Plant Physiol. (1971) 48, 163-165

Phosphoglycerate as a Hill Oxidant in a Reconstituted Chloroplast System[1]

Received for publication December 31, 1970

D. M. STOKES[2] AND D. A. WALKER
Department of Botany, University of Sheffield, Sheffield, S10, 2TN, England

ABSTRACT

The ability of intact chloroplasts to evolve oxygen when illuminated in the presence of 3-phosphoglycerate is lost following osmotic shock but may be largely restored by the addition of ATP, catalytic amount of nicotinamide adenine dinucleotide phosphate, ferredoxin, and a soluble fraction derived from chloroplasts. In the presence of uncoupling agents and ATP, the rate is faster than that supported by intact chloroplasts. In the absence of uncoupling agents, the ATP requirement may be partially met by photophosphorylation.

It has been established (8) that chloroplasts with intact envelopes will evolve oxygen when illuminated in the presence of PGA[3]. This reaction will commence in the absence of sufficient CO_2 to support normal photosynthetic oxygen evolution (7), and it is supposed that the added PGA enters the chloroplast and is converted to DPGA which then serves as the natural Hill oxidant (7, 8). The ability of chloroplasts to utilize either CO_2 or PGA as precursors of the natural oxidant is lost following exposure to osmotic shock (1, 6).

We wish to report that the activity of the PGA-dependent oxygen evolving system may be restored by the addition of coenzymes and protein fractions derived from chloroplasts.

MATERIALS AND METHODS

Spinach was grown locally or purchased from market sources in France or Spain. It was then used directly or after cold storage, but in either event it was illuminated in strong white light (about 6000 ft-c) in cold water for 30 to 45 min prior to maceration.

Chloroplasts were isolated in phosphate medium (6) containing sorbitol, 0.33 M; Na_2HPO_4, 50 mM; KH_2PO_4, 50 mM; $MgCl_2$, 5 mM; NaCl, 0.1%; and sodium isoascorbate, 0.2%, adjusted to pH 6.5 with HCl. The pellet was washed in 50 ml of 0.33 M sorbitol containing 2.0 ml of assay medium and finally resuspended in assay medium (below). These chloroplasts were used unchanged (Fig. 1a and Table IA) or osmotically shocked in the reaction vessel (1) (Fig. 1b and Table IB). Alternatively, the chloroplasts were osmotically shocked in 11 ml of assay medium diluted 25-fold. After a second centrifugation, the supernatant fraction was retained and designated CE, and the pellet of broken (envelope-free) chloroplasts was resuspended in assay medium and used in the experiments illustrated in Figure 2. The assay medium contained sorbitol, 0.33 M; EDTA, 2 mM; $MgCl_2$, 1 mM; $MnCl_2$, 1 mM; and HEPES, 50 mM, adjusted to pH 7.6 with KOH.

Oxygen was measured simultaneously in twin cells fitted with Clark-type electrodes and illuminated at 20 C with near saturating red light provided by quartz-iodine slide projectors (150 w, 24 v) fitted with heat filters and a broad red filter transmitting light above 600 nm.

Spectrophotometric examination (5) of the CE showed that following osmotic shock intact chloroplasts yielded approximately 15 mg of soluble protein/mg chl. At 24 C, 2.5 ml of assay medium at pH 7.6 containing CE, 3.75 μmoles of PGA, and 3.75 μmoles of ATP, oxidized 0.18 μmole of NADPH/mg protein·min, indicating the presence of an active phosphoglycerate kinase and triose phosphate dehydrogenase.

RESULTS

Figure 1 (see also Table I) shows the effect of osmotic shock on PGA-dependent oxygen evolution (*cf.* 1). In this experiment the only difference between the two reaction mixtures is that in Figure 1b the order of additions was such that the chloroplasts were briefly exposed to an osmotic shock (which renders them 100% envelope-free) while in Figure 1a the majority of the chloroplasts retained their outer envelopes. Photosynthetic O_2 evolution with 3 μmoles of R5P and 20 μmoles of bicarbonate as added substrate is similarly eliminated by osmotic shock (1, and Table I).

Table I shows that with 3 μmoles of PGA as the added substrate the ability to evolve oxygen was largely restored by the addition of CE (containing about 6 mg of protein), 7 nmoles of ferredoxin, 3 μmoles of ATP, and 0.1 μmole of NADP. In the presence of 120 μmoles of NH_4Cl (added as an uncoupling agent), the rate of oxygen evolution was doubled. In these circumstances there was no restoration of activity in the absence of PGA or ATP and very little in the absence of NADP. Ferredoxin and CE were not essential but each produced a marked stimulation in the presence of the other components. CE heated at 100 C for 1 min was without effect.

Illuminated ruptured chloroplasts will evolve O_2 when provided with NADP and ferredoxin alone (Fig. 2); the rates given in Table I are those recorded when O_2 evolution attributable to this source had ceased. The extent of O_2 evolution in the presence of NADP and ferredoxin alone (Fig. 2b) was very close to that predicted on the basis of the quantity of NADP added and was not substantially altered by the presence of CE (Fig. 2a), indicating that CE did not contain appreciable

[1] This work was supported by the Science Research Council and the Royal Society.

[2] Present address: Department of Botany, University of Melbourne, Parkville, Victoria, Australia.

[3] Abbreviations: CE: Chloroplast extract; DPGA: 1,3-diphosphoglycerate; PGA: 3-phosphoglycerate; R5P: ribose 5-phosphate.

NADP itself. It is probable, however, that CE did contain a small quantity of PGA because in the coupled system (*cf.* the uncoupled system summarized in Table I) it was able to support a slow rate of oxygen evolution in the absence of substrate. The addition of PGA then caused a marked acceleration (Fig. 2a) and a further increase followed the addition of NH_4Cl. In an initially coupled system, from which exogenous ATP was omitted (Fig. 2d), the rate of evolution was initially slower than in the complete mixture (Fig. 2c), and the addition of NH_4Cl was followed by a brief acceleration which quickly gave way to an inhibition. We interpret the acceleration as uncoupled electron transport with newly synthesized DPGA as the oxidant. As further formation of DPGA slows and ceases (because of inhibition of ATP formation in the uncoupled system) O_2 evolution also declines. The subsequent addition of ATP permits a resumption of DPGA formation from PGA. Oxygen evolution then restarts at the faster uncoupled rate (as in Fig. 2a).

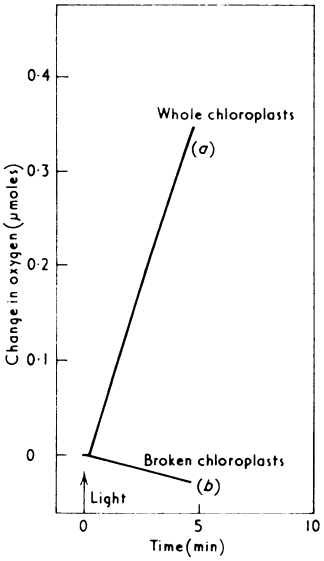

FIG. 1. Effect of osmotic shock on oxygen evolution by chloroplasts. In addition to 2 ml of assay medium and 100 μg of both reaction mixtures contained 3 μmoles of PGA.

Table I. *PGA-dependent Oxygen Evolution in a Reconstituted System*

For details of additions see text. All reaction mixtures contained 100 μg of chl and 2 ml of assay medium. The complete reconstituted system contained CE, ferredoxin, ATP, PGA, and NADP. No combination of these additives produced a significant restoration of rate when 3 μmoles of R5P and 20 μmoles of bicarbonate were substituted for PGA.

	Rate of Oxygen Evolution							
			Reconstituted system					
	PGA	R5P + bicarbonate	Complete	−PGA	−Ferredoxin	−NADP	−ATP	−CE
	μmoles/mg chl·hr							
A. Whole chloroplasts	51	54						
B. Chloroplasts osmotically shocked in electrode vessel	−4	−6	38					
Chloroplasts osmotically shocked in electrode vessel +NH_4Cl			91	−4	32	6	−6	40

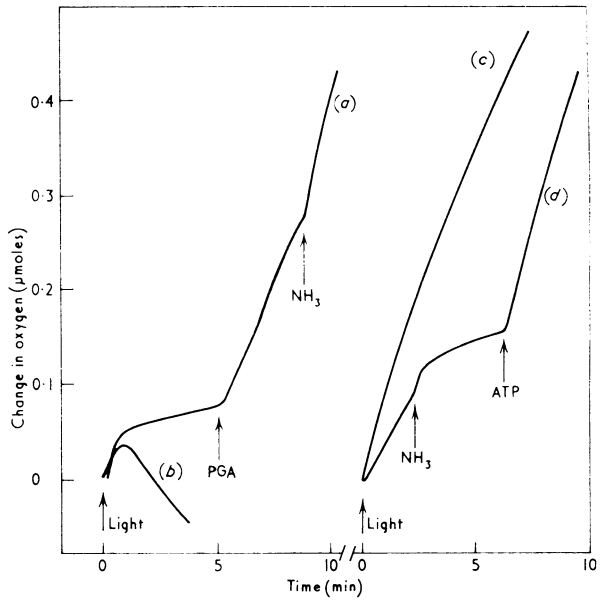

FIG. 2. Oxygen evolution in a reconstituted system containing washed envelope-free chloroplasts. Reaction mixture (b) contained: NADP, 0.1 μmole; ferredoxin, 7 nmoles; 2 ml of assay medium; 100 μg of chl. In addition (c) contained CE (20 mg protein); ATP, 3 μmoles; PGA, 3 μmoles; (a) CE (20 mg protein), ATP 3 μmoles and (d) CE (20 mg protein), PGA 3 μmoles. ATP (3 μmoles); PGA (3 μmoles) and NH_4Cl (120 μmoles) were added as indicated. Curves identical to (b) were obtained with mixtures as for (c) except that CE was boiled or replaced by water.

The ability of CE to support O_2 evolution in a system from which ATP is omitted implies that there is sufficient ATP/ADP within such a mixture to allow endogenous photophosphorylation to proceed at an appreciable rate; this ability is lost if CE is dialyzed and restored by the subsequent addition of a catalytic quantity of ATP. It should be noted, however, that CE in the quantities used did not contain enough ATP to allow a significant continuation of oxygen evolution beyond that attributable to NADP reduction when added to an uncoupled system (*cf.* Table I) from which ATP had been omitted. Catalytic ATP alone could not substitute for CE. It must be emphasized that in the experiments illustrated in Figure 2 the chloroplasts used were well washed, envelope-free chloroplasts which would not evolve oxygen (beyond that associated with the stoichiometric reduction of NADP) unless CE was present. Conversely, in Table I the chloroplasts used were osmotically shocked in the reaction mixture (see "Materials and Methods") and there was therefore no absolute requirements for added CE.

Essentially similar results were obtained with chloroplasts and CE prepared from pea leaves and with nigericin used as an uncoupling agent rather than NH_4Cl.

DISCUSSION

Our results are clearly consistent with the view that PGA can function as a Hill oxidant in a reconstituted chloroplast system, and that (in an ATP requiring reaction) PGA is initially converted to DPGA which, in turn, accepts electrons from NADPH.

In the presence of sufficient CE (see "Materials and Methods," spectrophotometric assay) to support a rapid conversion

of PGA to DPGA (and consequently an equally rapid regeneration of ADP from ATP and NADP from NADPH) enough ATP can evidently be supplied by photophosphorylation to support an appreciable rate of oxygen evolution (Fig. 2d). If substrate concentrations of ATP are added to an uncoupled system, the ruptured chloroplasts themselves contribute enough soluble (CE) factors to support some oxygen evolution (Table I), but added CE becomes an absolute requirement (Fig. 2) if the chloroplasts used have been washed free of readily soluble components.

Our results also go some way towards explaining the loss of PGA-dependent O_2 evolution which follows rupture of the chloroplast envelope. Ruptured chloroplasts will inevitably tend to lose to the surrounding medium those soluble components to which the intact envelope is relatively impermeable. If at the same time they lose their ability to carry out a photosynthetic process and this process may be then restored by the addition of soluble chloroplast components, it is reasonable to conclude that the change in the effective concentration of these components following envelope rupture is an important factor in the consequent loss of activity. The present results imply that for PGA-dependent O_2 evolution the dilution of endogenous NADP is more important than the dilution of soluble enzymes, although it is by no means certain these are not partly adsorbed on the thylakoid membranes. The possibility must also be borne in mind that the intact envelope may well control the concentration of substrates as well as that of enzymes and coenzymes (*cf.* 4). Thus, in the reconstituted system, externally added enzyme might offset any decrease in rate brought about by dilution of PGA normally within the chloroplast. The fact that the reconstituted system works with PGA but not with R5P plus CO_2 may relate to the low affinity of the carboxylase for CO_2 and adds credence to current suggestions that the intact envelope may help to maintain high internal concentrations of CO_2 (2, 3).

Acknowledgment—We are most grateful to Dr. David Hall for gifts of spinach ferredoxin.

LITERATURE CITED

1. Cockburn, W., C. W. Baldry, and D. A. Walker. 1967. Some effects of inorganic phosphate on O_2 evolution by isolated chloroplasts. Biochim. Biophys. Acta 143: 614–624.
2. Gibbs, M. 1967. Photosynthesis. Annu. Rev. Biochem. 36: 757–784.
3. Hatch, M. D. and C. R. Slack. 1970. Photosynthetic CO_2-fixation pathways. Annu. Rev. Plant Physiol. 21: 141–162.
4. Heldt, H. W. and L. Rapley. 1970. Unspecific permeation and specific uptake of substances in spinach chloroplasts. FEBS Letters 7: 139–142.
5. Kalckar, H. M. 1947. Differential spectrophotometry of purine compounds by means of specific enzymes. III. Studies of the enzymes of purine metabolism. J. Biol. Chem. 167: 461–475.
6. Walker, D. A. 1967. Photosynthetic activity of isolated pea chloroplasts. *In:* T. W. Goodwin, ed., Biochemistry of Chloroplasts, Vol. 2. Academic Press, New York. pp. 53–69.
7. Walker, D. A., W. Cockburn, and C. W. Baldry. 1967. Photosynthetic oxygen evolution by isolated chloroplasts in the presence of carbon cycle intermediates. Nature 216: 597–599.
8. Walker, D. A. and R. Hill. 1967. The relation of oxygen evolution to carbon assimilation with isolated chloroplasts. Biochim. Biophys. Acta 131: 330–338.