Hypothesis

A role for ribulose-1,5-bisphosphate carboxylase as a metabolite buffer

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1. INTRODUCTION

Ribulose-1,5-bisphosphate carboxylase, the most abundant protein known, is localized within the stromal phase of the chloroplast. The concentration of Ru-1,5-P_2 carboxylase in this compartment has been estimated to be 3–4 mM (active site) or 250–300 mg/ml [1,2]. In fact the concentration of Ru-1,5-P_2 carboxylase in the stroma is similar to the concentration of the enzyme in some crystals of the purified protein (type I crystals contain 266 mg protein/ml [3]). The extraordinary abundance of this protein is largely due to the requirement for high rates of photosynthetic carbon assimilation and the low catalytic turnover rate of the enzyme (2.8 μmol . min^{-1} . mg^{-1} or 3 s^{-1}, [4]). Some enzymes are present in tissues at greater concentrations than their substrates [5,6]. This is also the case for Ru-1,5-P_2 carboxylase where [CO_2] is 11 μM while Ru-1,5-P_2 levels normally range from 0.2 – 4 mM depending upon experimental conditions [7]. Conditions such as these do not fulfill conditions normally assumed for enzymic catalysis where the substrate concentration is far in excess of the enzyme concentration. The consequences of these conditions upon the kinetics of Ru-1,5-P_2 carboxylase have been studied in detail [8]. Thus, although the K_m of Ru-1,5-P_2 carboxylase for Ru-1,5-P_2 is 20–30 μM the half-maximal rate of carboxylation can only be achieved when there is ≥ 1 Ru-1,5-P_2/2 active sites, i.e., $\sim$ 2 mM (or 100-times the K_m for Ru-1,5-P_2). The sheer abundance of Ru-1,5-P_2 carboxylase can also shed light on potential allosteric regulation of the enzyme. Some metabolites including Sed-1,7-P_2 and NADPH may regulate Ru-1,5-P_2 carboxylase activity in vivo [9]. However, the concentration of enzyme is significantly greater than the concentration of most of the proposed effectors [1,10]. Thus, if all the Fru-1,6-P_2 of the chloroplast ($\sim$0.4 mM) was bound to Ru-1,5-P_2 carboxylase most of the Ru-1,5-P_2 carboxylase molecules would still not be affected.

What was not been explicitly discussed is the corollaly to these observations, i.e., what effect will the presence of mM Ru-1,5-P_2 carboxylase have upon the metabolism of these effectors? Ru-1,5-P_2 carboxylase could potentially bind a large fraction of the total chloroplast Fru-1,6-P_2, Sed-1,7-P_2 or NADPH which would effectively reduce the free concentration of these metabolites by $\geq$10-fold. This metabolite buffering would have profound effects upon photosynthetic carbon metabolism and our interpretation of it since the redox potential of the NADPH/NADP+ couple can influence numerous reactions directly and indirectly while the 2 bisphosphatase reactions are important sites for regulation of the photosynthetic carbon reduction cycle.

2. Ru-1,5-P_2 CARBOXYLASE AS A METABOLITE BUFFER

Fructose-1,6,-bisphosphate which binds to Ru-1,5-P_2 carboxylase with K_d 40 μM [11] occurs in

Abbreviations: Ru-1,5-P_2, ribulose-1,5-bisphosphate; Fru-1,6-P_2, fructose-1,6-bisphosphate; Sed-1,7-P_2, sedoheptulose-1,7-bisphosphate

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illuminated chloroplasts at ~400 μM [12]. In the presence of 4 mM Ru-1,5-P_2 carboxylase active sites it can be calculated that 98.9% of this Fru-1,6-P_2 will be enzyme-bound or alternatively the free (unbound) concentration of Fru-1,6-P_2 will be 4.4 μM. Thus, as little as 1.1% of the total Fru-1,6-P_2 pool may be available to participate in other metabolism. Similar calculations indicate that 97.6% of stromal Sed-1,7-P_2 (K_d 85 μM [11]; 1 mM in stroma [12]) or 97.7% of the stromal NADPH (K_d 70 μM [11]; ~1 mM in stroma [13] would be bound to Ru-1,5-P_2 carboxylase. In [11] the 'allosteric effectors of Ru-1,5-P_2 carboxylase bound to the active site, competing with Ru-1,5-P_2 but also stabilizing the Mg^{2+}–CO_2 activated form of the enzyme. Thus the active site of the enzyme may be occupied by the substrate Ru-1,5-P_2 or any of the other effectors including Fru-1,6-P_2, Sed-1,7-P_2 and NADPH, so it is not possible to predict the effect of Ru-1,5-P_2 carboxylase upon the free concentration of individual metabolites without considering the simultaneous interactions of other metabolites. Table 1 lists the distribution of some metabolites between bound and free states in a simulated chloroplast stromal environment. Here, 95% of the Ru-1,5-P_2 carboxylase subunits have ligand bound to them. In this case the free concentration of Fru-1,6,P_2, NADPH and Sed-1,7-P_2 are 4–6-fold lower than the total metabolite concentration. Appreciable amounts of 3-phosphoglycerate and P_i are bound to Ru-1,5-P_2 carboxylase but because of their relatively large total pool sizes the free pool is still 80% of the total metabolite pool. In the presence of Ru-1,5-P_2 the number of sites available to the other metabolites will be diminished. Because the K_d of Ru-1,5-P_2 carboxylase for Ru-1,5-P_2 is 0.6 μM [14] or less (the K_m Ru-1,5-P_2 is 20–30 μM, however) Ru-1,5-P_2 can effectively displace all the other metabolites from the carboxylase active site. [Ru-1,5-P_2] in chloroplasts ranges from 0.26 mM [12] to ≥4 mM [7]. The effect of different concentrations of Ru-1,5-P_2 upon the binding of some effectors is shown in fig.1. Increasing the concentration of Ru-1,5-P_2 progressively increases the free concentration of the other ligands such that when Ru-1,5-P_2 is equimolar with Ru-1,5-P_2 carboxylase the free pool of the other metabolites is ≥ 90% of the total metabolite pool.

Another view of metabolite buffering by Ru-1,5-P_2 carboxylase is provided in fig.2. Here, the free concentration of a metabolite is plotted against the total concentration of that metabolite in the presence of 0.26 mM or 2.5 mM Ru-1,5-P_2. At the low concentration of Ru-1,5-P_2 the Fru-1,6-P_2 concentration is strongly buffered such that for every 5 molecules of Fru-1,6-P_2 added to the system the number of free Fru-1,6-P_2 molecules increases by only 1. At high [Ru-1,5-P_2] the metabolite buffer capacity of Ru-1,5-P_2 carboxylase for Fru-1,6-P_2 is diminished such that 3 molecules of the 5 added remain in free solution. Buffering of other metabolites also occurs and depends upon the K_d: the higher the K_d the less metabolite buffering occurs.

Table 1

	K_d (mM)	[Metabolite] (mM) Total	Bound	Free	% Free
Fru-1,6-P_2	0.040	0.4	0.328	0.072	18.0
NADPH	0.070	1.0	0.721	0.279	27.9
Sed-1,7-P_2	0.075	1.0	0.707	0.293	29.3
3-Phospho-glycerate	0.84	3.35	0.594	2.76	82.4
P_i	0.90	5.8	0.971	4.83	83.3

Calculated distribution of metabolites of chloroplast stroma containing 3.50 mM Ru-1,5-P_2 carboxylase subunits. The K_d-values are those listed in [11] and the metabolite concentrations are those determined in [12,13]. It is assumed that [Ru-1,5-P_2] is zero. The basis of this calculation and those of fig.1,2 is that all bound ligands are in equilibrium with the same concentration of free Ru-1,5-P_2 carboxylase via the equation:

$$K_d = \frac{[\text{free ligand}] \cdot [\text{free Ru-1,5-P}_2\text{ carboxylase}]}{[\text{RU-1,5-P}_2\text{ carboxylase–ligand complex}]}$$

The other constraints satisfied are:
(i) That each active site can bind only one ligand at a time;
(ii) Conservation of ligand; and
(iii) Conservation of Ru-1,5-P_2 carboxylase.
The concentration of free Ru-1,5-P_2 carboxylase is adjusted until the conservation expressions are satisfied. The concentration of free Ru-1,5-P_2 carboxylase under these conditions is 0.18 mM

3. EVIDENCE FOR METABOLITE SEQUESTRATION

These calculations assume that the binding properties of Ru-1,5-P_2 carboxylase in vivo are the same as those determined in vitro. Because of the transient nature of the binding of ligands to Ru-1,5-P_2 carboxylase it will be difficult to show directly that the proposed sequestration does in fact occur. However, 2 independent lines of evidence support the proposal that Ru-1,5-P_2 carboxylase can act as a metabolite buffer.

One line of evidence suggests that ligands do bind to Ru-1,5-P_2 carboxylase in vivo. Thus, conditions found in the stroma (pH 8, 10 μM CO_2 and 5–10 mM Mg^{2+}) are not sufficient alone to keep Ru-1,5-P_2 carboxylase activated in vitro [11]. However, assays of freshly extracted ribulose-1,5-P_2 carboxylase show that the enzyme is largely activated in vivo. In [11] effectors such as NADPH were proposed to bind to the enzyme in vivo thereby stabilizing the activated form. In all the simulations presented here, one sort of ligand is bound to $\geq$ 95% of all Ru-1,5-P_2 carboxylase active sites.

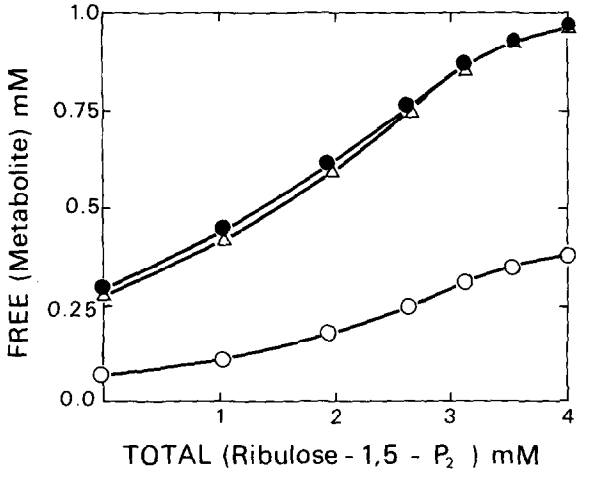

Fig.1. Change in free metabolite level with increasing ribulose bisphosphate. The K_d-values and metabolite concentrations are the same as those in table 1 while [Ru-1,5-P_2] is varied. Ru-1,5-P_2 is assumed to bind to Ru-1,5-P_2 carboxylase with a K_d of 0.6 μM [14]. [Free Ru-1,5-P_2 carboxylase] is 181, 100, 50, 25, 12.5, 6.2 and 3.1 μM at [Ru-1,5-P_2] = 0, 1.0, 1.9, 2.6, 3.1, 3.5 and 4.0 mM, respectively: (○), Fru-1,6-P_2; (●) Sed-1,7,-P_2; (△) NADPH.

The other line of evidence suggests that much of the stromal Fru-1,6-P_2 is not in equilibrium with the triose phosphate pool and may be sequestered. In the photosynthetic carbon reduction cycle the aldolase reaction proceeds in the direction:

Dihydroxyacetone phosphate + Glyceraldehyde-3-phosphate → Fructose-1,6-P_2

The apparent equilibrium constant for this reaction at 5 mM Mg^{2+} is 6210 M^{-1} [15]. If there is sufficient aldolase to catalyze a near equilibrium reaction then fructose-1,6-P_2 will approach the concentration predicted by the equilibrium constant however, since fructose-1,6-P_2 is the product of the reaction, the free fructose-1,6-P_2 concentration cannot exceed the predicted equilibrium concentration. Stromal [triose phosphates] (dihydroxyacetone phosphate + glyceraldehyde-3-phosphate) of 0.34 mM was reported in [12]. If the triose phosphate pool is at equilibrium, i.e., [dihydroxyacetone phosphate]/[glyceraldehyde-3-phosphate] = 22.2, [16], then the calculated [Fru-1,6-P_2] in equilibrium with this pool via the aldolase reaction is 0.03 mM. The measured [Fru-1,6-P_2] was 0.39 mM; i.e., an excess of 0.36 mM. Although the chloroplast contains considerable triose phosphate isomerase it may be that the triose

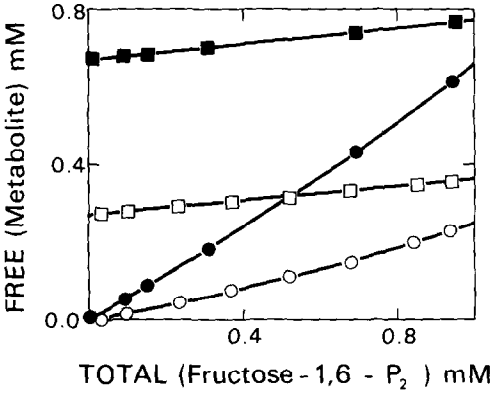

Fig.2. Change in free metabolite concentration with increasing concentration of fructose-1,6-bisphosphate. The simulated stroma was as in table 1 and fig.1 but Fru-1,6-P_2 was varied in the presence of 0.26 mM (○) or 2.5 (●) Ru-1,5-P_2. The rise in the free concentration of NADPH as a consequence of the increasing Fru-1,6-P_2 level is also shown in the system containing of 0.26 mM (□) or 2.5 mM (■) Ru-1,5-P_2.

phosphate pool is not in equilibrium. The least possible Fru-1,6-P_2 excess occurs when [dihydroxyacetone phosphate] = [glyceraldehyde-3-phosphate] = 0.17 mM. Even in this case the calculated Fru-1,6-P_2 at equilibrium is 0.206 mM, still leaving a 0.18 mM excess. This excess could be explained if the extra Fru-1,6-P_2 was bound and not directly in equilibrium with the triose phosphate pool. Fig.1. indicates that in the stromal environment in [12], i.e., 0.26 mM Ru-1,5-P_2, the bound Fru-1,6-P_2 is 0.31 mM which agrees well with the above estimates of excess Fru-1,6-P_2. It is possible that there is also Sed-1,7-P_2 in excess of that predicted for the aldolase reaction because estimates of [erythrose-4-phosphate] are quite low [16]. A similar analysis of chloroplast metabolite levels in spinach protoplasts [17] also reveals an apparent excess of Fru-1,6-P_2.

Direct estimates of the redox potential of the NADPH/$NADP^+$ couple are difficult to make. The glyceraldehyde-3-phosphate dehydrogenase reaction is closely coupled to the 3-phosphoglycerate kinase reaction and, during conditions of low photosynthetic carbon assimilation, may catalyze a near equilibrium reaction. Using total levels of metabolites [18] it was reported that this complex reaction sequence is removed from equilibrium by a factor of 7. If most of the chloroplast NADPH was bound, this reaction would be closer to equilibrium. The observation [13] that the NADPH/NADP ratio attained in an illuminated reconstituted chloroplast system was less than that of whole chloroplasts (1.1 compared to 2.3–2.5) could be explained by assuming that 0.68 mM NADPH (17 nmol NADPH/mg chl) was bound in whole chloroplasts but not in the reconstituted system. This excess NADPH is comparable to that calculated in table 1 and fig.1 to be bound to Ru-1,5-P_2 carboxylase.

4. CONSEQUENCES OF METABOLITE BUFFERING

This sequestration of metabolites by Ru-1,5-P_2 carboxylase must cause a revision of our understanding of the free metabolite levels that occur during photosynthesis. Estimates of free energy changes of the reactions of the photosynthetic carbon reduction cycle [16] are also compromised by this phenomenon.

Ribulose-1,5-P_2 carboxylase acting as a metabolite buffer, may have a significant role in stabilizing steady state photosynthesis. As shown in fig.2 changes in free metabolite levels cannot occur as readily as they would in the absence of a metabolite buffer. In the case of Fru-1,6-P_2, to achieve a 1% change in the free concentration, the total Fru-1,6-P_2 concentration must be changed by 5%. The effect of this buffer capacity will be to maintain steady-state metabolite levels and fluxes during transient perturbations to photosynthesis especially considering that metabolite pools in the photosynthetic carbon reduction cycle turn over with $t_{1/2} \leq 2$ s [17].

A question that remains however is what effect, if any, will this metabolite buffering have upon flux through the photosynthetic carbon reduction cycle? Ottaway [19] in simulations of glycolysis and the citric acid cycle has shown that the mere binding of a large proportion of a metabolite to an enzyme (or binding protein) need not influence the flux through a pathway provided that the metabolite can be replenished from an 'infinite reservoir of primary nutrient'. The pathway would be perturbed only as long as would be required to fill the bound pool.

In the case of chloroplasts the supply of carbon from the atmosphere is effectively infinite but the total phosphate content (inorganic plus esterified) of the photosynthetic carbon reduction cycle metabolites is constant – triose phosphate, the product of photosynthesis exported from the chloroplast is strictly exchanged for P_i by the phosphate translocator of the chloroplast envelope [12]. The data in [12] show that the chloroplast contains ~ 17 mM photosynthetically active phosphate (i.e., P_i, sugar monophosphates, ATP +2 phosphates/fructose and sedoheptulose bisphosphate molecule) which must be distributed between 14 metabolites of the photosynthetic carbon reduction cycle. Although well in excess of Ru-1,5-P_2 carboxylase sites, this amount of phosphate is clearly not infinite. It is possible that depletion of this phosphate pool by metabolite binding to Ru-1,5-P_2 carboxylase may influence flux through the pathway. Higher rates of photosynthesis may be attainable when fewer phosphate containing metabolites are bound to the enzyme. These speculations will require a more rigorous analysis than is possible here, to determine if the effect of phosphate depletion on

steady-state photosynthesis is trivial or not.

During short-term metabolism the size of the NADP + NADPH pool is constant and bound NADPH cannot be readily replaced like the other metabolites. While the free NADPH/NADP ratio could readjust to maintain the same redox potential, the free concentrations of both of these nucleotides must change in response to the variation in buffer capacity of Ru-1,5-P_2 carboxylase.

5. METABOLITE BUFFERING AND PHOTOSYNTHETIC INDUCTION

When plants or intact chloroplasts are illuminated abruptly, maximum CO_2 fixation does not occur until some minutes later in a phenomenon known as induction [20]. Several explanations of this behaviour have been offered including light activation of photosynthetic enzymes or, alternatively, the requirement for the build up of intermediates of the photosynthetic carbon reduction cycle to a critical level. Light activation of photosynthetic enzymes precedes the rising rate of CO_2 fixation therefore it seems unlikely that the induction phenomenon directly reflects light activation [21] but see [17]. On the other hand, measurements of total metabolite levels do not show the dramatic changes during the induction period that would be expected if rising total metabolite levels 'triggered' photosynthetic carbon assimilation [17]. However, as discussed above and illustrated in fig.1 the free metabolite concentration could change markedly without any change in total metabolite pools.

After illumination of a plant the following sequence of events may occur. Upon illumination enzymes including ribulose-5-phosphate kinase, Fru-1,6-P_2ase and Sed-1,7,-P_2ase are activated [22]. The Ru-1,5-P_2 generated by the now active ribulose-5-phosphate kinase will displace the sequestered Fru-1,6-P_2 and Sed-1,7,-P_2, and thereby enhance the substrate available to the 2 potentially rate-limiting phosphatases. These changes will be amplified by increases in the total metabolite pool which may exceed the binding capacity of Ru-1,5-P_2 carboxylase. The rising level of esterified phosphate lowers stromal levels of inorganic phosphate which inhibits some photosynthetic enzymes [23]. The overall effect would be to initiate a positive feedback control on CO_2 fixation until the 'metabolite buffer' capacity of Ru-1,5-P_2 carboxylase is overcome by the raised concentration of Ru-1,5-P_2 or other limitations on the rate of CO_2 fixation are reached. Ru-1,5-P_2 levels attained in illuminated intact chloroplasts are usually quite low (0.26 – 0.6 mM) whereas Ru-1,5-P_2 levels in spinach leaves may be equilomar with Ru-1,5-P_2 carboxylase [7]. Thus in intact leaves the metabolite buffer capacity of Ru-1,5-P_2 carboxylase may be largely abolished after illumination while isolated chloroplasts fix CO_2 at high rates in the presence of substantial metabolite buffer capacity.

6. OTHER POTENTIAL INFLUENCES ON METABOLITE BUFFERING

Many properties of Ru-1,5-P_2 carboxylase which might have significance for this metabolite buffering role are not yet known. Other metabolites may also bind to Ru-1,5-P_2 carboxylase and thereby compete with those metabolites discussed above. Thus 6-phosphogluconate binds tightly to Ru-1,5-P_2 carboxylase (K_d 8.5 μM [11]) but has not been considered in this analysis since its concentrations in conditions similar to those in [12] is not known. In *Chlorella* in the dark, it was estimated at 59 μM and presumably less in the light due to the light-dependent inhibition of glucose-6-phosphate dehydrogenase [16]. While binding of 6-phosphogluconate may significantly lower free 6-phosphogluconate concentrations such interactions are unlikely to seriously influence the interaction of the other metabolites discussed above. Other metabolites which also bind to Ru-1,5-P_2 carboxylase include ribose-5-phosphate and fructose-6-phosphate [9,24,25] but the K_d-values for these effectors are not known. Ru-1,5-P_2 carboxylase can exist in an inactive as well as a Mg^{2+}–CO_2-containing activated form. These forms may have different affinities for the same ligand. In fact effectors such as Fru-1,6-P_2, Sed-1,7,-P_2 and NADPH were proposed to activate Ru-1,5-P_2 carboxylase precisely because they bind more tightly to the active form of the enzyme, thereby pulling the inactive $\rightleftharpoons$ active equilibrium more towards the active form [11]. Negative effectors [24] such as ribose-5-phosphate and fructose-6-phosphate may also bind more tightly to the inactive form of Ru-1,5-P_2 carboxylase [11]. Since the equilibrium between active and inactive Ru-1,5-P_2 carboxylase is dependent upon pH, [Mg^{2+}] and [CO_2] [26] it is clear that the

'metabolite buffer capacity' of Ru-1,5-P_2 carboxylase will also be influenced by these variables.

An approximation used in discussion is to treat Ru-1,5-P_2 carboxylase solely as a binding protein ignoring any effects that its catalytic activity may have on this function. Since it is likely that Ru-1,5-P_2 binds first in the reaction cycle [27] this assumption seems reasonable. It is also assumed that all effectors bind to the same site, i.e., the Ru-1,5-P_2 binding site in the active centre. The presence of other sites will have significant consequence for potential free metabolite depletion in the chloroplast [14]. Another group of ligands [28] which bind to pea and bean Ru-1,5-P_2 carboxylase are the auxins. This high-affinity site (K_d 0.8 μM for indole-3-acetic acid) is, apparently, distinct from the Ru-1,5-P_2 binding site [29]. Ru-1,5-P_2 carboxylase may play a role in sequestering auxins [28]. This proposal finds support in [30] where intact chloroplasts accumulated more auxin than could be explained by the passive distribution of a weak acid.

7. CONCLUSIONS

Because of the high concentration of Ru-1,5-P_2 carboxylase in the chloroplast stroma the concentration in free solution of any metabolite which can bind to the enzyme will be lower than the total stromal concentration of that metabolite and moreover, changes in the total level of any such metabolite will alter the free concentrations of all the other ligands of Ru-1,5-P_2 carboxylase that can bind to the same site. The sequestration of metabolites by Ru-1,5-P_2 carboxylase may limit carbon assimilation under some circumstances but will also tend to stabilize flux through the photosynthetic carbon reduction cycle against transient perturbations to photosynthesis. The metabolite buffer capacity of Ru-1,5-P_2 carboxylase for particular metabolites may also be altered by changes in pH, and Mg^{2+} or CO_2 concentration and thus subject to regulation between conditions of illumination and darkness.

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