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Chlamydomonas reinhardtii cell preparation with altered permeability toward substrates of organellar reactions

(envelope-intact chloroplasts/mitochondria/photosynthetic CO_2 fixation)

WILLIAM R. BELKNAP AND ROBERT K. TOGASAKI

Department of Biology, Indiana University, Bloomington, Indiana 47401

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ABSTRACT ***Chlamydomonas reinhardtii*** **cells disrupted under low pressure in a Yeda press yielded a preparation ("pressate") with high permeability toward substrates for Class A chloroplasts and intact mitochondria. The stoichiometric rates of CO_2 uptake and O_2 photoevolution by the wild-type cell pressate were severely suppressed by 10 mM exogenous phosphate, and this suppression could be reversed by the addition of either 3-phosphoglycerate or dihydroxyacetone phosphate. A mutant, *F60*, which lacks phosphoribulokinase activity and hence CO_2-dependent O_2 photoevolution, was studied by using intact cells, pressate, and sonicated pressate. In the pressate, the rate of 3-phosphoglycerate-dependent O_2 photoevolution was high, whereas that dependent on $K_3Fe(CN)_6$ was low; the opposite was true of the sonicated pressate. *p*-Benzoquinone supported high rates of O_2 evolution in both the pressate and the sonicated pressate. The slow O_2 uptake in the dark by the dark-adapted wild-type pressate could be increased by the addition of succinate and further stimulated by ADP. Addition of KCN resulted in rapid but only partial suppression of this activity. Dark O_2 uptake by the unpressed preparation did not show similar responses. The procedure described here opens the possibility of *in situ* analysis of Class A chloroplasts from wild-type and mutant strains of *C. reinhardtii*.**

Chlamydomonas reinhardtii, a unicellular facultative autotroph, is a very useful organism for photosynthesis research. It shares many advantages with other algal systems, such as the ease of standardizing experimental material and ready amenability to many microbiological techniques (1). It is especially well suited for the mutational analysis of chloroplast functions because its nuclear and chloroplast genetic systems are well characterized and many mutant strains with altered chloroplastic phenotypes are available (2–5).

Past research efforts on mutational analysis of chloroplast functions in *Chlamydomonas* and other algae have focused on phenomena analyzable either with the intact organism, such as chloroplast biogenesis, or with broken thylakoid fragments, such as photosynthetic electron transport and photophosphorylation (6). In contrast, photosynthetic carbon metabolism, which is dependent upon stromal proteins enclosed in the chloroplast envelope, has received little attention. This uneven development was caused in part by the difficulty in obtaining mutant strains with lesions specifically localized in photosynthetic carbon cycle components as well as by the difficulty in isolating chloroplasts with functionally intact envelopes from algal sources, including *Chlamydomonas* (7).

We have initiated a research program on mutational analysis of photosynthetic carbon metabolism in *Chlamydomonas reinhardtii*. Along with appropriate mutant strains defective in photosynthetic carbon cycle components, such a program requires the capability to analyze complete or partial reactions of the photosynthetic carbon cycle in wild-type and mutant cells at the level of the envelope-intact chloroplast. Isolation of Class A chloroplasts from *Chlamydomonas* cells will be an obviously ideal solution to the second problem. Class A chloroplasts capable of high rates of photosynthetic CO_2 fixation have been extremely useful in analysis of chloroplast functions, but, to date, the bulk of research with Class A chloroplasts has been limited to higher plant systems such as spinach and peas (8). Though the isolation of chloroplasts capable of CO_2 fixation has been reported for several algae (9–17), their use has been confined mainly to the study of reactions mediated by broken chloroplasts (6, 18, 19).

During the course of our program to isolate such chloroplasts, we have succeeded thus far in making a cell preparation that has high permeability toward substrates of both chloroplast and mitochondrial reactions while retaining high activity in both organelles. Although we do not yet have an isolated Class A chloroplast, we now have a capability to analyze both the whole and partial reactions of the photosynthetic carbon cycle in wild-type and mutant strains of *Chlamydomonas*. Also, this cell preparation allows us to study mitochondrial functions more directly than before.

MATERIALS AND METHODS

Strains and Culture Conditions. *Chlamydomonas reinhardtii* wild-type (*Wt*) strain 137c(+) and mutant strain *F60*(−) were grown photoheterotrophically in Tris/acetate/phosphate medium as described (20), to final chlorophyll (Chl) concentrations of 10–15 μg/ml of medium. For photosynthetic O_2 photoevolution and $^{14}CO_2$ uptake assays the cells were harvested, resuspended in high-salt minimal medium (21), and further incubated in the dark on a rotary shaker for 16 hr. For dark O_2 uptake and enzyme distribution analysis the 16-hr dark incubation was omitted.

Cell Preparation. Cell suspensions containing 3 mg of Chl were harvested and resuspended in 35 ml of 20 mM Hepes buffer, pH 7.0, 25°C. This suspension was centrifuged at 700 × *g* for 4 min, and the resulting supernatant fluid was discarded. The pellet was resuspended in sufficient breaking medium [0.25 M sorbitol/50 mM 2-(*N*-morpholino)ethanesulfonic acid (Mes)–Tris buffer, pH 7.5/2 mM EDTA/1 mM $MgCl_2$/1 mM $MnCl_2$, adjusted to pH 7.5 with NaOH; immediately prior to use bovine serum albumin (Sigma A 6003) was added to 1% and the pH was adjusted to 7.2 at 25°C] to result in a final Chl concentration of 0.3 mg/ml. Because upon resuspension in breaking medium the cells showed an extremely long lag prior to the onset of CO_2-dependent O_2 photoevolution, the suspension was aerated in the dark for 1 hr at 25°C on a rotary shaker. During

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Abbreviations: Chl, chlorophyll; RuP_2, ribulose 1,5-bisphosphate; Mes, 2-(*N*-morpholino)ethanesulfonic acid.

this aeration period both the lag and final rate of CO_2-dependent O_2 photoevolution approached that of Tris/acetate/phosphate-grown *Wt* suspensions in a phosphate buffer. After aeration, the suspension (unbroken preparation) was cooled to 5°C in the dark. Samples (10 ml) were placed in a cold Yeda pressure cell (22) and were equilibrated for 3 min under a N_2 gas pressure of 230 pounds/inch^2 (1.6 megapascals). The suspension was then released from the Yeda cell (at a flow rate of approximately 5 ml/sec) into an ice-cold polypropylene tube. This preparation ("pressate") was kept on ice in the dark and used for subsequent $^{14}CO_2$ uptake and O_2 photoevolution analysis. The unbroken preparation and the pressate were analyzed with a Zeiss phase-contrast photomicroscope.

For the analysis of mitochondrial functions and enzyme profiles, the pressate was fractionated into a pellet and supernatant by centrifugation at 500 × *g* for 4 min. The supernatant was saved for further analysis, and the pellet was gently resuspended in cold breaking medium. Dark O_2 uptake was monitored in the unbroken preparation, pressate, resuspended pellet, and the supernatant. For enzyme distribution analysis of the pressate, pellet, and supernatant, samples of each were sonically disrupted, then centrifuged at 30,000 × *g* for 30 min to remove most of the particulate matter.

Where indicated, samples were sonicated at ice temperature, using a Fisher Dismembrator model 150 at 80% full strength.

O_2 Exchange and $^{14}CO_2$ Uptake. O_2 exchange was measured with a Clark oxygen electrode (Yellow Springs Instrument no. 5331) fitted into a water-jacketed cylindrical chamber designed by M. Hirosawa of the Institute of Applied Microbiology, University of Tokyo, which was purchased from Makuhari Scientific Glass (Chiba, Japan). The electrode is inserted into the lower portion of the reaction chamber, with the reaction volume controlled by vertical movement of a Teflon cylinder having a small central opening for sample retrieval and substrate injection. The O_2 reaction mixture consisted of 2.6 ml of stock solution (0.3 M sorbitol/50 mM Mes–Tris buffer, pH 7.5/2 mM EDTA/1 mM $MgCl_2$/1 mM $MnCl_2$, adjusted to pH 7.5 with NaOH; immediately prior to use, KH_2PO_4 was added as indicated and the pH was adjusted to 7.2) and 0.5 ml of the unbroken preparation, pressate, pellet, or supernatant. The substrates (or inhibitor) 3-phosphoglycerate (0.1 M), dihydroxyacetone phosphate (0.1 M), $NaHCO_3$ (0.5 M), *p*-benzoquinone (37 mM), $K_3Fe(CN)_6$ (30 mM), succinate (0.25 M), ADP (50 mM), and KCN (0.5 M) were added where indicated. The final concentrations of substrates in the assay mixtures are indicated in the legends. The resulting assay mixtures with a final Chl concentration of approximately 50 μg/ml were equilibrated in the dark for at least 2 min prior to illumination or addition of substrate.

For simultaneous measurement of O_2 exchange and $^{14}CO_2$ uptake the reaction mixture volume was increased by a factor of 1.4 without changing the chemical composition of the assay except for the replacement of the unlabeled bicarbonate with a lower concentration of radioactive substrate as indicated. Samples (0.13 ml) were withdrawn from the reaction vessel where indicated by lowering the central cylinder and were transferred to glass vials containing 0.05 ml of glacial acetic acid to terminate the reaction. The acid-stable radioactivity in each sample was determined by scintillation spectrophotometry. Sodium [^{14}C]acetate of known specific activity was added as an internal standard to determine counting efficiency.

Both O_2 exchange and $^{14}CO_2$ uptake were carried out at 25°C with an incident light intensity of 1.5 kW/m^2. When a CO_2-free assay mixture was required, the mixture was flushed with air that had been passed through a column of Ascarite and bubbled through a 5 M KOH solution.

Hepes, 3-phosphoglycerate, dihydroxyacetone phosphate, Mes, Tris, and ADP were purchased from Sigma. The specific activity of the $NaH^{14}CO_3$ obtained from New England Nuclear was accepted without verification.

Enzyme Assays. Activities of glyceraldehyde-3-phosphate dehydrogenase and isocitrate dehydrogenase, both NADP-dependent, were assayed at 20°C using a Gilford 240 spectrophotometer to monitor NADP reduction with final reaction volumes of 3 ml. Glyceraldehyde-3-phosphate dehydrogenase was determined by the method of Schulman and Gibbs (23); the reaction mixture contained 40 mM Tris at pH 8.5, 4 mM cysteine at pH 8, 9 mM arsenate, 0.5 mM NADP, 4 mM glyceraldehyde 3-phosphate, and extract. Isocitrate dehydrogenase activity was determined as by Hanson and Cox (24); the reaction mixture contained 3 mM Tris·HCl at pH 7.4, 1 mM $MnCl_2$, 0.072 mM NADP, DL-isocitric acid at 0.33 mg/ml, and extract.

Phospho*enol*pyruvate carboxylase and ribulose-1,5-bisphosphate (RuP_2) carboxylase activities were determined by the fixation of $^{14}CO_2$ into acid-stable compounds as determined by scintillation spectrophotometry in assays run at 20°C. Phospho*enol*pyruvate carboxylase was assayed as by Wong and Davies (25) and modified by Robinson and Walker (26), the reaction mixture containing 33 mM Tris·HCl at pH 8, 17 mM $MgCl_2$, 0.13 mM NADH, 3.3 mM $NaH^{14}CO_3$ (0.5 μCi/mmol; 1 Ci = 3.7 × 10^{10} becquerels), 70 units of malate dehydrogenase, 0.67 mM phospho*enol*pyruvate, and extract in a final volume of 3 ml. RuP_2 carboxylase activity was determined in a reaction mixture containing 0.3 M *N*-[tris(hydroxymethyl)methyl]glycine at pH 7.5, 10 mM $MgCl_2$, 5 mM $NaH^{14}CO_3$ (0.5 μCi/mmol), 0.7 mM RuP_2, and extract in a final volume of 0.5 ml. In both assays the reactions were stopped after 25 min by addition of 0.1 ml of the reaction mixture to 0.1 ml of glacial acetic acid. Formation of acid-stable ^{14}C-labeled compounds was determined as before. All substrates and reagents used in the enzyme analyses were purchased from Sigma.

RESULTS AND DISCUSSION

The presence of the intact envelope in Class A chloroplasts as isolated from higher plant systems allows retention of soluble stromal fractions and restricts substrates' entry into the stroma except for compounds that are relatively hydrophobic and those for which there are specific translocators in the membrane (27). Both CO_2 fixation and CO_2-dependent O_2 photoevolution by such chloroplasts are highly sensitive to the orthophosphate (P_i) concentration in the assay medium. The presence of 10 mM P_i is usually sufficient to cause nearly complete inhibition (28). Inhibition by P_i can be reversed by the addition of several photosynthetic carbon cycle intermediates, including 3-phosphoglycerate and dihydroxyacetone phosphate (29). Hydrophobic class III Hill oxidants such as *p*-benzoquinone can support O_2 photoevolution in these chloroplasts whereas hydrophilic class I Hill oxidants such as $Fe(CN)_6^{3-}$ cannot (30, 31). The pressate described in this paper resembles Class A chloroplasts in these properties,

CO_2-Dependent O_2 Photoevolution. Fig. 1 demonstrates that O_2 photoevolution by the *Wt* pressate was dependent upon illumination (curve D) and the presence of CO_2 (curve C). Prior removal of the CO_2 from the reaction mixture by flushing with CO_2-free air resulted in a significant loss of O_2 photoevolution (curve C), which could be restored by the addition of $NaHCO_3$ (curve B). The O_2 photoevolution showed a lag of 1–2 min even after preincubation with $NaHCO_3$ (curve A). Rates of O_2 photoevolution greater than 40 μmol (mg Chl)$^{-1}$ hr^{-1} were routinely obtained, more than 80% of this original activity being retained after 3 hr of storage on ice in the dark (data not shown).

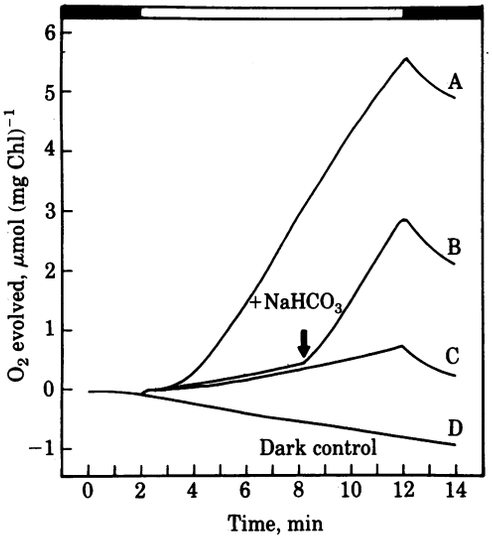

FIG. 1. CO_2-dependent O_2 photoevolution by the *Wt* pressate. All assays contained the standard reaction mixture with 0.5 mM added P_i and Chl at 40 μg/ml. The mixtures for curves A and D contained in addition 8 mM $NaHCO_3$. Mixtures for B and C were flushed with CO_2-free air for 4 min prior to illumination. $NaHCO_3$ (8 mM) was added to mixture B as indicated by the arrow. Dark and light periods are indicated by the shaded and open blocks at the top of the figure except that the mixture for D was not illuminated.

Inhibition of O_2 Photoevolution and $^{14}CO_2$ Uptake by P_i. Fig. 2 shows that the initial rate of CO_2-dependent O_2 photoevolution by the *Wt* pressate was sensitive to P_i at millimolar concentrations. While a substantial rate was observed in the absence of exogenously added P_i (curve A), maximal activity was observed in the presence of 0.5 mM added P_i (curve B). Further increases in the P_i concentration resulted in progressively greater suppression of O_2 photoevolution (curves C and D), with 10 mM added P_i resulting in greater than 80% inhibition of the inital rate (curve E).

Photoevolution of O_2 was associated with stoichiometric $^{14}CO_2$ uptake, as shown in Fig. 3 (curve A). Both $^{14}CO_2$ uptake and O_2 photoevolution were severely inhibited by 10 mM P_i (curve D). The rate of CO_2-dependent O_2 photoevolution by intact cells in aqueous suspension was not affected by this level of P_i (data not shown); the rate in the *Wt* unbroken preparation in the standard reaction mixture was suppressed 10–15% by 10 mM added P_i (data not shown).

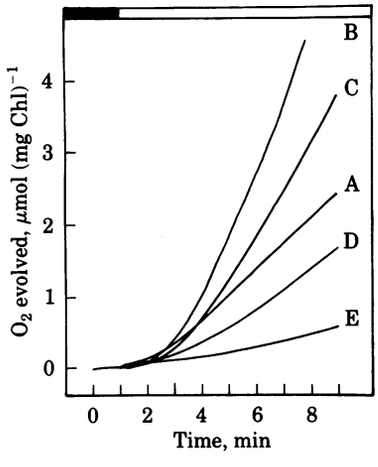

FIG. 2. Effect of P_i concentration on CO_2-dependent O_2 photoevolution by the *Wt* pressate. All assays contained the standard reaction mixture, 8 mM $NaHCO_3$, Chl at 47 μg/ml, and the following final concentrations of added P_i: curve A, 0 mM; curve B, 0.5 mM; curve C, 1 mM; curve D, 3 mM; curve E, 10 mM. Dark and light periods are indicated as in Fig. 1.

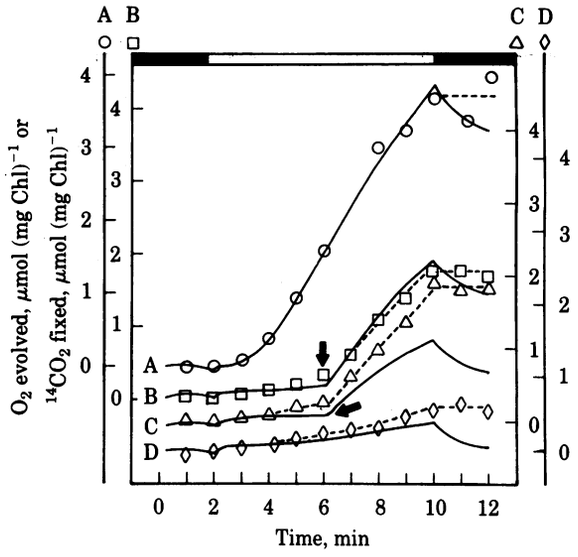

FIG. 3. Simultaneous measurements of O_2 photoevolution (——, curves A–D) and $^{14}CO_2$ uptake (----, ○, □, △, and ◇) by the *Wt* pressate. All assay mixtures contained the standard reaction mixture, 5 mM $NaH^{14}CO_3$ (0.5 μCi/μmol), and Chl at 46 μg/ml. In addition, the mixture for curves A and ○ contained 0.5 mM added P_i; all other mixtures contained 10 mM added P_i. Additions of 3-phosphoglycerate (1.44 mM) to mixture B and dihydroxyacetone phosphate (1.44 mM) to mixture C were made after 4 min of illumination as indicated by arrows. Dark and light periods are indicated as in Fig. 1.

Reversal of P_i Inhibition by 3-Phosphoglycerate and Dihydroxyacetone Phosphate. Fig. 3 shows that addition of 1.5 mM 3-phosphoglycerate (curve B) or dihydroxyacetone phosphate (curve C) partially reversed the inhibition of $^{14}CO_2$ uptake and O_2 photoevolution due to 10 mM added P_i. The rates of $^{14}CO_2$ uptake and O_2 photoevolution induced by 3-phosphoglycerate were stoichiometric (curves B), whereas the corresponding rates induced by dihydroxyacetone phosphate were not (curves C). These data suggest that the conversion of 3-phosphoglycerate to triose phosphate may have been rate limiting under the conditions used. Upon cessation of illumination there was no appreciable uptake or release of $^{14}CO_2$, whereas there was pronounced postillumination O_2 uptake. These observations are similar to those made by Walker and coworkers, using higher plant chloroplast systems (29, 32).

O_2 Photoevolution by the *F60* Pressate. The *C. reinhardtii* mutant *F60* shows little CO_2-dependent O_2 photoevolution, due primarily to a deficiency in phosphoribulokinase activity (33). Fig. 4*A* shows that an unbroken preparation of *F60* exhibited little CO_2-dependent O_2 photoevolution and was unaffected by the addition of 1 mM 3-phosphoglycerate, suggesting that this substrate did not enter the stromal space. Fig. 4*B* demonstrates that while the *F60* pressate also lacked CO_2-dependent O_2 photoevolution in the presence of 2 mM P_i, the addition of 1 mM 3-phosphoglycerate resulted in O_2 photoevolution at a rate approaching 20 μmol (mg Chl)$^{-1}$ hr^{-1}. This reflects the ability of this substrate to enter the stromal space and act as a Hill oxidant in the presence of requisite enzymes and cofactors.

Fig. 4*B* also shows the *F60* pressate's capacity to use *p*-benzoquinone as a Hill oxidant, but not $K_3Fe(CN)_6$. Fig. 4*C* shows that brief sonication of the *F60* pressate resulted in a decrease in 3-phosphoglycerate-dependent O_2 photoevolution and a con-

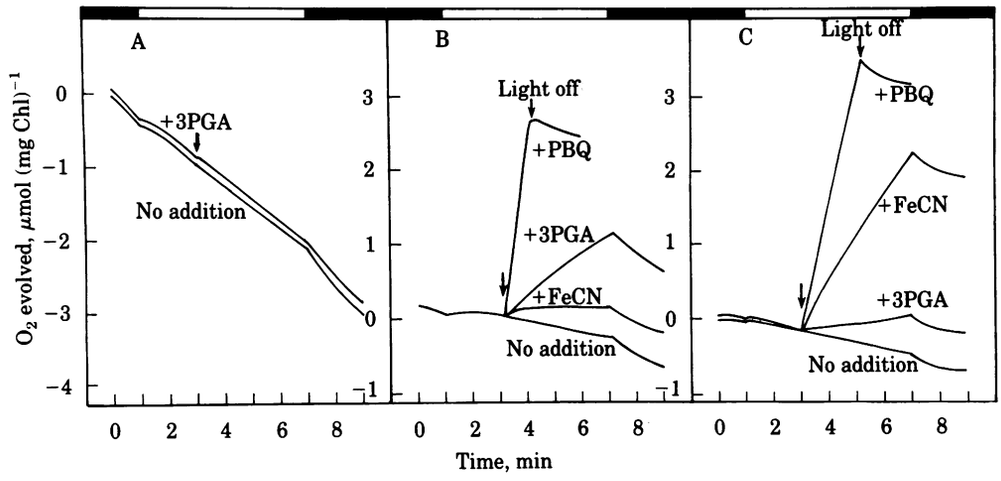

FIG. 4. O_2 photoevolution by the *F60* unbroken preparation (*A*), pressate (*B*), and sonicated pressate (*C*). All assay mixtures contained the standard reaction mixture, 8 mM $NaHCO_3$, 2 mM added P_i, and Chl at 46 μg/ml. 3-Phosphoglycerate (3PGA, 1 mM), $K_3Fe(CN)_6$ (0.46 mM), and *p*-benzoquinone (PBQ, 0.56 mM) were added where indicated by arrows. Dark and light periods are indicated as in Fig. 1, except where indicated.

comitant increase in $K_3Fe(CN)_6$-dependent activity. These data strongly suggest that the sonication of the pressate resulted in the breakdown of the limiting envelope barrier, which (*i*) resulted in the loss of stromal components required for 3-phosphoglycerate photoreduction, and (*ii*) allowed free access of $K_3Fe(CN)_6$ to the photoreducing system of the thylakoid membranes.

An uncoupler was not included in the tests with *p*-benzoquinone and $K_3Fe(CN)_6$ so that direct comparisons could be made to the 3-phosphoglycerate photoreduction rates, which require coupled electron transport. Thus the rates of $K_3Fe(CN)_6$- and *p*-benzoquinone-supported O_2 photoevolution represent underestimates for these reactions.

Dark O_2 Uptake by the *Wt* Pressate. While the above data show a close similarity between higher plant Class A chloroplasts and the pressate, some anomalies have emerged. Class A chloroplasts of higher plants can be easily lysed by osmotic shock, but similar lysis of the pressate was not observed. Even though the pressate showed a very low level of dark O_2 uptake, in comparison to the unbroken preparation, the addition of succinate resulted in marked stimulation of the O_2 uptake rate, which was enhanced further by the addition of ADP (Table 1). Addition of KCN inhibited the O_2 uptake rapidly and severely. Upon centrifugation of the pressate, only the pellet fraction showed these responses, not the supernatant. Active dark O_2 uptake in the unbroken preparation was affected very little by the addition of succinate and ADP and was inhibited less by KCN. The data in Table 1 indicate that the pressate contains

Table 1. Effect of sequential addition of succinate, ADP, and KCN on dark O_2 uptake by the *Wt* preparations

	O_2 uptake, μmol (mg Chl)$^{-1}$ hr^{-1}			
Addition	Unbroken preparation	Pressate	Pellet	Supernatant*
None	9.2	2.5	2.5	0.5
Succinate	12	11	17	3.3
ADP	12	20	30	3.3
KCN	6.9	5.0	3.6	0.0

All assay mixtures contained the standard reaction mixture. Succinate (4 mM), ADP (0.75 mM), and KCN (3 mM) were added at 2-min intervals. Rates of oxygen uptake were recorded and corrected for a low level of oxygen uptake due to breaking medium alone.
* Rate calculated by using Chl concentration of pressate.

active mitochondria and that exogenously added substrates can reach these organelles. The pellet fraction also exhibits high rates of 3-phosphoglycerate-dependent O_2 photoevolution (data not shown), demonstrating that the intact chloroplast activity is also located in the pellet.

Distribution of Marker Enzyme Activities After Pressate Fractionation. Because both intact chloroplasts and mitochondria were present in the pellet, it is more likely that the pressate contains cells with altered permeability than that it contains free organelles. To further define the status of the preparation, the pressate was fractionated by centrifugation into the supernatant and pellet and the distribution of activities of four marker enzymes was studied. RuP_2 carboxylase and glyceraldehyde-3-phosphate dehydrogenase(NADP) were chosen as markers for chloroplast stroma proteins, and isocitrate dehydrogenase(NADP) and phospho*enol*pyruvate carboxylase for cytoplasmic proteins.

As Table 2 indicates, nearly all of the chloroplast marker enzyme activities were found in the pellet, and more than half of the isocitrate dehydrogenase activity was lost into the supernatant. Though most of the phospho*enol*pyruvate carboxylase remained in the pellet, its percentage distribution in the supernatant was greater than for chloroplast marker enzymes.

These data strongly suggest that the pressate contains not cell-free isolated organelles but rather highly permeabilized cells. This suggestion is supported by phase-contrast photomicrographs of the unbroken preparation and the pressate that show well-dispersed and refractive particles of similar sizes, with no organellar clumps. These particles do not lyse upon transfer to medium of low osmotic strength (data not shown). The enzyme profiles imply that macromolecules such as cytoplasmic enzymes are partially retained, but that the permeability barrier against small molecules, such as substrates for organellar reactions, is diminished greatly. Because 55% of the isocitrate dehydrogenase activity and 16% of the phospho*enol*pyruvate carboxylase activity are found in the supernatant, it must be assumed that during the preparation of the pressate some release of these enzymes occurred, and then ceased. It is suggested that the plasma membrane is perturbed during the single pass through the Yeda pressure cell, and that the damaged membrane is only partially repaired. This would result in leakage and high membrane permeability towards small molecules. It should be noted that, while the addition of succinate stimulated the dark O_2 uptake of the pellet, the addition of equivalent amounts of 3-phosphoglycerate had no effect (data

Table 2. Distribution of enzyme activities and chlorophyll after pressate fractionation

Enzyme	Enzyme activity or Chl content*			% of (pellet + supernatant)		
	Pressate	Pellet	Supernatant	Pressate	Pellet	Supernatant†
Glyceraldehyde-3-*P* dehydrogenase	76 (±5.5)	76 (±2.8)	2.5 (±0.36)	97	97	3
Isocitrate dehydrogenase	54 (±1.0)	27 (±1.8)	33.6 (±3.2)	90	45	55
RuP_2 carboxylase	152 (±15)	148 (±5.5)	4.0 (±0.7)	100	97	3
Phospho*enol*pyruvate carboxylase	1.6 (±0.07)	1.4 (±0.07)	0.26 (±0.005)	96	84	16
Chl	298 (±2)	305 (±2.5)	<5	98	100	0

* Enzyme activities are given in μmol of NADP reduced (mg Chl)$^{-1}$ hr^{-1} for dehydrogenases and cpm × 10^{-3} fixed (mg Chl)$^{-1}$ for carboxylases. Chl is given as μg/ml. Values are reported as an average of two experiments in which all enzymes are analyzed simultaneously. Variations about the averages are shown in parentheses.

† Enzyme activities calculated by using the Chl concentration of pressate.

not shown). The stimulation of light-dependent O_2 photoevolution indicates that 3-phosphoglycerate is able to enter the stroma via the cytoplasm. Thus the absence of any stimulation of dark O_2 uptake by this glycolytic intermediate suggests that even partial leakage of the cytoplasmic proteins or cofactors may diminish the glycolysis rate severely. The pressate reported in this paper is not a cell-free Class A chloroplast preparation, but it still permits us to analyze whole and partial reactions of functional chloroplasts by the immediate access of exogenously provided substrates to the chloroplast. Thus with this preparation, the analysis of partial reactions of the photosynthetic carbon cycle in a mutant strain carrying a defect in the cycle is made possible.

In higher plant systems the preillumination of dark-incubated leaves results in significantly higher rates of CO_2 fixation as well as reduced susceptibility to P_i inhibition in the isolated Class A chloroplasts. Similarly, we have observed that the pressates prepared from continuously illuminated cultures often exhibited higher rates of CO_2 fixation and much less response to P_i than did the pressates from dark-incubated cells described here. Omission of both the 16-hr dark incubation and the recovery period resulted in a pressate with rates of CO_2 and 3-phosphoglycerate-dependent O_2 photoevolution in excess of 100 μmol (mg Chl)$^{-1}$ hr^{-1}. The 16-hr dark incubation was included in order to maximize the response to added P_i, reversible by 3-phosphoglycerate. The cells were allowed to recover after resuspension in breaking medium to facilitate comparison of chloroplast functions before and after breaking in the Yeda pressure cell. We hope that it will be possible to increase the rate of CO_2 fixation of the preparation as well as to prepare free Class A chloroplasts from the pressate.

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