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Emergence of new regulatory mechanisms in the Benson–Calvin pathway via protein–protein interactions: a glyceraldehyde-3-phosphate dehydrogenase/CP12/phosphoribulokinase complex

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Abstract

Protein–protein interactions are involved in many metabolic pathways. This review will focus on the role of such associations in CO_2 assimilation (Benson–Calvin cycle) and especially on the involvement of a GAPDH/CP12/PRK complex which has been identified in many photosynthetic organisms and may have an important role in the regulation of CO_2 assimilation. The emergence of new kinetic and regulatory properties as a consequence of protein–protein interactions will be addressed as well as some of the questions raised by the existence of these supramolecular complexes such as composition, function, and assembly pathways. The presence and role of small intrinsically unstructured proteins like the 8.5 kDa protein CP12, involved in the regulation and/or assembly of these complexes will be discussed.

Key words: Benson–Calvin cycle, CP12, glyceraldehyde-3-phosphate dehydrogenase, multienzyme complex assembly, phosphoribulokinase, protein–protein interactions.

Introduction

It is now clear that, *in vivo*, enzymes bind to other proteins in a highly specific manner to form more complex structures and interact with many components of the cell, such as the membrane. These organized structures have been isolated from prokaryotic organisms and from many organelles of eukaryotic cells, and are quite ubiquitous. The evidence supporting the existence of multienzyme complexes in sequential metabolic pathways is more compelling for some pathways (e.g. glycolysis) than for others (Srere, 1987). The term 'metabolon' has been introduced to describe supramolecular complexes of sequential metabolic enzymes and structural components (Srere, 1985). Recent experimental data clearly support the existence of metabolons especially for the Krebs tricarboxylic acid cycle (Velot and Srere, 2000). In the cell, proteins are packed together (Goodsell, 1991) and the mean distance between them is lower than the mean diameter of a protein (Srere, 1982). Thus, specific interactions between them, requiring both spatial and electrostatic complementarities, are very likely to occur *in vivo*. Recently, global studies on protein–protein interactions have been performed on the yeast *Saccharomyces cerevisiae* by using different methods, such as the TAP-tag (Tandem Affinity Purification), protein arrays, two-hybrid, or surface plasmon resonance (Biacore) coupled to the identification of proteins by mass spectrometry. The TAP-tag method used by Gavin *et al.* (2002) has identified complexes of which 58% had never been observed before and 33% involved proteins of unknown function (Gavin *et al.*, 2002; Ho *et al.*,

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Abbreviations, GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PRK, phosphoribulokinase; FBPase, fructose 1;6-*bis*phosphatase; RubisCO, ribulose *bis*phosphate carboxylase/oxygenase; SBPase, sedoheptulose 1;7-*bis*phosphatase; PRI, phosphoribose isomerase; PGK, phosphoglycerate kinase; DTT, dithiothreitol; PGA, 3-phosphoglyceric acid; BPGA, 1;3-biphosphoglyceric acid; RuBP, ribulose 1;5-*bis*phosphate; Ru5P, ribulose 5-phosphate; IUPs, intrinsically unstructured proteins.

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2002; Uetz *et al.*, 2000). These studies also indicate that the composition of these complexes *in vivo* may vary, depending on physiological conditions. Thus, proteins are not isolated but associate into dynamic complexes that may themselves interact to create a network of complexes. Predictions on the role of these higher order structures show that they participate in many cellular functions (Gavin *et al.*, 2002; Kumar and Snyder, 2002). Only recently, has the concept that metabolic pathways consist of multienzymes, been applied to plants.

The frequency and the dynamic behaviour of macromolecular complexes trigger new questions as to their composition, the cellular functions they are involved in and the advantage of such complexes compared with individual proteins. In recent years, the use of very sensitive analytical techniques has also revealed the presence of small proteins in macromolecular complexes. These small proteins are found in a wide variety of macromolecular complexes, such as pufX and the photosynthetic core complex of *Rhodobacter sphaeroides* (Jungas *et al.*, 1999; Francia *et al.*, 2002), complexins and SNARE complexes (Pabst *et al.*, 2000, 2002), and IF1 and ATPase (Cabezon *et al.*, 2000, 2001; Solaini *et al.*, 1997). The presence of such small proteins in higher order structures raises questions about their role in the formation and regulation of these complexes. One may also wonder what signal in the protein triggers the association with specific partners into stable or transitory complexes? Is it possible to predict such signals? Are there conformational changes upon the association of proteins to form higher order structures? These questions have stimulated research in this field and the study of protein–protein interactions now appears as one of the major issues for the years to come. This review focuses on recent advances in this domain and addresses specific aspects of the regulation of the metabolic pathway responsible for carbon dioxide assimilation, the so-called Benson–Calvin cycle, via protein–protein interactions. Recent studies on a glyceraldehyde-3-phosphate dehydrogenase/CP12/phosphoribulokinase complex involved in CO_2 assimilation will be emphasized, as it is considered to be a good model to answer some of the questions mentioned above.

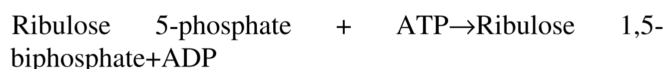
The Benson–Calvin cycle and its regulation

The photochemical stage of photosynthesis corresponds to the oxidation of a water molecule followed by an electron transfer in the photosynthetic chain of the thylakoid membrane. At the end of the electron transfer chain, NADP is reduced into NADPH. The electron transfer is also coupled with the translocation of protons through the thylakoid membrane, responsible for the formation of a proton gradient used by ATP synthase to synthesize ATP. Both NADPH and ATP are then used during the second stage of photosynthesis that corresponds to the CO_2 assimilation or Benson–Calvin cycle. This metabolic pathway is regulated by light and does not operate in the dark (Leegood, 1990).

Phosphoribulokinase (PRK), ribulose *bis*phosphate carboxylase/oxygenase (RubisCO), two enzymes specific to this cycle, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), fructose-1,6-*bis*phosphatase (FBPase), and sedoheptulose-1,7-*bis*phosphatase (SBPase) have been considered as key enzymes because their regulation by dark/light transitions is responsible for the regulation of the cycle. Extensive *in vivo* analyses have been carried out to study the role of these key enzymes in the regulation of the Benson–Calvin cycle. Experiments using antisense *rbc*S to decrease expression of ribulose *bis*phosphate carboxylase/oxygenase have been reviewed previously (Stitt and Schulze, 1994). The most important findings are that, in fairly low irradiance conditions, antisense plants with 40% less RubisCO than the wild type, are capable of almost the same rate of photosynthesis as the wild type. Nonetheless, at high irradiance, or when plants are grown on limiting inorganic nitrogen, RubisCO exerts more control. It was also shown that, in plants with decreased RubisCO, the amount of starch which is accumulated in the leaf, but not mobilized for plant growth, was decreased, probably by limiting 'wastage' of carbohydrate. Water use efficiency also decreased while the rate of transpiration and stomatal conductance remained unchanged (Stitt and Schulze, 1994).

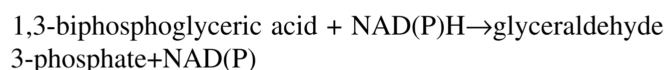
Recent analyses of transgenic plants also indicate that GAPDH, PRK, and FBPase have little control over photosynthetic carbon fixation (Raines, 2003). The antisense studies have revealed the importance of the levels of individual enzymes in controlling primary carbon flux and allocation. However, reductions in the level of some enzymes, for example, PRK and GAPDH, have little effect on carbon flux. The regulatory properties of these enzymes allow reductions in protein levels to be compensated for by increases in their activation state (Paul *et al.*, 1995; Price *et al.*, 1995; Banks *et al.*, 1999). The regulation of PRK and GAPDH is certainly complex.

PRK and GAPDH, respectively, use ATP and NADPH that have been synthesized during the first stage of photosynthesis. PRK catalyses the following reaction:



Ribulose 5-phosphate + ATP→Ribulose 1,5-biphosphate+ADP

Chloroplast GAPDH uses NAD(P)H according to the following equation:



1,3-biphosphoglyceric acid + NAD(P)H→glyceraldehyde 3-phosphate+NAD(P)

It also has the ability to use NAD(H) and thus contributes to glycolysis, whose first steps at least occur in the chloroplast (Plaxton, 1996).

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The properties of isolated PRK (Miziorko, 2000) and GAPDH (Baalmann *et al.*, 1995) are well known, but many questions remain as to their regulation and structure in supramolecular complexes.

Redox regulation of the Benson–Calvin cycle

The redox state of the key enzymes of the Benson–Calvin cycle is regulated by 12 kDa proteins, thioredoxins. Two isoforms (m and f) of thioredoxins are found in chloroplasts and have a high level of amino acid sequence similarity. Their active sites have a consensus sequence (WC(G/P)PC) (Jacquot *et al.*, 2002). By contrast, no consensus amino acid sequence motif has been found on target proteins.

The redox state of thioredoxins is also regulated by dark/light transitions. Their reduction is mediated by reduced ferredoxin, produced during the first stage of photosynthesis, and a specific enzyme, the ferredoxin-thioredoxin reductase. Other reducing agents such as dithiothreitol (DTT) or β-mercaptoethanol are often used *in vitro* to simulate the effects of thioredoxins. *In vitro* studies have shown that the effect of thioredoxins may be further modulated by other metabolites, such as NADPH, ATP, Mg^{2+}, or substrates (Faske *et al.*, 1995; Wolosiuk *et al.*, 1993).

Experiments performed on isolated PRK have demonstrated that this enzyme is inactive in its oxidized form. Its reduction by thioredoxin and DTT leads to its activation. Alkylation and site-directed mutagenesis studies have shown the existence of a regulatory disulphide bridge in this enzyme (Brandes *et al.*, 1996; Porter and Hartman, 1988). Higher plant GAPDH and FBPase have also been shown to be regulated by thioredoxins and redox transitions (Balmer *et al.*, 2001; Jacquot *et al.*, 1997; Li *et al.*, 1994; Qi *et al.*, 2001; Sparla *et al.*, 2002).

Regulation by other physiological parameters

Other parameters change in the chloroplast during dark/light transitions and may play a role in the regulation of the Benson–Calvin cycle. The pH of the stroma most probably increases from 7 to 8 upon dark to light transitions (Heldt *et al.*, 1973; Werdan *et al.*, 1975). Accordingly, it has been found that the optimum pH of most enzymes involved in the Benson–Calvin cycle is close to 8 (Heldt *et al.*, 1973). The proton translocation across the membrane also causes the movement of a counter-ion, Mg^{2+}, whose concentration in the stroma has been shown to increase upon dark to light transitions. CO_2 assimilation seems to be regulated by the concentration of this cation (Portis and Heldt, 1976).

Finally, dark/light transitions are also responsible for changes in the concentration of metabolites such as ATP, NADP(H), phosphoglyceric acid (PGA), and NAD(H). However, it is extremely difficult to obtain accurate measurements of the *in vivo* concentrations of metabolites. In addition, their distribution within the stroma is probably not homogeneous. They may be more concentrated in some parts, while almost absent in other parts of the stroma. One must therefore be extremely cautious when interpreting the regulation of enzymes by metabolites.

Specific regulatory mechanisms for RubisCO

Extensive study of the regulation of RubisCO (see the review of Houtz and Portis, 2003) has shown that, under steady-state conditions, its activity is regulated by variations in its activation state rather than by fluctuations in substrate concentration, ribulose *bis*phosphate (RuBP). *In vivo*, carbamylation of the residue Lys 101 permits the binding of the catalytically essential divalent metal (Mg^{2+}). Both carbamylation and an increase in activity are required when irradiance increases. The activation of RubisCO is mediated by the stromal protein Rubisco activase (Portis, 1992; Portis *et al.*, 1995) which acts by removing the otherwise inhibitory sugar phosphates, RuBP, and in some plants, 2-carboxyarabinitol 1-phosphate. Rubisco activase is a member of the ATPases associated with diverse cellular activities (AAA+) protein family that constitutes a wide variety of proteins with chaperone-like functions. It is a nuclear-encoded chloroplast protein that usually consists of two isoforms generated by alternative splicing of a pre-mRNA. These two isoforms differ only at the carboxyl terminus. The activity of Rubisco activase is regulated by the ADP/ATP ratio and the larger isoform is regulated through thioredoxin f. The presence of two Cys residues found only in the larger isoform is responsible for this activation (Zhang and Portis, 1999). RubisCO, therefore, can be regulated via the activase in response to light intensity. The major mechanism for compensation of a decrease in the amount of RubisCO in transgenic plants is an increase in the RubisCO activation state, presumably involving Rubisco activase (Stitt and Schulze, 1994).

Supramolecular complexes of the Benson–Calvin cycle

The idea that the enzymes involved in this metabolic pathway are not randomly distributed in the chloroplast stroma, but interact to give multienzyme complexes, has been forwarded because some of these proteins, for example, PRK and GAPDH, could not be easily isolated by conventional purification methods, such as ion exchange, gel filtration, and affinity chromatographies and co-purifies with other enzymes of the Benson–Calvin cycle. In addition, different authors have isolated multienzyme complexes from pea and spinach with varying compositions (Giudici-Orticoni *et al.*, 1992; Gontero *et al.*, 1988, 1993, 1994; Müller, 1972; Sainis and Harris, 1986; Sainis and Jawali, 1994; Sainis *et al.*, 1989), which may be

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linked to their dissociation during the purification procedure. Hence, smaller complexes may be considered as sub-complexes of higher order structures. Strikingly, Clasper *et al.* (1991) and, more recently, Scheibe *et al.* (2002) have isolated a PRK/GAPDH complex in spinach leaves, which is also found in green algae such as *Chlamydomonas reinhardtii* (Avilan *et al.*, 1997*b*; Wedel and Soll, 1998), *Scenedesmus obliquus* (Nicholson *et al.*, 1987; O'Brien *et al.*, 1976), and in the cyanobacterium *Synechocystis* PCC6803 (Wedel and Soll, 1998). This complex may, therefore, correspond to the core complex of a super-complex involved in CO_2 assimilation.

Lately, a small 8.5 kDa protein, named CP12, has also been identified in most of these complexes (spinach, pea, tobacco, *C. reinhardtii*, and *Synechocystis*) (Pohlmeyer *et al.*, 1996; Wedel and Soll, 1998; Wedel *et al.*, 1997).

Organization of the Benson–Calvin cycle in *C. reinhardtii*

Immunolocalization studies on *C. reinhardtii* have shown that phosphoribose isomerase (PRI), PRK, phosphoglycerate kinase (PGK), GAPDH, and FBPase co-localize and are located near the thylakoid membrane. Enzymes of the Benson–Calvin cycle in this green alga thus seem to be organized in supramolecular complexes, as the Benson–Calvin cycle of higher plants (Süss *et al.*, 1995).

In the laboratory, Avilan *et al.* (1997*b*) have isolated a 460 kDa complex that seemed to be composed of two homodimers of PRK and two homotetramers A_4 of GAPDH. Wedel and Soll (1998) have shown that this complex also had a third 8.5 kDa protein, CP12. Attempts by the laboratory to reveal the presence of this protein by immunochemical methods failed, until the complex was studied by MALDI-TOF mass spectrometry, finally showing that the complex isolated by Avilan *et al.* (1997*b*) was the same as that found by Wedel and Soll (1998). In higher plants, chloroplast GAPDH is present as an A_2B_2 tetramer. The B subunit carries a C-terminal extension responsible for the oligomerization of higher plant chloroplast GAPDH into an A_8B_8 regulatory form (Baalmann *et al.*, 1996; Li *et al.*, 1994). In green algae such as *C. reinhardtii* and *Scenedesmus obliquus*, GAPDH is only present as an A_4 homotetramer. Interestingly, CP12 shares sequence homologies with the C terminal extension of the B subunit of GAPDH (Pohlmeyer *et al.*, 1996), that contains two cysteine residues believed to be involved in the redox regulation of this enzyme (Baalmann *et al.*, 1995; Qi *et al.*, 2001; Sparla *et al.*, 2002).

Studies of the GAPDH/CP12/PRK complex

The complex described above is relatively simple compared with most higher plant complexes, as it is made up of only two enzymes and a small protein. This composition facilitates site-directed mutagenesis programmes to identify the residues involved in protein–protein interactions. Site-directed mutagenesis coupled to *in vitro* reconstitution experiments enables the characterization of chimeric complexes, since the oxidized partners are able spontaneously to reconstitute a complex *in vitro*, which is quite similar to the native state (Gontero *et al.*, 2001; Lebreton *et al.*, 1997*b*).

The GAPDH/CP12/PRK complex is dissociated by reducing agents such as DTT, which enables the purification of isolated PRK and a sub-complex of GAPDH and CP12, also called native GAPDH (Avilan *et al.*, 1997*b*). The kinetic properties of PRK and GAPDH, both embedded within the complex and as individual enzymes, have been analysed (Lebreton and Gontero, 1999; Lebreton *et al.*, 1997*b*; Graciet *et al.*, 2003*b*)

Studies on the complex have shown that PRK within the complex is active. The progress curve of the reaction catalysed by the PRK embedded in the complex displays a lag, which corresponds to the dissociation of the complex. If this complex is first incubated in the reaction mixture lacking the substrates of PRK, then no lag is observed when the reaction is started by adding the substrates (Lebreton *et al.*, 1997*b*).

Studies on PRK kinetics in this system suggest a model that links the activity of PRK to the dissociation of the complex. The equation that may be derived from the model fits the experimental results best, only if one assumes (as postulated in the model) that both the free PRK released upon dissociation of the complex and the bound PRK of this complex are active. If only the free PRK is assumed to be active, the fit is biased. One may therefore measure the reaction rate catalysed by the bound PRK of the complex by monitoring the reaction rate immediately after mixing this complex with its substrates in a suitable reaction medium. The active oxidized form of PRK that has just been released by the dissociation of the complex in the assay mixture is not stable, for it slowly loses its activity and becomes identical to the stable, almost inactive, form. This recently dissociated PRK is thus considered as a metastable form of the enzyme and its conformation changes have been characterized by fluorescence spectroscopy (Lebreton *et al.*, 1997*b*). Hence, there are three different forms that have different conformations and activities: the stable enzyme, the enzyme bound to GAPDH/CP12 sub-complex, and the metastable free enzyme. Each of these three forms exists under both oxidized and reduced states which differ in their K_m and k_{cat} values (Fig. 1; Table 1) (Lebreton and Gontero, 1999; Lebreton *et al.*, 1997*b*).

Mixing the stable, oxidized, and almost inactive, PRK with GAPDH/CP12 results in the formation of the bi-enzyme complex and in the increase of the catalytic activity of PRK (Avilan *et al.*, 1997*b*). From these data it seems that GAPDH/CP12 may give an instruction to PRK

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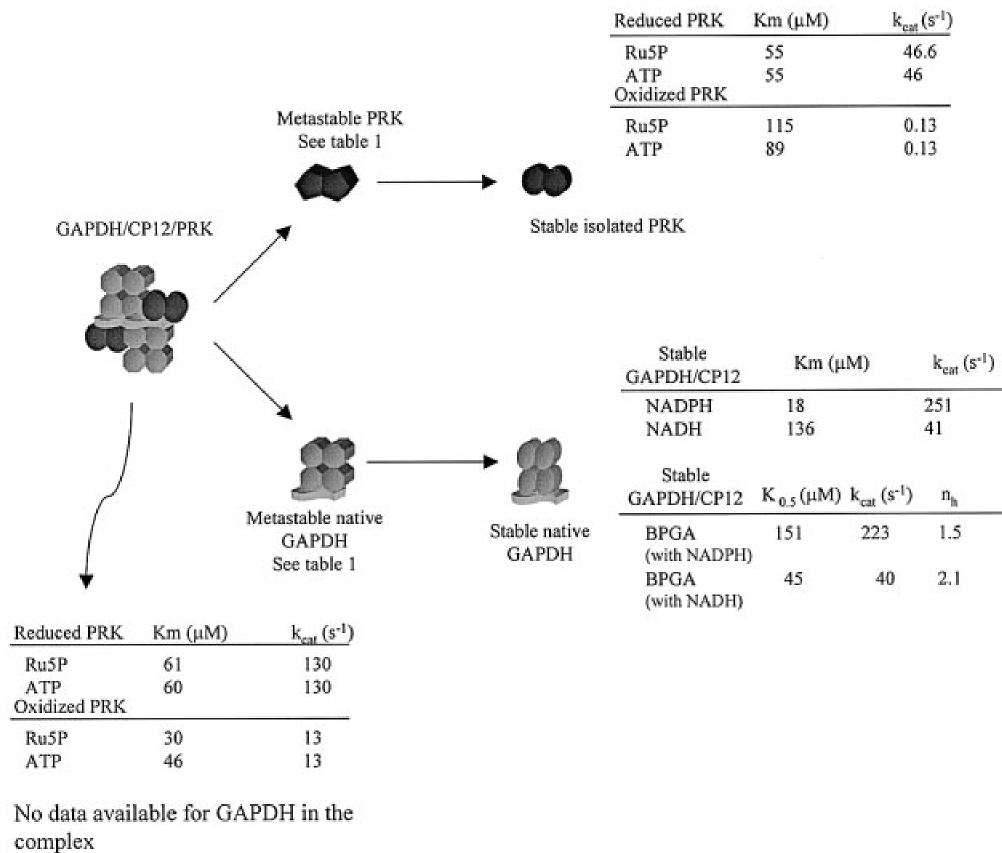

Reduced PRK	Km (μM)	k_{cat} (s^{-1})
Ru5P	55	46.6
ATP	55	46
Oxidized PRK		
Ru5P	115	0.13
ATP	89	0.13

Stable GAPDH/CP12	Km (μM)	k_{cat} (s^{-1})
NADPH	18	251
NADH	136	41

Stable GAPDH/CP12	$K_{0.5}$ (μM)	k_{cat} (s^{-1})	n_h
BPGA (with NADPH)	151	223	1.5
BPGA (with NADH)	45	40	2.1

Reduced PRK	Km (μM)	k_{cat} (s^{-1})
Ru5P	61	130
ATP	60	130
Oxidized PRK		
Ru5P	30	13
ATP	46	13

Fig. 1. Model explaining the origin of the metastable forms of PRK and native GAPDH. The inserted tables illustrate the different kinetic parameters obtained for different forms of PRK and GAPDH. It was not possible to characterize the kinetic parameters of GAPDH included in the complex as its dissociation was too fast in the presence of its substrates (Graciet *et al*., 2002). The dissociation of the complex results in the appearance of metastable forms that relapse into stable forms. Some of the kinetic parameters of these metastable forms have been characterized (see Table 1). The standard errors (SE) on all parameters were less than 10%. Kinetic parameters for PRK are from Lebreton and Gontero (1999).

and, as a consequence of their interaction, an increase of activity of PRK is observed.

Moreover, the metastable PRK which is released just after dissociation of the complex and whose activity and conformation evolve with time is very active compared with the stable isolated PRK (Table 1). This high activity has been linked to an imprinting exerted on PRK by GAPDH/CP12. This imprinting lasts even after dissociation of PRK from the GAPDH/CP12 sub-complex, as if the enzyme kept the memory of the conformation it had in the complex before slowly relapsing into the stable, isolated, inactive form. A possible explanation for the higher activity of metastable PRK compared with the same enzyme associated with GAPDH/CP12 is that the catalytic activity requires an important mobility of the enzyme which is favoured if it is in a free state.

Conversely, PRK may be expected to exert an imprinting effect on the GAPDH/CP12 sub-complex. Such an effect has been demonstrated and it affects the catalysis of GAPDH with NADH or NADPH as cofactors by decreasing the energy barrier of these reactions by 3.8±0.5 and 1.3±0.3 kJ mol^{-1}, respectively. This effect was quantified using the statistical thermodynamic theory mentioned below. With regard to CP12, its association with GAPDH is responsible for changes in the kinetic parameters of GAPDH, probably due to conformation changes (Graciet *et al.*, 2003*b*).

These associations also result in the modification of the regulatory properties of the enzymes. When PRK and GAPDH are within the complex, they are regulated by NADP(H), but not when they are in stable isolated states (Graciet *et al.*, 2002). The activities of the metastable PRK and GAPDH, that correspond to the forms released just after dissociation of the complex upon dilution and that slowly evolved into the stable forms, have been measured. The activities of the metastable PRK and GAPDH from the complex preincubated with NADP(H) are different from those of the metastable enzymes released from the untreated complex. NADP increases PRK and NADPH-GAPDH activities and decreases NADH-GAPDH activity; NAD(H) has no effect. NADPH slightly activates PRK and inhibits GAPDH using NADH. As a consequence of

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Table 1. *Kinetic parameters of the metastable forms of PRK and GAPDH*

The metastable forms were obtained from the complex after dilution. It was shown that the conversion (characterized by a dissociation rate constant, k^*) of the PRK embedded into the complex into the dissociated metastable form is 10-fold faster under reducing conditions than under oxidizing conditions (k^* is about 0.1 min^{-1} for the oxidized complex and 1 min^{-1} for the reduced one) (Lebreton and Gontero, 1999). For GAPDH, this rate constant is too fast and has not been determined. The values of K_m for NAD(P)H have also not been characterized for this form. The standard errors (SE) on all parameters were less than 10%. Kinetic parameters for PRK are from Lebreton and Gontero (1999). Kinetic parameters for GAPDH are from Lebreton et al. (2003).

	K_m (μM)	k_{cat} (s^{-1})
Oxidized PRK		
Ru5P	30	112.6
ATP	46	113
Reduced PRK		
Ru5P	94	1200
ATP	51	1205
Reduced GAPDH		
BPGA (NADPH constant)	262	650

NADP(H) binding, the imprinting effects of these two enzymes on each other are modified. Thus, incubation with NADP increases the energy barrier of the NADH-GAPDH dependent reaction by 1.8±0.2 kJ mol^{-1} and decreases that of the reaction catalysed by PRK by 3±0.2 kJ mol^{-1} and GAPDH using NADPH by 1.2±0.3 kJ mol^{-1} (Graciet *et al.*, 2002). These same metabolites have been shown to regulate an oligomeric 600 kDa form of GAPDH in spinach (Baalmann *et al.*, 1996; Wolosiuk and Buchanan, 1976) that is absent from *C. reinhardtii*.

As the Benson–Calvin cycle is redox-regulated, the activation of oxidized PRK, either free or as part of a complex, by reduced thioredoxin was studied. Reduced thioredoxin may act on PRK either within the complex or in the free states (stable or metastable forms). However, the time required to activate the enzyme as part of the complex is shorter than the one required to activate the free forms. Moreover, this time is similar to the induction time of the Benson–Calvin cycle upon dark–light transitions. With regard to GAPDH, incubation of a crude extract from *C. reinhardtii* with dithiothreitol increased GAPDH activity 3-fold (Li *et al.*, 1994). Surprisingly, the same increase in NADPH-dependent activity is obtained for GAPDH when it is part of the complex, but not for the individual enzyme. A titration of the thiol groups of GAPDH in the complex reveals 4 SH groups, whatever the redox state of the complex, while each PRK monomer in the oxidized complex has one disulphide bridge that is disrupted upon reduction. These results show that the modulation of GAPDH activity is not linked to disulphide reduction, but to heterologous interactions. The regulation of PRK may thus modulate the activity of GAPDH via a 'domino-like' effect (Lebreton *et al.*, 2003).

Protein–protein interactions are responsible for kinetic and conformational changes that may last, even after dissociation of the supramolecular structure. Such changes have also been shown to occur upon association of the proteins. As directly demonstrated by cryo-electron microscopy, GAPDH and PRK undergo important conformational changes upon association, compared with the enzymes in their isolated state (Mouche *et al.*, 2002).

Protein–protein interactions and information transfer

As mentioned above, association between enzymes may result in the change of their properties as enzymes may store some energy upon association. Kinetic studies have led to the development of a thermodynamic theory by J Ricard and coworkers to explain the origin of the energy required to generate these modifications (Ricard *et al.*, 1994, 1998). This theory states that an information transfer between PRK and GAPDH occurs within the GAPDH/CP12/PRK complex and it may be quantified. Imprinting effects have emerged from this theory, but they imply the dissociation of the complex and the existence of metastable forms of the enzymes, that have different kinetic properties and probably different conformations compared with the same enzyme in the complex or under an isolated state. The imprinting of GAPDH on PRK has been demonstrated and quantified in the GAPDH/CP12/PRK complex from *C. reinhardtii* (Lebreton and Gontero, 1999; Lebreton *et al.*, 1997*a*, *b*). PRK also exerts an imprinting on GAPDH, which is responsible for the decrease of the kinetic barriers of the reactions catalysed by GAPDH. Thus, protein–protein interactions are responsible for kinetic and conformational changes that may last, even after dissociation of the supramolecular structure. Many examples in the literature illustrate the emergence of novel functions as a consequence of protein–protein interactions such as the plasminogen-streptokinase, the Ras-GTPase activating protein, and the prions, to cite but a few (Ricard *et al.*, 1998).

Nature of protein–protein interactions and supramolecular complex assembly

The gene coding for PRK of *Chlamydomonas reinhardtii* has been isolated, cloned and expressed in *E. coli*. The recombinant protein may form, with native GAPDH (or GAPDH/CP12 sub-complex), a complex, which is apparently kinetically indistinguishable from that extracted from *Chlamydomonas* cells. A *C. reinhardtii* mutant (12-2B) previously isolated and shown to bear a R64C mutation in

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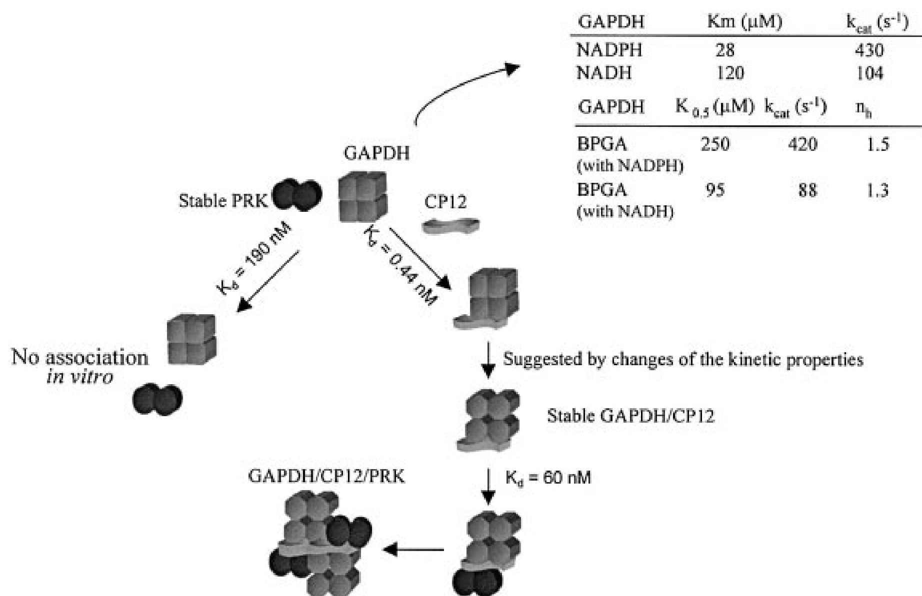

Fig. 2. Model describing the assembly process of GAPDH/CP12/PRK complex of *Chlamydomonas reinhardtii*. The dissociation constants (K_d) were obtained using surface plasmon resonance (Graciet *et al*., 2003*a*). The inserted table corresponds to kinetic parameters of the stable GAPDH. The standard errors (SE) on all parameters were less than 10%. Kinetic parameters are from Graciet *et al.* (2003*b*) and the assembly pathway of the GAPDH/CP12/PRK complex is from Graciet *et al*. (2003*a*).

PRK has been studied (Roesler *et al.*, 1992; Salvucci and Ogren, 1985). Avilan *et al.* demonstrated that the mutation of this residue impaired the interaction of PRK with GAPDH to form the GAPDH/CP12/PRK complex (Avilan *et al.*, 1997*a*). Whereas the R64A and R64E mutant PRKs are unable to form a complex with GAPDH, the R64K mutant does, although only slightly.

Protein–protein association is a process that is harder to simulate than ligand–protein association, as both interacting molecules are large and will, in general, have complex shapes and charge distributions (Sheinerman *et al.*, 2000). In order to study the nature of the protein–protein interactions within the GAPDH/CP12/PRK complex, heterologous expression systems have been developed for the two other partners, GAPDH and CP12 (Graciet *et al.*, 2003*a*, *b*). The role of CP12 in the assembly pathway of the GAPDH/CP12/PRK complex has been investigated, as no complex could ever be reconstituted *in vitro* using isolated native PRK and recombinant GAPDH (devoid of CP12). Reconstitution assays show that oxidized CP12, in addition to modifying GAPDH kinetic properties and, probably, conformation, also acts as a linker in the assembly of the GAPDH/CP12/PRK complex (Graciet *et al.*, 2003*b*). The presence of two disulphide bridges in oxidized CP12 seems to be essential for complex reconstitution. The lack of overall organization and the great flexibility of oxidized CP12 observed by nuclear magnetic resonance and circular dichroism are in good agreement with the role of this protein as a linker. Its behaviour resembles that of 'intrinsically unstructured proteins' or 'IUPs' (Dyson and Wright, 2002; Tompa, 2002; Wright and Dyson, 1999), often involved in protein–protein interactions. The flexibility of these IUPs as well as that of CP12, favours the binding of partners. In many cases, it has been shown that partly disordered conformations provide flexibility and adaptability. Both characteristics explain why these IUPs may be functionally important for the properties of biological macromolecules (Namba, 2001).

Data obtained from surface plasmon resonance binding experiments and *in vitro* reconstitution assays suggest a model for the assembly process (Fig. 2). The first step in the assembly is the association of CP12 with GAPDH, with a dissociation constant (K_d) close to 0.44 nM. This association is followed by a change of the conformation of GAPDH. This GAPDH/CP12 complex then binds PRK with a K_d close to 60 nM to form half-a-complex, defined as one unit. This unit dimerizes to give the native complex composed of two dimers of PRK, two tetramers of GAPDH and, probably two monomers of CP12 (Fig. 1) (Graciet *et al.*, 2003*b*).

The formation of this complex may be a prerequisite for efficient light activation of the enzymes and subsequent docking of the other enzymes of the Benson–Calvin cycle as the use of immunoelectron cryomicroscopy reveals that, in addition to PRK and GAPDH, phosphoribose isomerase, RubisCO, FBP aldolase, sedoheptulose bisphosphatase, nitrite reductase, ferredoxin NADP oxidoreductase, and

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ATP synthase are also found (Süss *et al.*, 1995), and also as higher supramolecular complexes have been described (Anderson *et al.*, 1995; Gontero *et al.*, 1988).

Conclusion

Transitory or stable interactions between proteins play extremely diverse functions in the cell's life, from signal transduction to an optimal and fine regulation of metabolic pathways. A better understanding of the biological roles of these supramolecular complexes would be facilitated by the study of 'simple' models. The work on a CO_2-assimilating GAPDH/CP12/PRK complex isolated from the green alga *Chlamydomonas reinhardtii* enables the diverse roles of protein–protein interactions in supramolecular complexes, as mentioned above, to be dealt with. Nonetheless, many questions regarding the existence, composition, and dynamics of the GAPDH/CP12/PRK complex *in vivo* remain to be answered. Is the composition of this complex the same whatever the physiological conditions? Are there physiological factors that trigger dissociation or association events? Can this complex be considered as the core of a bigger supramolecular complex, other enzymes having the possibility to dock to this core complex? The study of this complex and other complexes, although far from being trivial will clearly help in the understanding of how metabolic pathways are regulated.

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