W Junge (pp 571–595) in Govindjee et al. (2005).]

Relationship between photosynthetic and respiratory chains in *Rhodobacter capsulatus*

The study of the respiratory chain in *Rb. capsulatus* was pioneered in Bologna, partly in collaboration with Antony Crofts' laboratory, then in Bristol, UK, during the 1970s; these studies established the branched nature of the chain (Zannoni et al. 1976a), and the involvement in the two branches, respectively, of a cytochrome c_2 oxidase and of the UQH_2 (Ubiquinol) oxidase (Zannoni et al. 1976b). For these studies, the availability of mutant strains with lesions in different points of the respiratory chain, isolated by Barry Marrs (St. Louis University, USA) was essential. Further, these studies led also to the discovery that the cytochrome c_2 oxidase was associated with a cytochrome *b*-type heme (Zannoni et al. 1974) (today, the oxidase is known to be a cbb_3 oxidase) and that also the quinol oxidase contained a CO-inhibited cytochrome *b* (Zannoni et al. 1976b) (today referred as a bb_3 quinol oxidase). The sites of oxidative phosphorylation were also localized utilizing the then recently introduced technique of the quenching of fluorescent acridines (Baccarini-Melandri et al. 1973). The coincidence of several components of the photosynthetic and of the respiratory chain led to a scheme in which the quinone pool, the cytochrome bc_1 complex and the cytochrome c_2 pool are shared by the two electron transfer systems, whereas the NADH dehydrogenase complex and the two oxidases operate uniquely in respiration. The role of the photosynthetic Reaction Center (PRC) therefore is that of reversing the central sector of the respiration pathway from cytochrome c_2 to ubiquinone. The concept that quinone molecules directly connect respiratory and photosynthetic components in membranes from facultative phototrophs was further supported by studies of Davide Zannoni in collaboration with Barry Marrs, indicating that a strong quinol oxidase activity (Q-bb_3 pathway) was stimulated by light (LIOU-reaction or light-induced oxygen uptake) also in the absence of a functional cytochrome bc_1 complex (Zannoni et al. 1978). This overall scheme is, of course, in full agreement with the double role of the ATP synthetase in the respiratory and photosynthetic ATP synthesis (see above).

Membrane-anchored Cytochrome *c* (cy) and high-potential iron–sulfur proteins (HiPIP) as electron carriers in bacterial photosynthesis and respiration

Prior to the mid-1980s, models of bacterial photosynthetic (Ps) and respiratory (Res) electron transfer chains (ETC) always involved a soluble, freely diffusible protein, called cytochrome c_2 (Prince et al. 1975). Consistent with this dogma, a cytochrome *c* minus mutant of *Rhodobacter capsulatus* (MT113), isolated by Zannoni et al. (1980), was unable to grow, using only photosynthetic electron transport. However, our grasp of the actual role of cytochrome c_2 in photosynthesis had to await the availability of a mutant lacking only cytochrome c_2. Indeed, subsequent studies by Davidson and collaborators in Philadelphia, USA (Davidson et al. 1987) demonstrated that mutant MT113 lacked not only cytochrome c_2 but also the Cytochrome bc_1 complex including its cytochrome c_1 subunit. Thus the lack of photosynthesis (Ps) could not be attributed solely to the absence of cytochrome c_2. It is noteworthy that already in 1986, Fevzi Daldal, in collaboration with Roger Prince, demonstrated that a mutant, MT-G4/S4 that lacked only cytochrome c_2 was Ps^+ like its wild-type parent. This showed, for the first time, that bacterial photosynthesis in the absence of cyt c_2 is possible. Despite this progress, the molecular nature of

the critical component(s) of the cytochrome c_2-independent Ps growth remained obscure until 1990. Indeed, an important finding came from the work of J. (Baz) Jackson and his colleagues in Birmingham, UK (Jones et al. 1990). They discovered in a double mutant (constructed by F. Daldal), lacking both cytochrome c_2 and the Cytochrome bc_1 complex, photo-oxidation of a membrane-associated cytochrome *c* (which they called cyt c_x), and proposed it to be the electron donor to PRC alternative to cytochrome c_2. Resolution of this intriguing story awaited the discovery of the structural gene (*cycY*) of the membrane-associated cytochrome *c* (re-named cyt c_y) by F. Daldal and co-workers in Philadelphia and the demonstration by Zannoni and Venturoli, in collaboration with F. Daldal (Zannoni et al. 1992), that cytochrome c_y steady-state amount depended on the composition of the growth medium used. Finding cycY ended all controversies on the molecular nature of cytochrome c_y and focused the attention on the question of whether cytochrome c_2-independent pathways existed and to how such pathways operated. Zannoni in Bologna, in collaboration with the Daldal's group, undertook a series of detailed studies indicating that in *Rb. capsulatus*, unlike *Rb. sphaeroides*, two independent electron carriers with distinct properties operate simultaneously. The membrane-attached cytochrome c_y communicates with only a subset of Reaction Centers in *Rb. capsulatus* while it is not involved in Ps electron transport (ET) in *Rb. sphaeroides* although working efficiently in respiratory ET of both bacterial species (Myllykallio et al. 1999; see review by Daldal et al. 2001).

Ironically, after almost three decades, it became clear in the mid-1990s that high-potential iron–sulfur proteins (HiPIP) are the soluble electron carriers of choice in most photosynthetic bacteria. The first functional evidence of the role of HiPIP in bacterial photosynthesis was published in 1995 by Zannoni and collaborators (Hochkoeppler et al. 1995). Our research group in Bologna demonstrated that the rate of the light-induced oxygen uptake (LIOU) in membranes from *Rhodoferax fermentans*, a phototroph lacking cytochrome c_2 but expressing high amounts of HiPIP, was concentration-dependent on the HiPIP ($E^{\circ\prime}$ = +351 mV), isolated from the same microrganism. Subsequent studies by the Bologna research group supported and extended the previous report on the involvement of HiPIP in the photosynthetic electron transfer. In particular, kinetic evidence was provided, for the first time, that HiPIP rapidly re-reduces the Reaction Center (RC) following a single turnover excitation flash identifying the tetra-heme cytochrome RC-bound (THC-RC), cytochrome c556, as the electron acceptor (Hochkoeppler et al. 1995). The conclusive evidence that HiPIP was indeed the direct electron donor to the THC-RC of *R. fermentans* was reported in 1996 by our research group in collaboration with a group at the University of Arizona in Tucson (Hochkoeppler et al. 1996). Laser excitation of P to P*, and the subsequent oxidation to P^+, caused a rapid photo-oxidation of cytochrome c556 f; this was followed, in the presence of HiPIP, by a slower re-reduction of cytochrome c556 with a second-order rate constant of $4.8\times10^7\ M^{-1}\ s^{-1}$. The amplitude of this slower process was shown to decrease with increasing HiPIP concentration, with the amplitude of a faster phase ($3\times10^5\ s^{-1}$) increasing concomitantly and independently of the HiPIP concentration (a first-order process). These results were interpreted as evidence for the formation of a complex between HiPIP and THC-RC (K_d = 2.5 μM) prior to the excitation flash. Thus the multiphasic kinetics, observed for the reaction between soluble HiPIP and the THC-RC in *Rf. fermentans*, turned out to be similar to that of soluble cytochrome c_2 with the RCs from *Rb. sphaeroides* and *Rb. capsulatus*: the circle was closed. [For historical perspectives on electron transfer proteins and on membrane-anchored cytochrome *c*, see Meyer and Cusanovich (pp 455–470) and Daldal et al. (pp 471–478), respectively, in Govindjee et al. (2005).]

Chemiosmotic circuits in bacterial chromatophores

Over the period from the 1970s to the early 1990s systematic studies on chemiosmotic coupling to phosphorylation in chromatophores from *Rb. capsulatus*, which in many ways is an ideal model system, were performed. Bacterial 'chromatophores' from *Rb. capsulatus* possess several characteristics that make them an ideal model system for the study of chemiosmotic coupling in photophosphorylation with an excellent time resolution (Jackson et al. 1980). Due to the inverse polarity of the membrane with respect to its orientation in whole cells (Prince et al. 1975), the light-induced proton pumping is inwardly directed, producing an acidic lumen of the chromatophores (ΔpH) and a positive inside membrane potential difference ($\Delta\psi$). The extent of these two forces was continuously monitored, using 9-amino-acridine for ΔpH (Casadio et al. 1974), and the electrochromic spectral shift of endogenous carotenoids (for $\Delta\psi$). The responses of these two indicators are fast enough for following in real time the transients of $\Delta\mu_H^+$ (Turina et al. 1990), and have been conveniently calibrated by Baccarini-Melandri et al. (1977), Venturoli et al. (1986a) and Casadio (1991).

Systematic studies on chemiosmotic coupling were performed: the ''static head'' concentrations of substrates and products of phosphorylation were measured while the extent of steady-state $\Delta\mu_{\rm H}^{+}$ was varied by the addition of small concentrations of uncouplers or of electron transfer inhibitors. The results obtained were compatible with a H^+/ATP ratio in the range of 2–3 (Baccarini-Melandri et al. 1977). The rates of photophosphorylation were also measured under the same varied conditions and the results obtained were surprising: the rates of ATP synthesis appeared more related to the rates of electron transfer than to the extent of $\Delta\mu_{\rm H}^{+}$ (Baccarini-Melandri et al. 1977; Casadio et al. 1978), an observation hardly compatible with a totally delocalized chemiosmotic coupling (Westerhoff et al. 1984). Ensuing researches indicated that [ATP]/[ADP] [Pi] ratio was a decisive factor affecting the onset rate of photophosphorylation and its pre-steady-state kinetics (Melandri et al. 1980), and that effects of the titration of the electron transport chain with antimycin A or of the ATP synthase with DCCD (dicyclohexyl carbodiimide) suggested localized coupling (Venturoli and Melandri 1982). Subsequent studies, however, performed with 'chromatophores' from cells grown at very high light intensity (and therefore containing high concentrations of ATP synthases with respect to the photosynthetic antenna size), indicated delocalized coupling when the ATP synthase was titrated with DCCD (Virgili et al. 1986). A model in which the coupling unit for photophosphorylation is the entire vesicle, under certain growth conditions, can reconcile these apparently conflicting results. This aspect is being investigated at the moment.

Q-pool function and Q-cycle mechanism in 'chromatophores'

Starting from the initial paper (Baccarini-Melandri and Melandri 1977), the relevance of the Q-pool for electron transport has been extensively studied over the past 30 years. Chromatophores contain a large pool of ubiquinone, in a 30/60-fold molar excess over the Reaction Centers. The relevance of this pool for the electron transfer reactions has been the topic of extensive studies (Casadio et al. 1984). The optimal redox poise of the quinone pool, clamped at set redox potentials by the use of redox mediators and small addition of oxidants or reductants, was found to be around 100 mV for photophosphorylation (Baccarini-Melandri et al. 1979). Reversible extraction of ubiquinone from lyophilized 'chromatophores' and their reconstitution with ubiquinone-10, or with its lower molecular weight homologues, indicated the need of a large pool for an effective electron transfer rate and the relative unimportance of the length of polyprenyl lateral chain (for *n* (= or) >3) (Baccarini-Melandri et al. 1980). [For a personal historical perspective on the ''Q-cycle', see Crofts (pp 479–499) in Govindjee et al. (2005).]

Extraction and reconstitution of UQ-10 was also utilized for studying the kinetic behavior of the quinone pool at the Q_o and at the Q_i sites of the cytochrome bc_1 complex. The size of the pool was found to affect the optimal redox poise for cytochrome *b* reduction in the presence of antimycin, a reaction occurring through the Q_o site, and the duration of the lag between the firing of the flash and the onset of cytochrome *b* reduction (Venturoli et al. 1986b). Reactions at the Q_i site were followed indirectly by measuring the associated electrochromic signal as a function of the redox potential and in differently extracted chromatophores (Venturoli et al. 1988). The results demonstrated that the reaction rates depended on the concentrations of quinol or of quinone, respectively at the quinol-oxidizing (Q_o) or at the quinone-reducing (Q_i) site. These data offer a clear kinetic evidence for a Q-cycle operating in 'chromatophores', in which ubiquinone species act both upstream and downstream the cytochrome bc_1 complex.

The behavior of ubiquinone-10 was also studied in reconstituted systems. Purified complexes (Gabellini et al. 1982) and ubiquinone were incorporated into phospholipid liposomes containing cytochrome c_2 and the kinetics of electron transfer were studied, obtaining information on the pool behavior and on the exchange kinetics of cytochrome c_2 in its interaction with the Reaction Center (Venturoli et al. 1989a, b). These studies were extended to the successful reconstitution of photophosphorylation, obtained when purified ATP synthase was inserted into the lipid bilayer in addition to the electron transfer chain components (Gabellini et al. 1989).

In 1990, the level of reduction of the Q-pool of *Rhodobacter capsulatus* was determined voltametrically, for the very first time, by Zannoni in collaboration with Moore in Brighton, UK (Zannoni and Moore 1990), using a glassy carbon working electrode and a platinum electrode. The results obtained with this non-invasive technique indicated that unlike the cytochrome bc_1 complex, through which electron transport appears to be linearly related to the Q-pool redox state (between 4–5% and 30% of the quinol pool, i.e., reduced quinone, Qr), net flux through the Qr-oxidizing step of the alternative pathway is strongly limited until the Qr pool reaches ~25%. Based on a Q-pool

containing ~60 quinones per Reaction Center (RC), it was concluded that the K_m of QH_2 at the Q_o site is ~2.4–3 Q per RC while at the Q_o site (Q-bb_3 oxidase pathway) the K_m of QH_2 is ~15 Q per RC.

Subsequent studies were aimed at elucidating thermodynamic and mechanistic aspects of the interaction of the Reaction Center (RC) with its diffusive electron transfer partners, i.e., quinone at the Q_B acceptor site and cytochrome c_2. In collaboration with P. Mathis in Saclay, France, the docking of cytochrome c_2 and the electron transfer to the primary photooxidized donor P^+, within the preformed RC–cyt c_2 complex, were kinetically characterized, both in detergent suspensions and in proteoliposomes. This last intracomplex process was shown to involve a substantial structural re-organization, being totally impaired at 230 K (Venturoli et al. 1993). The reorganization energy involved in cytochrome c_2 oxidation could be determined by studying the reaction kinetics in a series of specifically mutated Reaction Centers characterized by altered midpoint redox potentials of the P^+/P couple. This analysis also showed that the semi-classical Marcus equation fully accounts for the temperature and free energy dependence of the kinetics, restricting the range of coupled vibrational modes to frequencies lower than 200 cm^{-1} (Venturoli et al. 1998). Information on the interaction of the ubiquinone pool with the Q_B site was obtained by studying the recombination kinetics of the flash-induced $P^+Q_B^-$ state in detergent suspensions and in different reconstituted systems, i.e., reverse micelles of phospholipids in hexane (Mallardi et al. 1997; Palazzo et al. 2000a) and lecithin vesicle (Ambrosone et al. 2002; Palazzo et al. 2000b). These comparative analyses, in which the dependence of the recombination kinetics upon the size of the quinone pool and upon temperature were simultaneously examined, allowed proper separation of the thermodynamic parameters governing the binding at Q_B and electron transfer from reduced Q_A. They clearly showed that in reverse micellar solutions, as well as in liposomes, quinone molecules are in fast exchange with the Q_B site on the time scale of electron transfer. A new model was developed which accounted for the non-exponential character of the recombination kinetics by considering the distribution of ubiquinone molecules in the vesicle populations and the size polidispersity of proteoliposomes, as probed by quasi-elastic light-scattering (Ambrosone et al. 2002; Palazzo et al. 2000b).

The operon in *Rb. sphaeroides* coding for the L and M subunits of the Reaction Center and the α and β subunits of the internal antenna system (LH I) contains an additional gene (*puf*X) whose deletion impairs the capacity of photosynthetic growth. The role of PufX in the photosynthetic electron transport chain was studied in details. PufX is associated with the Reaction Center–LHI complex in a stoichiometry of 1:1 (Francia et al. 1999). The PufX protein was demonstrated to have a role in facilitating the exchange reaction between the Reaction Center and the cytochrome bc_1 complex. In 'chromatophores' from cells carrying a deletion of pufX (Barz et al. 1995a, 1995b), or even truncation of its C-terminal sequence (residues 68–82) (Francia et al. 2002), the lag period encompassing the production of ubiquinol at the Q_B site of the Reaction Center and its oxidation at the Q_o site of the cytochrome bc_1 complex was markedly prolonged as compared to the wild-type (from ≤1 ms to 5–10 ms). A possible explanation of this phenotype, when the structural effects of PufX were demonstrated, was: PufX is necessary to form an S-shaped dimeric structure of the LHI–RC complex, a structure that allows a passage for fast diffusion of reduced and oxidized ubiquinone in exchange with the quinone pool (Scheuring et al. 2004). Consistent with this model, the effects of PufX deletion were alleviated, and ultimately eliminated, when the quinone pool was maintained largely reduced, both in vitro and in vivo. Surprisingly, the LHI–RC preparations, as isolated by sucrose gradient centrifugation, contained an amount of ubiquinone much larger than the average content in un-fractionated membranes (Francia et al. 2004a); this observation indicates the possibility that ubiquinone could be associated *in vivo* to specific protein complexes (in this case, the LHI–RC complex), casting some doubt on the accuracy of kinetic models assuming a totally delocalized diffusional behavior of the quinone pool.

Dynamics of the Reaction Center protein in trehalose glassy matrices

A new research program was initiated in 2002 in which RC protein dynamics were studied in trehalose glassy matrices. Trehalose, a non-reducing disaccharide of glucose, is found in large amounts in organisms (like yeast cells, soil-dwelling animals, and desert resurrection plants) that can survive extreme conditions (such as high temperatures (>60°C) and lack of water) in a state of suspended animation called anhydrobiosis. This property has been related to the extraordinary efficacy of dehydrated trehalose matrices in preserving biological structures (isolated proteins, membranes and tissues). Several experimental and simulative studies, mostly performed on myoglobin containing CO, have unambiguously shown that incorporation of a soluble protein into extremely dehydrated trehalose

glassy matrices severely inhibits internal protein dynamics at room temperature. Starting from these observations, we have shown that bacterial photosynthetic RCs can be functionally incorporated into trehalose matrices and that trehalose coating can be used to condition protein dynamics coupled to electron transfer in this large integral membrane protein (Palazzo et al. 2002). In extensively dehydrated trehalose matrices, RC relaxation from the dark-adapted to the light-adapted conformation and inter-conversion between conformational substates could be blocked at room temperature over the time scale of hundreds of milliseconds: the impairment was reflected in inhomogeneous kinetics of the $P^{+}Q_{A}^{-}$ charge recombination (Francia et al. 2004b). By exploiting a similar strategy we have shown that Q_{A}^{-}-to-Q_{B} electron transfer can be slowed down by orders of magnitude at room temperature when the water content of the trehalose matrix is decreased over a limited range. These findings were consistent with conformational gating of the reaction and demonstrated that the conformational dynamics controlling electron transfer to Q_{B} was strongly dependent on the structure and dynamics of the surrounding medium (Francia et al. 2003). As a whole, it appeared that incorporation into dried trehalose matrices mimics, at room temperature, several effects observed on electron transfer at cryogenic temperatures, when the protein dynamics is frozen. This approach has allowed us to uncouple temperature and solvent effects on electron transfer, yielding direct information on the dynamics of solvent–protein interactions (Cordone et al. 2005). The extraordinary stability at room temperature of RCs, when incorporated into trehalose-dehydrated glasses, could be of great relevance for biotechnological applications.

Figure 3 shows a 2005 photograph of some of the members of the photosynthesis research group in Bologna.

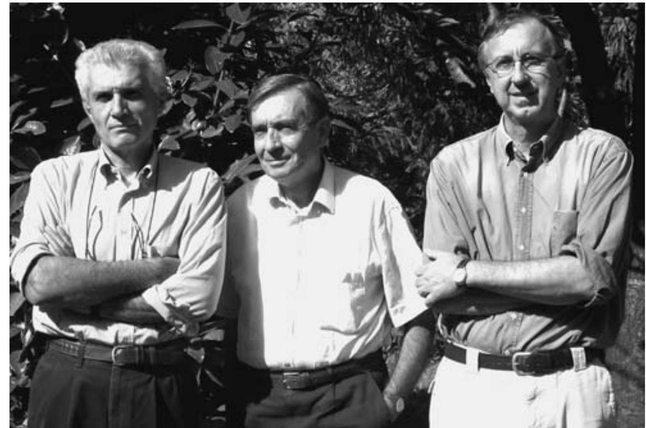


Fig. 3 A 2005 photograph of some of the staff members of the research group in Bologna. From left to right: Davide Zannoni, Andrea Melandri and Giovanni Venturoli. Photo taken on September 29, 2005

Radiation absorption, transfer of excitation energy and primary photochemical reactions: structure and function of the antennae and Reaction Centers of the two photosystems (by Robert C. Jennings, Flavio M. Garlaschi, and Gìuseppe Zucchelli)

Robert Jennings arrived in Italy in the December of 1972, fresh from a postdoctoral period in Itzhak Ohad's laboratory in Jerusalem, Israel, to take up a postdoctoral fellowship in Giorgio Forti's laboratory in Naples, Italy. This was the beginning of a long and fruitful association between Jennings and the Italian photosynthesis researchers. During 1973–1977, some of the first chlorophyll (Chl)*a* fluorescence induction experiments in Europe were performed with research directed towards an understanding of cation effects on the organization of the two photosystems in membranes and in particular on the ''spillover'' of excitation energy among the two photosystems. In 1974, an early study on cation effects on Chl fluorescence induction was published (Jennings and Forti 1974) and it was subsequently demonstrated, by measuring fluorescence induction at 682 nm, with selective excitation into the long wavelength band of Photosystem I at 722 nm, that an ''uphill'' energy transfer from Photosystem I to Photosystem II may occur (Jennings and Forti 1975). This was the first demonstration of thermally activated energy transfer in plant photosystems. (For a detailed discussion on Chl fluorescence as a probe of photosynthesis, see Papageorgiou and Govindjee 2004.)

Gotthard Krause (in Germany) had, at that time, discovered what is now known as ''non-photochemical quenching'' in isolated, intact chloroplasts and this was further studied in the Italian laboratory where it was demonstrated that the phenomenon almost exclusively affects the variable fluorescence of Photosystem II, with little or no effect on the minimum fluorescence level Fo (Jennings et al. 1976; also see Papageorgiou and Govindjee 2004). An early modeling study on fluorescence induction was also presented in which it was proposed that Mg^{2+} ions promote energy transfer between the Reaction Center and the main light-harvesting (antenna) complex of Photosystem II, LHC II. This investigation heralded the beginning of an almost lifelong collaboration between Robert Jennings and Flavio Garlaschi, which continues to the present day. The experimental demonstration that electrostatic screening by cations, including protons, promotes en-

ergy coupling between the Reaction Center and LHC II had to wait another 12 years (Zucchelli et al. 1988) and the arrival of Giuseppe Zucchelli as a new and lasting partner of the research group in 1985.

Returning to the late 1970s and the early 1980s, the main research effort concerned membrane stacking, which at that time was a very active field of photosynthesis research. Studies by Jim Barber's group (in UK) had established the electrostatic screening mechanism, which constituted the basis for grana formation and much effort was spent in Italy, and elsewhere, in an attempt to understand its biological implication. The close relation between grana formation and "spillover" interruption, previously suggested in the literature, was experimentally confirmed (Jennings et al. 1978) by the use of mild trypsin digestion to selectively unstack thylakoids. Subsequently, Charles Arnzten and his co-workers (at the University of Illinois at Urbana, Illinois) demonstrated that this mild trypsin digestion was due to the cleavage of a small N-terminal polypeptide segment from LHC II containing the phosphorylation site. Furthermore, Jennings et al. (1980b) demonstrated that grana formation establishes energy transfer between Photosystem II units (i.e., cooperativity). An interesting observation made in this period concerns the promotion of normal grana formation by protons at micromolar concentrations (Jennings et al. 1979b, 1981), which means that protons are orders of magnitude more effective than metal cations in bringing about this process. The lateral separation of the two photosystems, which is associated with grana formation, does not apply to plastoquinone, found to be uniformly distributed throughout the thylakoid membrane (Jennings et al. 1983) while the NADPH-ferredoxin oxidoreductase is restricted to the stromal membranes (Jennings et al. 1979a). These latter points were established using techniques that did not involve membrane fractionation, which may itself modify component distribution (Jennings et al. 1980a). An unexpected offshoot of the studies on grana function was the demonstration of the optical *sieve effect* associated with the formation of this structure (Jennings and Zucchelli 1985). This decreases light absorption specifically by Photosystem II by as much as 20%.

Following the investigation of State transitions in isolated spinach thylakoids (see e.g., Jennings et al. 1986), a novel research line of a completely different nature was begun and which involved the study of leaf optical properties. Garlaschi et al. (1989) concluded that the highly scattering internal leaf environment (*detour effect*) increases light harvesting by both the photosystems by 35–40%. As light is filtered across successive vegetation layers, however, there will be an increase in the relative Photosystem I absorption, associated with the long wavelength spectral forms (red forms) in this photosystem. Rivadossi et al. (1999) further analyzed the role of the Photosystem I red forms in light harvesting in crowded vegetation systems and established its relation with increased synthesis of Photosystem II antenna (LHC II). Further, Rivadossi et al. (2004) analyzed the influence of the leaf optical properties on the effective absorption spectrum of the four chlorophyll *a/b* proteins of Photosystem II. The most unexpected findings are that (i) in leaves, in the Q_y absorption region, it is the chlorophyll *b* absorption band, which dominates that of chlorophyll *a* in LHC II and (ii) the carotenoids would appear to have only a rather slight light-harvesting role. The vibrational bands coupled to the Q_y transition have a very significant light harvesting function in all complexes in a leaf, which is many times greater than one would expect from their weak oscillator strength.

From the earlier studies of excitation energy transfer processes between the photosystems, the main research effort of Jennings et al. group, over the past 15 years, has been on a detailed understanding of the biophysical characteristics of antenna structure and function of both the plant photosystems. Initially, the characterization and function of the different pools of spectral Chl forms in Photosystem II was examined. For LHC II, the lowest energy pigment pool with significant oscillator strength was identified at 683–684 nm by gaussian decomposition of absorption, fluorescence as well as linear dichroism spectra (Zucchelli et al. 1990, 1992, 1994). The small Stokes shift (2 nm) was subsequently demonstrated to be a general feature of most Photosystem II antenna chlorophylls and is due to the small reorganization energy of these pigments in the excited state (Zucchelli et al. 1996). The thermal broadening analysis of LHC II led to the suggestion that the red-most form of Chl *a* in LHC II is generated by coupling to a protein phonon frequency of the order of 120 cm^{-1}. Analysis of the Reaction Center trapping efficiency as a function of the different Chl spectral pools, present in the Photosystem II antenna, revealed that a 683–684 spectral form was most strongly coupled to the Reaction Center (Jennings et al. 1991b, 1992). This spectral pool was subsequently shown to be also an important component of the Reaction Center complex (Cattaneo et al. 1995) and compartment modeling of Photosystem II in terms of its component chlorophyll/protein complexes shows that this determines its strong coupling to Photosystem II primary photochemistry (Jennings et al. 2000).

A useful collaboration with the laboratory of Roberto Bassi (in Verona, Italy) developed which led to a detailed absorption and fluorescence analysis of all the Photosystem II chlorophyll/protein complexes (Jennings et al. 1993a, b). The main conclusion was that all the Photosystem II complexes are approximately isoenergetic with the main spectral pools having a similar distribution in all the chlorophyll/protein complexes. This demonstrated that energy funneling in Photosystem II antenna is almost absent. The ΔG^o for energy transfer from the outer antenna to the core was estimated as $-0.2\ k_BT$ at room temperature. (It is interesting to note that many Plant Physiology and Biochemistry text books written before these studies, and quite a number written since, show elegant little funnel shaped diagrams for Photosystem II antenna spectral forms though there was no experimental evidence at all for this and it has now been disproved.)

Jennings et al. (1991a) made the novel observation of photophysical activity associated with an isolated antenna complex (LHC II). The reversible, light-induced fluorescence quenching was subsequently studied in detail in collaboration with the laboratory of Gyozo Garab (Barzda et al. 1995, 1999) who had been analyzing light-induced circular dichroism signals in isolated LHC II. This opened up the interesting possibility that this antenna complex could, under certain light-induced conditions, down-regulate the excited state level in the Photosystem II antenna. This phenomenon is still under investigation in several laboratories.

In the mid-1990s, the study of photosynthetic antenna systems expanded to include Photosystem I with particular interest being dedicated to the low energy red Chl spectral forms. Of particular interest was the finding, based on steady-state fluorescence techniques that the excited state population of the red Chl forms accounts for 80–90% of the steady-state antenna population at room temperature. This means that most excited states generated in the antenna are trapped on the red forms (Croce et al. 1996a). Jennings et al. (2003b) subsequently demonstrated, on the basis of a time resolved fluorescence analysis, that energy transfer from the red antenna forms to ''bulk'' antenna molecules is energetically ''uphill,'' and is thermally activated. The activation energy increases with wavelength above 700 nm and lies in the range of 2–4 k_BT at room temperature. Furthermore, Croce et al. (1998) demonstrated that most of the red chlorophyll states are in the external antenna complexes and that they are almost absent in the core complex. In this paper an initial attempt was also made at establishing the optical reorganization energy of the red forms, which was shown to be much greater than for normal antenna chlorophylls. Subsequent studies, in which the lowest energy form in isolated LHC I (F735, fluorescence band at 735 nm, at 77 K) was selectively excited, demonstrated that the very high reorganization energy has the effect of producing a half absorption band width of more than 50 nm at room temperature (five times greater than for normal antenna chlorophylls) with a massive Stokes shift of 27 nm from a thermally equilibrated excited state manifold (Jennings et al. 2003a, 2004). The ''in band'' selective excitation technique, furthermore, demonstrated the presence of two low energy states in LHC I (F735, F713), both of which are present in the reconstituted Lhca4 monomer (Zucchelli et al. 2005).

The large energy shift of the red forms with respect to normal chlorophyll *a* antenna molecules was often suggested to be due to strong excitonic coupling between pigments, with the red form representing the low energy excitonic state. The first clear experimental evidence for this was obtained by selective bleaching combined with circular dichroism measurements of a red form of the cyanobacterial core complex (Cometta et al. 2000; Engelmann et al. 2001). The matrix interaction energy between the chlorophyll *a* dimer was estimated as about 300 cm^{-1}. Subsequently the laboratory of Roberto Bassi provided similar evidence for the F735 of plant LHC I.

The presence of the low energy chlorophyll states in the external antenna complexes of Photosystem I leads to a situation in which antenna energy equilibration is not fast, with respect to Reaction Center trapping, as is thought to occur in most other photosystems. Analysis of the first spectral moment of the fluorescence decay of isolated Photosystem I–LHC I and that of the spectral equilibration itself show that the two processes occur on a similar time scale (Croce et al. 2000b). Thus the antenna, due to the presence of the red forms, is kinetically limiting in Photosystem I primary charge separation.

Research on the Photosystem II antenna continued with the doctoral studies of Stefano Santabarbara on photoinhibition. It was shown that while this process followed the linear dose/response reciprocity rule, only a slight decrease in photoinhibition was observed, when the antenna excited state population was drastically reduced by added singlet excited state quenchers (Santabarbara et al. 1999). This phenomenon was also observed in intact algal cells (Santabarbara et al. 2003) and led to the suggestion that thylakoids contain a small number of energetically uncoupled chlorophyll molecules, which are active in photoinhibition of Photosystem II. Both chlorophylls *a* and *b* seem

involved with a model-based estimate of 1–3 uncoupled chlorophylls per Photosystem II (Santabarbara and Jennings 2005). In agreement with this the photoinhibition action spectrum for thylakoids is blue shifted with respect to the Photosystem II absorption spectrum (Santabarbara et al. 2001). The search for chlorophyll triplets in thylakoids, which are not associated with the Reaction Center recombination triplet, was successful (Santabarbara et al. 2002).

The first attempt at calculating the *in vivo*, protein bound, chlorophyll *a* band shape using the published vibrational frequency modes and Franck–Condon factors was successfully carried out (Zucchelli et al. 2002) and now a new project on the influence of antenna size and characteristics on the effective photosystem trapping rate has been initiated: Engelmann et al. (2005), using time resolved fluorescence techniques and antenna mutants, have shown, for the first time, that the outer antenna of this photosystem slows down the effective photochemistry by a factor of 2.5–3. Similar studies on Photosystem I are now being explored.

The most recent contribution, which is probably the most interesting of all, is the demonstration that the primary photochemistry of the core complexes of higher plants may occur with an overall decrease in the total entropy (Jennings et al. 2005). Thus, primary photochemistry in principle constitutes an exception to the second law of thermodynamics and this is basically due to its extremely high thermodynamic efficiency. The inevitable entropy increase associated with most energy (heat) conversion and transfer processes does not apply to the quantum machine of primary plant photochemistry.

[For historical perspectives on early studies on excitation energy transfer, see S.S. Brody (pp 165–170) and M. Mimuro (pp 171–176) in Govindjee et al. (2005).]

Figure 4 shows a 2005 photograph of the photosynthesis research group in the Laboratory of Plant Physiology at the University of Milano.

Fig. 4 A 2005 photograph of the members of the Laboratory of Plant Physiology at the University of Milano. From left to right: Giorgio Forti, Anna Paola Casazza, Flavio M Garlaschi, Enrico Engelmann, Giuseppe Zuccheli and Robert C Jennings

On the composition and structure of the photosynthetic apparatus (by Roberto Bassi)

Giorgio Forti, of Milano, initiated photosynthesis studies in Italy for plants and Assunta Baccarini and Andrea Melandri, of Bologna, for bacteria. These two laboratories were well connected with the international research community of photosynthesis research and contributed to the development of the basic aspects of photosynthetic electron transport. The organization of the International Congress of Photosynthesis in 1971 at Stresa was a seal for their status (see above). This built around scientists working in these groups kind of an ''aura'' made of respect and admiration. When a talk by one of them was scheduled at the meeting of the ''*Società Italiana di Fisiologia Vegetale*,'' even Italian scholars, notoriously noisy, were speaking less loudly, like in a church, waiting for the verb which could connect the Italian University plant community, rather provincial, to the wonderful world of Robin Hill, Peter Mitchell, and Melvin Calvin through the wire of a red fluorescence beam.

At the Botanical Institute of the University of Padua, one of the older Universities in the world (University of Bologna being the oldest), systematic and pharmaceutical botany were the subjects of study considered convenient for a young undergraduate student longing to study plant science. Electron microscopy (EM), though, was admitted, although considered a bit too fashionable for a serious botanist, since you could produce nice pictures that did not really need explanations in English. The idea of joining photosynthesis studies through the mainstream led by Giorgio Forti was unthinkable there, due to the lack of both the equipment and cultural background. This led the early undergraduate student Roberto Bassi, slightly disgusted from the bloody aspects of experimental pharmacology, where he had a previous experience, to observe plant tissues from C3 and C4 plants grown in shade and in full sunlight by EM. The network of thylakoid membranes was clearly affected by the light gradient, with preponderance of stroma-exposed membranes and grana stacks made up by merely two membranes in the chloroplasts of the first layer of cells facing full sunlight and stacks of up to 25 membranes in the shade (Bassi 1986). What was the difference in composition of stacked versus unstacked membranes

became an obvious question that could be answered by analyzing membranes in the bundle sheath tissue of the C4 plants, which did not have grana at all. Using selective cellulase and pectinase digestion of cell walls separated these cells. Green-gel fractionation of thylakoid pigment binding proteins showed that Photosystem II was absent from unstacked bundle sheath cell chloroplasts, thus suggesting that grana stacking was associated with Photosystem II Reaction Center rather than with LHC II, as previously suggested. LHC II, in fact, was found not only in grana but also in unstacked bundle sheath membranes (Bassi and Simpson 1986), where, most unexpectedly, it was associated with Photosystem I as shown by chlorophyll fluorescence excitation and emission spectra (Bassi and Simpson 1987).

The above-described work suggested two additional questions: the first was: what was the lonely PS I doing in stroma membranes without its companion PS II? The second asked if the LHC II could jump from grana to stroma membranes in mesophyll chloroplast, as it was being proposed by Bennett (1983). Finally, the two questions, together, showed that there was a strong need for the precise identification of the many subunits that made up PS I and PS II in order to elucidate both their function and regulation during the ever-changing conditions of photosynthesis in natural environment. A genetic approach was badly needed but no plant geneticists interested in photosynthesis were working in Italy at that time. This is why, a few months after being appointed assistant professor, Bassi undertook a long visit to the Carlsberg Research Center (Copenhagen), where Diter von Wettstein and David Simpson were constructing the barley collection of photosynthetic mutants. *Chlorina f2* had no chlorophyll *b* and lacked LHC II. Yet, EM showed it had grana larger than in wild-type plants, consistent with its higher PS II/PS I ratio (Bassi et al. 1985). Elucidation of the architecture of PS I, and of PS II, was the long range goal. The subunit composition of PS I–LHC I complex was defined as well as their spectral characteristics leading to a tentative model for their organization (Bassi and Simpson 1987), including a binding site for LHC II, which was found associated with the PS I complex in some conditions. During this period, Roberto Bassi became associated with the laboratory of Giorgio M. Giacometti, a young full professor who had made his career in Rome with hemoglobins, and later dedicated his life to the green side of the world. The topological work initiated with PS I was extended to PS II by isolating the most intact system and then dissociating it into the component subunits, one of which was the new component CP 26 (Bassi et al. 1987), later called Lhcb5 when the new nomenclature for light-harvesting protein was defined (X-P. Li et al. 1992). The problem of the double localization for LHC II in both PS I, and PS II (and with PS I in bundle sheath membranes) was of great interest and appeal since it was a sign of dynamics in the system. Following an overnight discussion with David Simpson, and in collaboration with Giacometti, State transition was obtained in vivo by illuminating dark-adapted leaves with PS I light. Leaves were cooled in ice water and stroma membranes isolated. LHC II was there and was able to transfer energy to PS I, while stroma membranes from control plants did not show any trace of it (Bassi et al. 1988a). Symmetrically, a LHC II isoform disappeared from grana membranes (Bassi et al. 1988b). Moreover, permanence of LHC II into stroma-exposed membranes was dependent on the presence of phosphatase inhibitors.

Although John Allen and John Bennet had defined State transitions ''*the carburettor of photosynthesis*'' (Allen et al. 1981), it did not sound right that this complex mechanism was activated just for increasing PS I antenna size by 10% (at least in higher plants). At that time, in Paris, Francis André Wollman was studying State transitions in *Chlamydomonas reinhardtii* while Pierre Joliot was studying cyclic electron transfer. This unicellular alga undergoes huge fluorescence changes following switch from PS I to PS II light. The fact that State transitions could be induced by changes in the level of ATP metabolic demand in *C.reinhardtii* (Bulté et al. 1990) led Wollman to propose that a switch from linear to cyclic electron transfer could be the 'underground' reason for State transitions. Of course, this could be the only possible explanation for the case of the agranal bundle sheath chloroplasts where the LHC II binding to PS I was first observed (Bassi et al. 1985). In fact, bundle sheath chloroplasts do not evolve oxygen and can only perform ATP synthesis required for CO_2 fixation by cyclic electron transfer around PS I. A joint French–Italian team performed the comparative analysis of thylakoid protein redistribution upon State transition in *C. reinhardtii* and *Zea mays* showing that in both organisms LHC II was not the only complex migrating from grana to stroma membranes: in particular, Cytochrome *b6/f* complex accumulated in the stromal regions thus making, together with LHC II and PS I, a supercomplex well suited for cyclic electron transport and ATP synthesis (Vallon et al. 1991). Like bacterial systems, cyclic electron transport needs a low-rate of electron donor for refilling lost electrons. Such a donor was found to be a monomeric PS II complex present at low level in both bundle sheath thylakoids and mesophyll

stroma-exposed membranes. This PS II complex was able to reduce plastoquinone pool at a 50 times lower rate with respect to dimeric PS II in grana stacks (Bassi et al. 1995). Thus, what had been long considered merely an example of chromatic adaptation of PS I versus PS II antenna size was, in fact, a more complex mechanism able to comply with the changes of the cellular needs for ATP versus NADPH.

At the end of the 1980s, the knowledge of the structure of higher plant photosystems was limited to indirect evidence and homology to bacterial systems. Cartoons of monomeric PS II core surrounded by Lhc proteins were the only pictures available. In this context, even low resolution EM data were welcome such as those obtained from 2D arrays self organizing in grana membranes (Bassi et al. 1989, Santini et al. 1994) showing a dimeric PS II core complex. Still, the architecture of the Lhc antenna system was unknown and had to wait for a low-resolution model by an integrated approach of cross-linking of near-neighbor subunits (Harrer et al. 1998) and single particle analysis (Boekema et al. 1999). At that time, our knowledge of PS II antenna system structure was still unsatisfactory.

While the overall structure of the photosystems was emerging, the structure and function of antenna proteins was a complete mystery, which allowed multiple and contradictory models derived from spectroscopic studies. Even the number of chromophores bound per protein subunit was uncertain till the non-denaturing isoelectric focusing method was devised for purification of individual subunits (Dainese et al. 1990) and the determination of their pigment to protein stoichiometry (Dainese and Bassi 1991) yielding a value of 12–13, 8, 9 and 10 chlorophyll molecules per polypeptide for, respectively, LHC II, CP 29, CP 26, and CP 24. Soon these data were confirmed by Kühlbrandt et al. (1994): it was the most remarkable paper published in the field of antenna proteins showing the overall structure of the LHC II trimer with two central carotenoids and location for 12 chlorophylls (Kühlbrandt et al. 1994). The availability of a structural model for antenna proteins allowed the study of structure–function relationships by using site-directed mutagenesis. The absence of a suitable expression system able to synthesize the different chromophores led us to try in vitro reconstitution of the apoproteins overexpressed in *E. coli* with purified pigments. (Gerald Plumley and Harald Paulsen had already established this method; they were the first to obtain green bands, in polyacrylamide gel electrophoresis by mixing recombinant proteins and pigments.) The procedure, once refined, yielded pigment–protein complexes identical to those purified from thylakoids, thus differential biochemistry and spectroscopy between wild-type and single point mutants on chlorophyll binding residues could be used for the determination of spectral properties, orientation and chemical identity of individual chromophores within the complex (Bassi et al. 1999; Remelli et al. 1999; Croce et al. 1999a, b; Simonetto et al. 1999). It was interesting to see that each chlorophyll is tuned to its specific wavelength of absorption, presumably for optimization of excitation energy transfer and that even carotenoid binding sites were not equivalent: not only lutein, e.g., absorbed at two different wavelengths when bound to one of the two central binding sites but also the occupancy of site L1 was indispensable for folding while site L2 could be empty or filled with any of the three xanthophyll species (Formaggio et al. 2001) that, in turn, would set LHC II protein to different conformations (Moya et al. 2001) having distinct efficiency in light harvesting versus heat dissipation. When recombinant pigment-proteins were used for ultra-fast measurements in collaboration with Alfred Holzwarth (in Germany), functional pattern of energy transfer could be elucidated and basic aspects such as the energy transfer rate between individual carotenoids and chlorophyll could be measured (Croce et al. 2000a, 2000c; Cinque et al. 2000).

The finding that binding of different xanthophyll species to allosteric binding sites of Lhc proteins could regulate ^{1}Chl* excited state lifetime had obvious importance for physiology and prompted us to study the dynamics of carotenoid binding in vivo and in vitro. An additional xanthophyll binding site was found in LHC II, called V1, whose affinity for violaxanthin was decreased at low lumenal pH (Caffarri et al. 2004) thus making the substrate available for violaxanthin de-epoxidase enzyme whose product could be rebound to the allosteric site L2 of Lhc proteins to a different extent and rate (Morosinotto et al. 2002a) thus contributing to the photoprotection of PS II in vivo (Dall'Osto et al. 2005). Further insight in the function of zeaxanthin was obtained by constructing a plant with zeaxanthin as the only xanthophyll (Havaux et al. 2004); it had a reduced antenna size by selectively affecting the major LHC II degradation. These results changed the view on carotenoid function in plants from accessory pigments for light harvesting to modulators of photosynthesis, in some instances, like violaxanthin bound to site V1, completely unable to transfer energy to chlorophyll.

During many years the laboratory focused on antenna proteins of PS II. Still, PS I had its own antenna

system made of homologous Lhc proteins which, surprisingly enough, had very similar chemical composition and very different spectroscopic properties. Actually, Lhca gene products confer to the PS I–LHC I complex its characteristic fluorescence emission at 735 nm, strongly shifted towards low energy if one considers that chlorophyll *a* absorbs at around 660 nm in organic solvent and the red-most chlorophyll bound to LHC II was found to have a 682 nm absorption (Remelli et al. 1999). The fact that these properties could be reconstituted in recombinant proteins (Croce et al. 2002) made them amenable to mutation analysis: Chl A5 was responsible for red-shifted spectral forms (Morosinotto et al. 2002b) by interacting with the neighboring Chl B5. Responsible for all this was a single amino acid substitution of histidine with asparagine implying a shorter coordination distance (Morosinotto et al. 2003) and a position for Chl A5 closer to B5 thus yielding into an excitonic interaction shifting absorption to lower energy.

The high-resolution structure of LHC II (Li et al. 2004) opens now the possibility to proceed to a far more detailed analysis of structure–function relations in antenna proteins. However, basic research in Western Europe is becoming more and more difficult: everything must be applied to short-term industrial productivity. In Italy, genomic and proteomic studies aimed at identifying genes involved in crop stress resistance and productivity are the main possibility of financial support offered by the government. In this context, part of the work in the laboratory has been dedicated to the molecular basis of abiotic stress resistance and hardening. It was shown that the ability of reversible phosphorylation of the CP29 (Lhcb4) PS II subunit correlates with cold-stress resistance (Bergantino et al. 1995) through the conformational change of this antenna subunit (Croce et al. 1996b; Testi et al. 1996). Further basis to the concept that Lhc genes could be stress-resistance factors came from the evidence of their differential expression under stress (Caffarri et al. 2004, 2005) while the concept emerged that constitutive expression of stress induced genes, although increasing resistance, decreases productivity and increased light energy dissipation (Dall'Osto et al. 2005) through mechanisms that involve the coordinated role of PS II Reaction Center (Finazzi et al. 2004) and the antenna proteins. These dissipative mechanisms were activated by low lumenal pH through a molecular trigger: PsbS (Li et al. 2000; Dominici et al. 2002). The analysis of chloroplast located stress-resistance proteins was extended to soluble, non-pigment binding components, in close collaboration with Luigi Cattivelli from Piacenza. Crosatti et al. (1999) and Dal Bosco et al. (2003) showed that cold-stress response needs light and originates in the chloroplast. This research is being developed now by using chloroplast development mutants and concepts derived from basic photosynthesis. Integration of the photosynthesis studies into general plant physiology and molecular biology is certainly a positive step for plant biology after so many years of virtual separation. Nonetheless, it is unwise to decrease research on the basic process of photosynthesis, as further applications will depend upon what we still learn from the newer details of this basic process. Many aspects of plant stress biology need the contribution of this unique combination of biophysics, biochemistry, photobiology and molecular biology that has identified the studies in photosynthesis during the last 50 years.

I acknowledge here the essential contribution of my former and present students: Paola Dainese, Roberta Croce, Stefano Caffarri, Tomas Morosinotto, Luca Dall'Osto, and many others: they did most of the work and often had the best ideas. Moreover, I want to thank my masters in science to whom I owe the little I have done: Diter von Wettstein, David Simpson, Pierre Joliot, and Jean-David Rochaix. Last but not the least, I want to mention Professor Mario Orsenigo who was in charge of the EM laboratory at the Botanical Garden of Padua University, where I was admitted for my graduation thesis work.

Figure 5 shows a 2004 photograph of the photosynthesis research group in the Laboratory of Plant Physiology at the University of Verona.

Photoinhibition of the donor side of Photosystem II (by Roberto Barbato)

Research in photoinhibition and D1 protein in Padova began in 1988, during discussions between Maria Teresa Giardi and Roberto Barbato. At that time, Maria Teresa was visiting the University of Padova, after she had worked at the Imperial College of Science in London, UK, in Jim Barber's laboratory. Soon after Roberto Barbato went, in 1989, to the Imperial College to work in Jim's laboratory with a project related to the isolation of a cytochrome b559-free Reaction Center complex, using sucrose gradient centrifugation instead of ionic exchange chromatography, with the aim to retain in the preparation at least the primary quinone Q_A. At that time, Cathie Shipton, at Imperial College, had just shown that, in the presence of artificial electron acceptors, light could promote degradation of the D1 protein in isolated Reaction Center complex, giving

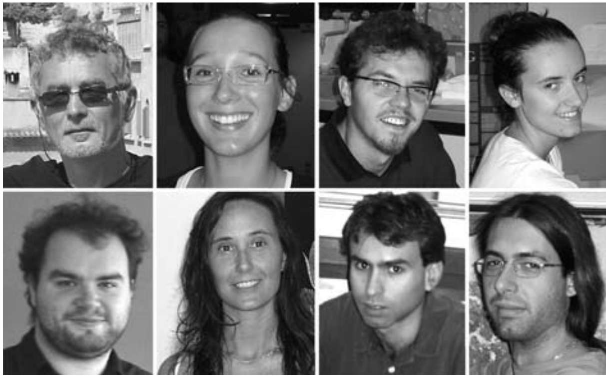


Fig. 5 A 2004 photograph of some of the members of the Laboratory of Plant Physiology at the University of Verona; several are also jointly at the Faculté des Sciences de Luminy, in Marseilles, France, or at Polotecnolgia in Trento. (Top) Left to right: Roberto Bassi, Sara Frigerio, Tomas Morosinotto and Giulia Bonente. (Bottom) Left to right: Luca Dall'Osto, Roberta Croce, Stefano Caffarri and Matteo Ballottari

rise to immunodetectable breakdown fragments with apparent molecular weight of about 24 kDa; in this condition, photoinhibition is said to be of the ''donor side'' type, as highly oxidizing cations are accumulated ($Tyr^{ox}{}_{Z}$, $P680^{+}$) which, by oxidizing both pigments and the protein environment, may induce damage to Photosystem II; this kind of photoinhibition has to be separated from the 'acceptor side' one, as the latter is linked to double reduction of Q_A which, leaving its binding site on D2 protein, could promote, as consequence of further charge separation and recombination, the formation of singlet oxygen. Cathie was also studying, with J. Marder, whether these findings were relevant to the in vivo mechanism of normal D1 protein turnover as in the classical studies of Marvin Edelman and co-workers in Israel.

One main question was to find out a way to relate the degradation pattern of D1 with a particular mechanism of photoinhibition: in Padova, the adopted strategy was the proteolysis with Lys-C of the isolated wheat D1 protein using a species, such as wheat, with a single lysine residue in its sequence. In this way, two well-defined D1 fragments could be produced, i.e., the N-terminus containing residues 2–235, and the C-terminus, consisting of residues 236–349. These fragments could then be used to raise antibodies with different specificity for different regions of the proteins. This approach was successful with the N-terminal proteolytic fragments (Barbato et al. 1991a), whereas it did not work well with the C-terminus one. Luckily, an antibody to D1 protein (the protein was isolated by electroendosmosis preparative electrophoresis from barley PS II core preparation) was produced some years before and left at −70°C: this antibody was proven to be essentially C-terminal specific (Barbato et al. 1991b). In this way, by the beginning of 1991 a couple of antibodies specific for different regions of D1 were available to characterize the white light induced, and later, the ultraviolet-B light induced, degradation products of the D1 protein. The first use of these antibodies was the characterization of D1 breakdown products in isolated PS II-RC irradiated in the presence of DBMIB, giving rise to donor side photoinhibition. For the first time, it was possible to show that a specific degradation pattern could be associated with a particular photoinhibition mechanism (Barbato et al. 1991a). This idea was further extended to other kinds of PS II preparations, and proven to be correct. Analysis of preparation with different structural complexity and functional capability showed that a 16 kDa C-terminus was consistently produced under donor side photoinhibitory conditions, and could be used as a marker for this kind of photoinhibition (Barbato et al. 1992a).

A second main point worked out in donor side photoinhibition was the effect of D1 breakdown in structural organization of PS II. At that time, work carried out in a number of laboratories supported the concept of a 'life cycle' or 'repair cycle' of PS II. According to this idea, PS II centers are photoinhibited in the grana domain of the thylakoid membrane; afterwards they migrate to the stroma-exposed region. Here, newly synthesized D1 proteins are inserted into the membrane, substituting the light-damaged ones. The cycle would end with the back migration to grana membranes of the repaired cores. A plan was made to study the spatio-temporal dynamics of PS II cores as well as variation in its structural organization in thylakoids subjected to donor side photoinhibition, using the 16 kDa C-terminus of D1 as a specific marker. This plan consisted of digitonin-based grana/stroma fractionation followed by a non-denaturing fractionation step based on sucrose gradient ultracentrifugation. Each gradient was fractionated and distribution of polypeptides analyzed by immunoblotting with a battery of polyclonal antibodies to PS II proteins. In this way we could study the structural organization of PS II Centers undergoing lateral migration. Some major points emerged (Barbato et al. 1992b): PS II is organized mainly as a dimer in control thylakoids; as a consequence of inactivation, PS II is converted to a monomeric form; broken D1 protein is retained in monomeric PS II cores; breakdown or damage of D1 (D1) brought about the dissociation of the CP43 subunit from the rest of the core (CP 47/D1/D2); independently of each other, the CP 47/D1(D1)/D2 core and free CP43 migrate to the stroma; lamellae

compartment: here, reassembly of PS II cores using newly synthesized D1 protein occurs.

The finding that it was possible to detect a complex consisting of damaged D1, D2, and CP47 and free CP43 has some structural implication. If we have to think that this complex could act as an acceptor for the newly synthesized protein, which could displace the damaged one, the only possible way to place subunits was CP47 on the D2 side and CP43 on the D1 side. Recently, 3D crystals analysis clearly shows that this indirectly derived topological information was correct. Later, a similar study was undertaken in collaboration with Eva-Mari Aro, in which the role of reversible phosphorylation of some subunits was investigated (Baena-Gonzalez et al. 1999).

As stated above, another type of donor-side related photoinhibition is the one observed under ultraviolet-B light (Renger et al. 1989). Investigation on this topic was started in 1993, first in collaboration with Imre Vass in Szeged, Hungary, and since the first experiment, it was clear that the donor side of the Photosystem II was involved (Friso et al. 1994). It was found that this light promotes degradation of D1 to a well defined 23 kDa fragment which, based on immunoreactivity to N- and C-terminus specific antibodies and behavior on Lys-C digestion, could be identified as the C-terminus of the protein. Later, it was found that, at least when low dose of pure ultraviolet-B light is used, this fragment is formed only when functional manganese is present (Barbato et al. 1995). The effect of ultraviolet B light on PS II repair cycle was also studied (Barbato et al. 2000), and the role of visible light in the recovery was recently shown to exist (Bergo et al. 2003).

The meaning of donor side photoinhibition in vivo has been a matter of discussion for a long time and it had never been accepted as a relevant physiological phenomenon. However, at least in some extreme environment, it could be the case. Species belonging to the ‘tribe’ of *Salicornia* live in retrodunal environment under extreme high salinity. Together with Flora Andreucci and Cristina Pagliano, we found that these plants perform photosynthesis in the early morning and in the late afternoon, and they are mostly switched off during the day when light intensity is high. By using site-specific antibodies to D1 and in this case also to D2, we had the indication that they suffer from donor side photoinhibition: when we tried to understand the reason for this, we found, to our surprise, that they completely lacked the PsbP and PsbQ subunits, whereas they retained the PsbO extrinsic subunit. As in the case of the Fud34 mutant of *Chlamydomonas*, the lack of the PsbP protein brings about donor side photoinhibition, but this time under natural conditions.

The work described above was done with the help of Giulia Friso and Elena. Bergo.

[For a historical perspective on ‘*photoinhibition*’, see N. Adir et al. (pp 931–958) in Govindjee et al. (2005).]

Figure 6 shows the members of the photosynthesis research at the University of Alessandria.

Studies on non-photochemical quenching (NPQ) (by Enrico Brugnoli)

The National Research Council (CNR) laboratory at Porano (a section of the Istituto di Biologia Agroambientale e Forestale), led by Enrico Brugnoli, was started in 1985. Initially, research activities were focused on physiological ecology of photosynthesis (gas exchange, stable isotope discrimination in plants, chlorophyll fluorescence and photoinhibition). In the late 1980s, a collaborative work was started with Olle Björkman (of the Department of Plant Biology of the Carnegie Institution of Washington in Stanford, California, USA) and Barbara Demmig (University of Würzburg, Germany). This work elucidated the role of the xanthophyll cycle components in photoprotection–photoinhibition under environmental stresses such as drought, salinity and unfavorable temperature (see e.g., Adams et al. 1988; Brugnoli and Björkman, 1992a, b; Brugnoli et al. 1994; Loggini et al. 1999). Brugnoli and Björkman (1992b); it showed that zeaxanthin

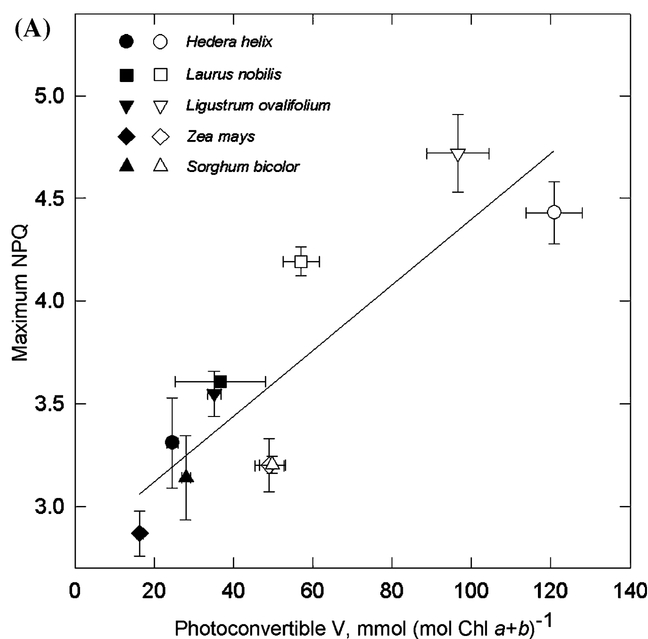
Fig. 6 A photograph of the members of the photosynthesis research group*, and others, at the University of Alessandria. Top, from left Elena Bergo, Giulia Friso and Cristina Pagliano*; bottom, Roberto Barbato* and Flora Andreucci*

formation plays a relevant role under stress conditions in preventing photoinhibition of photosynthesis and photo-oxidation: this photoprotective role contributed in maintaining the full photosynthetic capacity expressed as carbon fixation per unit leaf area or on chlorophyll basis.

In addition, the light-induced absorbance change caused by violaxanthin deepoxidation in intact leaves was studied by Brugnoli and Björkman (1992a). The correlation between the light-induced absorbance change at 508 nm and zeaxanthin formation in leaves is relevant for physiological and ecological applications. Light-induced chloroplast movements were found to cause changes in chlorophyll fluorescence emission and matching changes in leaf absorptance both in kinetics and in maximum extent. These results clearly showed that chloroplast movements could strongly influence the efficiency of light utilization in photosynthesis.

The capacity for non-radiative energy dissipation, measured as maximum non-photochemical quenching (NPQ), and its dependence on carotenoid composition were studied in sun and shade leaves of several species. The amount of photoconvertible violaxanthin was higher in the sun compared to the shade leaves. Hence sun leaves contain a greater pool size of xanthophyll cycle components and these are more convertible to zeaxanthin compared to that in shade leaves (Brugnoli et al. 1994). The amount of photoconvertible violaxanthin in vivo is strongly dependent on the degree of thylakoid membrane appression and on the stoichiometries of PS II antennae (LHC II) and minor PS II complexes. In a study on C3 and C4 plants (Brugnoli et al. 1998), shade-grown leaves showed more grana stacking, higher content of LHC II and lower contents of minor complexes compared to sun-grown leaves. This caused a much higher proportion of photoconvertible violaxanthin and a higher capacity for NPQ in the sun compared to the shade leaves (see Fig. 7A). Figure 7B shows members of the photosynthesis research group at Porano.

Photoinhibition, photoprotection and desiccation tolerance were studied in the poikilohydric (resurrection) plant *Ramonda serbica*. Changes in zeaxanthin and antheraxanthin contents and in the pools of reduced ascorbate and glutathione were evident during dehydration and rehydration and these changes were related to increases in NPQ (Augusti et al. 2000). In addition, fully dehydrated leaves maintained some zeaxanthin and this may be relevant to cause energy dissipation during subsequent rehydration. Reduced ascorbate and reduced glutathione were found to be most relevant for preventing photo-oxidation at the lowest water content. Changes in the pools of antioxidants and of zeaxanthin were found to be crucial for photoprotection and to confer desiccation tolerance to *Ramonda serbica* leaves.



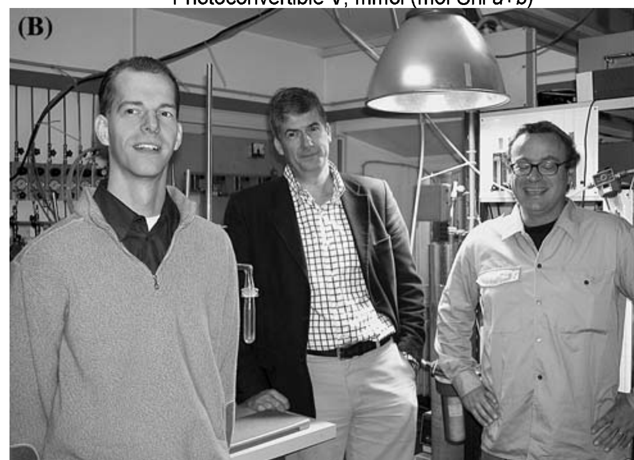


Fig. 7 (**A**) Relationship between photoconvertible violaxanthin (i.e., the maximum amount of violaxanthin converted to zeaxanthin under high light and low CO_2) and maximum non-photochemical quenching, which is related to the capacity for non-radiative energy dissipation. Experiments run on intact full sun exposed leaves (open symbols) and shade adapted leaves (closed symbols) in different species possessing the C3 (*Hedera elix* L., *Ligustrum ovalifolium* Hassk. and *Laurus nobilis* L.) and the C4 photosynthetic pathway (*Zea mays* L. and *Sorgum bicolor* Moench.). The correlation coefficient $r = 0.86$ is indicative of high level of significance. NPQ stands for non-photochemical quenching of the excited state of chlorophyll molecule (Source: Brugnoli et al. 1998). (**B**) A photograph of members of the photosynthesis research group in their laboratory in Porano. Enrico Brugnoli (middle), with Filip Volders (right) and Marco Lauteri (left)

[For a historical personal account of linking the xanthophyll cycle to thermal energy dissipation, see B. Demmig-Adams (pp 923–930) in Govindjee et al. (2005).]

Kinetics and energy modulation of the quinone acceptor complex in photosynthetic Reaction Centers from purple bacteria in different solubilizing environments (by Angela Agostiano and Massimo Trotta)

The research group on photosynthesis-related topics in Bari, Italy, was initiated by Angela Agostiano in the late 1980s, after she returned form the laboratory of Francis Fong at the Purdue University, West Lafayette, Indiana, USA, where she worked on the electrochemistry of aggregated species of chlorophyll *a* generated in several environmental conditions (Agostiano et al. 1990, 2002, 2003). Her research topic expanded to include bacterial photosynthesis in the 1980s in close collaboration with Massimo Trotta who had returned from a first 18-month stay at the University of Bologna collaborating with G. Venturoli, A Melandri and D Zannoni, working on the charge recombination from the final quinone acceptors in Reaction Centers isolated from the thermophilic bacterium *Chloroflexus aurantiacus* (Venturoli et al. 1991) and a subsequent second 2-year stay at the University of California at San Diego working with George Feher and Mel Okamura on NMR applied to Reaction Centers (Trotta et al. 1992). Francesco Milano, who had spent some time with Wolfgang Lubitz, and Livia Giotta, with Peter Rich, joined this group in 2001 and 2002, respectively. (Livia is presently at the University of Lecce, Italy.)

The photocycle and the associated electron transfer rate of the Reaction Center (RC) from the purple bacterium *Rhodobacter sphaeroides* were well established (Feher et al. 1989). The rate by which the loosely bound quinone (as well as the quinol) enters and leaves its binding site in the Reaction Center for closing the photocycle and restart the cascade of the electron transfer reactions was, and partially is, not yet clear. The investigation on the quinone exchange rate at Q_B site was performed in our laboratory by studying the role of different solubilizing environments of the enzyme. The first investigated environment was the zwitterionic detergent N,N-Dimethyl-N-dodecylamine oxide (LDAO) and in particular the influence on the functioning of the secondary electron acceptor played by the size, i.e., the number of LDAO monomers (in the polymer), of the solubilizing micelles surrounding the RC (Agostiano et al. 1999, Halmschlager et al. 2002). Subsequently the investigation was extended to the case of RC containing proteoliposomes formed by the zwitterionic phospholipid phosphatidylcholine (PC) (Trotta et al. 2002, Milano et al. 2003). These studies allowed us to assess kinetic constants for the ubiquinone 10 uptake into—and release from—the Q_B-binding site which were found to be 7×10^7 $M^{-1}s^{-1}$ and 40 s^{-1}, respectively.

The effect of the solubilizing environment was also studied in terms of the stability of the semiquinones at the Q_A and the Q_B binding sites in RC incorporated into proteoliposomes containing negatively charged phospholipids, namely phosphatidylglycerol (PG) and cardiolipin (CL). These studies were performed in collaboration with Péter Maróti and László Nagy (of the Department of Biophysics of the University of Szeged, Hungary) and allowed us to determine the standard free energy of the primary electron acceptor Q_A^- (with the neutral state Q_A, as a reference) by using the delayed luminescence technique (Turzo et al. 2000). This value was strongly dependent upon the solubilizing environment and it increased by as much as 46 meV when PG is added either to Triton X-100 micelles or to phosphatidylcholine proteoliposomes. An even larger increase was found in the free energy of Q_A^- upon addition of CL. On the other side, the free energy of Q_B^- was found to be rather insensitive to the presence of the negative charge on the phospholipids. The presence of both PG and CL as phospholipids of the Intra Cytoplasmic Membrane (ICM) of the photosynthetic system as well as their role in stabilizing the lifetime of the Q_B^- state (mostly by increasing the energy level of Q_A^-) was interpreted as possible regulating mechanism for Reaction Centers under physiological conditions (Nagy et al. 2004). This stabilization effect of negatively charged phospholipids was found to be extremely efficient in the case of proteoliposomes formed exclusively by PG or phosphatidylinositol (PI) or phosphatidylserine (PS), when the charge separated state generated by continuous illumination was found to last up to 60 min (Agostiano et al. 2005).

In close collaboration with researchers from Hungary, Agostiano et al. (2004) and Milano et al. (2005) have investigated the possibility of studying in the RC, the proton uptake or release associated with the quinone reduction and quinol oxidation, respectively, by using pyranine, a pH sensitive fluorescence probe. Preliminary information on this technique has shown its validity and further experiments are presently being performed.

Figure 8 shows members of the photosynthesis research group at the University of Bari.

Fig. 8 A 2005 photograph of members of the photosynthesis research group at the University and Istituto per i Processi Chimico Fisici in Bari. From left to right: Massimo Trotta, Francesco Milano, Angela Agostiano and Francesca Italiano

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