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Interactions between ribulose-1,5-bisphosphate carboxylase and stromal metabolites. II. Corroboration of the role of this enzyme as a metabolite buffer

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The capacity of ribulose-1,5-bisphosphate carboxylase to bind reversibly chloroplast metabolites which are the substrates for both thylakoid and stromal enzymes was assessed using spinach chloroplasts and chloroplast extracts and with pure wheat ribulose-1,5-bisphosphate carboxylase. Measurements of the rate of coupled electron flow to methyl viologen in 'leaky' chloroplasts (which retained the chloroplast envelope and stromal enzymes but which were permeable to metabolites) and also with broken chloroplasts and washed thylakoids were used to study the effects of binding ADP and inorganic phopshate to ribulose-1,5-bisphosphate carboxylase. The presence of ribulose-1,5-bisphosphate carboxylase significantly altered the values obtained for apparent K_m for inorganic phosphate and ADP of coupled electron transport. The K_m (P_i) in washed thylakoids was 60–80 μM, in 'leaky' chloroplasts it was increased to 180–200 μM, while in 'leaky' chloroplasts preincubated with KCN and ribulose 1,5-bisphosphate the value was decreased to 40–50 μM. Similarly, the K_m (ADP) of coupled electron transport in washed thylakoids was 60–70 μM, in 'leaky' chloroplasts it was 130–150 μM and with 'leaky' chloroplasts incubated in the presence of KCN and ribulose 1,5-bisphosphate a value of 45–50 μM was obtained. The ability of ribulose 1,5-bisphosphate carboxylase to reduce the levels of free glycerate 3-phosphate in the absence of ribulose 1,5-bisphosphate was examined using a chloroplast extract system by varying the concentrations of stromal protein or purified ribulose 1,5-bisphosphate carboxylase. The effect of binding glycerate 3-phosphate to ribulose-1,5-bisphosphate carboxylase on glycerate 3-phosphate reduction was to reduce both the rate an the amount of NADPH oxidation for a given amount of glycerate 3-phosphate added. The addition of ribulose 1,5-bisphosphate reinitiated NADPH oxidation but ATP or NADPH did not. Incubation of purified ribulose-1,5-bisphosphate carboxylase with carboxyarabinitolbisphosphate completely inhibited the catalytic activity of the enzyme and decreased inhibition of glycerate-3-phosphate reduction. Two binding sites with different affinities for glycerate 3-phosphate were observed with pure ribulose-1,5-bisphosphate carboxylase.

Introduction

The measurement of photosynthetically active metabolites in leaves [1,2], leaf protoplasts [3,4] and chloroplasts [5] has added to the understand-

Abbreviation: P_i, inorganic phosphate; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid.

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ing of the regulation of photosynthesis. It has aided the identification of regulatory reactions in the photosynthesis, particularly following transitions in environmental conditions such as temperature [6], light [7], CO_2 [8] and also in studies of stress [9]. The interpretation of such data often involves the assumption that measured amounts of metabolites reflect the free concentrations available to photosynthetic enzymes and processes. This assumption may not be valid in the case of the chloroplast stroma, as the stromal environment is a complex mixture of enzymes at extremely high protein concentrations [10]. Up to 90% of the stromal protein is ribulose-1,5-bisphosphate carboxylase which has an active site concentration of at least 4 mM [11]. From calculations based on the K_d values for binding various metabolites to the ribulose-1,5-bisphosphate binding site of the carboxylase, it has been proposed that a considerable proportion of certain stromal metabolites might be bound to this enzyme in vivo [12]. However, apart from the theoretical considerations of binding capacity, there is no direct evidence for such 'metabolite buffering' in the chloroplast stroma. If the kinetic parameters measured in in vitro studies using purified thylakoid and soluble enzyme systems are to be extrapolated to the in vivo situation, then the degree of metabolite buffering in various situations must be measured and assessed.

There are two major ways in which metabolite buffering may affect photosynthetic carbon assimilation in vivo. Firstly, ribulose-1,5-bisphosphate carboxylase may bind ADP, P_i and NADP required for photophosphorylation and NADP reduction. There is evidence from experiments with reconstituted chloroplasts that ribulose-1,5-bisphosphate carboxylase modifies the kinetics of photophosphorylation [13], possibly by interaction with the ATPase and its substrates. Secondly, ribulose-1,5-bisphosphate carboxylase may bind metabolites which are the substrates of stromal enzymes in the carbon reduction cycle. The effects of such binding on the catalytic activity of ribulose-1,5-bisphosphate carboxylase have been described previously [7]. The present study examines the effect of metabolite buffering by ribulose-1,5-bisphosphate carboxylase [12] on both thylakoid and stromal reactions using a modified 'intact' chloroplast system and chloroplast stromal extracts.

Materials and Methods

Intact chloroplast preparation. *Spinacia oleracea* L. cv virtuosa was grown in hydroponic culture in a glasshouse [14,15] and intact spinach chloroplasts prepared according to the procedures described by Walker [15]. Chloroplasts permeable to small molecules but retaining stromal protein [16] were prepared by resuspension in a reaction medium containing 0.1 M sorbitol, 1 mM $MgCl_2$, 50 mM Hepes/KOH buffer (pH 7.6) and 1 mM EDTA. Chloroplasts were incubated for 2 min in the dark at a chlorophyll concentration of 50 $\mu g \cdot ml^{-1}$ before assay. These chloroplasts (termed 'leaky' chloroplasts here) retained 90% of their stromal protein content after treatment, as judged by the detection of protein appearing in the supernatant after centrifugation at 5000–g for 5 min.

O_2-uptake measurements. Coupled electron flow to methyl viologen was measured in a reaction medium containing either 0.33 or 0.1 M sorbitol and 10 mM $MgCl_2$, 50 mM Hepes/KOH buffer (pH 7.6), 1 mM EDTA, ADP and P_i at the concentrations stated on the figure legends, 1 mM methyl viologen and chloroplasts equivalent to 50 μg chlorophyll in a total volume of 1 ml. O_2 uptake was meausred polarographically [15] using a Clark-type oxygen electrode (Hansatech Ltd., U.K.) at 25 °C illuminated with a quartz halogen projector lamp (150 W) with a Calflex C heat filter (Balzers, F.R.G.) and a red filter, giving an irradiance of 500 $\mu mol \cdot m^{-2} \cdot s^{-1}$.

Preparation and assay of stromal proteins. Stromal proteins were prepared from intact chloroplasts as follows. Intact chloroplast pellets were lysed in 2 ml of medium 1, containing 10 mM Hepes/KOH (pH 7.6), 1 mM $MgCl_2$, 1 mM EDTA and 5 mM dithiothreitol and centrifuged at $2000 \times g$ for 2 min. The supernatant (chloroplast stromal extract) was retained and passed through a PD 10 gel filtration column (Pharmacia Fine Chemicals, Uppsala, Sweden) eluted with medium 2, containing 2 mM EDTA, 2 mM $MgCl_2$, 4 mM ascorbate, 1 mM dithiothreitol, 10 mM KCl and 50 mM Hepes/KOH buffer (pH 7.9) and stored on ice. NADPH oxidation in stromal extracts was

assayed spectrophotometrically on a Perkin-Elmer (Beaksonfield, U.K.) 557 double-wavelength double-beam spectrophotometer, in medium 2 (total volume 1 ml), except that dithiothreitol was increased to 5 mM and $MgCl_2$ was 10 mM. NADPH (0.25 mM), ATP (4 mM), creatine phosphate (5 mM) and creatine phosphate-kinase (2 U) were included in each assay.

Purified ribulose 1,5-bisphosphate carboxylase from wheat leaves [17] was the gift of the Department of Biochemistry, Rothamsted Experimental Station, U.K. The specific activity was 1 μmol per mg protein per min (at 25°C) when activated prior to use for 40 min at 40°C in a medium containing 20 mm $MgCl_2$, 10 mM $NaHCO_3$ and 0.1 mM Hepes/KOH buffer (pH 8.0). Carboxyarabinitol bisphosphate was given by Dr. George Lorimer and was added to the incubation media at a concentration of at least 5-times the nominal active-site concentration.

Other assays. Protein was estimated by the dye-binding protein ssay (Bio-Rad Laboratories, München, F.R.G.). Chlorophyll was estimated by the method of Arnon [18].

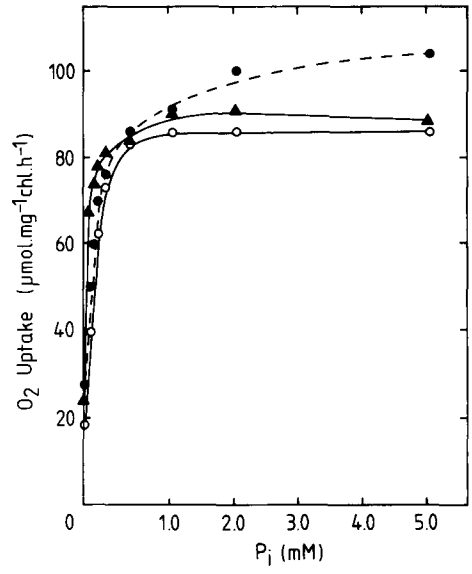

Fig. 1. The effect of P_i concentration on the rate of coupled electron flow to methyl viologen in 'leaky' chloroplasts (●), 'leaky' chloroplasts + KCN (0.5 mM) + ribulose-1,5-bisphosphate (0.5 mM) (▲) and washed thylakoids (○). These data have not been corrected for uncoupled rates of electron transport.

Results

Apparent K_m (ADP) and K_m (P_i) for photophosphorylation

A major difficulty in assessing the influence of stromal components on the kinetic behaviour of thylakoid-bound enzymes is the design of experiments in which the ratio of thylakoid membrane to stromal protein is similar to that in vivo, but in which the concentrations of stromal metabolites are easily manipulated. When isolated chloroplasts were subjected to a mild hypo-osmotic treatment, the envelope became permeable to small molecules, but stromal protein was largely retained within the chloroplast [16]. This property has been exploited previously [19] to demonstrate ATP-dependent quanching of chlorophyll *a* fluorescence in intact chloroplasts. Similarly, 'leaky' chloroplasts were used here to determine the K_m (ADP) and the K_m (P_i) of photophosphorylation in a system in which (unlike the reconstituted chloroplast system) the ratio of stromal protein to chlorophyll is conserved (Figs. 1–3).

The initial rate of O_2 consumption in the presence of methyl viologen was measured following the addition of ADP and P_i after 2 min preillumination. Values for the apparent K_m (P_i) and K_m (ADP) of photophosphorylation were obtained and were also derived in a similar manner

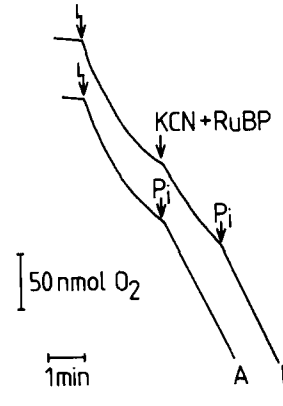

Fig. 2. Representatie O_2 electrode traces of coupled electron flow to methyl viologen where 0.5 mM KCN and 0.5 mM ribulose-1,5-bisphosphate were added to 'leaky' chloroplasts during illumination in the presence of ADP but with no added P_i.

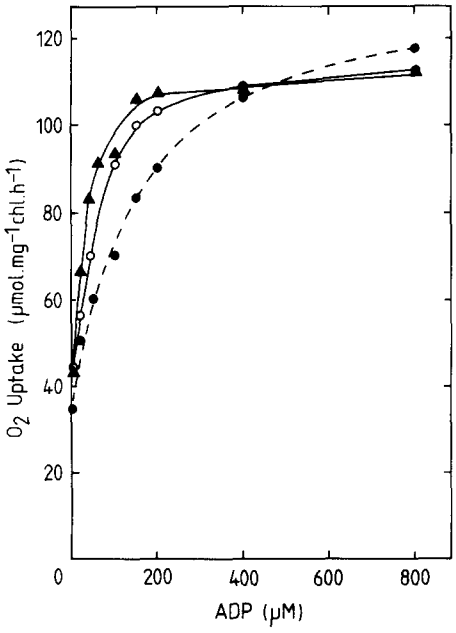

Fig. 3. The response of coupled electron flow in the presence of methyl viologen to ADP concentration in 'leaky' chloroplasts (●), 'leaky' chloroplasts + KCN (0.5 mM) + ribulose-1,5-bisphosphate (0.5 mM) (▲) and washed thylakoids (○).

following an initial treatment of 2 min preincubation in the dark in the presence of 0.5 mM KCN and 0.5 mM ribulose 1,5-bisphosphate. This treatment results in the formation of a tightly bound complex at the active site of ribulose-1,5-bisphosphate carboxylase and would be expected to exclude binding of metabolites to this portion of the enzyme [19]. The K_m values obtained by direct linear plots of the data in Figs. 1 and 3 were compared with those determined for washed thylakoids (Table I). For the determinations of K_m in Table I the basal coupled rate of electron in the absence of P_i or ADP was subtracted from that in the presence of added substrate.

TABLE I

CALCULATED K_m VALUES FOR ADP AND P_i FOR COUPLED ELECTRON FLOW TO METHYL VIOLOGEN

System	K_m (ADP) (μM)	K_m (P_i) (μM)
Thylakoids	60– 70	60– 80
Thylakoids + KCN + ribulose-1,5-bisphosphate	50– 60	60– 80
'Leaky' chloroplasts	130–150	180–200
'Leaky' chloroplasts + KCN + ribulose-1,5-bisphosphate	45– 50	40– 50

The apparent K_m for P_i for coupled electron transport in washed thylakoids was consistently found to be between 60 and 80 μM, while in 'leaky' chloroplasts this value rose to 180–200 μM. A K_m (P_i) of 40–50 μM was obtained in 'leaky' chloroplasts preincubated with KCN and ribulose-1,5-bisphosphate four determinations were made in each case). No effect was observed when KCN or ribulose-1,5-bisphosphate were added individually to 'leaky' chloroplasts. Washed thylakoids were unaffected by KCN and ribulose-1,5-bisphosphate addition. Fig. 2 shows a representative O_2 electrode trace in which KCN and ribulose-1,5-bisphosphate were added during illumination in the presence of ADP but in the absence of P_i. The rate of O_2 uptake immediately increased following KCN and ribulose-1,5-bisphosphate addition, but rapidly returned to the basal rate (unless additional P_i was supplied) suggesting that a small amount of bound P_i had been made available to the ATPase. Some ribulose 1,5-bisphosphate preparations, however, contained as much as 15% P_i as a contaminant. Thus, the K_m determinations of Table I were made by adding KCN and ribulose 1,5-bisphosphate prior to illumination and allowing endogenous P_i to become exhausted in the light before the addition of substrate for the K_m determinations. Similar trends in the K_m (ADP) were observed when coupled methyl viologen reduction in 'leaky' chloroplasts and thylakoids were compared (Fig. 3). In four separate determinations, the K_m (ADP) of coupled electron transport in washed thylakoids was found to be between 60 and 70 μM, while in 'leaky' chloroplasts the value rose to between 130 and 150 μM. However, with 'leaky' chloroplasts incubated in the presence of KCN and ribulose-1,5-bisphosphate lower values of between 45 and 50 μM were obtained (Table I).

Binding of sugar phosphates to ribulose-1,5-bisphosphate carboxylase

The influence of ribulose-1,5-bisphosphate carboxylase the effective concentrations of photosynthetically active sugar phosphates was followed spectrophotometrically in the presence of different

concentrations of stromal protein or purified ribulose-1,5-bisphosphate carboxylase. Binding of glycerate 3-phosphate to ribulose-1,5-bisphosphate carboxylase was estimated by assaying glycerate 3-phosphate reduction in this system in the presence of rate-saturating ATP and NADPH. Fig. 4 shows the effect of glycerate 3-phosphate concentration on the rate of glycerate 3-phosphate reduction at two stromal protein concentrations, 0.2 and 2.0 mg · ml^{-1}. The value obtained for the apparent K_m (glycerate 3-phosphate) of the glycerate 3-phosphate kinase was dependent on the stromal protein concentration used. The K_m at 0.2 mg · ml^{-1} was 2.8 mM rising to 7.6 mM at 2.0 mg · ml^{-1} while the V_{max}, expressed on a protein basis, remained unchanged (calculated from direct linear plots). Increasing the protein concentration also reduced the amount of NADPH oxidised for a given amount of glycerate 3-phosphate added. This is also shown to be the case in Table III in

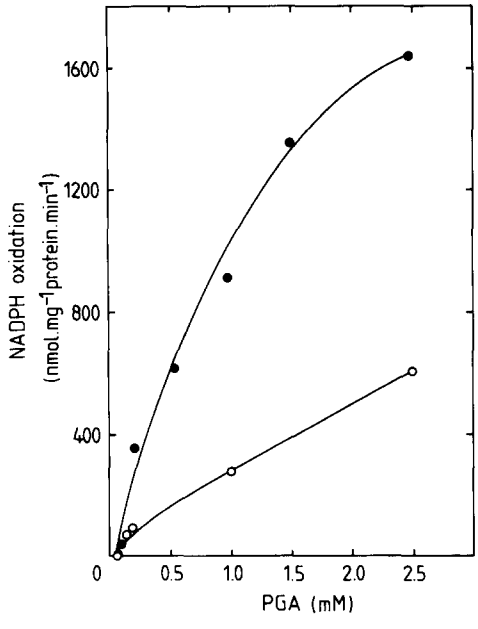

Fig. 4. The effect of glycerate 3-phosphate (PGA) concentration on the rate of glycerate 3-phosphate reduction. The rate of NADPH oxidation resulting from the action of glyceraldehyde 3-phosphate dehydrogenase coupled to that of phosphoglycerate kinase present in the stromal extracts was measured at two concentrations of stromal protein (0.2 (●) and 2.0 (○) mg · ml^{-1}). Glycerate 3-phosphate was added to initiate the oxidation of NADPH by the enzymes in the stromal protein extracts as described in methods.

TABLE II

VALUES FOR THE K_m (ADP) AND K_m (P_i) FOR PHOTOPHOSPHORYLATION (A) AND FOR THE STEADY-STATE LEVEL OF THESE METABOLITES IN ILLUMINATED CHLOROPLASTS (B) TAKEN FROM THE LITERATURE

(A) Values for the K_m (ADP) and K_m P_i for photophosphorylation

System	K_m ADP (μM)	K_m P_i (μM)	Reference
Thylakoids	85	600	33
Thylakoids	19 –105	–	34
Thylakoids	2.7– 7.0	–	39
Thylakoids	–	23	35

(B) Values for ADP and P_i levels in the chloroplast, assuming a stromal volume of 25 μl per mg chlorophyll

System	ADP (mM)	P_i (mM)	Reference
Leaves (non-aqueous fractionation)		4–25	36
	1.3–1.7		7
		20–35	37
Isolated chloroplasts	0.3–0.5	5–10	5
	0.4	8–10	38

TABLE III

NADPH OXIDATION RESULTING FROM GLYCERATE 3-PHOSPHATE (50 nmol) REDUCTION IN A REACTION WHERE STROMAL PROTEIN (200 μg per ml) WAS USED AS THE SOURCE OF THE ENZYMES PHOSPHOGLYCERATE KINASE AND NADP-GLYCERALDEHYDE PHOSPHATE DEHYDROGENASE

Purified ribulose-1,5-bisphosphate carboxylase was added in the activated form and in the inactivated form pretreated with 25 μM carboxyarabinitol bisphosphate.

Addition	Total NADPH oxidised (nmol)
None	43
Ribulose-1,5-bisphosphate carboxylase (0.5 mg · ml^{-1})	29
Ribulose-1,5-bisphosphate carboxyalse (3.5 mg · ml^{-1})	2
Ribulose-1,5-bisphosphate carboxylase (3.5 mg · ml^{-}) with carboxyarabinitol bisphosphate (25 μM)	18

which purified ribulose-1,5-bisphosphate carboxylase was added to a reaction mixture containing 0.2 mg · ml^{-1} stromal protein. Although 50 nmol glycerate 3-phosphate was added in each case, the amount of NADPH oxidised decreased with increasing ribulose-1,5-bisphosphate carboxylase concentration. The addition of more ATP or NADPH did not result in recovery of the remainder of the 'unused' glycerate 3-phosphate, but ribulose-1,5-bisphosphate (or additional glycerate 3-phosphate) reinitiated NADPH oxidation, suggesting that ribulose-1,5-bisphosphate carboxylase was not directly inhibiting glycerate 3-phosphate kinase. When carboxyarabinitol bisphosphate was included in the reaction mixture in the presence of 3.5 mg · ml^{-1} added ribulose-1,5-bisphosphate carboxylase, the amount of glycerate 3-phosphate reduced increased to approx. 50% of that observed in the absence of added ribulose-1,5-bisphosphate carboxylase. Catalytic activity of ribulose-1,5-bisphosphate carboxylase was completely inhibited by carboxyarabinitol bisphosphate treatment. This suggests that the effect is not entirely due to glycerate 3-phosphate binding at the ribulose-1,5-bisphosphate binding site of ribulose-1,5-bisphosphate carboxylase.

The dependence of the amount of glycerate 3-phosphate available to the glycerate 3-phosphate kinase on the concentration of ribulose-1,5-bisphosphate carboxylase present was determined for ribulose-1,5-bisphosphate carboxylase concentrations over the range 0–10 mg · ml^{-1} in the presence and absence of carboxyarabinitol bisphosphate (Fig. 5). As seen in Table III, higher ribulose-1,5-bisphosphate carboxylase concentrations progressively reduced the amount of glycerate 3-phosphate available for utilisation by glycerate 3-phosphate kinase in the assay mixture (assayed as glycerate-3-phosphate reduction with a constant stromal protein level of 0.2 mg · ml^{-1}). In Fig. 5, the data show the affinity of glycerate 3-phosphate binding and indicate that their two binding sites for glycerate 3-phosphate are present on ribulose-1,5-bisphosphate carboxylase; a high-affinity site which is sensitive to carboxyarabinitol bisphosphate and a low-affinity site insensitive to carboxyarabinitol bisphosphate addition. The unconventional approach of varying enzyme concentration rather than metabolite level was used to facilitate extrapolation of these data to in vivo protein concentrations. Nevertheless, such a treatment of the data is valid, since the measurements of NADPH oxidation were made in quasi steady-state conditions when the level of remaining NADPH decreased so slowly that it could not be measured within the time course of the experiment.

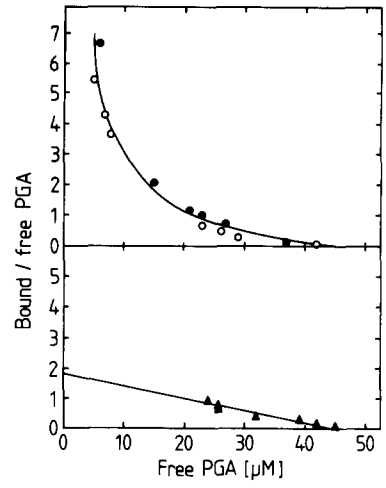

Fig. 5. Scatchard plot for the binding of glycerate 3-phosphate (PGA) to activated (●) and non-activated (○) wheat ribulose-1,5-bisphosphate carboxylase (A) and in the presence of 25 μM carboxyarabinitol bisphosphate (B).

Discussion

It is evident that binding of stromal metabolites to ribulose-1,5-bisphosphate carboxylase has a considerable inhibitory action on the reactions for which these metabolites are substrates. This is of importance in attempting to interpret measurements of photosynthetically active metabolites made on intact leaves and chloroplasts or protoplast systems. For example, measurements of ADP and P_i levels in photosynthesising tissue suggest that chloroplast concentrations of these metabolites never fall low enough to limit photophosphorylation, with measured values in the illuminated chloroplast in vivo up to 1000-fold higher (if measured values are meaningful) than the K_m values obtained in vitro (Table II). Also, photophosphorylation potentials attained during electron transport in thylakoid preparations are at

least an order of magnitude higher than those measured in vivo [20]. Despite this observation, much of the current theory concerning photosynthetic regulation requires that photophosphorylation should be ADP and P_i limited under many circumstances, mediating a feedbck control on electron transport via ΔpH [8,14,19–23]. The data of Figs. 1–3 suggest that it is inappropriate to apply kinetic parameters obtained in stroma-free thylakoid preparations to in vivo observations. The 'leaky' chloroplast system would at best provide an underestimate of metabolite binding by ribulose-1,5-bisphosphate carboxylase as hypoosmotic treatment will increase the stromal volume more than 2-fold [16]. This complication is difficult to overcome, because a correction factor cannot be determined, but a doubling of the K_m values obtained here is conceivable. The reported apparent K_m (ADP) and P_i values for photophosphorylation measured in vitro vary enormously in the literature (Table II) depending on the electron acceptor and the preparation used. This has recently been attributed to the dependence of these K_m values on the magnitude of the ΔpH at the time of measurements and hence the electron-transport rate [24,25]. This interaction must also be considered as a factor which could substantially increase the appropriate K_m values for photophosphorylation in vivo.

Reversal of ADP and P_i binding by carboxyarabinitol bisphosphate suggests that these metabolites are bound to the ribulose-1,5-bisphosphate binding site. However, we have previously shown that ribulose-1,5-bisphosphate carboxylase can inhibit photophosphorylation in the reconstituted chloroplast system, apparently via an Mg^{2+}-dependent binding of ribulose-1,5-bisphosphate carboxylase to the thylakoid membrane [13]. It is possible that both these processes contribute to higher K_m (ADP) and P_i values for photophosphorylation in vivo. If ribulose-1,5-bisphosphate carboxylase exerts its effect by screening the ATPsynthase from its substrates in the 'leaky' chloroplast system used here, then the thylakoid-protein-metabolite interaction is dependent on the ribulose-1,5-bisphosphate binding site (as carbxyarabinitol bisphosphate reverses the effect). The mechanism of such an interaction remains unclear.

Binding of metabolites by ribulose-1,5-bisphosphate carboxylase not only affects thylakoid processes, but from the data of Figs. 4–8 it is apparent that effective metabolite concentrations available to stromal enzymes are also modified. Chloroplast extracts were used in this study as they provide an opportunity to work at high stromal protein concentrations in a membrane-free system. Using this system it was possible to examine the competition between ribulose-1,5-bisphosphate carboxylase and glycerate 3-phosphate kinase for glycerate 3-phosphate. Glycerate 3-phosphate is present in high concentrations in the chloroplast stroma and its concentration varies enormously during transient changes in the rate of photosynthesis [8]. At the highest ribulose-1,5-bisphosphate carboxylase concentration which was possible in our experimental system (around 10 $mg \cdot ml^{-1}$) over 95% of added glycerate 3-phosphate was bound and unavailable for catalysis by glycerate 3-phosphate kinase. This suggests a stoichiometry of 7 glycerate 3-phosphate per ribulose-1,5-bisphosphate carboxylase molecule. It has previously been suggested that glycerate 3-phosphate binds at the ribulose-1,5-bisphosphate binding site of the ribulose-1,5-bisphosphate carboxylase in a competitive fashion, as evidenced by measurements of inhibition of catalysis with the purified enzyme [25–29]. The data shown here indicate that this is the case but that at least one other binding site is present, indicated by persistence of binding in the presence of carboxyarabinitol bisphosphate in concentrations sufficient to occupy all ribulose-1,5-bisphosphate binding sites. This is consistent with another report that three glycerate 3-phosphate binding sites are present on ribulose-1,5-bisphosphate carboxylase [27]. We detected little difference in glycerate 3-phosphate binding capacity between activated and non-activated carboxylase despite suggestions that glycerate 3-phosphate may be bound more tightly to the active enzyme [28].

Fig. 4 indicates that the kinetic behaviour of the glycerate 3-phosphate kinase/glyceraldehyde 3-phosphate dehydrogenase is influenced by the stormal protein concentration in the assay. The apparent K_m (glycerate 3-phosphate) for this reaction incrased considerably with increasing protein concentration, despite the inclusion of an

ATP-generating system and saturating NADPH. It is dififcult to envisage how simple metabolite binding could explain this phenomenon as the ratio of glycerate 3-phosphate kinase active sites to ribulose-1,5-bisphosphate carboxylase sites is conserved.

These results have several implications for the regulation of carbon assimilation as binding of glycerate 3-phosphate to the ribulose-1,5-bisphosphate carboxylase produces effective inhibition of catalysis [26,28] particularly at sub-saturating ribulose-1,5-bisphosphate concentrations (unpublished results). The presence of a number of glycerate 3-phosphate binding sites presents the possibility that one of these sites may be an allosteric site. Also, if these other binding sites accept glycerate 3-phosphate it is likely that other sugar phosphates, including ribulose-1,5-bisphosphate, may bind. Although the role of these other binding sites is not apparent from this study, the possibility of additional regulation of activation or catalytic activity via metabolite binding is intriguing.

We may now asign a physiological significance to the binding of Calvin cycle metabolites to ribulose-1,5-bisphosphate carboxylase, that of synchronising the catalytic activity of ribulose-1,5-bisphosphate carboxylase with other stromal enzymes and with the rate of photophosphorylation. Calvin cycle intermediates modulate ribulose-1,5-bisphosphate (Ref. 26), and by stabilising activation state [26]. feedback inhibition of ribulose-1,5-bisphosphate carboxylase by glycerate 3-phosphate ensures a close correspondence between the rate of energy production in the thylakoid and ribulose-1,5-bisphosphate carboxylase catalysis during transient changes in irradiance, for example. When light intensity is reduced, glycerate 3-phosphate rapidly accumulates, reducing the catalytic activity of ribulose-1,5-bisphosphate carboxylase and conserving the ribulose-1,5-bisphosphate pool so that catalysis can quickly increase when light intensity increases [7]. This is particularly significant for short-term regulation of ribulose-1,5-bisphosphate carboxylase during transients because regulation via a modification of activation state is too slow to provide regulation over a timescale of 30 s to a few minutes [8].

The dual role of ribulose-1,5-bisphosphate carboxylase as a catalytic enzyme and a metabolite 'buffer' provides a rapid mechanism for modifying biochemically active pools of intermediates. We have shown here that the presence of ribulose-1,5-bisphosphate carboxylase can substantially alter the kinetic behaviour of photophosphorylation in vitro via a binding phenomenon dependent on the ribulose-1,5-bisphosphate binding site of the enzyme. Hence, the apparent K_m (P_i) and K_m (ADP) of photophosphorylation in vivo may be substantially modified by pools of other metabolites which change radically during changes in photosynthetic flux such as ribulose-1,5-bisphosphate and glycerate 3-phosphate, as these metabolites will displace adenylates and P_i from the carboxylase [27–29]. This may alternatively be viewed as a modification to 'effective' levels of metabolites. Metabolite 'buffering' and metabolite regulation of ribulose-1,5-bisphosphate carboxylase may also be important during photosynthetic induction, a situation where both enzyme activity and metabolite levels are rapidly changing. Many Calvin cycle intermediates interact with ribulose-1,5-bisphosphate carboxylase at the active site and stabilise the activator carbamate [26] maintaining the activation state of the enzyme at least in the short term. There is much less ribulose-1,5-bisphosphate in the stroma in the dark than in the light [30]. While glycerate 3-phosphate also decreases in the dark, the change is not marked and the level of stromal P_i increases [29]. Thus binding of these metabolites will maintain the activation state of ribulose-1,5-bisphosphate carboxylase in the dark but inhibit catalysis immediately after illumination, possibly explaining lack of dark inactivation of ribulose-1,5-bisphosphate carboxylase in some studies [30]. This would allow photosynthetic metabolites in the regenerative phase of the Cavlin cycle to accumulate and may be particularly important in species lacking the inhibitor, carboxyaabinitolphosphate [31]. In addition, a bound pool of glycerate 3-phosphate slowly released as ribulose-1,5-bisphosphate accumulates may be beneficial to photosynthesis, since the length of induction phase of photosynthesis following a transition from darkness to light has been shown to be influenced by the level of glycerate 3-phosphate in leaves in the dark [32].

Due to the technical difficulties involved, there

are few examples in the literature where biochemical measurements have been made on photosynthetic enzymes at physiological protein concentrations. However, the data presented here indicate that if plant biochemists are to extrapolate in vitro kinetic data to the in vivo case, the fact that the chloroplast stroma is almost a 'liquid crystal' of protein cannot be ignored.

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