Vol. 88, No. 1, 1979 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
May 14, 1979 Pages 154-162

THE ATP/2e RATIO DURING PHOTOSYNTHESIS IN INTACT SPINACH CHLOROPLASTS

Luciana Rosa

Istituto di Botanica, Università di Napoli, via Foria 223, Naples, Italy and *Botany School, University of Oxford, South Parks Road, Oxford OX1 3RA, U.K.

Received March 20, 1979

Summary. Addition of ribose-5-phosphate to intact spinach chloroplasts in the absence of added P_i resulted in a conversion of part of the Benson-Calvin cycle into a linear sequence so that triose phosphate accumulated during CO_2 fixation stoichiometrically with the O_2 evolved (triose phosphate / O_2 ratio was 2.0). The fortunate consequence of this effect is that the ATP/2e ratio may be calculated from the 3-phosphoglycerate and triose phosphate accumulated and the O_2 evolved. In this way the ATP/2e ratio was shown to be 2.0, with cyclic or pseudocyclic phosphorylation contributing less than 9% to the total phosphorylation.

It has long been known that CO_2 fixation by the Benson-Calvin cycle requires 3ATP per 2 NADPH (1). However there is still much controversy as to whether the ATP requirement for CO_2 fixation by the Benson-Calvin cycle is met wholly by non cyclic phosphorylation (with an ATP/2e ratio $\geq$ 1.5) or whether ATP generated by cyclic or pseudocyclic electron transport supplements the ATP generated by non cyclic electron transport (with an ATP/2e ratio $<$ 1.5) (review, 2-4).

In broken chloroplasts direct determinations indicate maximum ATP/2e ratios of 1.5 - 2.0 for non cyclic phosphorylation, where no correction has been made for a basal rate of electron transport (review 5). A limiting ATP/2e ratio of 2.0 would be in agreement with the presence of two phosphorylation sites, a H^+/e ratio of 2, and a H^+/ATP ratio of 2 (6-10). However in intact chloroplasts comparison of the quantum requirements for the reduction of different metabolites, which use different amounts of ATP per

Abbreviations: HEPES, N-2,hydroxyethylpiperazine-N^1-ethane sulphonic acid; PGA, 3-phosphoglycerate; 1,3-PGA, glycerate 1,3-bisphosphate; Rbu-5-P, ribulose 5-phosphate; RbuBP, ribulose 1,5-bisphosphate; Rib-5-P, ribose 5-phosphate; TP, triose phosphate.

*address for correspondence

0006-291X/79/090154-09$01.00/0

NADPH, indicates that the ATP/2e ratio for non cyclic electron transport is <1.5 and that it may be variable (11, 12). Furthermore there is evidence to indicate that cyclic (13-15) and pseudocyclic (16-17) phosphorylation contribute significantly to the ATP synthesised during CO_2 fixation. A more direct determination of the ATP/2e ratio during CO_2 fixation in intact chloroplasts is difficult to make since ATP, as well as the intermediates of the Benson-Calvin cycle, turn over rapidly in the stroma rather than accumulate, and the inner membrane of spinach chloroplasts is only slowly permeated by ATP (18).

This problem has been overcome in the present work, which presents determinations of the ATP/2e ratio in intact chloroplasts during CO_2 fixation. As a result of adding Rib-5-P to the chloroplasts in the absence of added P_i, TP is shown to accumulate in the light in amounts stoichiometric with the O_2 evolved. Thus a part of the Benson-Calvin cycle has been converted to a linear sequence (Fig.1). From simultaneous measurements of the O_2 evolved and of the PGA and TP formed, an ATP/2e ratio of 2.0 has been determined. The contribution from cyclic or pseudocyclic phosphorylation during CO_2 fixation is shown to be relatively small.

METHODS AND MATERIALS. Intact chloroplasts were isolated from outside grown spinach essentially as described by Heldt and Sauer (19). Percentage intactness was determined by the ferricyanide method (20) and the preparations routinely contained 50-80% intact chloroplasts. Chlorophyll was determined as in (21).

The reactions were carried out at 25° in duplicate in twin oxygen electrodes of the type described in (22). One electrode was used for the determination of O_2; from the other electrode vessel, samples were taken for the determination of TP and PGA. Illumination was provided by Prinz 300 W tungsten lamps in slide projectors with built-in heat filters in the slide compartment. The light intensity was saturating for non-cyclic electron-transport at chlorophyll concentrations up to 150 µg/ml.

For determinations of TP and PGA, 100 µl samples were taken at intervals, added to 50 µl of 5% perchloric acid, neutralised with K_2CO_3, and centrifuged to clear. A sample of the supernatant was added to a cuvette which contained in a final volume of 1 ml: 0.1 M Tris-HCl (pH 7.5), 2mM sodium EDTA, 5 mM $MgCl_2$, 5 mM ATP and 0.1 mM NADH. Addition of glycerol 3-phosphate dehydrogenase and triose phosphate isomerase caused an absorbance change at 340 nm which was proportional to the TP concentration. A subsequent addition of phospho-glycerate kinase and triose phosphate dehydrogenase gave an additional absorbance change at 340 nm which was proportional to the PGA concentration.

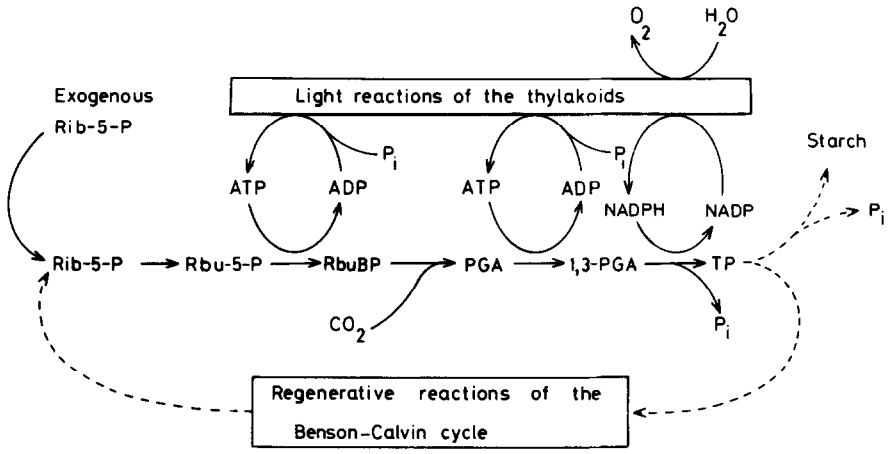

Fig. 1. Scheme showing the reactions (unbroken lines) operating when Rib-5-P is added to intact chloroplasts in the absence of added P_i.

Biochemicals and enzymes were from Sigma London Chemical Co. Ltd., Poole, U.K., and from Boehringer Corp. (London), Ealing, London.

RESULTS. Fig.2 shows the characteristic O_2 evolution traces observed when intact chloroplasts are illuminated in the presence of bicarbonate. An addition of catalase to chloroplasts under the conditions of Fig.2 gave no detectable difference in the rates of O_2 evolution. Furthermore, H_2O_2 added to the chloroplasts in the absence of added catalase was rapidly broken down. Thus it appears that the chloroplast preparations used in the present study contained a significant catalase activity (cf refs. 23, 24). In the absence of added P_i (Fig.2a) the rate of O_2 evolution decreased with time. This decrease was accompanied by a depletion of the endogenous P_i (25) and an increase in the PGA/TP ratio (Fig.2a). These results are consistent with previous work (26) where PGA and TP levels were measured only in the presence of exogenous P_i. From this previous work (26) it is apparent that as the concentration of added P_i was decreased so the PGA/TP ratio increased. The PGA/TP ratio is higher in the absence of added P_i largely as a result of a low ATP/ADP ratio in the stroma (27), a condition known to inhibit phosphoglycerate kinase activity (28, 29). Chloroplasts from the same preparation as that used for Fig.2a gave, in the presence

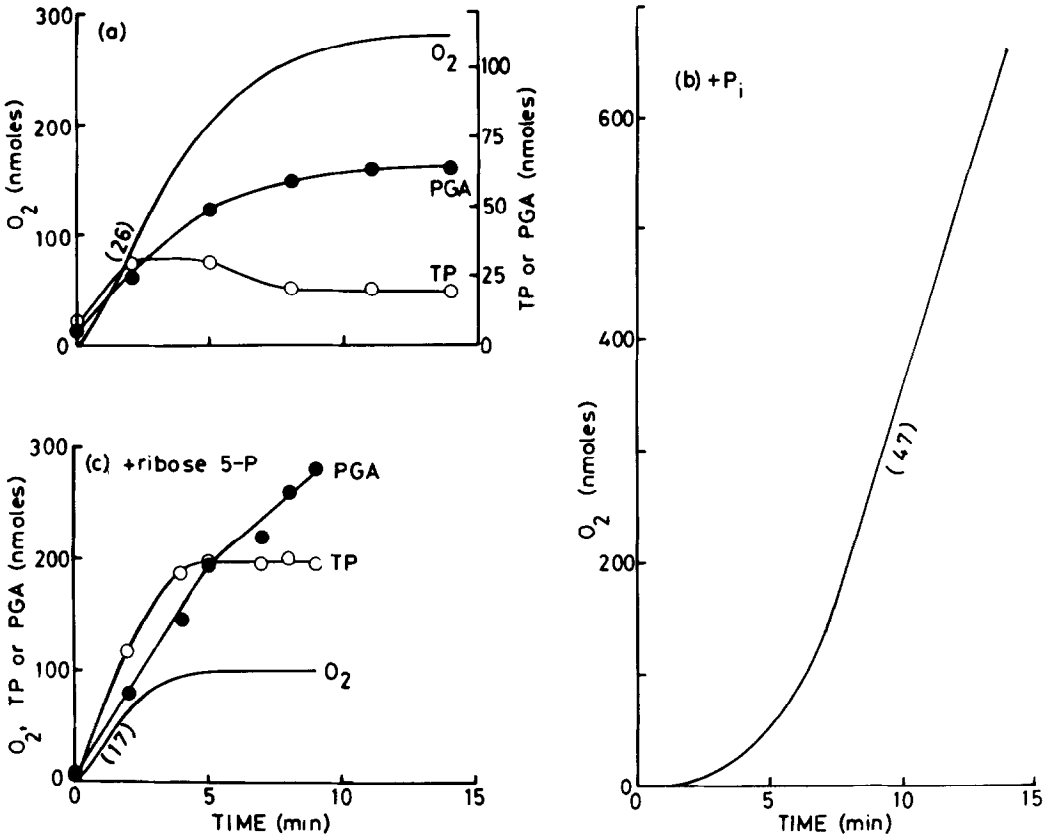

Fig. 2. Simultaneous measurements of O_2 evolution, TP and PGA accumulation during photosynthesis in spinach chloroplasts. Reaction mixtures contained in 1 ml:0.33M sorbitol, 50 mM HEPES/NaOH (pH 7.6), 1 mM $MgCl_2$, 1mM $MnCl_2$, 2 mM sodium EDTA, 10 mM $NaHCO_3$. In addition (b) contained 0.5 mM KH_2PO_4 and (c) contained 5 mM Rib-5-P. Chloroplasts were added to a concentration of either 125 µg/ml in (a) and (c) or of 97 µg/ml in (b). The numbers in brackets are the rate of O_2 evolution expressed in µmoles O_2/h per mg chlorophyll.

of 0.5 mM P_i (Fig.2b), an O_2 evolution trace which did not come to a stop, but, after an initial lag, proceeded at a linear rate.

It has been reported previously (30) that the addition of Rib-5-P to intact chloroplasts, in the absence of added P_i, causes an inhibition of O_2 evolution which is overcome by an addition of P_i. From subsequent experiments with a reconstituted system it was shown (31) that the Rbu-5-P derived from the added Rib-5-P acts as an ATP sink, and that it is the resulting low ATP/ADP ratio which contributes to inhibit O_2 evolution by an inhibition

of the phosphoglycerate kinase. Fig.2c shows the amounts of TP and PGA accumulated and the O_2 evolved in the presence of Rib-5-P and absence of added P_i. The concentration of TP increased until there was no further O_2 evolution, while the PGA concentration continued to rise even after O_2 evolution had come to a stop. Therefore O_2 evolution stopped not because there was no more P_i available for ATP synthesis (since PGA continued to be formed) but presumably because of both a low ATP/ADP ratio (27, 30-32) and a high TP concentration (Fig.2c, Table I) which combined to inhibit the reduction of PGA to TP. Indeed an appreciable P_i level was determined to be present during the time course of the experiment of Fig.2c and Table I, and this P_i was shown to originate from the Rib-5-P present (25). Table I shows that there is a strict relationship between the amount of O_2 evolved and the amount of TP accumulated, indicating that the TP accumulated can account for the O_2 evolved with a TP/O_2 ratio near 2. This ratio was found to be highly reproducible: when the experiment of Fig.2c and Table I was repeated 9 times over a period of 5 months a mean TP/O_2 ratio of 2.05 (with a S.D. of 0.16) was obtained. The implication of this is that the only reactions of the Benson-Calvin cycle that occurred in the chloroplasts under these conditions are those indicated in Fig.1 and listed below:

ribose 5-phosphate → ribulose 5-phosphate .....(1)

ribulose 5-phosphate + ATP → ribulose 1, 5-bisphosphate .....(2)

ribulose 1, 5-bisphosphate + CO_2 → 2 3-phosphoglycerate .....(3)

3-phosphoglycerate + ATP + NADPH → glyceraldehyde 3-phosphate (TP) + ADP + P_i + NADP .....(4)

glyceraldehyde 3-phosphate (TP) → dihydroxyacetone phosphate (TP) ....(5)

It is assumed that RbuBP did not accumulate to any significant extent (cf. 26, 33, 34) and therefore from reactions (2) - (5) above it follows that the amount of ATP formed is given by:

$$\Delta ATP = 1.5 \Delta TP + 0.5 \Delta PGA \quad \text{.....(6)}$$

Thus it was possible to determine under these conditions the ATP/2e ratio

Table I. Stoichiometry of phosphorylation coupled to O_2 evolution during photosynthesis in the presence of Rib-5-P

Time of illumination (min)	PGA	TP	O_2 evolved	ATP	ATP/2e ratio
	(μmoles per mg chlorophyll)				
0	0.05	0.07	-	-	-
2	0.64	1.08	0.46	1.81	2.0
5	1.52	1.59	0.80	3.02	1.9
7	1.76	1.52	0.80	3.03	1.9
8	2.08	1.59	0.80	3.29	2.1
9	2.24	1.52	0.80	3.27	2.1

Experiment of Fig.2c. The amounts of ATP formed were calculated according to Equation (6) from the observed increments of TP and PGA.

during O_2 evolution in intact chloroplasts. The calculated ATP/2e ratios for different times of illumination are shown in Table I. From 9 different chloroplast preparations examined over a period of 5 months a mean ATP/2e ratio of 1.96 (with a S.D. of 0.17) was observed using values obtained during the first 4 min of illumination.

It is clear from Table I that the ATP/2e ratio did not change significantly over the illumination time of 9 min. Yet after 5 min of illumination PGA accumulation continued without a measurable evolution of O_2. The ATP requirement for this PGA accumulation (see reactions (2) and (3) above) was presumably met by cyclic or pseudocyclic phosphorylation.

From the data of Table I it can be calculated that during O_2 evolution (first 2 min of illumination) the rate of phosphorylation was 55 μmoles/h/mg chlorophyll, but after O_2 evolution had ceased (after 5 min of illumination) the rate of phosphorylation (calculated from the observed increment of PGA) was 5 μmoles/h/mg chlorophyll. Thus the rate of cyclic or pseudocyclic phosphorylation after O_2 evolution had ceased was only 9% of the total rate during O_2 evolution. To determine the ATP/2e ratio it is necessary to take into account the amount of cyclic or pseudocyclic phosphorylation during O_2

evolution. It is well-established from studies made with a variety of photosynthetic systems that both cyclic (35, 36) and pseudocyclic (2, 16, 37) phosphorylation decrease when linear electron transport occurs. Furthermore addition of NADPH has been shown to increase more than 2-fold the rate of ferredoxin-dependent cyclic phosphorylation in broken chloroplasts (38). In the present study NADPH must have accumulated when O_2 evolution ceased. Thus it is concluded that the contribution of cyclic or pseudocyclic phosphorylation to the total phosphorylation determined during O_2 evolution was less than 9%.

CONCLUSIONS. The results reported in the present paper show that, in the absence of added P_i, it is possible to measure the rate of ATP synthesis in isolated, intact chloroplasts during photosynthetic O_2 evolution. This has been accomplished by the conversion of part of the Benson-Calvin cycle into a linear path (Fig.1) so that ATP synthesis could be calculated from equation (6) above. During the first 4 min of illumination, when O_2 evolution was occurring at a measurable rate, an ATP/2e ratio of 2.0 has been obtained. From the rate of cyclic or pseudocyclic phosphorylation measured after O_2 evolution had ceased it has been concluded that these contribute less than 9% to the ATP synthesised during O_2 evolution. Therefore the ATP/2e ratio coupled to photosynthetic O_2 evolution is between 1.8 and 2.0 in intact chloroplasts which are fixing CO_2 in the absence of exogenous P_i. It is significantly higher than the ratio of 1.5 which is the minimum ratio required for the operation of the Benson-Calvin cycle (1). An ATP/2e ratio $>$ 1.5 in intact chloroplasts, is in agreement with the many reports of an ATP/2e ratio $>$ 1.5 in broken chloroplasts, and with previous work which suggests that in whole algae, cyclic phosphorylation does not contribute to the steady-state CO_2 fixation (4, 39-41). Furthermore, the relatively low rate of H_2O_2 accumulation during CO_2 fixation by intact chloroplasts (in the absence of catalase activity) (24) may indicate a correspondingly low rate of pseudocyclic electron transport under these conditions. An ATP/2e ratio $>$ 1.5

might help to explain why in intact chloroplasts, NH_4Cl can stimulate photosynthesis, while it decreases the ATP steady-state level (42-44). If the chloroplasts are tightly coupled, with an ATP/2e ratio of 2.0, and are incapable of utilizing the "extra" 0.5 ATP/2e, this ATP would create a back-pressure on the electron transport which in turn would reduce the rate of O_2 evolution. This back-pressure would be relieved by partial uncoupling due to the NH_4Cl. Evidence for an ATP/2e ratio < 1.5 for intact chloroplasts comes largely from measurements of quantum requirements (11, 12), and evidence for the operation of cyclic and pseudocyclic phosphorylation in intact chloroplasts comes largely from inhibitor studies (13-15, 17). It may well be that under conditions different from those employed in the present paper the ATP/2e ratio is lower than 2.0, and that the redox poise is more conducive to the operation of cyclic and pseudocyclic phosphorylation.

As well as for determination of the ATP/2e ratio in intact chloroplasts, the system described in the present paper could be used to determine the effect of various factors on that sequence of the Benson-Calvin cycle between Rib-5-P and TP (Fig.1).

Acknowledgements. During part of this work I was the recipient of an Accademia Nazionale dei Lincei-Royal Society Fellowship. Professor G. Forti and Dr. A. Fuggi are thanked for stimulating discussions. I am grateful to Professor F.R. Whatley for his constant encouragement.

References

1. Bassham, J.A. (1964) Annu. Rev. Plant Physiol., 15, 101-120.
2. Heber, U. (1976) J. Bioenergetics and Biomembranes, 8, 157-172.
3. Krause, G.H. and Heber, U. (1976) The Intact Chloroplast (Barber, J., ed.) pp. 171-214, Elsevier, Amsterdam.
4. Raven, J.A. (1976) The Intact Chloroplast (Barber, J. ed.) pp.403-443, Elsevier, Amsterdam.
5. Hall, D.O. (1976) The Intact Chloroplast (Barber, J. ed.) pp.135-70, Elsevier, Amsterdam.
6. Trebst, A. and Reimer, S. (1973) Biochim. Biophys. Acta, 305, 129-139.
7. Izawa, S., Gould, J.M., Ort., D.R., Felker, P. and Good, N.E. (1973) Biochim. Biophys. Acta, 305, 119-128.
8. Gimmler, H. (1973) Z. Pflanzenphysiol., 68, 289-307.
9. Junge, W. and Auslander, W. (1974) Biochim. Biophys. Acta, 333, 59-70.
10. Gould, J.M. and Izawa, S. (1974) Biochim. Biophys. Acta, 333, 509-524.
11. Heber, U. (1973) Biochim. Biophys. Acta, 305, 140-152.
12. Heber, U. and Kirk, M.R. (1975) Biochim. Biophys. Acta, 376, 136-150.
13. Slovacek, R.W. and Hind, G. (1977) Plant Physiol., 60, 538-542.
14. Heber, U. and Egneus, H. (1977) Abstracts of 4th International Congress on Photosynthesis (Coombs, J. ed.) Reading, U.K.

15. Urbach, W. and Kaiser, W. (1977) Abstracts of 4th International Congress on Photosynthesis (Coombs, J. ed.) Reading, U.K.
16. Egneus, H., Heber, U., Mathiesen, U. and Kirk, M. (1975) Biochim. Biophys. Acta, 408, 252-268.
17. Forti, G. and Gerola, P. (1977) Plant Physiol. 59, 859-862.
18. Heldt, H.W. (1969) FEBS Lett., 5, 11-14.
19. Heldt, H.W. and Sauer, F. (1971) Biochim. Biophys. Acta, 234, 83-91.
20. Heber, U. and Santarius, K.A. (1970) Z. Naturforsch., 25b, 718-728.
21. Arnon, D.I. (1949) Plant Physiol., 24, 1-15.
22. Delieu, T. and Walker, D.A. (1972) New Phytol., 71, 201-225.
23. Kaiser, W. (1976) Biochim. Biophys. Acta, 440, 476-482.
24. Allen, J.F. and Whatley, F.R. (1978) Plant Physiol., 61, 957-960.
25. Rosa, L. (1979) Plant Sci. Lett. (to be submitted).
26. Lilley, R. Mc., Chon, C.J., Mosbach, A. and Heldt, H.W. (1977) Biochim. Biophys. Acta, 460, 259-272.
27. Kaiser, W. and Urbach, W. (1977) Biochim. Biophys. Acta, 459, 337-346.
28. Slabas, A.R. and Walker, D.A. (1976) Biochem. J., 154, 185-191.
29. Lavergne, D., Bismuth, E. and Champigny, M.L. (1974) Plant Sci. Lett., 3, 391-397.
30. Cockburn, W., Baldry, C.W. and Walker, D.A. (1967) Biochim. Biophys. Acta, 143, 614-624.
31. Slabas, A.R. and Walker, D.A. (1976) Biochim. Biophys. Acta, 430, 154-163.
32. Cockburn, W. (1974) J. Exo. Bot., 23, 111-120.
33. Bassham, J.A., Kirk, M. and Jensen, R.G. (1968) Biochim. Biophys. Acta, 153, 211-218.
34. Schacter, B. and Bassham, J.A. (1972) Plant Physiol., 49, 411-416.
35. Reeves, S.G. and Hall, D.O. (1973) Biochim. Biophys. Acta, 387, 306-319.
36. Maxwell, P.C. and Biggins, J. (1976) Biochemistry, 15, 3975-3980.
37. Elstner, E.F. and Heupel, A. (1973) Biochim. Biophys. Acta, 325, 183-188.
38. Arnon, D.I. and Chain, R.K. (1975) Proc. Nat. Acad. Sci. USA, 72, 4961-4965.
39. Biggins, J. (1973) Biochemistry, 12, 1165-1169.
40. Raven, J.A. (1971) J. Exp. Bot., 22, 420-433.
41. Klob, W., Kandler, O. and Tanner, W. (1973) Plant Physiol., 51, 825-827.
42. Forti, G., Rosa, L., Fuggi, A. and Garlaschi, F.M. (1975) Proc. 3rd Int. Congr. Photosynthesis (Avron, M. ed.) pp. 1499-1505, Elsevier, Amsterdam.
43. Werdan, W., Heldt, H.W. and Milovancev, M. (1975) Biochim. Biophys. Acta, 396, 276-292.
44. Tillberg, J., Giersch, C. and Heber, U. (1977) Biochim. Biophys. Acta, 461, 31-47.