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LIGHT-DEPENDENT ANION TRANSPORT IN ISOLATED SPINACH CHLOROPLASTS

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SUMMARY

A rapid centrifugation technique is described which provides direct measurements of transient anion movements in illuminated chloroplasts. The results obtained by the technique are consistent with proposed mechanisms of chloroplast anion transport in which light-induced proton uptake determines the movement of other ions in chloroplasts.

1. Direct measurements of weak acid anion transport (succinate, phosphate) during illumination support the hypothesis that proton association with weak acid anions within the chloroplasts produces concentration gradients of the uncharged weak acid species and results in net anion efflux.

2. Influx of dissociated anion (Cl^-) occurs in the presence of dissociated cations (Na^+, K^+) and weak base cations (NH_4^+). In both instances Cl^- influx is presumably necessary to maintain charge balance across the membrane during proton uptake.

Both the direction and quantity of anion transport in illuminated chloroplasts are predictable from the principles of charge balance and pH-dependent shifts in weak acid concentrations. Sufficient quantities of osmotically active ions are transported to account for the observed volume changes in grana by osmotic mechanisms.

INTRODUCTION

Ion transport in chloroplasts and mitochondria is integral to a number of proposed mechanisms by which these organelles may control their volume and physiological function. Measurements of cation transport have been particularly relevant in this regard, and are successful for several reasons. Cell membranes are relatively impermeable to cations and enough time is available for measurements of cation content before significant diffusion losses occur. More important is the ready availability of cation specific electrodes which allow small changes in concentration to be continuously followed in time.

Anion measurements have been neglected for the opposite reasons. Cell membranes are typically more permeable to anions. For instance, Cl^- and HCO_3^- exchange across red cell membranes in less than a second, whereas K^+ diffusion rates are measured

in hours. Secondly, anion-sensitive electrodes cannot accurately measure concentration changes in the micromolar range.

In previous studies[1–4] we have proposed that anion transport contributes to light-dependent volume changes in chloroplasts, and indirect evidence for anion transport was presented. However, no direct evidence for light-dependent anion transport in chloroplasts is available. We have therefore developed a technique for rapid separation of chloroplasts from a labelled solution with subsequent measurement of anion content. The present communication describes the results of these anion-transport studies in chloroplasts and their relation to proposed mechanisms of light-dependent ion transport.

METHODS

Preparations and assays

Chloroplasts were prepared from commercially obtained spinach (*Spinacea oleracea*) by homogenizing 100 g of deveined leaves in 250 ml of 0.35 M NaCl at 0°, followed by filtration through cheesecloth. No buffer was present and the final pH was 6.3. An initial centrifugation was carried out at 300 × *g* for 1 min, and the pellet was discarded. The supernatant was centrifuged 5 min at 700 × *g*, and the resulting pellet was washed once in 0.1 M NaCl. (This procedure reduced the osmotic shock when chloroplasts were later suspended in 0.1 M test solutions.) After determining the capacity for proton transport in 0.1 M NaCl at pH 6.0, using *N*-methylphenazonium methosulfate as an electron transport catalyst, the chloroplasts were used immediately. Only preparations that showed proton uptake greater than 0.6 μmole per mg chlorophyll were utilized. Chlorophyll was determined by the method of MacKinney[5].

Rapid separation technique

This procedure was carried out in polyethylene microcentrifuge tubes (Thomas) as shown in Fig. 1. Aliquots of the chloroplast suspension (10–20 μl) were mixed with 0.1 ml of the test solution containing labeled anions ($^{36}Cl^-$, $^{32}PO_4^{2-}$ or [^{14}C]succinate) at pH 6.0. The test solutions were separated from the subphase by an air bubble. The subphase contained 4% dextran (mol. wt. 200–275000), 0.1 M $NaNO_3$ and 5 mM $AgNO_3$. The presence of Ag^+ was necessary for rapid pellet formation. This effect is probably due to aggregation of chloroplasts as they enter the subphase containing Ag^+. If the centrifuge is stopped before 10 sec have elapsed small clumps of chloro-

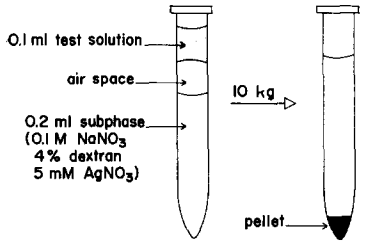

Fig. 1. Technique for rapid separation of chloroplasts from radioactive solutions. Centrifugation at 10000 × *g* results in pellet formation within 10 sec when Ag^+ is present. Anions external to the chloroplasts remain at the top of the dense dextran subphase. This technique has also been successfully employed to separate mitochondria and red cells from labeled solutions. See text for details.

plasts may be seen partially descended in the lower phase. In the absence of Ag^+, pellet formation was incomplete even after 5 min centrifugation. $NaNO_3$ (0.1 M) was present to osmotically balance chloroplasts as they descended from the 0.1 M test solutions.

The tubes were placed in a Coleman microcentrifuge (model 6-811) and were centrifuged for 10 sec after varying intervals of dark incubation or illumination with broad-band red light (1000 foot-candles). The microcentrifuge develops 10000 $\times$ g in 4 sec and stops 15 sec after being turned off. The particles appeared as a pellet at the bottom of the dense subphase and were thus separated from the lighter radioactive test solution, which remained on top. The bottom of the tube, containing the pellet, was immediately cut off and the upper portion was discarded. The tube bottom was placed in 1 ml of water in a glass scintillation tube and sonicated briefly to disperse the pellet. 4 ml of scintillation cocktail (5 g 2,5-diphenyloxazole and 100 g napthalene in 1 l of dioxane) were added, and the sample was counted to the 2 % error level (10000 total counts) in a Beckman LS 100 liquid-scintillation counter. Control tubes containing aliquots of the chloroplasts and test solution were also counted, together with blanks in which the same procedure was carried out but without particles present. Since chlorophyll had a heavy quenching effect on ^{14}C and ^{32}P scintillation, care was taken to maintain equivalent chlorophyll concentrations in all tubes. Thus, standardization and background counts included the same amount of chloroplast chlorophyll as the test samples.

The following time intervals were employed prior to centrifugation: chloroplasts were added to 0.1 M $Na^{36}Cl$ and immediately illuminated for 1 min or dark-incubated for 1 min. 1 min was chosen as a convenient period of illumination since most of the proton uptake or conformational changes have occurred during that interval. Chloroplasts were dark-incubated in 0.1 M [^{14}C]succinate or $^{32}PO_4^{2-}$ for 10 min to allow exchange with internal Cl^-, and then illuminated or kept dark for 1 min. Chloroplasts added to $NH_4{}^{36}Cl$ were immediately illuminated for 15 sec or dark-incubated for 15 sec, since 1 min of illumination caused such gross swelling that pellet formation did not occur.

A number of tests were carried out to establish that no gross artifacts were contributing to the final anion content of the pellet.

(i) If polystyrene spheres (6–13 μ^3) were used instead of chloroplasts the count in the resulting pellet was elevated only 20–30 counts over background in $Na^{36}Cl$ and not at all in phosphate or succinate test solutions. This indicated that extraneous label which might precipitate with a chloroplast pellet was minimal.

(ii) Dark diffusion rates of $^{36}Cl^-$ and $^{32}PO_4^{2-}$ were readily determined with the above method. These rates were measurable both as influx and efflux, and indicated that the anion content of an internal chloroplast volume was being determined. Cl^- flux had a half-time of 30 sec, and $^{32}PO_4^{2-}$ flux had a half-time of 1 min at 25°.

(iii) Chloroplasts were placed in varying osmolarities of sucrose and their volumes were fixed by addition of glutaraldehyde (2 % final concn.). Aliquots of the fixed suspensions were then placed in 0.1 M $Na^{36}Cl$ and centrifuged as usual into the subphase. The count in the pellets was linearly proportional to the inverse of sucrose concentration. This result confirmed that the measured count was proportional to chloroplast osmotic volume[6].

(iv) Electron micrographs of chloroplasts which had undergone the separation

process showed that grana and stromal membranes were intact. No gross distortion of chloroplast structure had occurred, and there was little or no electron dense material external to the chloroplasts.

(v) If individual chloroplast volume was calculated from the total pellet count, the resulting average volume (50 μ^3/chloroplast) was similar to that obtained experimentally (45 μ^3) from Coulter counter studies of chloroplast volume in 0.1 M NaCl[1,4].

We concluded from the above tests that the method gives semi-quantitative estimates of total anion content of chloroplasts, and that differences in anion content between dark and illuminated chloroplast pellets represent light-dependent anion transport when measured by this technique.

RESULTS

Results and calculations from a typical experiment in 0.1 M NaCl are described below. Four tubes were illuminated for 1 min after addition of chloroplasts (0.06 mg chlorophyll per tube) and four were kept in the dark for 1 min. The incubation periods were followed by immediate centrifugation for 10 sec. The pellets from the dark chloroplasts contained 679 counts/min (range 556–800, S.D. $\pm$ 82). This figure reflects the amount of label which exchanges with interior Cl^- during a 1 min interval. The pellets from the illuminated chloroplasts contained 884 counts/min (range 759–1047, S.D. $\pm$ 94). The difference of the means of these two values was 205 counts per min. Four aliquots (10 μl each) of the 0.1 M NaCl–chloroplast test solution contained 4724 $\pm$ 150 counts/min. Sufficient chloroplasts were added to the scintillation tube to give an amount of chlorophyll equivalent to that of the pellets. The following calculation was then carried out: (i) 10 μl of 0.1 M NaCl = 1 μmole Cl^- and contained 4724 counts/min. (ii) (205 counts/min)/(4724 counts/min) $\times$ 1.0 μmole = 0.043 μmole difference in Cl^- content between dark and light chloroplast pellets. (iii) (0.043 μmole)/(0.06 mg) = 0.7 μmole transported per mg chlorophyll. Since the difference between dark and illuminated chloroplast pellets was used, background count and small diffusion losses would not significantly affect the above calculations.

From the figures given above it is apparent that the difference between light and dark counts within a single experiment may contain a large standard deviation. This error was greatest in NaCl with a range of 30–85 % of the mean, and more acceptable in PO_4^{2-}, succinate and NH_4Cl, with a range of 20–30 % of the mean. At present we must accept this error as inherent in the method.

Since there were large variations in specific activity of the labeled anion solutions, it was convenient to express each final result as the mean of the means of 4–5 experiments $\pm$ S.D. These results are summarized in Fig. 2. About 0.7 μmole Cl^- per mg chlorophyll are transported inward during 1 min illumination in solutions of NaCl. About 0.9 μmole Cl^- per mg chlorophyll are transported inward during 15 sec illumination in 0.1 M NH_4Cl. Anions were transported outward during illumination in sodium succinate and PO_4^{2-} solutions. Approx. 0.9 μmole of succinate and 0.6 μmole of PO_4^{2-} per mg chlorophyll were transported during 1 min of illumination. Although the S.D. of the mean of the means was small (10–15 %) the results are approximations and are considered reliable only to $\pm$ 50 % of the mean for NaCl and $\pm$ 25 % of the mean for the other anion solutions.

In the presence of 100 μM Triton X-100, a concentration of detergent which

inhibits proton uptake[7] or 100 μM 3-chlorocarbonyl cyanide phenylhydrazone which similarly inhibits proton uptake, no anion transport was detected by the present method.

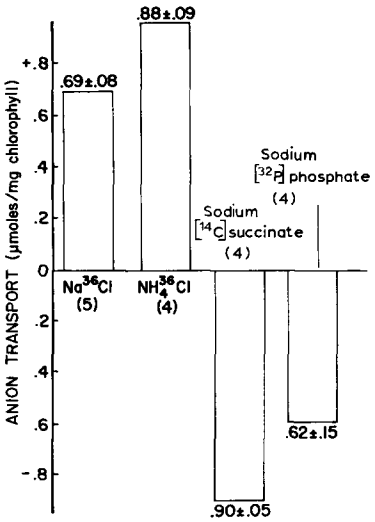

Fig. 2. Light-dependent anion transport in chloroplasts. Chloroplasts were illuminated 1 min in 0.1 M solutions of sodium salts of various anions. (In NH_4Cl, 15 sec illumination was used.) This was followed by centrifugation into the subphase, as described in METHODS. Anion transport represents the difference in anion content between 4 illuminated samples and 4 dark controls, and is expressed as the mean of the means of 4–5 experiments ± S.D.

DISCUSSION

We may now discuss the total ion transport of chloroplasts in various ionic environments. It is important to note that these conclusions are so far valid only for chloroplast inner membrane preparations. Intact chloroplasts may have quite different transport mechanisms. Furthermore, seasonal and age variations in spinach may affect transport, particularly in solutions of dissociated ions where no permeant cation or anion is available.

(i) In 0.1 M NaCl solutions, approx. 0.7 μmole of protons and 0.7 μmole of Cl^- are transported into the chloroplast interior. It is presumed that Cl^- enters to balance charge that develops across the membrane as a result of proton uptake. The entry of chloride into the inner space produces an osmotic imbalance and may result in mild swelling. Protons binding to anionic sites on inner membrane surfaces[8] may also release bound K^+ or Mg^{2+} which would further contribute to the osmotic imbalance. However, if Cl^- does enter the chloroplast inner membrane volume in a 1:1 stoichiometric ratio with protons, there would be no electrical imbalance under these conditions and K^+ and Mg^{2+} would not necessarily efflux.

(ii) In solutions of weak acid anions, such as sodium succinate and phosphate, protons are transported inward and exchange with cations as described by DILLEY AND VERNON[8]. Approx. 0.6 μmole of cations efflux per mg chlorophyll and this results in a pH gradient. The gradient causes an efflux of weak acid anions by driving the weak

acid anion equilibrium within the inner chloroplast volume toward the uncharged form of the acid. The uncharged species then diffuses outward down a concentration gradient, as suggested by CROFTS *et al.*[2]. The resulting osmotic imbalance produced by the efflux of 0.6 μmole cations and 0.9 μmole anions results in shrinkage of the osmotically active volume.

(iii) In solutions of weak base cations such as NH_4Cl, protons again are transported inward. The resulting pH gradient displaces the weak base equilibrium of the inner volume toward the charged form ($NH_3 + H^+ \rightarrow NH_4^+$), and uncharged NH_3 diffuses inward as demonstrated by CROFTS in our laboratory[9]. Cl^- again enters to balance the charge, as originally suggested by HIND AND JAGENDORF[10]. Since most of the protons which enter the interior volume combine with NH_3 to form NH_4^+, there is no 'back pressure' to limit proton uptake, and swelling will proceed until the pump mechanism or limiting membranes are disrupted.

It is important to determine whether sufficient ions are transported to account for the observed shrinking or swelling in illuminated chloroplasts. The shrinkage observed in weak acid anion solutions is most amenable to this calculation, since estimates of cation transport are available[8] together with the anion values presented here. Only the volume change for the grana space will be calculated, since proton uptake and resulting ion transport probably occur exclusively within the grana. The most significant electron-microscopic changes in chloroplasts take place in grana membranes[3] and GROSS AND PACKER[11] have demonstrated that isolated grana undergo volume changes very similar to those in intact chloroplasts.

DILLEY AND VERNON[8] demonstrated a cation efflux of approx. 0.6 μmole per mg chlorophyll, and the present results show that under similar conditions approx. 0.9 μmole of weak acid anion is transported outward. This would result in a total efflux of 1.5 μmoles of osmotically active ions.

DILLEY[12] has further shown that the sucrose-impermeable space is approx. 20 % of the total chloroplast volume in 0.1 M solutions. This is equivalent to about 10 μl per mg chlorophyll. A volume of 10 μl of 0.1 M monovalent salt solution contains 2.0 μmoles of osmotically active ions. Therefore, the total ionic efflux of 1.5 μmoles represents 75 % of the total osmotically active ions within the grana volume, and the grana would shrink to one-fourth (2.5 μl per mg chlorophyll) of their original volume during illumination. This result is entirely consistent with the dramatic light-dependent shrinkage of grana seen by electron microscopy[3,13].

Similar calculations for chloroplasts in NH_4Cl show that grana volume would increase from 10 to 20 μl during the first 15 sec of illumination. Again, this is in agreement with the gross swelling which occurs when chloroplasts are illuminated in solutions of weak base cation[3,14]. In solutions of dissociated salts grana would swell from 10 to 14 μl after 1 min illumination, assuming only Cl^- is osmotically active within the chloroplast.

We conclude from the above considerations that ion transport resulting from a proton uptake in chloroplasts could result in volume changes in grana by an osmotic mechanism. Both the direction and quantity of ion transport are predictable from the principles of charge balance and pH-dependent shifts of weak base and weak acid ions, and apparently enough osmotically active species are transported to account for the observed volume changes in grana.

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