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Irradiance Effects on Thylakoid Membranes of the Red Alga *Porphyridium cruentum*. An Immunocytochemical Study

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Immunogold labelling on ultrathin sections of the red alga *Porphyridium cruentum* (ATCC 50161) was used to assess changes in the density and distribution of polypeptide components of photosystem I, photosystem II, phycobilisomes, and ATP synthase within the thylakoid membrane as a function of growth irradiance. In cells grown under a low, limiting quantum flux (6 microeinsteins per square meter per second of continuous white light) thylakoid membrane density and total thylakoid area per cell are 2 1/2 times greater than in cells grown under a high, saturating quantum flux (280 microeinsteins per square meter per second). Immunogold labelling data indicate that concentrations of photosystem I, photosystem II and phycobilisomes in thylakoids of low light-grown cells are only slightly greater than in cells grown under high light. In contrast, the concentration of ATP synthase within the thylakoid membrane is nearly ten times greater in high light-grown cells. Photosystem I polypeptides were detected in those portions of the thylakoid membrane which traverse the pyrenoid, but photosystem II and phycobilisomes appeared to be absent from these membranes. Ribulose-1,5-bisphosphate carboxylase was restricted primarily to the pyrenoid, and its concentration in the stroma or pyrenoid was little affected by the photon flux density. Quantitative estimates of photosystems I and II, phycobilisomes, and ATP synthase by spectroscopy or by immunoelectrophoresis are in accord with the immunogold results and lend support to the use of immunogold labelling for quantifying changes in relative amounts of membrane proteins.

Key words: ATP synthase — Immunocytochemistry — Photoacclimation — Photosystems I and II — Phycobilisome — *Porphyridium cruentum*.

Exposure of photosynthetic organisms to relatively high PFD results in a host of characteristic changes in the structure, composition, and function of their photosynthetic apparatus (see Anderson et al. 1988 for a recent review). The unicellular red alga *Porphyridium cruentum* (ATCC 50161) has a relatively low effective range of PFD for growth (3 to 280 μE m^{-2} s^{-1}), but exhibits the same general response to high irradiance as the much more intensively studied green plants and algae whose light requirements can be an order of magnitude higher. In *P. cruentum* we have previously observed significant and reproducible increases in such properties or parameters as P_{max}, light compensation point, respiration rate, growth rate (Levy and Gantt 1988), and resistance to photoinhibition (Vonshak, Cunningham and Gantt, in preparation). Concomitant with and at least partly responsible for these changes in performance, are structural and compositional changes which include reductions in cell content of thylakoid area, protein, light-harvesting antenna pigments, reaction centers, and a reduction in thylakoid membrane density

Abbreviations: APC, allophycocyanin; ATPase, the chloroplast ATP synthase; D2, PS II reaction center polypeptide and product of the *psbD* gene; L_{CM}, phycobilisome core linker polypeptide and product of the *apcE* gene; P_{700}, reaction center of PS I; PBsome, phycobilisome; PFD, photon flux density; P_{max}, light-saturated rate of photosynthesis; Q_A, primary quinone acceptor of PS II; RUBISCO LSu; large subunit of ribulose-1,5-bisphosphate carboxylase.

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(Cunningham et al. 1989). At the same time, cell size (Cunningham et al. 1989) and cell content of certain key photosynthetic enzymes (Cunningham, Vonshak and Gantt, in preparation) are increased.

To gain further insight into the process whereby *P. cruentum* and other plants acclimate to the prevailing light environment, we sought information on possible changes in thylakoid membrane architecture which may accompany the previously observed compositional changes. To this end, we have been exploring the use of immunocytochemical techniques to ascertain the subcellular distribution and concentration of various polypeptide components of the photosynthetic apparatus.

Immunocytochemical techniques, and in particular immunogold labelling, are routinely used to determine the tissue or subcellular distribution of polypeptides or other antigenic molecules for which a specific antiserum is available (e.g. Herman 1988). These methods have been used to determine the lateral distribution of various thylakoid components in stromal and granal lamellae of higher plant and green algal chloroplasts (e.g. Allred and Staehelin 1985, Shaw and Henwood 1985), and to ascertain on which side of the thylakoid membrane certain thylakoid-associated polypeptides (e.g. Hinshaw and Miller 1989) or portions of those polypeptides (Vallon et al. 1989) are exposed. In a recent paper we showed that this technique can also be used to obtain quantitative information on relative amounts of thylakoid membrane polypeptides (Mustárdy et al. 1990).

In this paper we use immunogold labelling to ascertain the relative abundance and distribution of various apoprotein components of PS I, PS II, PBsomes, and ATP synthase within the thylakoid membrane of *Porphyridium cruentum* as a function of the growth irradiance. We then compare the immunogold data with data obtained by spectroscopic and activity measurements and by other immunological methods. These combined approaches reveal that the concentrations of the reaction centers and light-harvesting antenna complexes are not greatly affected by PFD, but the concentration of ATP synthase within the thylakoid membranes of *P. cruentum* increases by nearly an order of magnitude with increase in growth irradiance.

Materials and Methods

Cell culture—Cultures of *Porphyridium cruentum* (ATCC 50161) were grown under continuous light (provided by Sylvania VHO daylight fluorescent tubes) in one liter batches of Jones' medium (Jones et al. 1963) in low form culture flasks (2.5 liter capacity) on a rotary platform shaker at 80 cycles min^{-1}. The temperature was kept constant at 18°C, and cultures were bubbled with 5% CO_2 in air to ensure that supply of CO_2 did not limit growth. All samples were taken from exponentially-growing cultures. Inocula for the experimental cultures were from cultures which had been maintained in the log phase and fully acclimated to the chosen PFD.

Antisera—Antisera to the *beta* subunit of chloroplast ATP synthase, the large subunit of RUBISCO, and the D2 reaction center polypeptide of PS II, all from spinach, were generous gifts of Drs. A. T. Jagendorf of Cornell University, S.-D. Kung of the University of Maryland at College Park, and W. J. F. Vermaas of Arizona State University, respectively. All other antisera were produced in our laboratory using polypeptide antigens purified from *P. cruentum*. Purification of antigens and production, affinity purification and specificity of the antisera are described in Mustárdy et al. (1990).

Immunocytochemical quantitation—These procedures are described in detail in Mustárdy et al. (1990). Briefly, cells were fixed in glutaraldehyde, dehydrated, and embedded in Lowicryl K4M resin. Ultrathin sections were incubated with a primary rabbit antiserum (IgG fraction for most, but an immunoaffinity purified serum fraction for the L_{CM} and PS I polypeptides) which was specific for the polypeptide(s) of interest. After washing to remove unbound primary serum, the sections were incubated with goat anti-rabbit serum coupled to 10 nm colloidal gold particles (GAR G10 from Janssen Life Science Products), washed again, and finally stained with uranyl acetate. Numbers of gold particles per section area were determined for chloroplast, pyrenoid, and "nonplastid" (including cytoplasm, nucleus, and mitochondria) compartments. Occasional clumps of gold particles were counted as one particle (i.e. a single immunoreactive site). All data are the mean and standard deviation of three experiments. Because of an age-dependent loss of antigenicity, all comparisons were made using sections cut at the same time and always less than two days old. Data from different experiments were normalized to give equivalent specific label density for the chloroplast region in sections of cells grown under the lowest light intensity (6 μE m^{-2} s^{-1}), except that the RUBISCO data was normalized to the label density for the pyrenoid of low light-grown cells. Each of the proteins examined in this study is expected to be encoded in the plastid genome (discussed in Mustárdy et al. 1990) and thus the density of gold particles over the nonplastid region was generally considered to reflect nonspecific background binding of the primary antiserum. For quantitative comparisons of specific labelling, the mean density of gold particles over the nonplastid region was subtracted from those over the pyrenoid and plastid except in the case of the ATP synthase antigen. Because of the presence of homologous ATPase subunits in other compartments, the uncorrected chloroplast data was used. Thylakoid length per cross-section area was determined as described by Weibel and Bolender (1973) using sections of cells embedded in Epon rather than in Lowicryl K4M (as in Mustárdy et al. 1990).

Chloroplast and pyrenoid volumes were estimated from cross-sectional areas of central sections with the assumption that each approximates a sphere.

Results

Subcellular distribution of labelling—Subcellular distributions of PS I, PS II, PBsomes, RUBISCO, and ATPase were examined using antisera specific for polypeptide components of these multiprotein complexes in *Porphyridium cruentum*. The monospecificity of the antisera was previously shown by immunoblotting (Mustárdy et al. 1990). Representative electron micrographs of immunogold-labelled ultrathin sections are shown in Fig. 1 for several of these antisera. The subcellular distribution of labelling was determined by counting gold particles bound over three compartments: pyrenoid, chloroplast (excluding the pyrenoid) and cytosol (which included all portions of the cell outside of the chloroplast and pyrenoid). Labelling over the cytosol region was generally considered to be a reflection of nonspecific or background labelling since all of the antisera used in this work are specific for polypeptides which are encoded in the chloroplast genome of other plants which have been examined (e.g. see Gray 1988). A "nonimmune" rabbit serum (IgG fraction) used at high concentration gave low density of labelling with no apparent specificity in the three regions (data not shown).

Immunogold labelling for the various antigens examined was largely restricted to the chloroplast and pyrenoid compartments of *P. cruentum*, as expected (Figs. 1 and 2). The PS II core polypeptide D2 (Fig. 1B), the 45 kDa Chl-binding protein of PS II (homologous to spinach CP47), the PBsome core antenna proteins APC (Fig. 1A) and L_{CM}, and the chloroplast ATP synthase *beta* subunit all are present in the chloroplast but are apparently excluded from the pyrenoid (Fig. 2).

The distribution of gold particles indicated that the RUBISCO large subunit was heavily concentrated in the pyrenoid region of the plastid. Labelling density over the rest of the plastid was only slightly greater than that over the nonplastid region (Figs. 1C and 2). We estimate that the pyrenoid occupies at least 10% and perhaps as much as 25% of the total chloroplast volume in cells grown under our experimental conditions. Assuming that specific label density is proportional to the concentration of RUBISCO, a conservative estimate is that at least 85% of the RUBISCO is in the pyrenoid.

The 60 kDa core Chl-antenna proteins of PS I were also detected in the pyrenoid, and appeared to be concentrated over those regions where thylakoid membranes passed through the pyrenoid (Fig. 1D). The specific immunogold label density (particles per area corrected for nonplastid background density) for PS I over the pyrenoid was generally about 40% of that over the rest of the plastid, but this value rose to more than 70% under the highest irradiance (see Fig. 2).

Irradiance effects on immunogold label density—From the data presented in Fig. 2 it is evident that a change in PFD results in a change in the density of immunogold labelling for each of the thylakoid membrane polypeptide antigens. Specific labelling data for polypeptide components of PS I, PS II, and PBsomes indicate a large reduction (60–70%) in the density of these complexes as irradiance is increased from a very low, limiting PFD (6 μE m^{-2} s^{-1}) to a high, saturating one (280 μE m^{-2} s^{-1}). Since the size of the single plastid in *P. cruentum* does not vary greatly with PFD (Cunningham et al. 1989), these results compare favorably with the 60–70% reduction in PS I, PS II, and PBsome per cell determined previously by spectroscopic methods (Cunningham et al. 1989).

The large reductions in label density for components of PS I, PS II, and PBsomes in cells grown under high light presumably reflect either a reduction in the packing density of thylakoid membranes or a reduction in the concentration of these components within the membrane, or a combination of these two. We have previously reported that the density of thylakoid membranes in *P. cruentum* declines progressively as irradiance increases (Cunningham et al. 1989). Cells grown under low PFD have nearly 2 1/2 times the thylakoid membrane density (thylakoid length per section area) observed in cells grown under high PFD (Fig. 3A). When the specific immunogold particle density data is expressed on the basis of thylakoid length (Fig. 3B) it can be seen that concentrations of the light-harvesting complexes (PS I, PS II, and PBsomes) in the membrane are not greatly affected by PFD. There may be a small increase in density per thylakoid length at the lowest irradiance. In contrast, the concentration of ATP synthase is nearly ten times greater in cells grown under the highest irradiance than in those grown under the lowest PFD. Thus the amount of this enzyme complex per PS I, PS II, or PBsome is also about ten times greater in the high light-grown cells.

Some small changes in the densities of labelling for RUBISCO were observed in response to change in PFD, but no obvious pattern is discernable either for pyrenoid or stromal compartments (Fig. 2). There is no indication of any substantial redistribution of RUBISCO between the pyrenoid and the stroma compartments with change in irradiance.

Comparison of immunogold labelling with other techniques—In order to verify our results, we compared the immunogold labelling data with data obtained previously using other methods to quantify PS I, PS II, PBsomes and ATP synthase in cells of *P. cruentum* as a function of growth irradiance. These earlier data are expressed on the basis of cell number or Chl, and thus they can not be directly compared with the immunogold labelling data which is

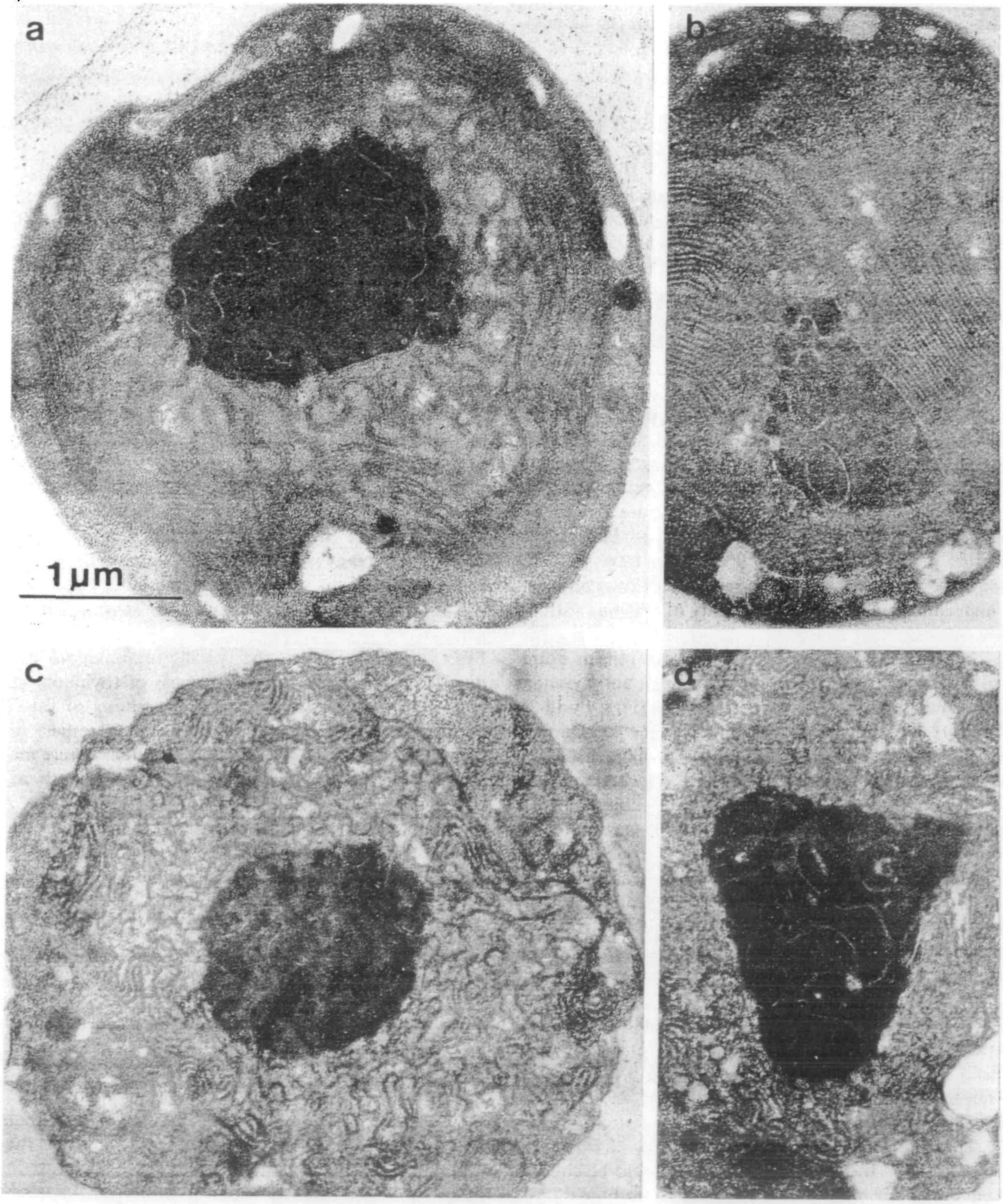

Fig. 1 Representative electron micrographs of *P. cruentum* cell sections immunolabelled with a primary antiserum, as designated, followed by gold-labelled goat anti-rabbit serum. A. Anti-APC (PBsome), cell grown under 35 μE m^{-2} s^{-1}. B. Anti-D2 (PS II), 6 μE m^{-2} s^{-1}. C. Anti-RUBISCO, 180 μE m^{-2} s^{-1}. D. Anti-60 kDa PS I polypeptides, 180 μE m^{-2} s^{-1}.

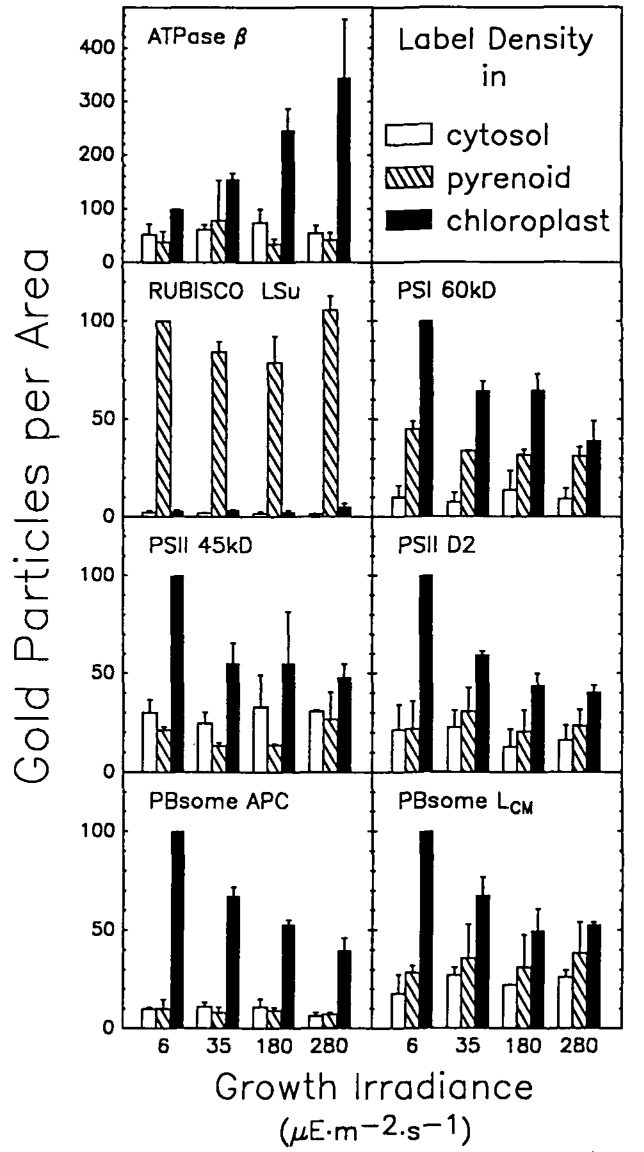

Fig. 2 Immunogold labelling densities over different cell compartments. Data is given as gold particles per section area and is normalized to a value of 100 particles per area in chloroplasts of cells grown under the lowest PFD (6 μE m^{-2} s^{-1}), except that the data for RUBISCO is normalized to 100 particles per area of pyrenoid.

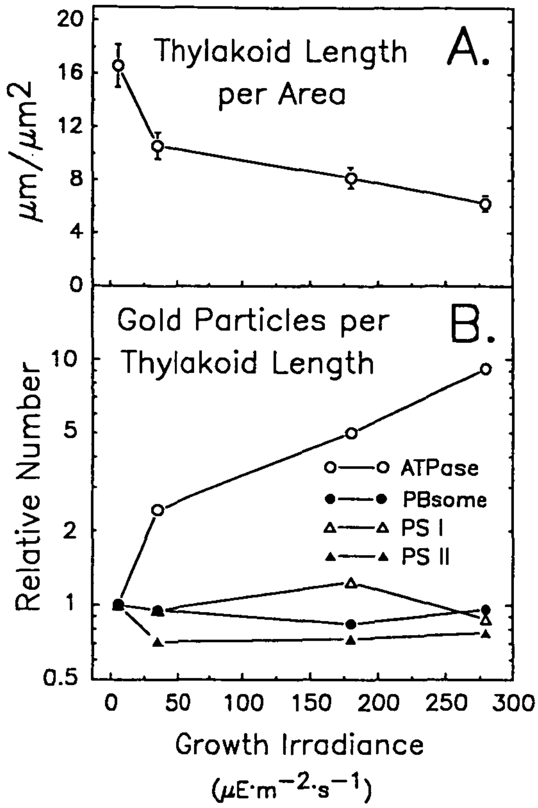

Fig. 3 A. Density of thylakoid membranes (μm thylakoid length per μm^2 section area) in ultrathin sections of *P. cruentum* as a function of PFD. B. Immunogold labelling density per μm thylakoid length as a function of PFD. Data for PS II is the average of data for the D2 and 45 kDa polypeptides of this photosystem, while that for the PBsome is the average of data for the L_{CM} and APC polypeptides.

obtained and expressed in terms of cross section area or thylakoid length. We therefore calculated ratios of two different components and compared the effects of a change in the growth irradiance on these ratios. In Fig. 4A are plotted the relative amounts of spectroscopically-assayed PS II and PS I in cells of *P. cruentum* as a function of PFD (data from Cunningham et al. 1989), and the relative immunogold label density of PS II antigens (average of data for the D2 and 45 kDa polypeptides) per PS I antigen under the same conditions. The coincidence of the plots is quite good. In Fig. 4B, the effect of PFD on the relative number of ATPase per PBsome, each quantified by rocket immunoelectrophoresis (data for PBsomes is from Cunningham et al. 1989; that for ATP synthase is from Cunningham, Vonshak and Gantt, in preparation) is likewise compared to immunogold estimates. Again the coincidence of the two plots is good. The close agreement obtained using these very different techniques provides additional support for our immunogold results and the conclusions based on them.

Discussion

Irradiance effects on thylakoid membrane composition—We have previously shown that the total numbers of PS I, PS II, and PBsomes in cells of *P. cruentum* are greatly affected (up to a threefold change) by the growth irra-

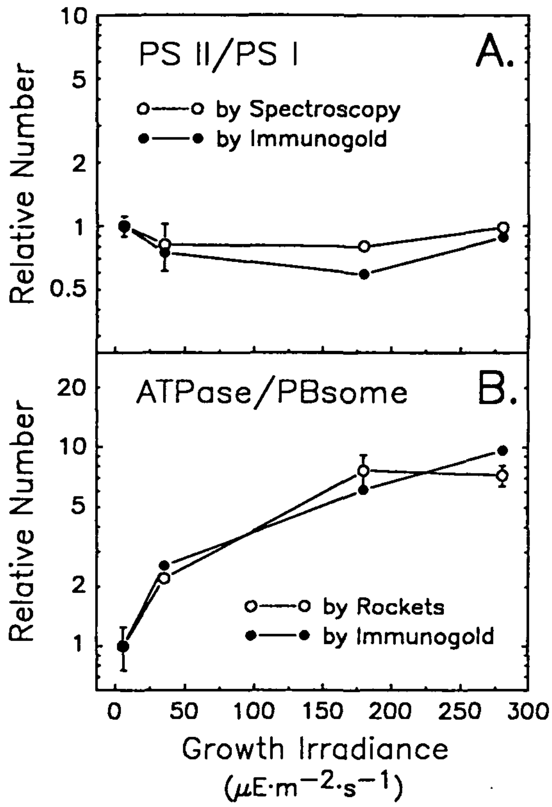

Fig. 4 Comparison of quantitative immunogold data with data obtained previously by spectroscopy and rocket immunoelectrophoresis. A. Relative numbers of photosystems I and II as a function of PFD. Spectroscopic estimates of PS I and PS II were based on the light-induced absorption changes at 701 nm (P_{700}) and 325 nm (Q_A), respectively (Cunningham et al. 1989). B. Relative numbers of PBsomes and ATP synthase as a function of PFD. Rocket estimates of PBsomes and ATP synthase were based on relative amounts of APC and the *beta* ATPase subunit, respectively. The spectroscopic and rocket data are plotted with error bars indicating one standard deviation. All ratios were normalized to a value of 1 at the 6 μE m^{-2} s^{-1} data point. A log scale was used for the ordinate so that the shape of the plots was not affected by normalization of the data. The same log scale is used in each panel.

diance, but their stoichiometric relationship changes little over the range 6 to 280 μE m^{-2} s^{-1} (Cunningham et al. 1989). One of our goals in the present work was to determine how the changes in cell content were manifested at the level of the thylakoid membrane. Our data indicate that numbers of PS I, PS II and PBsomes per thylakoid area are not substantially affected by change in PFD (white light) under nutrient sufficient conditions (Fig. 3B).

In stark contrast to the relatively constant concentration of the light-harvesting complexes is the massive increase in the concentration of ATP synthase under high irradiance. The number of quanta absorbed per thylakoid area should be roughly proportional to the PFD, thus the infrastructure of thylakoid membranes in cells grown under 280 μE m^{-2} s^{-1} of white light is obliged to accomodate nearly fifty times as many photons per unit area of membrane (or per Chl or reaction center) as that in cells grown under only 6 μE m^{-2} s^{-1}. The tenfold increase in the thylakoid membrane concentration of ATPase undoubtedly reflects one facet of an acclimatory response to this demand.

Irradiance effects on RUBISCO content and concentration—We found that specific immunogold labelling densities for RUBISCO over the pyrenoid and stroma regions of the chloroplast were somewhat affected by growth irradiance (Fig. 2), but the differences were not large and no clear relationship to irradiance is apparent. What is clear is that, regardless of the growth irradiance, the preponderance of the RUBISCO resides in the pyrenoid. Little or no redistribution of RUBISCO from pyrenoid to stroma occurs even as the growth irradiance (and the CO_2 fixation rate) is increased from a very low, limiting quantum flux (6 μE m^{-2} s^{-1}) to a high, saturating one (280 μE m^{-2} s^{-1}). Our findings lead us to conclude, as have others (Kuchitsu et al. 1988, McKay and Gibbs 1989, Osafune et al. 1990), that RUBISCO in the pyrenoid is probably functional in photosynthesis and not simply stored there.

The unique composition of membranes which traverse the pyrenoid—There are no indications of specialized thylakoid domains in *P. cruentum* comparable to the granal and stromal thylakoid membranes found in chloroplasts of higher plants and green algae. We have no evidence to suggest other than a homogeneous distribution of light-harvesting complexes within the bulk of the thylakoids. However, our results (Fig. 2) provide strong evidence that those membranes which traverse the pyrenoid do constitute a specialized thylakoid domain and contain polypeptide components of PS I but not of PS II or PBsomes. Our data complement those of McKay and Gibbs (1990) who detected PS I activity but not PS II activity or the peripheral PBsome antenna component phycoerythrin in the pyrenoid region of *P. cruentum* chloroplasts.

There are few clues as to the role or function of these PS I-containing membranes in the pyrenoid. Photoproduction of ATP to support a CO_2 concentrating mechanism and provide substrate for RUBISCO (without also increasing the concentration of O_2 from the operation of PS II) is one speculation. The recent report of Okabe and Okada (1990) that the pyrenoid of *Bryopsis maxima* contains substantial nitrate reductase activity suggests another role for these membranes.

Immunogold labelling as a quantitative technique—Immunocytochemical methods are predicated on the assumption that the binding of antibodies under certain, defined conditions can be specific and proportional to the number of antigens present in the sample. The data presented in this paper appear to conform reasonably well to this requirement. However, a number of theoretical and practical constraints (discussed by Griffiths and Hoppeler 1986 and by Kellenberger et al. 1987) can result in severe deviation from ideal behavior and could prevent reliable quantitative use of this technique. The fixation and embedding required to preserve fine structure may be inimical to the maintenance of antigenicity, and only a few of the antigenic sites present in a section are exposed on the surface and accessible to antibodies. Also, antigenicity can be lost as a result of oxidation or proteolysis on the surface of the section. The possibility of a false negative reaction is thus a very real one. False positive reactions may also arise depending on antiserum specificity and the presence of related antigenic determinants on other molecules and in other parts of the cell. The advantages of using the most specific antiserum available are substantial.

Many of the factors which limit the sensitivity and impede the reliability of immunogold quantitation in fixed and embedded ultrathin sections can be avoided, at least for membrane proteins, by using intact membranes which are spread on grids and reacted with antisera before fixation. Such samples also do not require subsequent embedding. In theory, absolute quantitation of membrane components may be possible in this way, with the additional advantage that information on the two-dimensional organization of membrane components, with resolution at the level of individual multiprotein complexes (e.g. PS I, PS II, PBsome, Cyt b_6/f and ATP synthase), may be obtainable. The potential of this technique is currently being evaluated in our laboratory.

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