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Elementary modes analysis of photosynthate metabolism in the chloroplast stroma

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We briefly review the metabolism of the chloroplast stroma, and describe the structural modelling technique of elementary modes analysis. The technique is applied to a model of chloroplast metabolism to investigate viable pathways in the light, in the dark, and in the dark with the addition of sedoheptulose-1,7-bisphosphatase (normally inactive in the dark). The results of the analysis show that it is possible for starch degradation to enhance photosynthetic triose phosphate export in the light, but the reactions of the Calvin cycle alone are not capable of providing a sustainable flux from starch to triose phosphate in the dark. When reactions of the oxidative pentose phosphate pathway are taken into consideration, triose phosphate export in the dark becomes possible by the operation of a cyclic pathway not previously described. The effect of introducing sedoheptulose-1,7-bisphosphatase to the system are relatively minor and, we predict, innocuous *in vivo*. We conclude that, in contrast with the traditional view of the Calvin cycle and oxidative pentose phosphate pathway as separate systems, they are in fact, in the context of the chloroplast, complementary and overlapping components of the same system.

Keywords: Calvin cycle; computer modelling; elementary modes analysis; oxidative pentose phosphate pathway; photosynthesis.

Introduction

Photosynthate metabolism

The Calvin cycle is a set of some 13 enzyme catalysed reactions that serve to fix external CO_2, making the carbon available to the rest of metabolism, and using energy stored in the form of ATP and NADPH harvested by the light reactions. The entry point is the well-known Rubisco reaction (see legend of Fig. 1 for abbreviations):

$$\mathrm{RuBP} + \mathrm{CO_2} \rightarrow 2\,\mathrm{PGA}$$

and the carbon thus fixed has three possible destinations: export into general metabolism, storage in the form of transitory starch, or uptake into the regenerative limb of the cycle resulting in the synthesis of ribulose 1,5-bisphosphate, continuing the cycle.

In eukaryotic organisms the Calvin cycle is located within the chloroplast stroma, and export of intermediates is thus restricted to those metabolites that can be transported across the chloroplast envelope, or to pathways that are also contained (or at least whose initial step is) within the stroma. The best known transport mechanism is the triose phosphate-phosphate translocator that is able to exchange 3-phosphoglycerate, dihydroxyacetone phosphate or glyceraldehyde 3-phosphate for cytosolic P_i [1,2]. Pathways known to start within the stroma include the shikimate pathway (starting with erythrose 4-phosphate and phospho*enol*pyruvate) [2] and nucleotide synthesis, starting with ribose 5-phosphate. Phosphate translocators for glucose 6-phosphate (or in some species glucose 1-phosphate) are known in nonphotosynthetic plastids [3], but do not appear to be present in chloroplasts under normal conditions [4,5]. A more recently discovered chloroplast translocator is the phospho*enol*pyruvate-phosphate translocator [2,6]. However, as chloroplasts lack significant enolase activity, export from this is unlikely to represent a carbon sink. Rather, as phospho*enol*pyruvate is an initial substrate for the shikimate pathway, it seems likely that an apparently paradoxical situation exists in which the import of phospho*enol*pyruvate into the chloroplast stroma is part of a net carbon sink from the Calvin cycle.

A second set of enzymes known to be present in the chloroplast stroma, sharing many reactions and metabolites with the Calvin cycle, is that belonging to the oxidative pentose phosphate pathway [7–9]. This pathway is generally described as consisting of an oxidative limb, comprising the reactions catalysed by glucose 6-phosphate dehydrogenase, lactonase, and 6-phosphogluconate dehydrogenase, catalysing the net reaction:

$$\mathrm{G6P} + 2\,\mathrm{NADP} \rightarrow \mathrm{R5P} + 2\,\mathrm{NADPH} + \mathrm{CO_2}$$

followed by a reversible limb comprising many of the reactions of the regenerative limb of Calvin cycle with the addition of transaldolase. The function of this pathway is less clearly defined, and may be more varied than that of the Calvin cycle, but certainly it reduces NADP to NADPH, and

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is capable of supplying various sugar phosphates as end products.

Enzymes of the Calvin cycle and the oxidative pentose phosphate pathway are known to be under the common, but opposing, influence of a third system: the thioredoxin system [10,11]. Thioredoxin is a small, redox active protein, capable in turn of reducing or oxidizing disulphide bonds in proteins. In chloroplasts, thioredoxin is reduced by ferredoxin, itself a component of the electron transport chain of the light reactions. The net effect of the system is such that the Calvin cycle enzymes Rubisco (via the reduction of Rubisco activase), glyceraldehyde-3-phosphate dehydrogenase, fructose 1,6-bisphosphatase, sedoheptulose-1,7-bisphosphatase, and ribulose-5-phosphokinase are up-regulated in the light and down-regulated in the dark, whereas the oxidative pentose phosphate pathway enzymes glucose 6-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase, and transaldolase [12] are up-regulated in the dark and down-regulated in the light.

Thus it is that the common intermediates of the Calvin cycle and the oxidative pentose phosphate pathway are being turned over and sugar phosphates exported [13] in both light and dark conditions, and a number of potential consumers of these compounds exist: either via chloroplast transport proteins to the cytosol, or biosynthetic pathways contained within the chloroplast stroma. In this paper we describe and use the computer modelling technique of elementary modes analysis to determine pathways by which carbon that originates from CO_2 and/or transitory starch can exit this group of reactions, and enter the rest of metabolism.

Approaches to modelling

At its most general level, a metabolic (or biochemical) model is simply a list of reactions. The information used to specify the individual reaction determines the nature of the information that can subsequently be extracted from the model.

To date, the majority of modelling effort has been concentrated on the kinetic approach, in which reactions are specified by their stoichiometries and reaction kinetics (i.e. rate equations). From this input it is possible to determine both time-course and steady-state characteristics of the model. More sophisticated analysis of the model can then be performed in terms of sequences of changes to the model and time-course or steady-state determination. This approach can be extremely powerful: it provides the scientist with a 'virtual laboratory' in which any aspect of the system under study may be modified and/or measured in the complete absence of experimental error. The disadvantages of kinetic modelling stem primarily from the uncertainty in the definition of the kinetics, both in terms of the form that the rate equation should take, and in the values to be assigned to the associated kinetic parameters. If a large model does not exhibit the expected behaviour it is extremely difficult to determine if this is due to some general property of the model or to some inauspicious choice of parameter values: the large number of parameters in any realistic model obviates the possibility of a systematic search of the space thus defined.

An alternative to kinetic modelling is the structural approach, in which information concerning the kinetics of individual reactions is discarded, and the model is constructed solely from reaction stoichiometries. Loosely speaking a structural approach identifies possible pathways within a system, and related properties and relationships of and between those pathways. The technique used and described here is elementary modes analysis, developed by (some of) us and coworkers [14,15].

Elementary modes analysis is concerned with identifying certain subsets of reactions, so-called elementary modes, within a system. These may be defined in terms of modes thus: a mode of a system is a set of reactions whose net stoichiometry (i.e. in terms of external substrates and products) is balanced and within which all internal reactions are also stoichiometrically balanced. Thus at steady-state a mode has no net consumption or production of any internal substrate. Given this definition, an elementary mode is a mode that cannot be subdivided into further modes.

An elementary mode can thus be thought of as a minimal independent pathway within a network of reactions. An advantage of the analysis is that it is unambiguous: a mode exists, or it does not. If the mode exists then the system is capable of supporting the net conversion defined by that mode. The extent to which such a flux is actually maintained would require further investigation. Conversely if a given mode converting some particular input to a particular product does not exist, then the system is incontrovertibly unable to sustain such a steady-state flux, and if such a flux is observed in actuality, this must be taken as proof that other reactions are present in the system.

Another factor to be taken into consideration is the reversibility of the component reactions. If an elementary mode contains irreversible reactions, they can only be utilized in the forward direction. Defining some reactions as irreversible within the network reduces the total number of elementary modes that can be determined, as only elementary modes in which all irreversible reactions operate in their forward direction can be accepted.

Previous model/SBPase results

We have previously reported various aspects of our analyses of a detailed kinetic model of the Calvin cycle [16–18], and extended the analysis to incorporate results from sedoheptulose-1,7-bisphosphatase antisense experiments [19]. An unexpected result from these studies is that sedoheptulose-1,7-bisphosphatase, both *in silico* and *in vivo* has a high (in the range ≈ 0.5–1.0) flux control coefficient over CO_2 assimilation.

Another observation seen in the model, but not addressed experimentally, is that under certain circumstances the steady-state rate of carbon export via the triose phosphate-phosphate translocator could exceed the rate of CO_2 assimilation via Rubisco, with the deficit being made up by starch degradation. This observation gave rise to the question of whether or not this represents a contribution to daytime photosynthesis from the same pathway of starch breakdown that would be active at night, i.e. is it possible for the export flux to exceed the assimilation flux if the assimilation flux is zero?

This would appear to be a straightforward question to answer, given the existing kinetic model of the Calvin cycle:

the modeller has simply to reduce the value of the parameter representing light to zero and determine the steady state flux within the model. However, when this simple investigation was carried out, all fluxes in the model fell to zero, immediately giving rise to the much more difficult (for reasons discussed above) question as to whether this was due to an incorrect choice of kinetic functions and/or parameters, or, whether the system was incapable of sustaining flux under any circumstances in the absence of light. This observation was made, in the first instance, using a model that did not have any representation of the thioredoxin system: enzymes normally assumed to be rendered inactive in the dark by the action of the thioredoxin system remained active.

This problem is particularly awkward, as it was already known [17,20] that the model is capable of entering a 'dead' state under which no flux is carried, and the possibility exists that the observed absence of flux is another manifestation of this, rather than an absolute restriction.

The deregulation of SBPase

Given the apparently significant role that sedoheptulose-1,7-bisphosphatase plays under light conditions, and its control by the thioredoxin system, we are investigating the relationship between the two by producing genetically modified plants in which the coupling between them was removed, by the expression of a version of a wheat sedoheptulose-1,7-bisphosphatase in which the regulatory cysteines were mutated to serines, rendering the resulting product insensitive to thioredoxin (unpublished data).

Such a change will impact in two ways on the system: in the light total sedoheptulose-1,7-bisphosphatase activity will be increased, and in the dark the topology of the network will be altered by the addition of a new reaction (sedoheptulose-1,7-bisphosphatase being otherwise rendered inactive by the thioredoxin system). In this paper we restrict our attention to the second of these, and consider the likely outcomes of changing the topology of stromal metabolism in the dark.

Thus it is that the goals of this investigation are threefold. By applying the technique of elementary modes analysis to a model of chloroplast photosynthate metabolism we aim to determine: (a) whether or not the reactions of the Calvin cycle can support triose phosphate export from starch degradation in the absence of ATP regenerating light reactions; (b) the possible pathways made available from the combination of the enzymes of the oxidative pentose phosphate pathway and those of the Calvin cycle not deregulated by the thioredoxin system, the exported metabolites from such pathways, and any constraints to which such export may be subject; (c) the structural impact of freeing sedoheptulose-1,7-bisphosphatase from the thioredoxin system, causing it to be active in the dark.

Model definition

The model was constructed using SCRUMPY (see below); the model description file is publicly available (in both SCRUMPY and SBML format) from http://mudshark.brookes.ac.uk/Models. SCRUMPY model description files are plain ASCII text, and it is relatively easy to convert them for use with other modelling software that also accepts plain text input. The reaction list from which the model is constructed is given in Table 1 and presented schematically in Fig. 1.

Although in principle, all reactions are reversible, in this case the assumption gives rise to a great many elementary modes that would either be considered physiologically incorrect (e.g. depend on fructose 1,6-bisphosphatase running in the reverse direction), or irrelevant to the problem currently under consideration (e.g. elementary modes synthesizing starch via importation of triose phosphate).

In order to eliminate such spurious modes certain reactions are assumed to be irreversible (see Table 1). It is worth emphasizing that the elementary modes thus eliminated are neither artefactual, nor necessarily physiologically irrelevant: it is simply that a knowledge of them does not contribute to a solution of this particular problem.

Modelling software

We have been developing software, SCRUMPY, in which modelling functionality is implemented in the form of a 'PYTHON' (http://www.python.org) language module, rather than as a stand-alone software application. PYTHON is a high level, object oriented language which can be used interactively. Thus PYTHON itself provides a language based, interactive, user interface to the modelling facilities. Although a programming language is used as the interface, users do not to need any programming experience in order to use the basic modelling functions, as these are accomplished either via single commands, or a GUI.

SCRUMPY models are defined in the form of a simple, plain ASCII file, containing a list of reaction names, their stoichiometries and their kinetic functions. If, as in this case, only a structural analysis is to be applied to the model, reactions are assigned a default rate equation.

SCRUMPY is open source (Gnu Public License) and can be downloaded, along with documentation, from http://mudshark.brookes.ac.uk/ScrumPy. Interested readers are directed there, or should contact MGP for further details. At time of writing SCRUMPY is only available for Unix (including Linux) platforms, although, depending on demand, versions for other operating systems may become available. The METATOOL program (http://www.bioinf.mdc-berlin.de/projects/metabolic/metatool/) of Schuster *et al.* is also capable of performing the analysis described here.

Results

Elementary modes in the light

The main purpose of our structural analysis of the system in the light (i.e. in the absence of oxidative pentose phosphate pathway reactions) was to investigate starch metabolism and the export of triose phosphate species. The analysis identified a total of eight such elementary modes, whose net stoichiometries are presented in Table 2. These elementary modes can be classified as: (a) three elementary modes producing one each of 3-phosphoglycerate, glyceraldehyde 3-phosphate, and dihydroxyacetone phosphate from three CO_2; (b) three elementary modes producing three each of 3-phosphoglycerate, glyceraldehyde 3-phosphate, and dihydroxyacetone phosphate from three CO_2 and a glucose 6-phosphate moiety from starch; (c) one elementary mode

Table 1. Stromal enzymes and their reaction stoichiometries as used to construct the model. Bidirectional arrows indicate reversible reactions and unidirectional arrows indicate irreversible reactions. All metabolites are considered stromal unless they have the subscript *cyt* denoting cytosolic metabolites. Starch, CO_2, NADP and NADPH and all cytosolic metabolites are considered external (i.e. have fixed concentrations). The 'Thio' column represents the response of the enzyme to the action of the thioredoxin system: ↑, activated by light; ↓, inactivated by light; –, not affected. See legend to Fig. 1 for definitions of abbreviations.

Enzyme	Label in Fig. 1.	Stoichiometry	Thio
		Unique to the Calvin cycle	
Rubisco	1	CO_2 + RuBP → 2 PGA	↑
PGK	2	PGA + ATP → BPGA + ADP	↑
G3Pdh	3	BPGA + NADPH ↔ NADP + GAP + P_i	↑
FBPase	6	FBP → *F*6P + P_i	↑
SBPase	9	SBP → S7P + P_i	↑
Ru5Pk	13	Ru5P + ATP → RuBP + ADP	↑
StSynth	16	G1P + ATP → ADP + 2 P_i + starch	–
Light reaction	–	ADP + P_i → ATP	–
		Shared reactions	
TPI	4	GAP ↔ DHAP	–
Aldo	5	DHAP + GAP ↔ FBP	–
TKL	7	F6P + GAP ↔ E4P + X5P	–
Aldo2	8	E4P + DHAP ↔ SBP	–
TKL2	10	GAP + S7P ↔ X5P + R5P	–
R5Piso	11	R5P ↔ Ru5P	–
X5Pepi	12	X5P ↔ Ru5P	–
PGI	14	F6P ↔ G6P	–
PGM	15	G6P ↔ G1P	–
StPase	17	Starch + P_i → G1P	–
		Export processes	
TPT	18	PGA + $P_{i_{cyt}}$ → P_i + PGA_{cyt}	–
TPT	18	GAP + $P_{i_{cyt}}$ → P_i + GAP_{cyt}	–
TPT	18	DHAP + $P_{i_{cyt}}$ → P_i + $DHAP_{cyt}$	–
		Unique to oxidative pentose phosphate pathway	
Oxid	19	G6P + 2 NADP → 2 NADPH + R5P + CO_2	↓
TAL	20	E4P + F6P ↔ S7P + GAP	↓

synthesizing starch from CO_2; (d) a futile cycle synthesizing and degrading starch.

The elementary modes producing glyceraldehyde 3-phosphate from the above list are illustrated in Fig. 2.

There are no elementary modes capable of producing triose phosphate solely from the degradation of starch. The elementary modes for which there is net starch degradation also involve CO_2 assimilation; it follows that although the system can use starch degradation to support CO_2 assimilation, starch degradation cannot supplant assimilation. Furthermore, all of these elementary modes depend upon the light reactions to regenerate ATP and NADPH, and all contain enzymes that are deactivated by the thioredoxin system in the dark. There is thus no possibility of the reactions of the Calvin cycle, as described by this model, generating triose phosphate from starch in the dark.

In addition to the elementary modes producing triose phosphate, exactly one elementary mode each was found for the unique production of erythrose 4-phosphate, ribose 5-phosphate, and glucose 6-phosphate from the assimilation of CO_2. Various other elementary modes were also found that produced these in combination with other products and/or with starch degradation. All of them (with the exception of glucose 6-phosphate production and export from starch degradation) were dependent on the light reactions.

Elementary modes in the dark

When the light reaction and light-activated reactions were removed, and the dark active reactions included in the model, exactly one elementary mode each was found producing glyceraldehyde 3-phosphate, dihydroxyacetone phosphate, erythrose 4-phosphate, ribose 5-phosphate, and glucose 6-phosphate. The inclusion of sedoheptulose-1,7-bisphosphatase in the dark model gave rise to one new elementary mode, completely oxidizing glucose 6-phosphate from starch, with a concomitant reduction of NADP. The overall stoichiometries of these elementary modes are presented in Table 3, and the modes producing glyceraldehyde 3-phosphate, and the oxidative sedoheptulose-1,7-bisphosphatase elementary mode are illustrated in Fig. 3. The elementary modes producing C_3 and C_4 species are cyclic schemes involving the transketolase reactions and the pentose phosphate isomerase/epimerase reactions. The elementary mode producing ribose 5-phosphate does not utilize these reactions and requires only the oxidative part of the oxidative pentose phosphate pathway and ribose-5-phosphate isomerase. The elementary mode producing

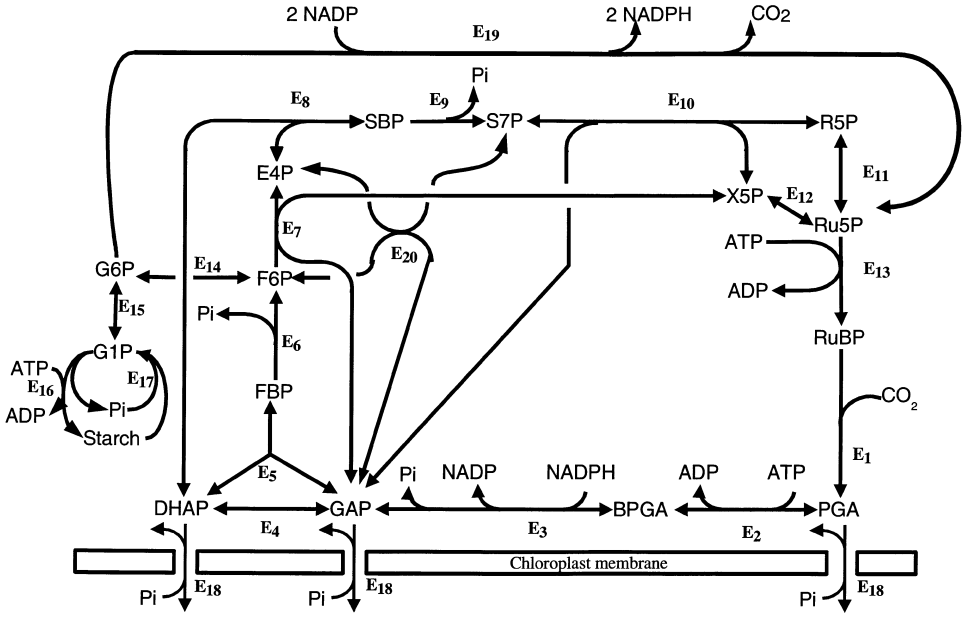

Fig. 1. Reactions of the Calvin cycle and oxidative pentose phosphate pathway as considered in this paper. Bidirectional arrows indicate reversible reactions and unidirectional arrows, irreversible reactions. The light reactions, assumed to catalyse ADP + P_i → ATP, and processes consuming E4P, Ru5P, or G6P are omitted for clarity. See Table 1 for enzyme names. Metabolite abbreviations: PGA, 3-phosphoglycerate; BPGA, glycerate 1,3-bisphosphate; GAP, glyceraldehyde 3-phosphate; DHAP, dihydroxyacetone phosphate; FBP, fructose 1,6-bisphosphate; F6P, fructose 6-phosphate; E4P, erythrose 4-phosphate; SBP, sedoheptulose-1,7-bisphosphate; S7P, sedoheptulose 7-phosphate; R5P, ribose 5-phosphate; X5P, xylulose 5-phosphate; Ru5P, ribulose 5-phosphate; RuBP, ribulose 1,5-bisphosphate; G6P, glucose-6-phosphate; G1P, glucose-1-phosphate.

glucose 6-phosphate utilizes only starch phosphorylase. The purely oxidative elementary mode comprises the greatest number of reactions, and involves the transketolase reactions, the pentose phosphate isomerase/epimerase reactions, the sedoheptulose-1,7-bisphosphate aldolase reaction, and triose phosphate isomerase, in addition to sedoheptulose-1,7-bisphosphatase.

Discussion

One of the original goals motivating this structural investigation of the Calvin cycle was to determine whether or not the traditional reactions of the Calvin cycle are capable of sustaining a triose phosphate output flux in the dark, using transitory starch as a starting point. The results show that such a flux is not possible; those elementary modes that do degrade starch also involve Rubisco, and thus depend on ATP from the light reactions. Even if a source of ATP were available, triose phosphate still could not be produced in this manner, as the elementary modes degrading starch all involve reactions that are down-regulated at night by the thioredoxin system.

In addition to establishing this fact, our analysis also explains how starch degradation can serve to support the Calvin cycle: the elementary modes degrading starch do not utilize fructose 1,6-bisphosphate aldolase or fructose 1,6-bisphosphatase; the flux that these reactions would otherwise have carried is supplied via the degradation of transitory starch, and thus becomes available for export via the triose phosphate-phosphate translocator.

Although the exact physiological role for these assimilatory elementary modes supported by starch degradation is not certain at present, a reasonable initial hypothesis is that they play a role in low light conditions. The demand these modes make upon the light reactions (in terms of ATP or NADPH) per mole of triose phosphate exported is one-third that of the conventional, nondegrading modes. The system is then effectively recouping both the carbon and the energy investment made when the starch was synthesized. The starch degrading modes can thus be expected to operate either in conditions where, although light is low (at least in respect to triose phosphate demand from the cytosol), it is not low enough for the thioredoxin system to have fully deactivated the relevant Calvin cycle enzymes, or, during a

Table 2. Overall stoichiometries of elementary modes (excluding C_4 and C_5 export) of the Calvin cycle in the light. External species P_{iext}, NADP, and NADPH are omitted here for clarity, but were included in the analysis. 'Starch' is interpreted as one glucose unit arising from stromal starch. The last elementary mode in the table is a futile cycle comprising starch synthase and starch phosphorylase driven by ATP from the light reaction.

Substrate(s)	Product
3 CO_2	PGA_{cyt}
3 CO_2	$DHAP_{cyt}$
3 CO_2	GAP_{cyt}
3 CO_2 + Starch	3 PGA_{cyt}
3 CO_2 + Starch	3 $DHAP_{cyt}$
3 CO_2 + Starch	3 GAP_{cyt}
6 CO_2	Starch
Starch	Starch

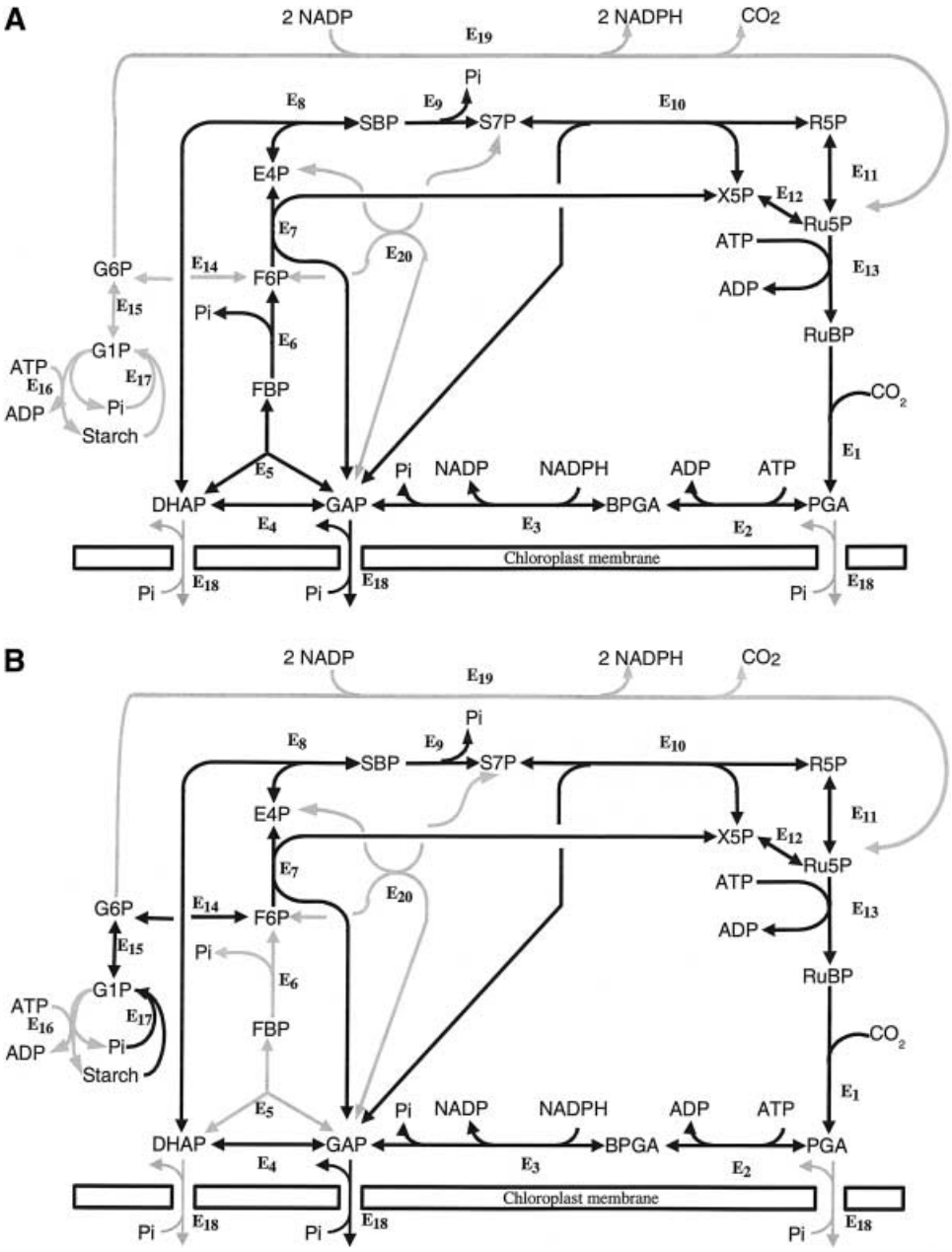

Fig. 2. Elementary modes of the Calvin cycle producing glyceraldehyde 3-phosphate from CO_2 assimilation. (A) By CO_2 assimilation alone. (B) CO_2 assimilation supported by starch degradation. Greyed out reactions do not take part. Elementary modes producing other triose phosphate species differ only in their degree of utilization of E_2, E_3, and E_4.

light–dark transitions, but before the thioredoxin system has had sufficient time to fully deactivate the Calvin cycle.

The existence of the oxidative pentose phosphate pathway has been known since the 1950s and there is little room for discussion as to the reactions of which it is comprised. There is an emerging consensus that chloroplasts possess an intact oxidative pentose phosphate pathway in plastids. Schnarenberger *et al.* [7] demonstrated a complete pathway in spinach chloroplasts; Debnam and Emes [9] reported a complete oxidative pentose phosphate pathway in spinach, pea and tobacco chloroplasts, and Thom *et al.* [8] demonstrated the existence of the pathway in sweet pepper fruit chloroplasts.

However, there is rather less consensus concerning the topology of the pathway, particularly with respect to final product, and the physiological role of the oxidative pentose phosphate pathway. Davies *et al.* [22] proposed a cyclic topology allowing for the complete oxidation of glucose 6-phosphate to CO_2; however, this proposal required fructose 1,6-bisphosphatase activity and so, as noted previously, cannot be present in dark chloroplasts. Bidwell [23] suggested an arrangement very similar to the elementary mode producing glyceraldehyde 3-phosphate shown in Fig. 3A the only difference being that the starting point is glucose rather than starch and thus requires the presence of hexokinase. ap Rees [21] describes 'the conventional view'

Table 3. Overall stoichiometries of elementary modes in the dark. All metabolites in this table are, by necessity, external in the modelling sense, that is that they can act as sinks or sources. Those metabolites subscripted 'ext' are those that have an internal counterpart. The last, purely oxidative elementary mode depends on the presence of SBPase.

Substrate(s)	Product
Starch + P_{iext}	$G6P_{ext}$
Starch + P_{iext} + 2 NADP	$R5P_{ext}$ + 2 NADPH + CO_2
Starch + P_{iext} + 4 NADP	$E4P_{ext}$ + 4 NADPH + 2 CO_2
Starch + P_{iext} + 6 NADP	GAP_{ext} + 6 NADPH + 3 CO_2
Starch + P_{iext} + 6 NADP	$DHAP_{ext}$ + 6 NADPH + 3 CO_2
Starch + 12 NADP	12 NADPH + 6 CO_2

of the oxidative pentose phosphate pathway as a branched, noncyclic pathway, starting with glucose 6-phosphate, and generating glyceraldehyde 3-phosphate and fructose 6-phosphate as the end products. He also sketches out a tentative cyclic scheme for starch oxidation in chloroplasts producing triose phosphate, but involving fructose 1,6-bisphosphatase or phosphofructokinase. Mohr and Schopfer [24] describe the oxidative pentose phosphate pathway as a cycle, not dependent on phosphatase activity, and utilizing storage starch as the starting point, with erythrose 4-phosphate or ribose 5-phosphate as the end product.

The authors cited above attribute the main functions of the oxidative pentose phosphate pathway as being some combination of the following: (a) production of redox potential in the form of NADPH; (b) production of glycolytic intermediates, reducing the demand put upon phosphofructokinase; (c) production of erythrose 4-phosphate and ribose 5-phosphate to provide initial substrate for the shikimate pathway and nucleotide synthesis, respectively.

It has also been proposed [25] that a 'swamp' analogy is an appropriate view of the oxidative pentose phosphate pathway. That is, that there are many, ill defined and interconnected flows and anything can be an input or an output. We feel that this is a view that should not be taken seriously: not only does it duck the intellectual challenge of understanding what is indeed a quite complex system, but the constraints imposed by the reaction stoichiometries (themselves a consequence of the law of mass conservation) are such that individual pathways within the system are limited in number and precisely defined [15].

Our results show that there is only one elementary mode for the net production of each of the C_3, C_4, C_5, and C_6 sugar phosphate species. Furthermore, the production of the C_5 and C_6 species did not involve the reversible reactions of the oxidative pentose phosphate pathway (see Fig. 3). Although these species are intermediates in this part of the pathway, they cannot be withdrawn from it in a sustainable fashion.

As far as the topology of the oxidative pentose phosphate pathway is concerned, elementary modes analysis reveals a number of points. Firstly, the reactions traditionally assigned to the oxidative pentose phosphate pathway are indeed capable of providing a steady-state flux of sugar phosphate, utilizing starch as an initial substrate, assuming appropriate consuming reactions. Although other reactions were present in the model (the two aldolase reactions and triose phosphate isomerase) they were not found to be present in any elementary mode (with the trivial exception of triose phosphate isomerase being used by elementary modes generating dihydroxyacetone phosphate).

The elementary modes also show that to generate C_3 or C_4 species the oxidative pentose phosphate pathway has to operate in a quite complex cycle, so that when generating C_3, 3 mol of CO_2 are produced – one arising from a starch glucose moiety, and the other two coming from recycled hexose phosphate. For the C_4 species, the ratio is 1 : 1. It is not possible for the oxidative pentose phosphate pathway to supply C_3 or C_4 as a noncyclic pathway. As noted above, the mode by which ribose 5-phosphate is produced is a simple linear pathway, not involving the reversible reactions of the oxidative pentose phosphate pathway, and glucose 6-phosphate is produced only via starch phosphorylase and phosphoglucomutase. There are no elementary modes by which the model is able to operate in a purely oxidative fashion, unless, as described below, sedoheptulose-1,7-bisphosphatase activity is included.

Another point, emphasized rather than revealed by our analysis, is that the net production of material is subject to two obligatory constraints: for every molecule produced there must be a concomitant import of a free phosphate moiety, and (with the exception of C_6 export) there is a tight coupling of export to the reduction of NADP to NADPH. For C_3 production this occurs in a 6 : 1 ratio (NADPH:TP), C_4 4 : 1 and C_5 2 : 1. As NADP and NADPH form a conserved total this implies a coupling between non-C_6 export and the oxidation of NADPH; in the absence of this coupling the oxidative reactions of the oxidative pentose phosphate pathway would rapidly exhaust their supply of cosubstrate, NADP. The nature of such a link cannot be determined on the basis of this study, but a promising starting point would be to extend the current model to incorporate nucleotide synthesis and the shikimate pathway, to determine precise ratios of NADPH : carbon demand, relative to that supplied by the oxidative pentose phosphate pathway. In addition to such a direct coupling, redox potential can be effectively exported independently from the mass flux via various shuttle mechanisms [26], and would have to be included in any model aiming to be complete.

The experimental observations of Neuhaus and Schulte [13] are qualitatively consistent with the *in vivo* operation of elementary modes of dark stromal metabolism described here. The authors investigated dark stromal metabolism in chloroplasts isolated from *Mesembryanthemum crystallinum*. This plant is interesting in that it is capable of operating C_3 or CAM (crassulacean acid metabolism) photosynthesis. The metabolites exported from both C_3 and CAM chloroplasts, when incubated in a variety of media, were determined. In C_3 chloroplasts the majority ($\approx$ 80%) of exported sugar phosphate was in the form of C_3 metabolites. Interestingly, the addition of oxaloacetate to the media resulted in a substantial increase in production of these species. The response is significant, as it shows that increasing the NADPH demand (presumably via the mechanism of the oxaloacetate–malate shuttle) leads to increased triose phosphate export, as would be predicted if the stromal metabolism was operating the cyclic elementary modes of Fig. 3.

In the CAM chloroplasts most ($\approx$ 65%) sugar phosphate was produced in the form of glucose 6-phosphate. However the addition of oxaloacetate still led to increased triose

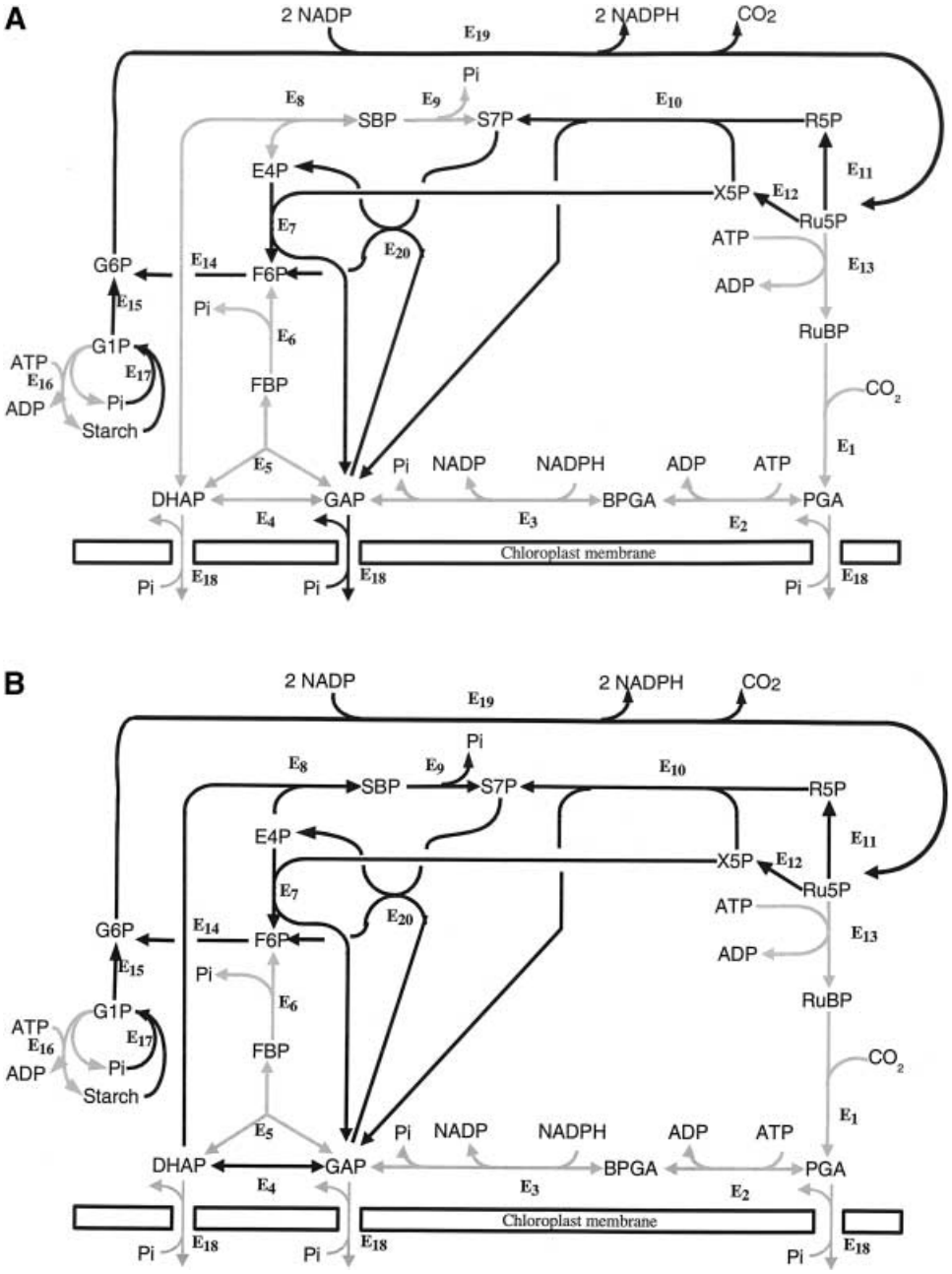

Fig. 3. Elementary modes of the system in the dark. (A) GAP producing elementary mode, elementary modes producing other C_3 or C_4 species use essentially the same set of reactions. (B) The purely oxidative mode introduced if sedoheptulose-1,7-bisphosphatase is made active in the dark. In these diagrams reversible reactions are illustrated by unidirectional arrows, indicating the direction in which flux is carried.

phosphate export. In one experiment the authors also determined the CO_2 release from CAM chloroplasts. This too was stimulated by oxaloacetate, and by approximately the same proportion as the triose phosphate export.

A consequence, in our model, of deregulating sedoheptulose-1,7-bisphosphatase from the thioredoxin system, rendering it active in the dark, is to introduce one new, cyclic, elementary mode completely oxidizing glucose 6-phosphate from starch, with the concomitant reduction of 12 mol NADP per mole of glucose 6-phosphate. This mode is similar to text-book schemes of the oxidative pentose phosphate pathway involving aldolase and fructose 1,6-bisphosphatase which also completely oxidize glucose [27]. If the observation that stromal fructose 1,6-bisphosphatase has sedoheptulose-1,7-bisphosphatase activity [28] holds true for the cytosolic isozyme, the existence of this elementary mode may have implications for the operation of the oxidative pentose phosphate pathway in the cytosol. However, exploring the significance of this is beyond the scope of the current study.

Of more immediate importance is the relevance of the inclusion of sedoheptulose-1,7-bisphosphatase into our current model. In addition to sedoheptulose-1,7-bisphosphatase the new elementary mode also uses the

sedoheptulose-1,7-bisphosphate–aldolase and triose phosphate isomerase reactions. The other reactions are the same as those in the C_3 and C_4 exporting modes, and they run in the same direction. Thus, apart from a subtle, possibly undetectable, rearrangement of intermediate metabolite concentrations there is unlikely to be a great impact on the internal biochemistry of the oxidative pentose phosphate pathway itself.

What is more likely to be significant is the fact that the new mode partially breaks the relationship between sugar phosphate utilization, NADP reduction, and NADPH oxidation described above. Although sugar phosphate utilization is still tightly coupled to NADP reduction, the reverse is no longer the case, and NADPH oxidation can proceed without the production of sugar phosphate. It is hard to predict the precise physiological consequences of this partial decoupling, especially when we consider, as noted previously, that NADP/H reduction and oxidation must anyway be tightly coupled. An immediate consequence would appear to be that a certain amount of decoupled NADP/H redox activity will be competing with the coupled activity leading to a lowering of efficiency, reduced starch at the end of the dark period, and ultimately slower growth in affected plants.

The conclusion that there will be little impact on stromal physiology from the activation of sedoheptulose-1,7-bisphosphatase in the dark is not particularly surprising as many studies of genetically modified organisms have reported only modest phenotypic changes. We suggest that this particular case is an example of the robustness of the thioredoxin system: in the model described here, the number of elementary modes, many apparently pathological, increases greatly with the number of reactions rendered insensitive to thioredoxin. Deregulating only one has only limited consequences. Furthermore this is not to say that there is no biological advantage to the thioredoxin sensitivity of sedoheptulose-1,7-bisphosphatase; selection pressures act over many generations in a natural environment, and our observations do not allow the prediction that a deregulated mutant would be as fit as the wild-type organism, in the natural environment.

Initial analysis of the transgenic plants described in the introductory section reveals no gross phenotype, although there were small but detectable increases in photosynthetic assimilation, qualitatively consistent with our previous report of a high flux control coefficient of sedoheptulose-1,7-bisphosphatase over assimilation. Interestingly, levels of starch as determined by iodine staining, suggest that at the end of the light period these plants have detectably lower levels of starch. This observation is at variance with our previous work in which we have reported a positive flux control coefficient for sedoheptulose-1,7-bisphosphatase over net starch synthesis. It may be that this is due to the disruption to the stromal metabolism in the dark affecting the metabolism in the light, although this is an issue that cannot be addressed until more results are available.

Conclusion

Although long in its theoretical gestation, the technique of elementary modes analysis has been relatively underexploited in comparison with kinetic modelling. We have shown that the technique can be used both as a tool complementary to kinetic modelling, and to analyse systems in the absence of any kinetic data.

Applying the technique to the reactions of the Calvin cycle and oxidative pentose phosphate pathway in the chloroplast shows that although the Calvin cycle can, at least potentially, supplement CO_2 fixation with the degradation of transitory starch, it nonetheless cannot perform pure starch degradation in the absence of other reactions. However, it appears that the plant very elegantly overcomes this restriction with the inclusion of the oxidative pentose phosphate pathway and the thioredoxin system which combine to ensure that both sugar phosphates and NADPH are available in light or dark. The analysis also shows that, in the dark chloroplast, the oxidative pentose phosphate pathway must operate cyclically for the production of C_3 and C_4 species, that only the oxidative part is involved in the export of C_5 species, and that the production of C_3, C_4, and C_5 sugar-phosphates is tightly coupled to NADP/H redox activity. The oxidative pentose phosphate pathway, in this context, can play no role in the production of C_6 species, despite the fact that these are intermediates of the cycle. It is perhaps a surprising observation, made clear by this application of elementary modes analysis, that the fact that a compound is an intermediate within a pathway, does not necessarily mean that it is may be withdrawn from the system.

Moreover, it can also be seen (for example by the comparison of Figs 2 and 3) that the oxidative pentose phosphate pathway and Calvin cycle play essentially complementary roles; we propose that they should possibly be regarded not as separate pathways, but overlapping sets of components whose operation is selected by the thioredoxin system in response to ambient light intensity.

The reactions of the oxidative pentose phosphate pathway and the Calvin cycle were elucidated in the 1950s, and conclusions as to their role, to be found in today's textbooks, drawn not long after. This considerably predates the localization of the reactions of the oxidative pentose phosphate pathway to the chloroplast stroma, the discovery of the thioredoxin system, the development of modern theoretical tools such as elementary modes analysis, and software that implements them. We have shown here that the application of such tools and experimental data, even to systems as extensively investigated as carbohydrate metabolism, can yield much new and useful insight.

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