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# THE POLYPEPTIDES OF STACKED AND UNSTACKED *CHLAMYDOMONAS REINHARDI* CHLOROPLAST MEMBRANES AND THEIR RELATION TO PHOTOSYSTEM II ACTIVITY

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## SUMMARY

When cells of the *ac*-5 mutant strain of *Chlamydomonas reinhardi* are cultured mixotrophically, their chloroplast membranes are unstacked and they lack a group of membrane polypeptides that have been reported to be associated with a membrane fraction enriched for Photosystem II activity. On the other hand, the chloroplast membranes of cells grown phototrophically are stacked and they possess the membrane polypeptides. Since the unstacked membranes possess Photosystem II activity, we suggest that the polypeptides must be present in the chloroplast membrane if stacking is to occur

## INTRODUCTION

Biological membranes are most often found to be separated by distances of at least 100–200 Å (ref. 1). When, however, plasma membranes are in close apposition, the regions of juxtaposed membrane are believed to serve specialized functions which, in the case of tight junctions, may be concerned with permeability[2,3] or, as in the case of gap junctions, with electronic coupling between cells[4,5]. Furthermore, it has been established[6] that the regions of membrane involved in gap junctions possess a structure and chemical composition distinct from the rest of the plasma membrane.

The most extensive union between biological membranes occurs in the chloroplasts of green algae and higher plants where numerous membranes are apposed into stacks or grana. These membranes are known from freeze-etch electron microscopy to be structurally distinct from unstacked chloroplast membranes[7], but their function in photosynthesis is not clear Certain chemical distinctions also characterize chloroplast membranes, for it has been shown that membrane fractions enriched for Photosystem II activity possess a profile of polypeptides that is distinct from the membrane fraction enriched for Photosystem I activity[8]. In this paper we show that the Photosystem II-associated group of chloroplast membrane polypeptides is affected in unstacked chloroplast membranes of the unicellular green alga, *Chlamydomonas reinhardi*, and we propose that the presence of these polypeptides is essential in order

for membranes to join and to form stacks or grana*. We also show that neither the presence of these polypeptides nor the stacking of the chloroplast membranes is required for Photosystem II activity *in vivo*.

## METHODS

The wild-type strain of *C. reinhardi* and the mutant strain *ac*-5 were used in the experiments described here. The mutant strain is chlorophyll deficient[7] and its chloroplast membranes are unstacked when cells are cultured mixotrophically (in the light with acetate and $CO_2$ as carbon sources) but stacked when they are cultured phototrophically[7]. Mixotrophically and phototrophically grown cells are equally chlorophyll deficient[7].

For the experiments described here, cells were cultured either phototrophically in minimal medium[9] or mixotrophically in minimal medium supplemented with 0.2 % sodium acetate. Cultures were maintained at a light intensity of 4000 lux from daylight fluorescent lamps and at a temperature of 27 °C. Cells were harvested from cultures that were in the logarithmic phase of growth.

The analysis of chloroplast membrane polypeptides by electrophoresis was carried out using chloroplast membranes prepared according to the procedure of Hoober[10]. For certain experiments the membranes were unstacked by washing them 5 times in 0.05 M Tricine–NaOH buffer (pH 7.3)[11]. When the polypeptides from total cell membrane protein, soluble cell protein, and total cell protein were analyzed, the cells were disrupted in a French press[12].

Chloroplast membrane polypeptides were obtained from purified chloroplast membranes by the method of Hoober[10] except that the proteins were solubilized in sodium dodecylsulfate and urea by placing the mixture in a boiling water bath for 90 s. Electrophoresis on sodium dodecylsulfate polyacrylamide gels was carried out as previously described[8]. Total cell protein, total membrane protein, and soluble cell proteins were prepared according to the method described by Hoober[12].

The amino acid analysis of chloroplast membrane proteins was carried out with the aid of a Beckman Model 120c amino acid analyzer[13].

The chlorophyll content of cells or of chloroplast membranes was determined by a modification[14] of the procedure of Maccinney[15]. Protein was determined by the method of Lowry *et al.*[16] Cell number was determined with the aid of a hemacytometer.

Samples of chloroplast membranes were tested for protease activity according to the method of Kunitz[17] in which the membranes were placed in a solution of casein and bovine serum albumin and any digestion of the proteins was detected by measuring the absorbance of the supernatant at 280 nm after the solution had been exposed to the membranes for various lengths of time.

$O_2$ evolved in a Hill reaction by whole cells using *p*-benzoquinone as an electron acceptor was measured with a Yellow Springs Instrument Co. oxygen monitor and Clark electrode. Light was supplied from a tungsten 1000-W projection lamp, and it was attenuated using neutral density filters or screens.

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Whole cells and preparations of chloroplast membranes were monitored by electron microscopy using fixation and embedding procedures described elsewhere[7,18].

## RESULTS

The photosynthetic properties of chloroplast fragments of *ac*-5 and of wild type have been described elsewhere[7]. We have measured the *in vivo* Hill reaction using *p*-benzoquinone as the electron acceptor, and Fig. 1 depicts rates typical for wild-type cells and cells of the mutant strain. None of the observations with *ac*-5 suggest that there is any major difference in the photosynthetic capacity of stacked *versus* unstacked membranes. The lower *in vivo* Hill reaction rate on a cell basis obtained for the mutant strain can be attributed to either a deficiency of the number of reaction centers per cell or to an impairment of photosynthetic electron transport.

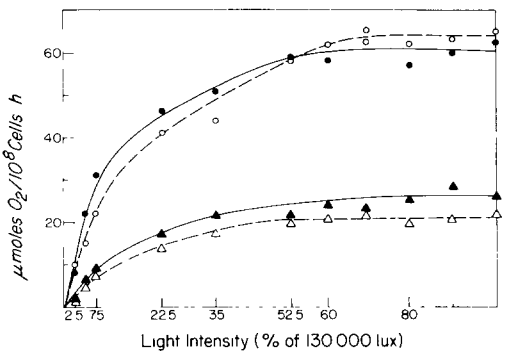


Fig 1 Photosystem II activity of mixotrophically and phototrophically grown cells of wildtype and *ac*-5 *C reinhardi* ●–●, mixotrophically grown wild-type ○- - -○, phototrophically grown wild type. ▲–▲, mixotrophically grown *ac*-5. △- - -△, phototrophically-grown *ac*-5. The reaction mixtures were 6 2 mM phosphate buffer (pH 7.0) and 6.4 mM KCl. They contained 1 32 μmoles of *p*-benzoquinone and cells equivalent to 10–15 μg chlorophyll. The volume of each reaction mixture was 2.2 ml. Samples were placed in a water-jacketed vessel and the temperature was maintained at 25 °C A separate sample was used for measurements at each light intensity Each sample was allowed to equilibrate in the dark for at least 1 min before the light was turned on. $O_2$ evolution in the light was recorded for 3 min, and the rate of $O_2$ evolution was calculated from the initial slope of the recording. An Esterline–Angus Speed Servo recorder was used. It was calibrated for 20 mV full scale.

The profiles of polypeptides obtained from the chloroplast membranes of mixotrophically and phototrophically grown cells of the wild-type strain are shown in Fig 2, in both cases the membranes join and form stacks[19], and in both cases the polypeptide profiles are decidedly similar. The polypeptides labeled IIa, IIb, and IIc in the profiles are of particular interest for they comprise the group of polypeptides that are associated with a membrane fraction rich in Photosystem II activity[8].

Fig. 3a shows a typical gel scan of chloroplast membrane polypeptides obtained from mixotrophically-grown *ac*-5 cells whose chloroplast membranes are not stacked[7], and it is seen that Polypeptides a, b, and c are deficient. In contrast, these polypeptides are readily apparent among the chloroplast membrane polypeptides

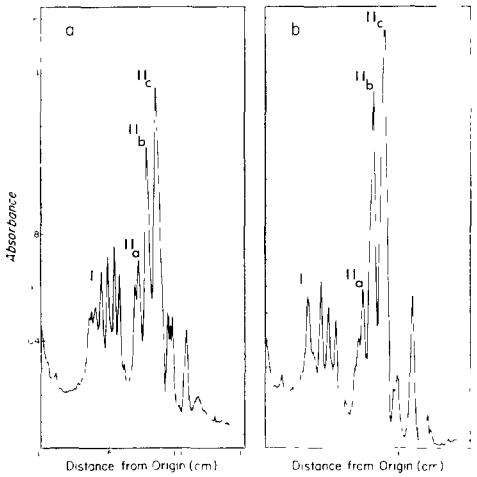


Fig 2 Electrophoretic separation of chloroplast membrane polypeptides from wild-type *C reinhardi*. a Mixotrophically grown cells, 80 $\mu$g protein b Phototrophically-grown cells, 90 $\mu$g protein Protein was subjected to electrophoresis on sodium dodecylsulfate polyacrylamide gels as described by Hoober[10] except that power was applied by a pulsed power supply operating at 160 V and 25 mA and 105 pulses/s. The gels were stained with Coomassie Blue and scanned at 555 nm I, Group I polypeptides, IIa, IIb, IIc, Group II polypeptides

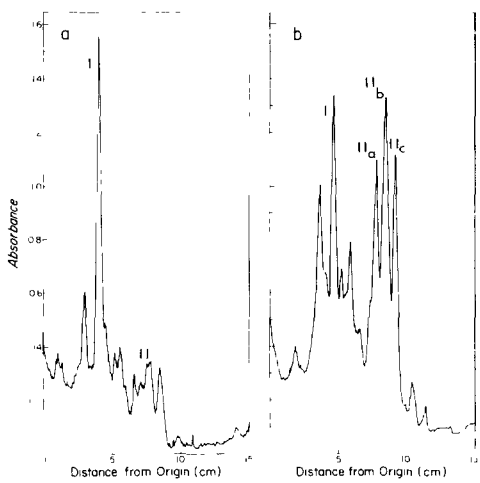


Fig 3 Electrophoretic separation of chloroplast membrane polypeptides from the *ac*-5 mutant strain of *C reinhardi* a Mixotrophically grown cells, 92 $\mu$g protein b. Phototrophically grown cells, 100 $\mu$g protein

obtained from phototrophically-grown *ac*-5 cells (Fig 3b) whose chloroplast membranes are stacked[7].

In order to determine whether Polypeptides IIa, IIb, and IIc were in some fraction other than the one containing chloroplast membranes, the polypeptides obtained from soluble cell proteins, total cell membrane proteins, and total cell proteins of

mixotrophically grown wild-type cells were compared by electrophoresis. The three polypeptides were not detected in any of the protein fractions that were obtained from mixotrophically grown *ac*-5.

Since the surfaces of the unstacked membranes obtained from the mixotrophically grown cells of *ac*-5 are more exposed than those of stacked membranes, it was considered possible that some or all of the polypeptides in question might be solubilized and lost during the isolation of the chloroplast membranes. In order to test for this possibility chloroplast membranes isolated from mixotrophically grown cells of wild type and phototrophically grown *ac*-5 were unstacked according to the method of Izawa and Good[11] by washing them 5 times in 0.05 M Tricine–NaOH buffer (pH 7 3). An aliquot of washed membrane preparation was examined by electron microscopy and it was seen that the membranes were unstacked. The remaining material was used for electrophoresis. As Fig. 4 shows, there is no significant loss of Polypeptides IIa, IIb, or IIc.

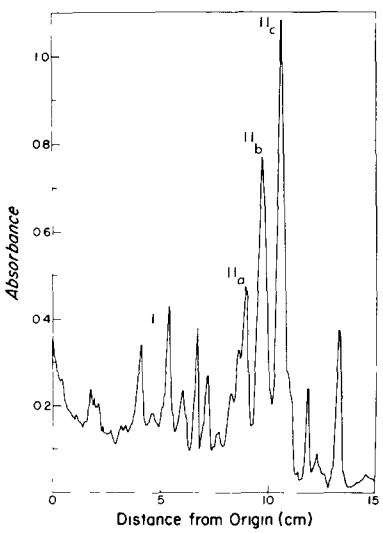


Fig 4 Electrophoretic separation (92 $\mu$g protein) of polypeptides from unstacked chloroplast membranes of mixotrophically grown wild-type cells. Chloroplast membranes were unstacked by washing them 5 times in 0 05 M Tricine–NaOH buffer (pH 7.3)

The absence of the three polypeptides from cells with unstacked membranes might be accounted for by the greater exposure of the membrane surfaces to proteolytic enzymes during the isolation procedure. However, when stacked wild-type membranes and unstacked *ac*-5 membranes were incubated with bovine serum albumin and casein for times up to 19 h at room temperature, there was no significant effect upon these substrates when measured spectrophotometrically as the release of small peptides.

Since cations appear to play a role in the union and stacking of chloroplast membranes[11,20], they may interact with or affect anionic sites on the membrane surfaces The free carboxyl groups of aspartic and glutamic acids and the phosphate groups of phospholipids are likely candidates for these anionic sites. An analysis of

the amino acid composition of chloroplast membranes obtained from mixotrophically grown wild-type and *ac*-5 cells revealed no significant difference in either aspartic or glutamic acids· the wild-type membranes were found to contain 6.77 and 8.08 mole $^{o}_{o}$ aspartic and glutamic acids, respectively; the values found for *ac*-5 membranes were 7.53 and 8.86 mole $^{o}_{o}$. The phospholipid composition of the membranes is currently under investigation.

## DISCUSSION

Studies of the chloroplast membranes of *ac*-5 permit us to make several statements regarding the stacking of membranes and the membrane polypeptides that are affected in the mutant strain. Arntzen *et al*[21] published a study of the internal structure of spinach chloroplast membranes as revealed by freeze–etch electron microscopy. It was known from the studies of Park and Branton[22] that chloroplast membranes possess tightly packed arrays of unusually large particles, and Arntzen *et al.*[21] demonstrated that when spinach chloroplasts were treated with digitonin to yield several membrane fractions, a fraction rich in Photosystem II activity was also the exclusive location of the large particles. This observation suggested that the large particles corresponded to Photosystem II. Since the Photosystem II-rich fraction was also rich in stacked membranes, however, it was recognized[7] that the large particles might instead represent a structural conformation assumed during membrane fusion. A test of these alternatives was possible by studying the freeze–etch structure of *ac*-5 membranes, since it was known that the membranes possessed the full Photosystem II activity whether stacked or unstacked. The results of this study[7] showed that the large particles were in fact absent from the unstacked membranes and present in the stacked membranes

The work of Goodenough and Staehelin[7] therefore indicates that stacked membranes contain arrays of large particles that can be visualized by freeze–etch microscopy and that unstacked membranes of *ac*-5 do not contain these particles. The unstacked membranes of the mutant strain also lack at least three principal chloroplast membrane polypeptides. When the membranes of the mutant strain become stacked in cells growing phototrophically, the particles and the polypeptides are both present. Thus there is a correlation between the stacking of the membranes, the presence of large particles, and the presence of certain polypeptides. The relationship between these features of membrane structure is as yet unknown, but it seems reasonable to adopt as a working hypothesis the concept that the polypeptides must be present in the chloroplast membranes if stacking is to occur and if large particles are to form as a consequence of the stacking process.

What little is known about the stacking of chloroplast membranes suggests that the ionic nature of the membrane surface plays an important role, since the stacking of chloroplast membranes *in vitro* depends in part on cation concentration[7,11,20] and the separated membranes can be re-stacked upon the addition of $MgCl_2$, NaCl, methylamine hydrochloride, or KCl. Also, unpublished work from this laboratory shows that the addition of the polycation, protamine sulfate, to a suspension of unstacked wild-type chloroplast membranes of *C. reinhardi* is effective in bringing about their stacking, perhaps in a manner similar to the union that occurs between mammalian cell membranes when they are exposed to polycations[24,25].

In view of the effects that cations have on the stacking of chloroplast membranes, it is tempting to assume that the membrane surfaces are studded with anionic sites whose charge or conformation must be altered in order for membranes to stack. It is further tempting to assume that the anionic sites are the free carboxyl groups of aspartic and glutamic acid residues of chloroplast membrane proteins and that proteins rich in these amino acids are affected in *ac*-5. If such is the case, however, the situation in mixotrophically grown *ac*-5 is not a simple one, for the amino acid analysis of its chloroplast membrane proteins does not show any striking difference when compared with that of wild-type chloroplast membranes.

The relationship between the stacking of chloroplast membranes and photosynthesis remains controversial. So far it has not been possible to find any distinctive qualitative differences between the photosynthetic properties of stacked and unstacked chloroplast membranes; this might suggest that stacking is irrelevant to the photosynthetic process. The fact that blue–green algae possess unstacked membranes and perform normal photosynthesis would support this point of view. It has been suggested[26] that the *in vitro* efficiency of Photosystem II is lower in the unstacked chloroplast membranes of bundle sheath cell chloroplasts of certain grasses than it is in the stacked membranes of the chloroplasts from mesophyll cells from the same plants; this does not appear to be the case for *C. reinhardi*, at least for the *in vivo* Photosystem II activity of *ac*-5, since the rate *versus* light intensity curves for the *p*-benzoquinone Hill reaction are identical for phototrophically and mixotrophically grown cells. Despite the fact that the existing experimental evidence does not yet support such a view, it seems reasonable to assume that the gathering of membranes into stacks or grana would enhance the probability of trapping photons, for the stacks would provide for relatively high local concentrations of photosynthetic pigments as well as the reaction centers where excitation energy is utilized to drive the photosynthetic electron transport chain. It is of interest in this regard to note that the grana stacks are exceedingly large in the chloroplasts of several species of plants that grow on the floor of tropical rain forests[27] where the light energy in the waveband available for photosynthesis is but a very small fraction of the energy that reaches the leaves of the trees that comprise the forest canopy[28].

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REFERENCES

1 Fawcett, D (1966) *The Cell, Its Organelles and Inclusions*, Saunders, Philadelphia
2 Farquhar, M G. and Palade, G. E (1963) *J. Cell Biol* 17, 375
3 Frompter, E. and Diamond, J. (1972) *Nat. New Biol* 235, 9
4 Davey, M M and Barr, L. (1964) *J. Cell Biol.* 23, 553
5 Pappas, G. D., Asada, Y. and Bennett, M. V. L. (1971) *J Cell Biol.* 49, 173
6 Goodenough, D A. and Stoeckenius, W. (1972) *J Cell Biol* 54, 646

7 Goodenough, U. W. and Staehelin, L A. (1971) *J Cell Biol* 48, 594
8 Levine, R. P., Burton, W G. and Duram, H. A (1972) *Nat New Biol* 237, 176
9 Sueoka, N. (1960) *Proc Natl. Acad Sci U S* 46, 83
10 Hoober, J. K (1970) *J Biol. Chem.* 245, 4327
11 Izawa, S. and Good, N. E. (1966) *Plant Physiol* 41, 544
12 Hoober, J. K (1972) *J Cell Biol* 52, 84
13 Spackman, D. H , Stein, W. H and Moore, S (1958) *Anal. Biochem* 30, 1190
14 Arnon, D (1949) *Plant Physiol.* 24, 1
15 Mackinney, G. (1941) *J Biol. Chem* 140, 315
16 Lowry, O H., Roseborough, N. J , Farr, A L. and Randall, R J (1951) *J Biol Chem.* 193, 265
17 Kunitz, M. (1947) *J. Gen. Physiol.* 30, 291
18 Goodenough, U. W and Levine, R P. (1970) *J. Cell Biol* 44, 547
19 Goodenough, U. W. and Levine, R. P (1969) *Plant Physiol* 44, 1001
20 Anderson, J. M. and Vernon, L P (1967) *Biochim Biophys Acta* 143, 363
21 Arntzen, C. J , Dilley, R A and Crane, F L (1969) *J Cell Biol.* 43, 16
22 Park, R. B and Branton, D. (1966) *Brookhaven Symp Biol.* 19, 341
23 Anderson, J M. and Boardman, N K (1966) *Biochim Biophys Acta* 112, 403
24 Katchalsky, A , Danon, D., Nevo, A and De Vries, A (1959) *Biochim Biophys Acta* 33, 120
25 Quinton, P. M. and Phillpott, C. W (1973) *J Cell Biol* 56, 787
26 Smillie, R. M., Bishop, D. G and Andersen, K. S. in *IInd Int Cong. Photosynthesis* (Forti, G , Avron, M and Melandri, A., eds), Vol I, p 779, W Junk, The Hague
27 Goodchild, D J , Bjorkman, O and Pyliotis, N A (1971–1972) in *Annual Report of the Director, Dept of Pl. Bio., Carnegie Institution*, p 102
28 Bjorkman, O and Ludlow, M M (1971–1972) in *Annual Report of the Director, Dept. of Pl Bio , Carnegie Institution*, p 85