PHOTOSYNTHETIC CONTROL IN ISOLATED SPINACH CHLOROPLASTS WITH ENDOGENOUS AND ARTIFICIAL ELECTRON ACCEPTORS

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SUMMARY. Spinach chloroplasts, isolated rapidly in isotonic media will reproducibly give photosynthetic control rates (State 3/State 4) of 4 - 6, and ADP/O ratios (equivalent to ATP/$2e^-$) of 1.4 - 2.1 when assayed in slightly hypotonic media. Photosynthetic control can be followed as oxygen evolution with either ferricyanide or NADP as electron acceptors, or as oxygen uptake in the presence of azide, which blocks chloroplast catalase, either alone (endogenous catalyst) or with added methyl viologen. This control can be triggered either by added ADP or by added Pi in all cases. Optimum concentrations of Mg, Pi and EDTA are required; the pH is also critical. Excess EDTA results in an inhibition of electron transport on addition of ADP.

The demonstration by West and Wiskich (1) of photosynthetic control in isolated pea chloroplasts opened up new approaches to the study of the stoichiometry of photophosphorylation and to the integrity of isolated chloroplasts. These workers obtained photosynthetic control ratios averaging 2.5 (i.e. State 3/State 4 ratios, using terminology (1) analagous to respiratory control in mitochondria). These values were obtained with ferricyanide as the electron acceptor and with carefully prepared pea chloroplasts. The average ADP/O ratio (equivalent to the ATP/$2e^-$ ratio) was found to be 1.2.

Using "Class I" (whole) spinach chloroplasts, Kraayenhof (2) obtained average photosynthetic control ratios of 2 and ADP/O ratios of 0.8 with NADP as the electron acceptor. Recently Telfer (3), using "Class II" (broken) spinach chloroplasts made according to Horton and Hall (4), obtained control ratios of 2.9, and control ratios of 2.1 with broken chloroplasts (P_1S_1) prepared according to Whatley and Arnon (5). Telfer followed light-induced O_2 uptake in a non-cyclic electron flow to methyl viologen (6).

All of these investigations have used chloroplasts which were freely permeable to ADP, NADP and ferricyanide in order to obtain photosynthetic

control. Thus the chloroplasts in the reaction mixtures would not satisfy the requirements of intactness defined in Walker's review (7) and experimentally documented by workers in several laboratories (8-11) who have shown that ADP and NADP penetrate intact chloroplasts only very slowly.

In this paper we report the isolation of spinach chloroplasts which will reproducibly give photosynthetic control ratios of 4 - 6, and ADP/O ratios of 1.4 - 2.1. These ratios can be obtained by following oxygen evolution with ferricyanide or NADP as electron acceptors, or by following oxygen uptake in the presence of azide, a catalase inhibitor, either alone (endogenous catalyst) or with added methyl viologen. We believe these methods will provide a rapid and useful technique for assaying many biochemical reactions and the biochemical integrity of isolated chloroplats and also facilitate spectrophotometric investigations.

Methods. Following the techniques of Walker (12, 9) and Jensen and Bassham (13) for the rapid isolation of chloroplasts in isotonic sorbitol we used the following procedure: 15 g. of fleshy, dark green, greenhouse grown spinach leaves were preilluminated in iced water beneath a 60 W desk lamp, then rapidly cut into strips and homogenised for 3 seconds in 50 ml of grinding medium in a 1 L. Virtis or 200 ml. MSE "Atomix" homogeniser at full speed. The grinding medium consisted of 0.4 M Sorbitol, 0.05 M MES (2-(N-morpholino) ethanesulphonate), 0.01 M NaCl, 5 mM $MgCl_2$, 1 mM $MnCl_2$, 2 mM EDTA, 2 mM Na Ascorbate, 1% crystalline BSA (bovine serum albumin), adjusted to pH 6.5.at room temperature; the ascorbate and BSA were added just before use. The resulting slurry was squeezed through 16 layers of cheesecloth and the filtrate centrifuged in a 6 x 100 ml. angle rotor in an MSE 6L "Mistral" centrifuge. Rapid acceleration to 5,000 rpm (ca. 4,000 x g) and immediate braking allowed a total elapsed time of 1 minute from rest, to top speed and back to rest again. The chloroplast pellet was gently resuspended with the aid of cotton wool on the end of a glass rod in 1 ml. of resuspending medium consisting of 0.4 M Sorbitol, 0.05 M HEPES (N-2-hydroxyethylpiperazine-N^1-2-ethanesulphonate), 0.01 M

NaCl, 5 mM $MgCl_2$, 1 mM $MnCl_2$, 2 mM EDTA, 1% crystalline BSA (added just before use), adjusted to pH 7.5. All operations were carried out at 0-4°C and the whole procedure took about 3 minutes. The concentration of chlorophyll in the chloroplast suspension was about 2.5 mg/ml, measured according to Arnon (14), and consisted of about 55-60% "Class I" chloroplasts, as defined by Spencer and Unt (15), when examined under the phase contrast microscope.

The experiments were carried out at 15°C in a water-jacketed vessel using a Clark-type O_2 electrode (Yellow Springs Instruments Co., Yellow Springs, Ohio) or a Rank O_2 electrode (Rank Bros., Bottisham, Cambridge). The latter is preferable since the electrode is at the base of the vessel which has a closely fitting conical cap through which additions can easily be made into the reaction mixture. Illumination was either with a tungsten lamp or with a Quartz-iodine projector plus an orange filter (Cinemoid, 5A; Strand Electric, London, W.C.2); in both cases saturating light intensities were provided to the reaction.

The reaction mixtures consisted of 0.1 M Sorbitol, 0.05 M Tricine, pH 7.5, 5 mM $MgCl_2$, 0.02 M NaCl, 0.01 M K_2HPO_4, 2 mM EDTA, pH 7, chloroplasts equivalent to 50-100 µg chlorophyll. $K_3Fe(CN)_6$, 2.5 mM; NADP, 2 mM together with saturating amounts of spinach ferredoxin; methyl viologen, 0.2 mM; NaN_3, 2 mM; ADP, pH 7, 0.1 mM, were added where indicated. Final volume of reaction mixture = 2 ml.

The concentration of O_2 in the 2 ml. reaction mixture was determined by the method of Robinson and Cooper (16) and also checked by successive additions of limiting amounts of ferricyanide which also confirmed the linearity of the response of the O_2 electrode. A complete, air-saturated reaction mixture was found to be 0.30 µM in O_2 at 15°C. The ADP concentration was checked by the enzymatic analysis method of Adam (17).

All reagents were "Analar" grade or the highest purity commercially available. Ferredoxin was prepared from spinach according to Rao, Cammack, Hall and Johnson (18).

Results and Discussion. Photosynthetic control with ferricyanide as the

electron acceptor is shown in Figure 1. The ferricyanide was added to the reaction mixture at the same time as turning on the light to prevent any damage to the chloroplasts which can occur in the dark in the presence of ferricyanide (19). A State 3 rate of electron transport can be induced by adding limiting amounts (0.1 mM) of ADP in the light (Fig. 1a) or having ADP present in the reaction mixture and then turning on the light (Fig. 1b). The addition of excess (10 mM) Pi to a reaction mixture containing ADP also brings the chloroplasts into State 3 (Fig. 1c); the apparent high ADP/O is probably due to endogenous Pi using up some of the previously added ADP. In Fig. 1d the inhibition of electron transport by ADP (in the absence of Pi; see refs. 4 and 20) is shown; the addition of Pi then induces a State 3.

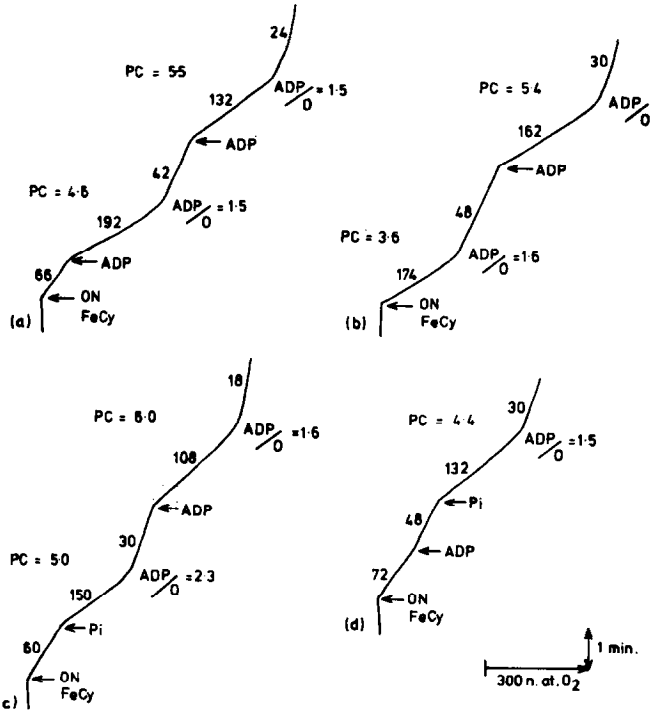

Figure 1. Photosynthetic control with ferricyanide (added simultaneously with turning on the light) : (a) ADP added twice to induce State 3 in reaction mixture containing Pi. (b) ADP present in reaction mixture with Pi; further ADP induces another State 3. (c) Pi added to reaction mixture containing ADP; further ADP induces another State 3. (d) ADP inhibits basal electron transport in the absence of Pi; addition of Pi induces State 3. The figures along the traces are μatoms oxygen per mg. chlorophyll per hour. PC = photosynthetic control, i.e., State 3/State 4. ADP/O = μmoles ADP added/μatoms oxygen evolved.

Optimal concentrations of Mg, Pi and EDTA are necessary for the best photosynthetic control.

Essentially similar results can be obtained with NADP as the electron acceptor (Fig. 2). However, as shown in Fig. 2a, the addition of ferredoxin is essential for electron transport (8). Figure 2b shows an immediate State 3 rate when the light is turned on if ADP, NADP and ferredoxin are already present in the reaction mixture. At pH 8.5 the photosynthetic control was low, as shown in Fig. 2c. Lower control ratios were observed in all the systems studied at pH 8.5 and 6.5 than at pH 7.5. A more accurate pH optimum for each system has not yet been determined. Fig. 2d shows the inhibitory effect of excess EDTA on electron transport on the addition of ADP (Pi present). This striking effect has been noted in all the electron transport systems studied. The ability of EDTA washing to remove coupling factors (21, 22) may

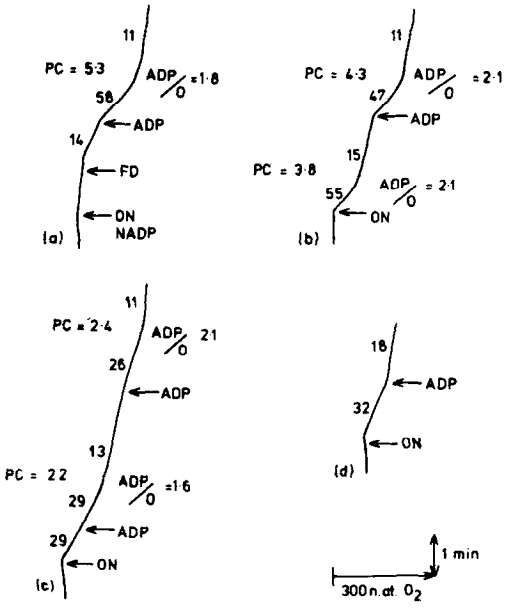

Figure 2. Photosynthetic control with NADP as the electron acceptor: (a) NADP added when the light is turned on but no O_2 evolution occurs until a saturating amount of ferredoxin is added; ADP added to induce State 3 in a reaction mixture containing Pi. (b) ADP present in reaction with NADP, ferredoxin and Pi; further ADP induces another State 3. (c) conditions as for (a) but pH = 8.5. (d) excess EDTA (20 mM) reverses the State 3 transition usually obtained on the addition of ADP to a reaction mixture containing Pi plus NADP and ferredoxin.

be the cause but the immediate inhibition by ADP, in the presence of Pi and Mg, may result from the formation of an inhibitory complex or the removal of Mg necessary for phosphorylation. We are investigating this phenomenon.

Instead of following O_2 evolution in non-cyclic electron transport, Telfer et al (6) showed that electron transport catalyzed by methyl viologen could be measured as an O_2 uptake by reoxidizing the reduced methyl viologen with O_2 to form H_2O_2 (a Mehler reaction). The H_2O_2 accumulates when the chloroplast catalase is inhibited by azide. The net result is light-induced

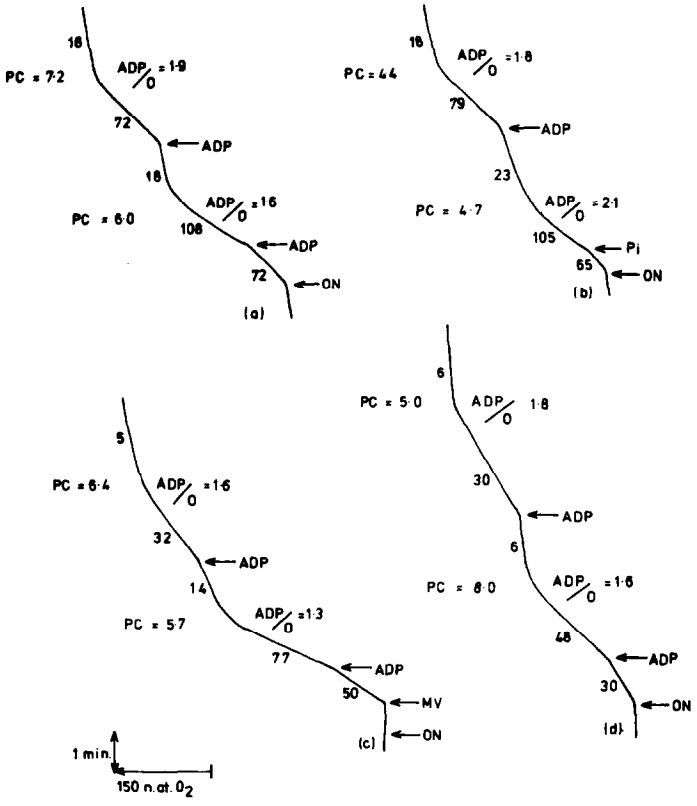

Figure 3. Photosynthetic control with an endogenous catalyst or added methyl viologen as the electron acceptor which is oxidised by oxygen to form H_2O_2 (trapped by inhibiting chloroplast catalase with azide): (a) no added catalyst; ADP added twice to induce State 3 in reaction mixture containing Pi. (b) Pi added to reaction mixture containing ADP; further ADP induces another State 3. (c) "aged" chloroplasts showing requirement for catalytic amounts of methyl viologen before oxygen uptake could occur; ADP added twice to induce State 3. (d) added methyl viologen; ADP added twice to induce State 3 in reaction mixture containing Pi.

O_2 uptake linked to a non-cyclic electron flow which Telfer (3) showed has photosynthetic control. In this investigation we have shown that the addition of an exogenous catalyst is not necessary if the chloroplasts are fresh (Figs. 3a and 3b); however, on ageing at 0° the isolated chloroplasts require the addition of methyl viologen in order to catalyze light-induced O_2 uptake (Fig. 3c). In Fig. 3 photosynthetic control is shown to be induced by ADP (Fig. 3, a, c and d) or by Pi (Fig. 3b).

A very rapid method of separating the chloroplasts from the cytoplasm seems to be essential for good photosynthetic control. Thereafter the chloroplast preparation is stable for 3 - 4 hours if the suspension is concentrated and kept at 0° (23). Our preparations of isolated chloroplasts were usually about 55-60% "Class I", but were disrupted when placed into the hypotonic reaction mixture. Completely intact chloroplasts, i.e. those capable of high rates of CO_2 fixation, are impermeable to NADP and ATP (24); a slightly hypotonic reaction mixture was therefore chosen to obviate any penetration problems.

The ADP/O ratios of 1.4 - 2.1 confirm our previous suggestion that ATP/$2e^-$ ratios of 1.5 - 1.6 for ferricyanide and NADP (4) indicate two sites of ATP synthesis in non-cyclic photophosphorylation. We now have a simple, reproducible technique to investigate the different types of non-cyclic electron transport and the different "states" of coupled or uncoupled electron flow in each system. The action of electron flow inhibitors, uncouplers and energy transfer inhibitors is under investigation.

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