Phosphorylation of small subunit plays a crucial role in the regulation of RuBPCase in moss and spinach

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RuBPCase has been purified to electrophoretic homogeneity from moss and spinach. On denaturing SDS-polyacrylamide gels the purified enzyme revealed two discrete bands, thereby indicating the presence of large and small subunits. The phosphoprotein nature of RuBPCase was proved by in vivo labelling of enzyme with [^{32}P]orthophosphate. Autoradiographic analysis of ^{32}P-labelled RuBPCase on SDS-PAG demonstrated that phosphorylation was restricted to the small subunit. Dephosphorylation of purified RuBPCase with alkaline phosphatase resulted in a dramatic decline (70% decrease) in the biological activity of the enzyme. Fractionation of the dephosphorylated enzyme on denaturing gels revealed only the presence of large subunits of RuBPCase. Thus it became evident that dephosphorylation of RuBPCase brings about the dissociation of small subunits from the catalytic large subunits (octamer). The dephosphorylated small subunits were isolated as dimers. These results clearly indicate that phosphorylation of small subunits is mandatory for the reconstitution of holoenzyme and hence crucial for the activation of RuBPCase.

Phosphoprotein *Small subunit* *Enzyme regulation* *Phosphorylation* *Ribulose-1,5-bisphosphate carboxylase*

1. INTRODUCTION

Phosphorylation of proteins during post-translational modification plays a significant role in the regulation of enzyme activity in eukaryotes [1]. Among plants, phosphorylation of chloroplast proteins has been reported in spinach [2–5], pea [6] and maize [7,8]. One of the proteins undergoing phosphorylation has been shown to be RuBPCase. The in vitro phosphorylation of both the small and the large subunits of RuBPCase has been reported in spinach chloroplasts [2–5]. However, the physiological significance of phosphorylation of RuBPCase has remained quite enigmatic. Furthermore, it was not even explicitly ascertained whether both the subunits of RuBPCase occur in a phosphorylated state in vivo.

We now present evidence to show that RuBPCase in moss and spinach occurs as a phosphoprotein and that there is selective phosphorylation of the small subunit under in vivo conditions. Dephosphorylation of the purified RuBPCase significantly lowered its catalytic efficiency. Concomitantly, there was dissociation of the small subunit from the catalytic large subunit of RuBPCase. Thus, phosphorylation of the small subunits seems quite vital for the assemblage of the large subunits with the small subunits. To our knowledge, this is the first report which elucidates the precise role of phosphorylation in the regulation of RuBPCase activity.

2. MATERIALS AND METHODS

Materials: moss, *Funaria hygrometrica* J.2, was

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Abbreviations: RuBPCase, ribulose-1,5-bisphosphate carboxylase; RuDP, ribulose 1,5-diphosphate; SDS-PAG, SDS-polyacrylamide gel; 3-PGA, 3-phosphoglyceric acid

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cultured in minimal medium [9] at 25°C in continuous light (3500 ± 100 lux). Spinach (*Spinacea oleracea*) was collected from our botanical garden. $NaH^{14}CO_3$ and [^{32}P]orthophosphoric acid (carrier-free) were procured from Bhabha Atomic Research Centre, Bombay. [γ-^{32}P]ATP was obtained from Amersham. RuDP was purchased from Sigma (USA).

2.1. *Purification of RuBPCase*

RuBPCase from moss and spinach was purified to electrophoretic homogeneity by the modified method given in [10]. Essentially, we omitted the sucrose zonal centrifugation step; instead, the DE-52 fraction was subjected to molecular sieving on Sepharose CL-6B for the ultimate purification of RuBPCase. Protein concentration was determined by the method of Bradford [11]. The purified enzyme was employed for studying the role of phosphorylation in the regulation of RuBPCase.

2.2. *In vivo labelling of RuBPCase with [^{32}P]orthophosphate*

Moss protonemal filaments and spinach leaf discs were incubated in carrier-free [^{32}P]orthophosphate (5 mCi) in sterile distilled water for 24 h. The tissue was harvested and ^{32}P-labelled RuBPCase was purified to electrophoretic homogeneity. The extraction and purification of ^{32}P-labelled RuBPCase were carried out in the presence of NaF (1 mM) with a view to inhibit the activity of endogenous phosphatases.

2.3. *Treatment of purified RuBPCase with alkaline phosphatase*

Purified RuBPCase (30 mg) was treated with purified alkaline phosphatase (2 mg) in Tris-HCl buffer (25 mM, pH 8.0) at 4°C for 12 h. The dephosphorylated enzyme was then fractionated on a Sephadex G-150 column (1.8 × 33.5 cm) equilibrated with Tris-HCl buffer (10 mM, pH 8.0) for the separation of the large and small subunits. The peak fraction (25 mg protein) containing large subunits (octamer) and alkaline phosphatase was subsequently fractionated on Sepharose CL-6B (2.4 × 50 cm) using Tris-HCl (25 mM, pH 8.0) containing 2-mercaptoethanol (8 mM), EDTA (0.2 mM) and $MgCl_2$ (10 mM) at 4°C. The untreated, phosphorylated enzyme served as a control. Both phosphorylated and dephosphorylated RuBPCase were used for determining their subunit structure and kinetic properties. The alkaline phosphatase (Sigma) was purified before use by gel filtration on a Sephadex G-150 column (1.8 × 33.5 cm) using Tris-HCl (10 mM, pH 8.0).

2.4. *Assay of RuBPCase activity*

The purified enzyme (25–50 μg/assay) was preincubated with $NaH^{14}CO_3$ (2.5 μmol), Tris-HCl (100 μmol, pH 7.8), Na_2EDTA (0.1 μmol), $MgCl_2$ (2.5 μmol) and 2-mercaptoethanol (4 μmol) at 26°C for 5 min. The assay of RuBPCase was then initiated by the addition of RuDP (0.15 μmol) in a final volume of 500 μl. The reaction was terminated after 10 min at 26°C with the addition of acetic acid (6 M, 100 μl) [12]. An aliquot (100 μl) was plated on to a Whatman 3MM disc and the radioactivity determined as in [13].

2.5. *Polyacrylamide gel electrophoresis*

Native gel electrophoresis of RuBPCase was carried out according to [14]. Both phosphorylated and dephosphorylated RuBPCase were also fractionated on SDS-PAG (10%) by the method of Laemmli [15]. The gels were stained with Coomassie brilliant blue R250 for visualization of the protein bands [16]. Autoradiography was performed by exposing the dried gels to Sakura X-ray film using intensifying screens at −50°C.

2.6. *In vitro dephosphorylation of purified RuBPCase*

In vitro phosphorylation of purified RuBPCase was achieved by incubating the enzyme (50 μg) with wheat protein kinase (10 μg) in the presence of Tris-acetate (20 mM, pH 7.6), magnesium acetate (20 mM) and [γ-^{32}P]ATP (2200 dpm/pmol, 40 μM) in a final volume of 50 μl for 30 min at 25°C. The radioactivity was determined by plating an aliquot (25 μl) onto a phosphocellulose (P-81) strip (1 × 1 cm) as described in [17]. Protein kinase used in this study was purified from germinating wheat embryos in our laboratory (unpublished).

3. RESULTS

3.1. *In vivo phosphorylation of RuBPCase*

RuBPCase was purified to electrophoretic homogeneity from moss and spinach. In both

plant systems, this enzyme occurs as a phosphoprotein. This was conclusively proved by labelling the enzyme in vivo with [^{32}P]orthophosphate. The purified ^{32}P-labelled RuBPCase revealed a single protein band on native acrylamide gels. Autoradiography of this gel gave a single radioactive band that corresponded with the protein band of RuBPCase (fig.1). Fractionation of ^{32}P-labelled enzyme on SDS-PAG showed two protein bands (fig.2b): a large subunit (54 kDa) and a small subunit (12 kDa) (fig.2a,b). However, autoradiography of these gels revealed only a single radioactive band in the zone of the small subunit (fig.2c). This clearly indicated that phosphorylation of RuBPCase occurs selectively on the small subunits. Acid hydrolysis of ^{32}P-labelled RuBPCase, followed by autoradiography of chromatographically separated phosphoamino acids, showed that phosphorylation is restricted to the serine and the threonine residues of the small subunits (not shown).

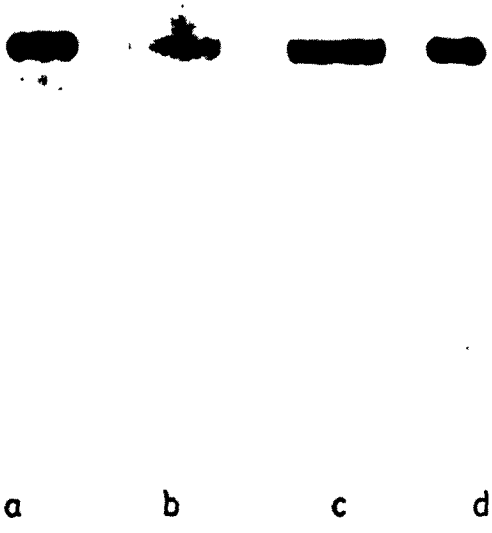

Fig.1. Electrophoretic gel pattern under nondenaturing conditions of ^{32}P-labelled RuBPCase, purified from moss and spinach. Coomassie brilliant blue R-stained protein band of purified RuBPCase from moss (a) and spinach (c); lanes b and d represent autoradiograph of a and c, respectively.

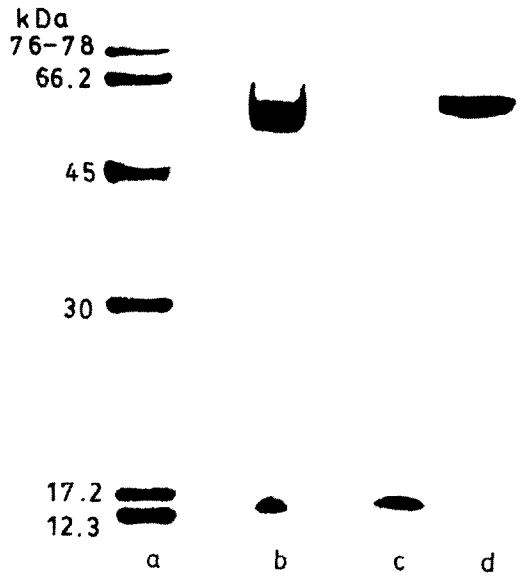

Fig.2. SDS-PAG pattern of purified phosphorylated and dephosphorylated moss RuBPCase depicting its subunit structure and phosphorylation of the small subunit. (a) Molecular mass markers stained with Coomassie brilliant blue R; (b) large and small subunits of RuBPCase stained with Coomassie brilliant blue R on SDS-PAG; (c) autoradiograph of b; (d) dephosphorylated RuBPCase stained with Coomassie brilliant blue R.

3.2. *In vitro dephosphorylation of RuBPCase*

The precise significance of phosphorylation of the small subunit was so far unknown [2–5]. With a view to resolving this issue, we adopted a strategy that should enable us to dephosphorylate RuBPCase in vitro and then compare the biological activity of the phosphorylated and dephosphorylated enzyme. This approach has provided an important clue for elucidating the role of phosphorylation in the metabolic regulation of RuBPCase. We were able to achieve complete dephosphorylation of purified RuBPCase by treatment with highly purified alkaline phosphatase. The dephosphorylated RuBPCase was conveniently separated from alkaline phosphatase by molecular sieving on Sepharose CL-6B. To prove that phosphatase treatment does indeed dephosphorylate purified RuBPCase, we also treated ^{32}P-labelled RuBPCase

with alkaline phosphatase under identical conditions. We observed complete loss of ^{32}P label in the acid-precipitable protein fraction following phosphatase treatment. There was no loss of radioactivity in the acid-precipitable enzyme fraction of the untreated control (not shown).

3.3. *Role of phosphorylation in the regulation of RuBPCase*

A dramatic decrease in enzyme activity was witnessed with the dephosphorylated RuBPCase (fig.3A) in comparison to the phosphorylated enzyme. Early kinetic data showed a distinct lag in

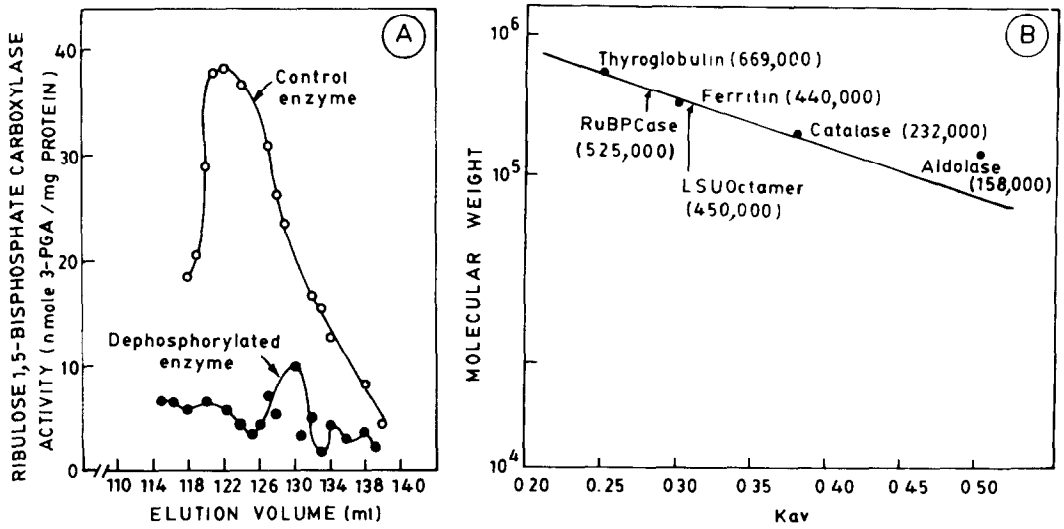

Fig.3. Molecular sieving of control (phosphorylated) and dephosphorylated purified RuBPCase from moss. The purified RuBPCase was treated with alkaline phosphatase. The dephosphorylated enzyme was fractionated on Sepharose CL-6B for the removal of alkaline phosphatase. (A) Elution profiles of control and dephosphorylated RuBPCase on Sepharose CL-6B; (B) calibration curve of Sepharose CL-6B.

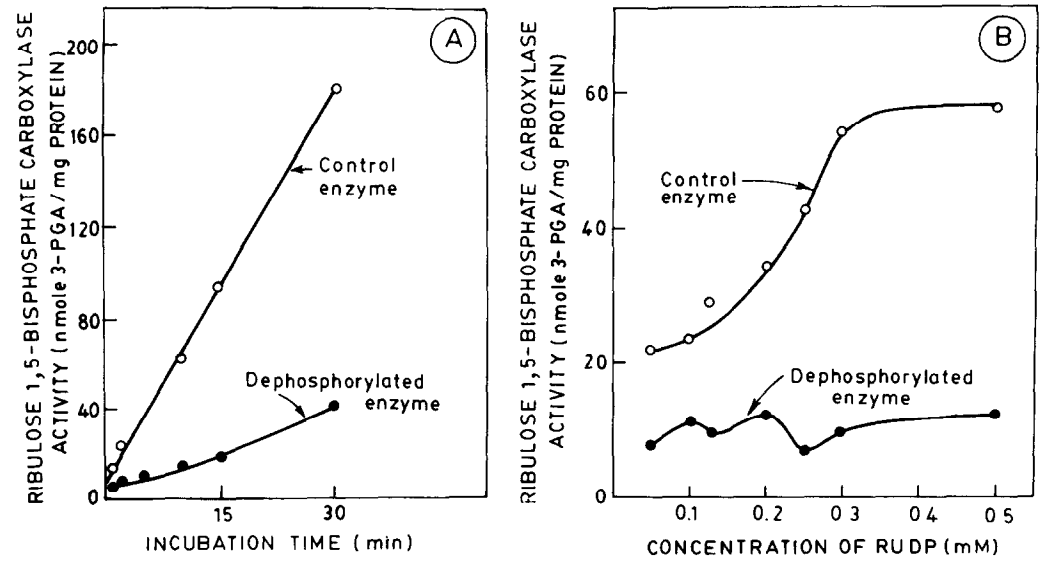

Fig.4. Differential catalytic activity of control (phosphorylated) and dephosphorylated RuBPCase in moss. Enzyme activity as a function of (A) time and (B) RuDP concentration.

the activity of the dephosphorylated enzyme as a function of time. In contrast, no lag phase was observed in the activity of phosphorylated enzyme (fig.4A). The dephosphorylated enzyme also exhibited decreased activity as a function of substrate (RuDP) concentration (fig.4B).

Curiously, the dephosphorylated RuBPCase (450 kDa) differed from the phosphorylated enzyme (525 kDa) in its molecular mass. This was revealed by determining the molecular mass of the enzyme fractions by molecular sieving on Sepharose CL-6B (fig.3A,B). In addition, the dephosphorylated enzyme also exhibited altered electrophoretic mobility on native gels in comparison to the control (not shown). The decrease in molecular mass of dephosphorylated RuBPCase could not be ascribed to any proteolytic activity associated with alkaline phosphatase. This was proved by the fact that phosphatase treatment failed to hydrolyze radiolabelled proteins. Analysis of purified dephosphorylated RuBPCase on SDS-PAG showed only the presence of the large subunit (fig.2d). Thus it turns out that dephosphorylation of RuBPCase resulted in dissociation of the small subunit from the holoenzyme. The small subunit is eliminated when alkaline phosphatase-treated RuBPCase is refractionated on Sepharose CL-6B for the separation and removal of the alkaline phosphatase. Judging from the molecular mass of the large subunit (54 kDa) and that of the dephosphorylated RuBPCase (450 kDa), it was obvious that the dephosphorylated enzyme is an octamer of large subunits. Clearly, the aggregating ability of the large subunits was not affected by the dissociation of the small subunits. Subsequently, the dephosphorylated small subunits were isolated on Sephadex G-150. Fig.5A depicts the profile of phosphorylated enzyme (control), dephosphorylated enzyme and also a profile of alkaline phosphatase. Two absorbance peaks were observed in the phosphatase-treated enzyme fraction. Peak I which eluted soon after the void volume represented a mixture of high molecular mass oligomeric large subunits and alkaline phosphatase. [The dephosphorylated RuBPCase fraction (peak I) was refractionated on Sepharose CL-6B before being electrophoresed on SDS-PAG. This step was necessary for removing alkaline phosphatase.] Peak II represented the dephosphorylated small subunits of RuBPCase

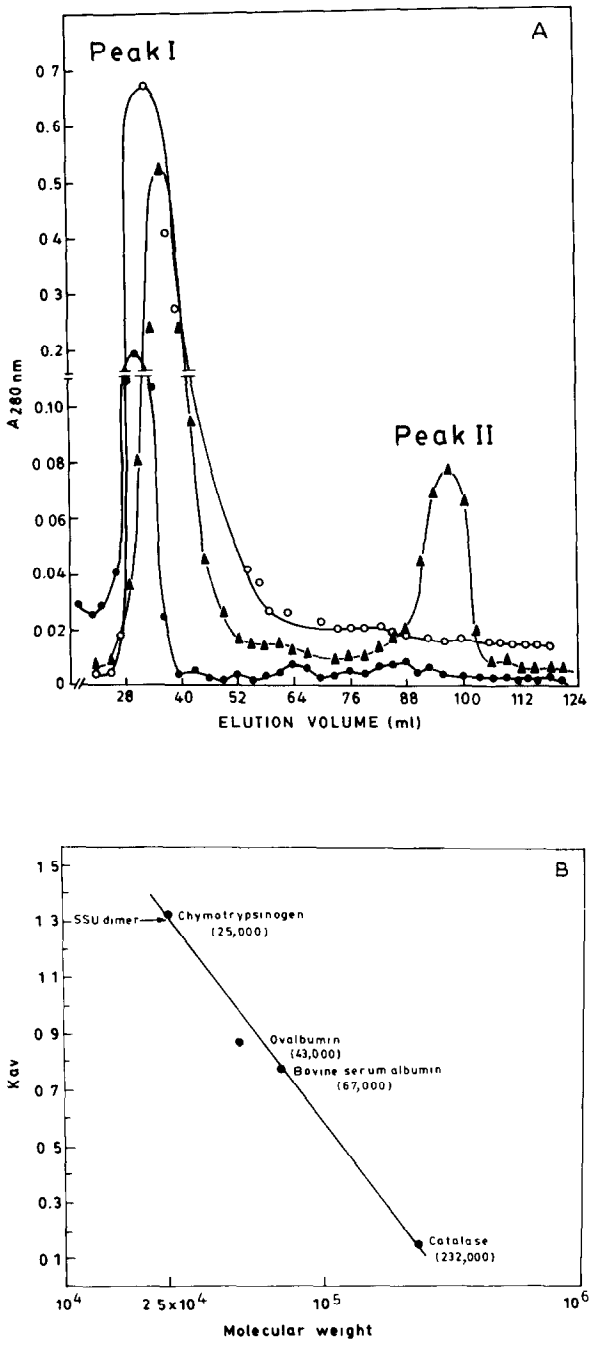

Fig.5. Isolation of dephosphorylated small subunit dimers of spinach RuBPCase on Sephadex G-150. (A) Elution profile of alkaline phosphatase (○—○), phosphorylated RuBPCase (●—●) and alkaline phosphatase-treated RuBPCase (▲—▲); (B) calibration curve of Sephadex G-150.

Fig.6. SDS-PAG pattern of the peak fractions obtained from Sephadex G-150 (see fig.5A). The protein bands were stained with Coomassie brilliant blue R. (a) Control, phosphorylated RuBPCase (peak I); (b) dephosphorylated RuBPCase (peak I) after fractionation on Sepharose CL-6B for the removal of alkaline phosphatase; (c) small subunit fraction (peak II).

(fig.5A). The molecular mass of the fractions in peak II is 28 kDa and hence it is a dimer of small subunits (fig.5B). The SDS-acrylamide gel pattern of peak activities (peak I, II) clearly depicted that dephosphorylation results in the dissociation of small and large subunits of the holoenzyme (figs 5 and 6). These observations strongly support our contention that phosphorylation of small subunits is a prime requirement for the reconstitution of active RuBPCase.

3.4. *In vitro phosphorylation of RuBPCase*

Additional phosphorylation of purified RuBPCase was achieved by incubating the holoenzyme with wheat protein kinase and [γ-^{32}P]ATP. This is tenable, since partial dephosphorylation of proteins can occur during enzyme purification [18]. The in vitro phosphorylation occurred only on the small subunit. All attempts to phosphorylate the dephosphorylated RuBPCase, under in vitro conditions showed no significant incorporation of ^{32}P label into the enzyme fraction (table 1). This was expected as the dephosphorylated RuBPCase was devoid of small subunits. The large subunit is evidently not phosphorylated in vitro, as also observed in our in vivo experiments.

We also phosphorylated purified RuBPCase in vitro with unlabelled ATP with a view to determining whether its biological activity is altered in any manner. However, additional phosphorylation of the small subunit, already associated with the large

Table 1

In vitro phosphorylation of purified RuBPCase in moss and spinach

Additions	^{32}P incorporation (dpm/mg protein)	Relative radioactivity	RuBPCase activity (nmol 3-PGA/mg protein)	Relative activity
Control moss RuBPCase	212635	1.00	36	1.00
Dephosphorylated moss RuBPCase	43620	0.20	10	0.28
Control spinach RuBPCase	309380	1.00	212	1.00
Dephosphorylated spinach RuBPCase	56520	0.18	40	0.19

The dephosphorylated enzyme showed no significant phosphorylation and exhibited lowered biological activity

catalytic subunit, failed to enhance the enzyme activity, thereby indicating that phosphorylation per se is not directly responsible for the modulation of RuBPCase (not shown).

4. DISCUSSION

The present investigation has revealed that phosphorylation of RuBPCase in moss and spinach plays a crucial role in the assembly of small and large subunits and is consequently vital for the regulation of enzyme activity. Although phosphorylation of both small and large subunits of RuBPCase was reported in crude enzyme preparations from other laboratories [1–8], it was not ascertained whether both the subunits occur in a phosphorylated form in nature. Further, nothing was stated about the physiological significance of phosphorylation of the two subunits. We have now conclusively demonstrated that RuBPCase in moss and spinach is a phosphoprotein by labelling the enzyme with [^{32}P]orthophosphate in vivo. The ^{32}P label was selectively incorporated into the small subunit of the holoenzyme. This was revealed by autoradiography of the labelled enzyme after fractionation on SDS-PAG. Furthermore, the ^{32}P label was recovered as phosphoserine and phosphothreonine from the purified RuBPCase. Thus, the earlier reports [1–8] which claimed phosphorylation of small and large subunits under in vitro conditions do not obviously represent the physiological state of the enzyme.

A novel approach was designed to ascertain the possible role of phosphorylation of the small subunit of RuBPCase. The structure-function relationship was determined by dephosphorylating purified RuBPCase with alkaline phosphatase. Dephosphorylation of RuBPCase resulted in the dissociation of small subunits from the catalytic large subunits. Concomitantly, there was a dramatic loss of biological activity of the enzyme. This is in agreement with earlier observations in pea and spinach where reconstitution experiments strongly suggested that the large subunits are catalytically more competent when present in association with the small subunits [19]. However, the true relevance of phosphorylation of the small subunit was altogether missed in these studies. Our findings have clearly shown that association of large catalytic subunits with small subunits is feasible only when the small subunits are present in the phosphorylated state. Dephosphorylation resulted in the dissociation of large and small subunits. Such dissociation did not affect the aggregating ability of the large subunits as these could be isolated as octamers. On the other hand, the dephosphorylated small subunits dissociated into a dimeric form. We propose that the reconstitution of the two types of subunits, though crucial to enzyme activation, is inherently dependent on the selective phosphorylation of the small subunit of RuBPCase. Nevertheless, phosphorylation per se does not seem to modulate RuBPCase activity, since additional phosphorylation of the small subunits already associated with the large subunits (holoenzyme) failed to alter the enzyme activity. Thus, the prime function of phosphorylation of the small subunit is to promote tight binding with the large subunits. We predict that the protein-protein interaction is responsible for the enhancement of catalytic activity of the large subunits of RuBPCase.

It is pertinent to mention the recent reports of light-mediated activation of RuBPCase in *Phaseolus* and *Nicotiana* [20,21]: the activity of RuBPCase was inhibited in the dark. This was ascribed to the binding of a phosphate ester at the catalytic site of the active form of the enzyme. Treatment of enzyme-inhibitor complex with alkaline phosphatase restored RuBPCase activity, possibly by hydrolysing the phosphate ester. However, this mechanism of regulation of RuBPCase involving dark-light conditions was not observed in spinach, maize and pea [20].

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