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Pettersson, Gösta; Ryde-Pettersson, Ulf

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On the regulatory significance of inhibitors acting on non-equilibrium enzymes in the Calvin photosynthesis cycle

Gösta PETTERSSON and Ulf RYDE-PETTERSSON

Avdelningen för Biokemi, Kemicentrum, Lunds Universitet

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Control analyses and kinetic model studies have been performed in order to obtain quantitative information on the regulatory significance of 12 experimentally well-documented inhibitory interactions of Calvin cycle intermediates with the four non-equilibrium cycle enzymes. Evidence is presented to show that none of these interactions contributes significantly to the cycle flux control over the range of external orthophosphate concentrations where the reaction cycle shows close to optimal activity. Contrary to what has been generally supposed, the examined inhibitions appear to be of little interest for our understanding of the biological regulation of the Calvin photosynthesis cycle under conditions of light and carbon dioxide saturation.

The reductive pentose phosphate pathway (the Calvin cycle) for photosynthetic carbohydrate formation in the chloroplast of C_3 plants involves 13 enzymes acting on 16 metabolites in a complex network of reactions. Four of the Calvin cycle enzymes catalyse reaction steps that are much displaced from equilibrium during the steady-state operation of the cycle. These non-equilibrium enzymes (fructose bisphosphatase, sedoheptulose bisphosphatase, ribulose-5-phosphate kinase, and ribulosebisphosphate carboxylase) are considered to be of particular regulatory interest [1], and their kinetic properties have been examined in considerable detail. Such studies have revealed that the non-equilibrium cycle enzymes are subjected to product inhibition as well as to inhibition by Calvin cycle intermediates distinct from the products of the respective enzyme. Several authors have proposed that these inhibitory interactions may be of importance for regulation of the Calvin cycle activity according to the principles of feed-back or feed-forward control [2–8], but no evidence has been presented to show that such is actually the case.

Particular attention has been paid to the regulatory role played by inorganic (ortho)phosphate, which acts as an inhibitor of all of the above non-equilibrium enzymes. Experiments performed with isolated chloroplasts have established that the Calvin cycle activity is strongly dependent on the external concentration of orthophosphate [9, 10], and experimental [9] as well as theoretical [11] data are available which indicate that changes of the external orthophosphate concentration cause corresponding and amplified changes of the internal (i.e. stromal) level of the metabolite. Consequently, there is strong reason to believe that the stromal orthophosphate concentration represents a crucial control variable which may contribute most significantly to the observed effects of external orthophosphate on the cycle activity [7–11]. The detailed mechanism for such regulation of the cycle activity has remained obscure [12, 13], however, and it is not known to what extent the regulation actually reflects the inhibitory interaction of orthophosphate with different cycle enzymes.

We have recently put forward a kinetic model for the operation of the Calvin photosynthesis cycle and ancillary pathway of starch production under conditions of light and carbon dioxide saturation [11]. This model has now been subjected to control analysis in order to obtain detailed information on the regulatory significance of the interaction of orthophosphate and other inhibitory Calvin cycle metabolites with the four non-equilibrium cycle enzymes. The results provide clear evidence that the fundamental dynamic behaviour of the Calvin cycle is determined by the stoichiometric structure of the reaction system rather than by control mechanisms based on product or feed-back/feed-forward inhibitions.

MATERIALS AND METHODS

We have previously described in detail a kinetic model for photosynthetic carbohydrate formation in the chloroplast of C_3 plants [11]. This model considers the 13 enzymically catalysed steps of the reductive pentose phosphate pathway (the Calvin cycle) and treats ATP synthesis as a system-dependent input step. Starch production within the chloroplast and photosynthate export to the external reaction medium are included as output processes. The model defines the time dependence of the concentration of 18 stromal metabolites (13 phosphorylated carbohydrates representing Calvin cycle intermediates, glucose 6-phosphate, glucose 1-phosphate, orthophosphate, ATP, and ADP) as a function of various parameters, including inhibition constants (K_i) for the interaction of different inhibitors with the four non-equilibrium cycle enzymes. For given values of these parameters, steady-state concentrations of all concentration variables and the corresponding rate of photosynthetic carbon dioxide fixation (v) become determined by the model, which can be applied also for numerical calculation [11] of flux control coefficients **C** defined [14] by

Correspondence to G. Pettersson, Avdelningen för Biokemi, Kemicentrum, Lunds Universitet, Box 124, S-221 00 Lund, Sweden

Enzymes. Fructose bisphosphatase (EC 3.1.3.11); sedoheptulose bisphosphatase (EC 3.1.3.37); ribulose-5-phosphate kinase (EC 2.7.1.19); ribulosebisphosphate carboxylase (EC 4.1.1.39).

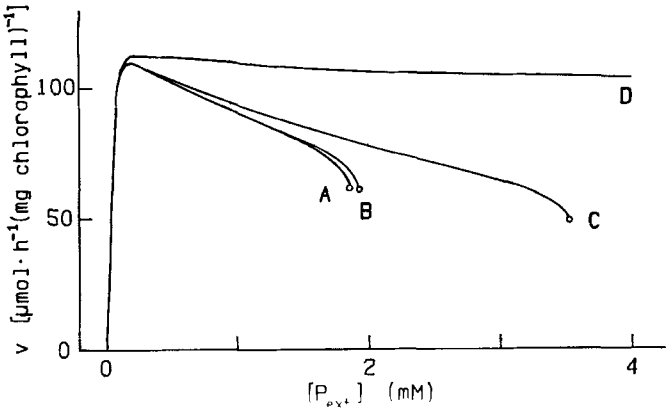

Fig. 1. *Load characteristics for the Calvin cycle activity.* Dependence on the concentration of external orthophosphate (P_{ext}) of Calvin cycle velocities (v) predicted by our kinetic model [11] as originally defined (A), and by modified models that do not include any inhibitions of (B) ribulosephosphate carboxylase, (C) fructose bisphosphatase by orthophosphate, and (D) anyone of the four non-equilibrium cycle enzymes. Circles at the right-hand side end of curves A – C indicate the bifurcation point obtaining for the maximum value of $[P_{ext}]$ than can support a steady-state mode of operation of the Calvin cycle

$$\mathbf{C}^{v}_{K_i} = \frac{\partial \ln v}{\partial \ln K_i} . \qquad (1)$$

Such control coefficients provide dimensionless measures of the change in cycle activity caused by variation of the magnitude of a certain inhibition constant K_i. Since an increased magnitude of the inhibition constant is formally equivalent to a decreased level of the corresponding inhibitor, control coefficients defined by Eqn (1) will provide direct measures of the regulatory significance of the respective inhibitory interactions with regard to the Calvin cycle reaction flux.

Unless otherwise stated, all data reported below were calculated by application of the above kinetic model using our previously detailed estimates of kinetic constants and other parameters of the model [11]. These parameter estimates have been selected to be representative for the operation of isolated chloroplasts in a reaction medium containing orthophosphate at pH 7.6 (assumed to correspond to a stromal pH of 7.9 [15]) under conditions of light and carbon dioxide saturation.

RESULTS

Orthophosphate load characteristic of the Calvin cycle

Curve A in Fig. 1 shows the dependence of the steady-state Calvin cycle activity on the concentration of external orthophosphate (P_{ext}), as predicted by our kinetic model for photosynthetic carbohydrate formation in chloroplasts. This theoretical load characteristic for the reaction system has been previously shown to provide a most satisfactory description of that observed experimentally [11]. Attention should be drawn in the present context to the model prediction that there is a critical value of $[P_{ext}]$ (about 2 mM) above which the reaction system cannot operate in a steady state. This critical value corresponds mathematically to a bifurcation point [16], and for external orthophosphate concentrations above the critical value the system will exhibit overload breakdown [17]; an excessive rate of photosynthate export will arise such that the chloroplast becomes deprived of Calvin cycle intermediates and the cycle activity approaches zero without reaching a steady state [11].

Flux control coefficients

Table 1 lists all experimentally well-documented inhibitory interactions of Calvin cycle metabolites with the four non-equilibrium cycle enzymes. Effects of these interactions are considered in our kinetic model according to rate equations that have been detailed previously [11]; notations of inhibition constants in Table 1 agree with those used previously. Two inhibition constants (K_{i134} and K_{i135}) are required for description of the interaction of ribulosephosphate kinase with ADP, which acts as a mixed-type inhibitor [6]. Other inhibitions listed in Table 1 are assumed to be kinetically competitive with the carbohydrate substrate for the respective enzyme [6–8, 18] and can be characterized by a single inhibition constant.

Calvin cycle flux control coefficients $\mathbf{C}^{v}_{K_i}$ for the respective inhibitory interactions were calculated as described in Materials and Methods. Results obtained for representative values of the external concentration of orthophosphate are given in Table 1. They establish that flux control coefficients for all of the inhibitors are of insignificant magnitude (much less than unity) at external orthophosphate concentrations up to 0.5 mM, i.e. over the concentration range were the Calvin cycle shows optimal activity (Fig. 1).

When the external orthophosphate concentration is raised above 1.5 mM, one approaches the critical value (1.87 mM) representing the upper limit of the concentration range over which steady-state conditions may obtain (Fig. 1). Appreciable flux control is then gained by fructose 6-phosphate and orthophosphate in their action on fructose bisphosphatase, and by orthophosphate in its action on sedoheptulose bisphosphatase and ribulose-5-phosphate kinase (Table 1).

Effect of inhibitors on the orthophosphate load characteristic

Results in Table 1 provide clear evidence that none of the ribulosebisphosphate carboxylase inhibitors considered contributes significantly to the control of the Calvin cycle activity. This means that the dynamic behaviour of the reaction system should be essentially the same whether or not the inhibitory interactions are at hand. To test this prediction, the kinetic model was modified such that all kinetic inhibition terms were deleted from the rate equation characterizing the velocity of the ribulosebisphosphate carboxylase step [11]. As indicated by curve B in Fig. 1, the load characteristic calculated for the modified model was found to be practically indistinguishable from that predicted by the original model, except in the neighbourhood of the bifurcation point; the assumed absence of inhibitory interactions raises the critical value of $[P_{ext}]$ from 1.87 mM to 1.94 mM, with consequent minor effects on the load characteristic.

The kinetic significance of other inhibitory interactions considered in Table 1 was similarly examined. Curve C in Fig. 1 shows the load characteristic obtained in the assumed absence of any orthophosphate inhibition of fructose bisphosphate ($K_{i62} = \infty$). Curves A and C differ mainly at high concentrations of external orthophosphate (as might be expected from data in Table 1), and that difference can be attributed to an increased stability of the reaction system in the absence of the inhibitory interaction; the value of $[P_{ext}]$ at the bifurcation point is raised to about 3.5 mM following elimination of the K_{i62} term. Similar effects were caused by elimination of the kinetic terms accounting for the inhibition of fructosebisphosphatase by fructose 6-phosphate or for the inhibition of ribulosephosphate kinase and ribulosebisphos-

Table 1. *Inhibition of non-equilibrium Calvin cycle enzymes*
Extent of inhibition of the non-equilibrium enzymes at different concentrations of external orthophosphate (P_{ext}), as indicated by the quotient between metabolite concentrations obtaining according to the kinetic model and the respective inhibition parameter K_i. Control coefficients $C^v_{K_i}$ calculated for the respective inhibitory interaction are shown within parentheses insofar as they exceed 0.02

Enzyme	Inhibitor (I)	Parameter	$[I]/K_i$ at $[P_{ext}]$ =				
			0.05 mM	0.2 mM	0.5 mM	1.5 mM	1.8 mM
Ribulosebisphosphate carboxylase	phosphoglycerate	K_{i11}	2.0	2.0	0.7	0.2	0.1
	fructose bisphosphate	K_{i12}	0.3	0.8	0.6	0.3	0.3
	sedoheptulose bisphosphate	K_{i13}	0.2	2.6	1.8	0.6	0.4
	orthophosphate	K_{i14}	0.9	5.6	9.0	12.5	13.3 (0.05)
	NADPH	K_{i15}	3.0	3.0	3.0	3.0	3.0
Fructose bisphosphatase	fructose 6-phosphate	K_{i61}	1.9	2.7	1.9	1.0 (0.14)	0.7 (0.43)
	orthophosphate	K_{i62}	0.0	0.4	0.7	0.9 (0.14)	1.0 (0.58)
Sedoheptulose bisphosphatase	orthophosphate	K_{i9}	0.0	0.4	0.7	0.9 (0.16)	1.0 (0.46)
Ribulosephosphate kinase	phosphoglycerate	K_{i131}	0.9	0.8	0.3	0.1	0.0
	ribulose bisphosphate	K_{i132}	0.0	0.2	0.2	0.2	0.1 (0.03)
	orthophosphate	K_{i133}	0.1	1.3 (0.04)	2.0	2.8 (0.24)	3.0 (0.70)
	ADP	K_{i134}	0.2	0.1	0.0	0.0	0.0
	ADP	K_{i135}	1.2 (−0.03)	0.6	0.3	0.1	0.0

Table 2. *Calculated metabolic levels in the absence and presence of phosphoglycerate inhibition of ribulosephosphate kinase*
Stromal reactant concentrations at an external orthophosphate concentration of 0.2 mM, as predicted by our original kinetic model [11] and by a modified model that does not include any phosphoglycerate inhibition of the kinase

Metabolite	Original model	Modified model
	mM	
Ribulose 5-phosphate	0.031	0.025
ATP	0.244	0.230
3-Phosphoglycerate	1.65	1.90
Ribulose 1,5-bisphosphate	0.142	0.152
Orthophosphate	5.06	5.31
ADP	0.256	0.270

phate carboxylase by orthophosphate. Effects on the load characteristic caused by elimination of the terms reflecting phosphoglycerate, ribulosebisphosphate, or ADP inhibition of ribulosephosphate kinase, however, were similar to or less pronounced than those indicated by curve B.

Curve D in Fig. 1 shows the load characteristic obtained when all inhibitory interactions in Table 1 are assumed to be absent. Under such conditions, the reaction system gains unrestricted stability and cannot be brought to overload breakdown by increasing the concentration of external orthophosphate.

Effect of phosphoglycerate on stromal metabolite levels

Photosynthetic carbon dioxide fixation in isolated chloroplasts occurs at an optimal rate when the external orthophosphate concentration is about 0.2 mM [9, 10]. Table 2 shows the steady-state concentrations predicted by our original kinetic model under such conditions for metabolites known (and assumed in the model) to affect the activity of ribulosephosphate kinase. These data are consistent with other evidence [6] indicating that concentrations of effectors such as phosphoglycerate are high enough to cause extensive inhibition of ribulosephosphate kinase during the steady-state operation of the Calvin photosynthesis cycle. With the hypothetical assumption that steady-state levels of all metabolites in Table 2 remain unchanged, elimination of the inhibitory effect of phosphoglycerate would enhance the rate of the ribulosephosphate kinase step by 30% according to reported kinetic data for the enzyme [6, 11].

The calculated steady-state metabolite concentrations actually obtaining following elimination of the phosphoglycerate inhibition ($K_{i131} = \infty$) are included in Table 2. The assumed absence of phosphoglycerate inhibition leads to increased levels of the remaining inhibitors and to decreased levels of the two substrates, such that the net increase of the steady-state rate of the ribulosephosphate kinase step (and of the cycle activity) will be less than 1%. This explains why phosphoglycerate inhibition of the enzyme, even though quite extensive, does not contribute significantly to the control of the Calvin cycle flux.

DISCUSSION

Inhibitors exerting insignificant flux control

Observations that individual enzymes in a metabolic pathway are inhibited by certain metabolites usually lead to the conclusion that the observed interactions may be of regulatory significance. Such inferences provide information on what control factors may have to be analytically considered, but otherwise do not contribute to our understanding of the regulation of intermediary metabolism. We know à priori that all interactions affecting reaction steps in a metabolic pathway are of potential regulatory interest, and the actual control problem one faces is to estimate the quantitative significance of the interactions in specific regulatory contexts. The present investigation addresses that problem with regard to the Calvin cycle flux control exerted by stromal metabolites acting as inhibitors of the four non-equilibrium cycle enzymes.

The view has been expressed that the competitive inhibition of ribulosebisphosphate carboxylation by Calvin cycle

intermediates such as phosphoglycerate could have a dramatic influence on net reaction flux in the cycle under steady-state conditions [2], and such interactions have been proposed to be of importance for modulation of the cycle activity when the activation state of the carboxylase remains constant [3]. The present results lend no support to these ideas, but corroborate the conclusion drawn in previous studies [19] of a kinetic model accounting also for the stoichiometric effects of inhibitor binding to ribulosebisphosphate carboxylase: inhibitors acting on the latter enzyme exert no significant flux control under conditions of light and carbon dioxide saturation, i.e. in the fully activated reaction system. This is quantitatively documented by the control measures in Table 1, and convincingly illustrated by the model data in Fig. 1 which show that cycle activities remain essentially the same whether or not the inhibitory interactions are assumed to be present.

Similar inferences can be drawn with regard to the proposals [4–6] that feed-back inhibition of ribulosephosphate kinase by its primary products ADP and ribulosebisphosphate, or by its secondary product phosphoglycerate, may be of importance for regulation of the light activity of the Calvin cycle. Results now presented (cf. Table 1) provide clear evidence that none of these interactions contributes significantly to the flux control of the examined reaction system. The cycle activity remains essentially unaffected in the hypothetical absence of the inhibitory interactions.

The latter observation might seem difficult to reconcile with the report of Gardemann et al. [6] that ribulosephosphate kinase should be extensively inhibited by phosphoglycerate at the concentrations of the metabolite that have been measured in the stromal solution of photosynthesizing chloroplasts; it would seem reasonable to believe that elimination of such inhibition should have a profound effect on the cycle activity. As indicated by data in Table 2, however, elimination of the inhibitory interaction of phosphoglycerate with ribulosephosphate kinase will lead to readjustment of the steady-state levels of other metabolites in a direction which opposes the relief of phosphoglycerate inhibition. This response of the reaction system is so strong that the cycle activity will remain essentially the same in the absence and in the presence of phosphoglycerate inhibition. Results in Table 1 and 2 illustrate an important, but not yet generally recognized, fact which represents a major complication in attempts to analyse the control situation in metabolic networks by intuitive reasoning: observations that physiological concentrations of an effector cause a very significant modification of the activity of an individual enzyme are not sufficient to establish that the interaction is of regulatory significance with regard to the control of reaction fluxes.

Flux control associated with orthophosphate inhibition

The competitive inhibition of fructose bisphosphatase and sedoheptulose bisphosphatase by orthophosphate has been supposed to be of considerable importance for regulation of the Calvin cycle activity and for the partitioning of reaction flux between starch production and photosynthate export to the cytosol [7, 8]. The present results confirm that such inhibition under certain conditions may contribute significantly to the cycle flux control (Table 1) and indicate that similar flux control contributions are provided by the inhibition of ribulosephosphate kinase by orthophosphate and the inhibition of fructose bisphosphatase by fructose 6-phosphate.

Two main circumstances, however, argue against the possibility that these interactions may be of regulatory interest from a physiological point of view. Firstly, none of the interactions is of regulatory significance over the range of external orthophosphate concentrations (0.1–0.5 mM) where the Calvin cycle shows optimal activity and where starch production occurs at an appreciable rate [9–11]. The inhibitors gain strong flux control only in the concentration region where the rate of starch production is close to zero and where the reaction system is close to overload breakdown. This is a region characterized by exceptionally high control strengths of most of the non-equilibrium enzymes [11], such that the system will respond strongly to any change in reaction conditions. It would seem reasonable to assume that the Calvin cycle under physiological conditions operates far from such an instability region where the reaction system could be readily brought to overload breakdown by external perturbations.

Secondly, load characteristics in Fig. 1 indicate that all of the inhibitory interactions now considered are operationally disadvantageous; the inhibitions lead to decreased rates of carbon dioxide fixation, as well as to a decreased stability of the reaction system such that it becomes more susceptible to overload breakdown. It is difficult to envisage any situation where such disadvantageous effects could be physiologically advantageous and lead to an evolutionary design of control mechanisms based on the inhibitions. The presence of the inhibitory interactions more probably reflects the fact that the inhibitors are structurally related to the substrates for the inhibited enzymes and hence may combine to sites that have been evolutionary designed for the binding of substrates rather than effectors.

This raises the question why evolution has not led to elimination of the disadvantageous inhibitory interactions. One possible answer is that it may have been more easy to increase the amount and/or catalytic efficiency of the enzymes than to increase their binding specificity. Alternatively, the interactions may actually have been eliminated as far as evolution can go, i.e. to such an extent that their disadvantageous effects no longer are of dynamic significance for the operation of the Calvin cycle under physiological conditions. The latter alternative would seem to be strongly favoured by the present observation that none of the inhibitors exerts any significant flux control under conditions where the Calvin cycle shows optimal activity.

Inhibitor contributions to the orthophosphate load characteristic

Results now reported establish that all inhibitors examined have the same basic effect on the orthophosphate load characteristic for the Calvin cycle: the reaction system becomes more susceptible to overload breakdown. This observation can be well understood in view of our previous analyses of the interplay between output and recycling of metabolites in reaction cycles leading to net synthesis of a cycle intermediate [20, 21]. Overload breakdown in the Calvin cycle may occur when the catalytic capacity for photosynthate output exceeds that for cyclic carbon dioxide fixation, and the latter capacity will be decreased by all inhibitors considered in Table 1.

Since the Calvin cycle obviously cannot operate in the overload breakdown region under physiological steady-state conditions, the existence of such a region represents a relatively uninteresting feature of the orthophosphate load characteristic from a regulatory point of view. Attention rather should be drawn to the experimental and theoretical observations that the load characteristic exhibits a maximum

[9–11], such that external conditions exist under which the stromal process of carbon dioxide fixation shows optimal activity. Data reported in Fig. 1 (curve D) establish that this fundamental feature of the load characteristics obtains whether or not any inhibitory interactions are assumed to be at hand. This indicates that the Calvin photosynthesis cycle, like many other metabolic pathways [17], is controlled mainly by the stoichiometric structure of the reaction system.

The present results, therefore, lead us to conclude that the examined inhibitory interactions of Calvin cycle metabolites with non-equilibrium cycle enzymes appear to be of little interest for our understanding of the biological regulation of the Calvin photosynthesis cycle under conditions of light and carbon dioxide saturation.

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