

Regulation of Fructose 1,6-Bisphosphatase Activity of *Chlorella* by Mole Mass Change

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Dedicated to Professor Wolfgang Kowallik on the occasion of his 65th birthday

Chlorella, Fructosebisphosphatase, Oligomerization, Activation, Kinetic Properties

Fast protein liquid chromatography on Superose 6 of partially purified FBPase II from *Chlorella* reveals a 1350 kDa-form at pH 6.0 and a 67 kDa-form at pH 8.5. Treatment of the large enzyme form with 5mM concentrations of Mg^{2+} , F1,6P₂, DTT or ATP leads to dissociation into smaller ones of 215–470 kDa.

Aggregation/dissociation is a reversible process, as has been shown for the effect of F1,6P₂ and of pH, by rechromatography.

The change in mole mass results in alterations of the activity and of the kinetic properties of the enzyme forms, obtained.

Dissociation results in a 4–6 fold increase in activity, as can be shown for F1,6P₂-treated samples.

Halfsaturation constants, as well as the degree of cooperativity of the 67- and the 1350-kDa form, are different for substrate affinity, activation by Mg^{2+} and DTT, and for inhibition by ATP. Both enzyme forms hydrolyse fructose 1,6 bisphosphate and seduheptulose 1,7 bisphosphate better than other phosphorylated compounds. The ratio of F1,6P₂- to SDP-cleavage is 100:58 for the small enzyme form and 100: 84 for the large one.

Activation of FBPase II in the light and inactivation in the dark is discussed on the basis of different oligomeric forms of the enzyme, generated by changes in the concentration of intermediates and effectors in the chloroplast stroma, leading to dissociation or aggregation. The conclusion is drawn that oligomerization of key enzymes, resulting in enzyme forms with different activities and different kinetic properties, might provide an effective mechanism for enzyme regulation in vivo.

Introduction

In autotrophic organisms the metabolism has to be adapted to the continuous changes of light and darkness. In the light, reserve carbohydrates are accumulated, whereas in darkness these reserves have to be metabolized for energy support. Probable candidates of this regulation are enzymes, which are rate limiting in metabolic sequences. Alterations in their activities can be achieved by different mechanisms. The most prominent ones concern

the rate of synthesis and degradation, as well as quick changes in the level of effectors.

Recently, aggregation and dissociation processes have been described, which lead to alterations in activities and kinetic properties of *Chlorella* enzymes (Grotjohann *et al.*, 1991; 1993; Ruyters *et al.*, 1991). Large enzyme forms contain an identical species in some complex probably held together by ionic forces (Frieden, 1971). One of those enzymes is fructose 1,6-bisphosphatase (FBPase), two forms of which can be separated in extracts of *Chlorella* (Grotjohann *et al.* 1993). One of them (FBPase I) participates in the gluconeogenesis in the cytoplasm, the other form (FBPase II) in the regeneration of ribulose 1,5 bisphosphate in the chloroplast (App and Jagendorf, 1964; Pedersen *et al.*, 1966; Buchanan *et al.*, 1967).

In several organisms, the chloroplast enzyme has been found to be modulated by pH-dependent monomer-dimer (Buchanan *et al.*, 1976) or dimer-tetramer interconversion in vitro (Lazaro *et al.*,

Abbreviations: FPLC, fast protein liquid chromatography; DTT, dithiothreitol; G1P, glucose 1 phosphate; G3P, glyceralate 3 phosphate; G6P, glucose 6 phosphate; F6P, fructose 6 phosphate; F1,6P₂; fructose 1,6 bisphosphate; SDP, seduheptulosebisphosphate; R5P, ribulose 5 phosphate; AMP adenosin monophosphate; ATP, adenosin triphosphate.

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1975; Zimmermann *et al.*, 1976; Gerbling *et al.*, 1985). In crude extracts of *Chlorella*, the influence of pH on the mole mass is much stronger. Dissociation of a 1350 kDa FBPase II, induced by alkaline pH, results in 67 kDa-enzyme forms with enhanced activity and altered substrate affinity (Grotjohann *et al.*, 1993).

In the following article we report about the influence of $F_1,6P_2$, Mg^{2+} , ATP, DTT and pH on the mole mass of partially purified FBPase II and about the properties of the respective enzyme forms. A reversible process is discussed, which leads to the activation of FBPase II by dissociation during photosynthesis and to inactivation due to aggregation in the dark.

Material and Methods

Organism and growth conditions

The experiments were performed with the unicellular green alga *Chlorella kessleri* Fott et Nováková no. 211–11h of the Culture Collection of Algae of the Institute for Plant Physiology at Göttingen University, Germany. The algae were grown in an inorganic medium according to (Ruppel, 1962) with iron supplied as an EDTA-complex. Culture tubes (4 cm diameter, 45 cm length), aeration with air +2% CO_2 and the light thermostat used (30 ± 1 °C), have been described by Kuhl and Lorenzen (1964).

Light regimes

Cells were grown in strong white light of three fluorescent tubes each, warm white (Osram L 36W/30–1) and cool white (Osram L 36W/20–1). The light intensity used was $245 \mu mol \cdot m^{-2} \cdot s^{-1}$, approximating 10 000 lux.

Preparation of crude cell extracts

Chlorella cells were washed twice with distilled water ($4300\times g$) and the resulting sediments were resuspended in 50 mM Hepes-NaOH buffer pH 8.5 or 6.0 (as indicated in the text) to a final density of 200 μl packed cell volume ml^{-1} , determined with microhematokrit tubes. The algae suspension was mixed with 1.6 volumes of glass beads (0.5 mm) and shaken for 10 min at 0–4°C in a Vibrogen cell mill (Bühler, Tübingen, Germany). The resulting homogenate of broken cells was centrifuged in a

Sorvall refrigerated centrifuge RC-5 (DuPont, Newton, CT, USA) at $27\ 000\times g$ for 20 min at 4°C and the supernatant (crude extract) was used for further purification.

Heat precipitation

Crude extracts prepared with 50 mM Hepes buffer pH 6.0 were incubated at 60°C for 10 min.. Approximately 40% of batch protein was precipitated and removed by subsequent centrifugation $27\ 000\times g$ for 15 min at 4°C. Sorvall refrigerated centrifuge RC-5), leaving FBPase protein in the supernatant.

Ion exchange chromatography

The FBPase form, assumed to originate from the chloroplast (Grotjohann *et al.*, 1993), was separated by ion exchange chromatography on DEAE cellulose 23 SN (column 2.2 x 8 cm) equilibrated with Hepes buffer pH 6.0 (50 mM) containing 5 mM DTT. After washing with two bed volumes of this buffer, the column was developed with a linear KCl gradient (0–0.6 M). Two ml fractions were collected at a flow rate of 80 ml/h. KCl density in the fractions was calculated from the index of refraction, by using an Abbe universal refractometer (Schmid and Haensch, Berlin).

The most active fractions of FBPase II were pooled, concentrated and desalted by washing with Hepes buffer pH 6.0 (50 mM), using a stirred ultrafiltration cell (Modell 8050), Amicon Inc. Beverly, MA 01915 USA with diaflow ultrafiltration membranes PM 10. Alkaline buffers were avoided because of the low stability of desalted FBPase fractions.

Fast protein liquid chromatography

For fast protein liquid chromatography (FPLC) superose 6 columns have been used as described in Kowallik *et al.* (1990). The flow rate was 20 ml $\cdot h^{-1}$, 0.25 ml fractions were collected and assayed for enzyme activity. For native mole mass determination the column was calibrated with the following standards: Blue dextran 2000 kDa, thyroglobulin dimer 1340 kDa, thyroglobulin 670 kDa, ferritin 440 kDa, catalase 232 kDa, aldolase 160 kDa, bovine serum albumin 67 kDa, ovalbumin 47

kDa, chymotrypsinogen 25 kDa, and ribonuclease 13.7 kDa.

The buffer systems applied for the equilibration of the columns and for the elution of the protein are given in the respective figure legends. Effector treated FBPase samples were incubated for 10 min on ice prior to separation.

Enzyme assay

FBPase activity was measured in a coupled test by following the reduction of NADP⁺ by glucose-6-phosphate dehydrogenase. Glucose 6-phosphate was produced by phosphoglucose isomerase from fructose 6-phosphate, deriving from the FBPase reaktion. Assay mixture concentrations: Hepes buffer pH 8.25 (69 mM), EDTA 0.5 mM, MgSO₄ 16 mM, NADP⁺ 0.5 mM, DTT 2 mM, phosphoglucose isomerase 2U, glucose 6-phosphate dehydrogenase 1U, crude extract 25 µl or separated fractions 200 µl respectively, fructose 1,6-bisphosphate 0.5 mM (start).

For to test the specificity of FBPase forms, P_i release was determined according to (Lanzetta and Alvarez, 1979), deriving from different phosphorylated compounds, hydrolysed in the test system above.

Results and discussion

Influence of different effectors on the mole mass of FBPase II

To prove the regulatory competence of the aggregation/dissociation process for the chloroplast FBPase II, possible effectors, whose concentrations change during light and darkness, as well as pH, were tested with the partially purified enzyme.

Without effector treatment, the mole mass of concentrated and desalted FBPase II fractions is identical with the one in crude extracts. Analysed by superose 6 FPLC at pH 6.0, a FBPase form of 1350 kDa can be separated, while extraction and separation at pH 8.5 reveals a 67-kDa form. As has been shown previously for different pH-values (Grotjohann *et al.* 1993), FBPase II of *Chlorella* may exist in various oligomeric forms. Addition of Mg²⁺, F1,6P₂, DTT or ATP to FBPase II samples, prepared at pH 6.0, all cause dissociation of the 1350 kDa FBPase into smaller forms of. 470–215 kDa, (Fig 1). The smallest form of 67 kDa could not be obtained by effector treatment alone.

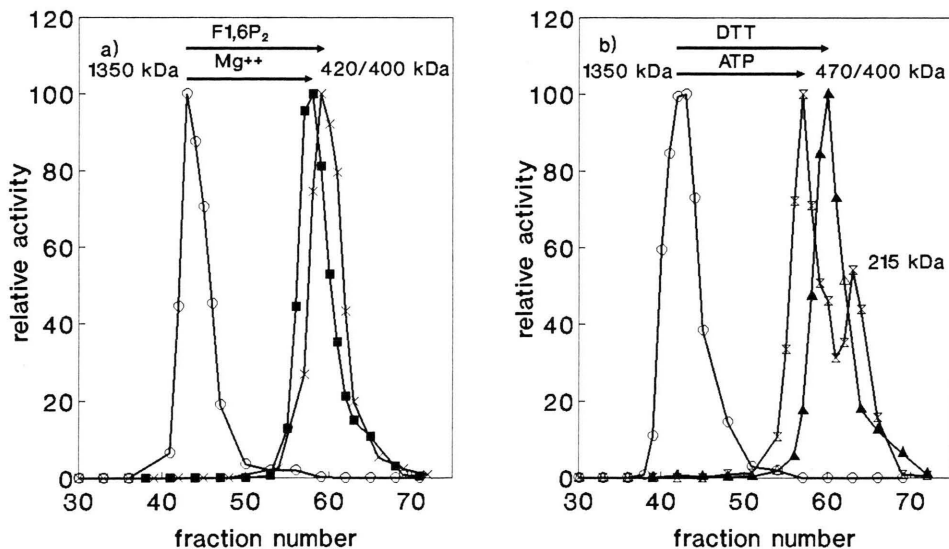


Fig. 1. Influence of F1,6P₂ (—×—), Mg²⁺ (—■—), DTT (—▲—) or ATP (—⊗—) on the mole mass of FBPase II of *Chlorella kessleri*. The figure shows the distribution of the relative enzyme activity after FPLC on superose 6, performed with 50 mM Hepes buffer pH 6.0, without (—○—) or with effectors at 5 mM concentrations. Specific activities are given in the text.

Is aggregation/dissociation a reversible process?

Assuming a biological relevance of the described mole mass change, reversibility of the process should be expected. This was tested for the influence of pH and F1,6P₂. Concentrated fractions of the large form of FBPase II, obtained FPLC at pH 6.0 were adjusted to pH 8.5 by addition of Tris (0.5M) and rechromatographed at this pH. The shift in pH resulted in a dissociation of the 1350 kDa enzyme to the low mole mass form of 67 kDa (Fig 2a). Vice versa, aggregation occurred, when the pH of concentrated FPLC samples of the small form were adjusted from pH 8.5 to pH 6.0 by addition of Hepes (0.5M), (Fig 2b).

Comparable results were found for the influence of F1,6P₂. The small enzyme forms obtained by F1,6P₂ treatment, were pooled and dialysed in an ultrafiltration cell. Rechromatography in the absence of substrate results in the large enzyme form again (Fig. 3a). Subsequent addition of F1,6P₂ to pooled fractions of this large enzyme form reveal the 67 kDa enzyme again (Fig. 3b). At this stage of purification no non-enzyme protein could be detected by UV-absorption during separation (Fig. 3b). Large enzyme forms are probably held together by ionic forces, which in contrast to covalent bounds can easily be influenced by effec-

tors. The observed reversibility of oligomerization indicates the process as a fast and economical means of regulation for the chloroplast FBPase of *Chlorella* which should have consequences for the properties of the enzyme, too.

Properties of oligomeric forms of FBPase II

Catalytic activity

Dissociation of FBPase induced by addition of effectors (Fig. 1), resulted in an increase in activity from app. $1.300 \mu\text{mol} \cdot \text{min}^{-1} \cdot \text{mg protein}^{-1}$ of the control, to 5.800- for F1,6P₂-, to 2.400- for Mg²⁺-, to 1.600- for ATP- and to 2.800 $\mu\text{mol} \cdot \text{min}^{-1} \cdot \text{mg protein}^{-1}$ for DTT-treatment. Activities given are mean values of the five best fractions of each separation. A comparable enhancement (5.6-fold) could be shown for the F1,6P₂ treated samples of Fig. 3b after rechromatography. No increase was found for the small enzyme form separated at pH 8.5 (Fig. 2), because of low stability of desalted enzyme-fractions at alkaline pH.

The effector-induced dissociation of the 1350-kDa FBPase into smaller forms is one possible way to activate the *Chlorella* enzyme. Further regulatory competence of the oligomerization process

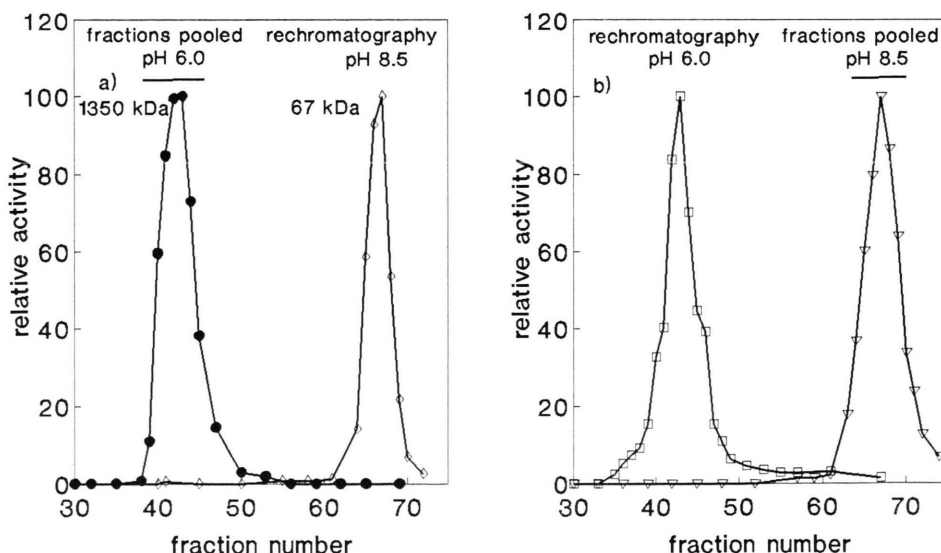


Fig. 2. Rechromatography of pooled and concentrated fractions of FBPase II ($1.280 \mu\text{mol} \cdot \text{min}^{-1} \cdot \text{mg protein}^{-1}$) on superose 6. a) samples obtained at pH 6.0 (—●—), were adjusted and rechromatographed at pH 8.5 (—◇—) b) samples obtained at pH 8.5, $1.307 \mu\text{mol} \cdot \text{min}^{-1} \cdot \text{mg protein}^{-1}$ (—▽—) were adjusted and rechromatographed at pH 6.0 (—□—). FPLC was performed with 50 mM Hepes buffer.

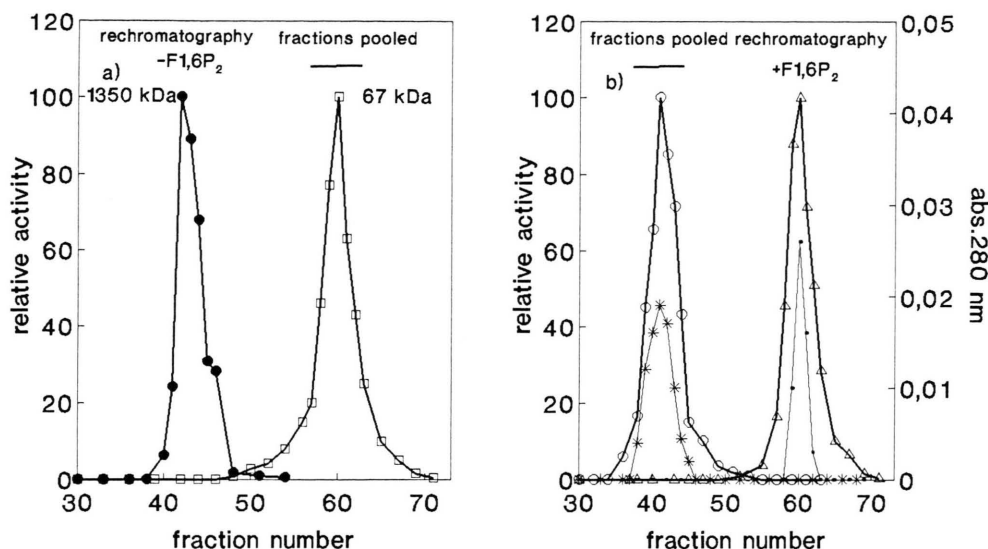


Fig. 3. Rechromatography of pooled and concentrated fractions of FBPAse II from *Chlorella kessleri* on superose 6. a). Samples obtained at pH 6.0 ($6.261 \mu\text{mol} \cdot \text{min}^{-1} \cdot \text{mg protein}^{-1}$) in the presence of 5 mM F1,6P₂ (\square) were dialysed and rechromatographed at the same pH without substrate (\bullet). b) samples obtained at pH 6.0 (\circ) specific activity $1.119 \mu\text{mol} \cdot \text{min}^{-1} \cdot \text{mg protein}^{-1}$ were treated with 5 mM F1,6P₂ and rechromatographed at pH 6.0 in the presence of 5 mM substrate (\triangle). FPLC was performed with 50 mM Hepes buffer. Protein pattern of samples separated at pH 6.0 (\ast) and after F1,6P₂ treatment (\bullet).

could be expressed by different kinetic properties of the enzyme forms.

Kinetic properties

Halfsaturation constants and Hill coefficients were determined for the 1350-kDa form and for the 67-kDa form, respectively, separated by FPLC at pH 6.0 and 8.5, (Tab. I). From the known regulatory factors of FBPAse, we chose F1,6P₂, DTT, Mg²⁺ and ATP. Both enzyme forms differ in K_m - ($S_{0.5}$)- values for the substrate F1,6P₂ and for Mg²⁺. For F1,6P₂, Hill coefficients indicate positive cooperativity (1.8) for the small form and menten kinetics for the large one. At saturating F1,6P₂ concentrations, the requirement for Mg²⁺ for half maximal activity is lower for the large form (0.58 mM), with slightly negative cooperativity ($n=0.73$), than for the small one (1.52 mM) with $n=1.1$. At high concentrations of Mg²⁺, both enzyme forms were inhibited. No significant differences in the degree of cooperativity were found ($n=2.4-2.3$).

For to be reduced to half maximal activity, the 67 kDa-forms need app. 55% more Mg²⁺, than the large form. Inhibition of the latter by ATP re-

quires app. half the amount (2.86mM) as for the 67 kDa-enzyme (6.07mM).

Assuming that the FBPAse II originates from the chloroplast, the *Chlorella* enzyme should be activated by the reductant DTT (Grotjohann 1993; Grotjohann *et al.*, 1993). This activation could only be shown for the small form, which reached half maximal activity at a concentration of 0.05 mM

Table I. Kinetic and regulatory properties of the large (1350 kDa) and the small form (67 kDa) of FBPAse II, separated from crude extracts of *Chlorella kessleri* by FPLC on superose 6. $S_{0.5}$ = concentration for half maximal velocity, $I_{0.5}$ = concentration for 50% inhibition, n (n_i) = Hill coefficient. Data are mean values of 5–9 experiments. no resp. = no influence on FBPAse activity.

positive effector/ substrate	Large form		Small form	
	$S_{0.5}$ [mM]	n	$S_{0.5}$ [mM]	n
F1,6P ₂	0.08	1.10	0.23	1.80
DTT	no resp.	no resp.	0.05	0.90
Mg ²⁺ low	0.58	0.73	1.52	1.10
negative effector	$I_{0.5}$ [mM]	n_i	$I_{0.5}$ [mM]	n_i
Mg ²⁺ high	43.70	2.40	68.40	2.30
ATP	2.80	0.90	6.07	2.00

DTT, while the large FBPase was unaffected. Thioredoxin modulation seems to be an important mechanism for to regulate the enzyme, since DTT is capable for both dissoziation and activation. *In vivo* this might be mediated by the thioredoxin system, which has been shown to be present in *Chlorella* (Tsang, 1981).

Summing up, these results demonstrate that changes in mole mass of FBPase II cause pronounced differences in the kinetic properties of the respective enzyme forms.

Substrate specificity

For higher plants, a change in the specificity of chloroplast FBPase for SDP only, to both SDP- and F1,6P₂ has been observed during monomer/dimer interconversion *in vitro* (Buchanan *et al.*, 1976). The 1350-kDa and the 67-kDa form of the *Chlorella* enzyme, tested for P_i-release from different phosphorylated compounds, hydrolysed F1,6P₂ best, followed by SDP, (Fig.4). The ratio of F1,6P₂ to SDP cleavage was 100:58 for the small enzyme form and 100:84 for the large one. Compared with the 1350-kDa form, the 67 kDa-enzyme possesses higher specificity towards fructose- and seduheptulosebisphosphate, than to the other phosphorylated substances.

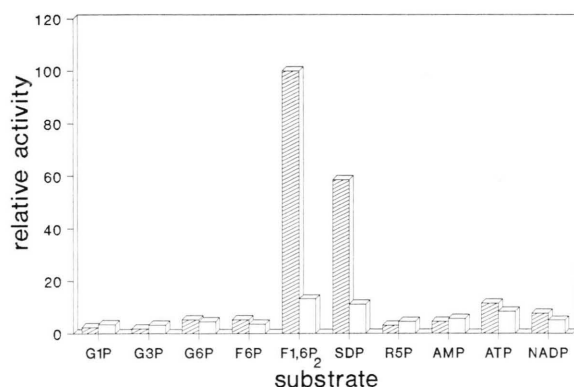


Fig. 4. Specificity of the small (▨) and the large (□) FBPase II form, separated from crude extracts of *Chlorella kessleri* by FPLC superose 6 at pH 8.5 or pH 6.0 using 50 mM Hepes buffer. Activity was determined by P_i-release, deriving from different phosphorylated substrates. Activity of the small enzyme form (217 μmol · min⁻¹ · mg protein⁻¹) was set 100.

Conclusion

Altogether, the results presented clearly demonstrate that the oligomerization of FBPase II is a reversible process, generating enzyme forms with pronounced differences in their activities and in their regulatory properties.

Under the influence of light/darkness, the concentration of regulatory factors, capable of dissociating/aggregating FBPase, are changed (Lilley *et al.* 1977), and thereby might cause the activation/inactivation of the enzyme in the chloroplast stroma. Since intact chloroplasts cannot be isolated from the *Chlorella* strain used, we have no data on pool sizes of enzyme effectors in the stroma, or changes in pH during light and darkness. Only comparison with data from other organisms can be used as an indirect approach. In spinach chloroplasts the pH in the stroma increases, because of proton transfer into the locus (Heldt *et al.*, 1973), whereas the Mg²⁺-content is increased in the stroma (Portis and Heldt, 1976; Heldt, 1977). Reducing power is available from the thioredoxin system, and F1,6P₂ is derived from the Calvin cycle.

The effector concentration consequently used for all experiments was 5mM, which is, in the case of ATP and F1,6P₂, far above the *in vivo* concentration (Lilley *et al.*, 1977). In our *in vitro* system, lowering the concentration of ATP or of F1,6P₂ to 1 mM, leads to the same results (dissoziation of FBPase II).

We have no information on the combined action of the effectors acting together, which might occur in the stroma. We expect, that in the light dissociating conditions lead to the activation of FBPase II, a process that might be reversed easily in the dark.

For other *Chlorella* enzymes like phosphofructokinase and pyruvatekinase, the influence of metabolites on the mole mass of the enzymes has been shown previously (Grotjohann *et al.*, 1991; Ruyters *et al.*, 1991). The light-dependent changes of effectors, found in whole cell extracts a few minutes after the onset of illumination (Kowallik and Scheil, 1976; Ruyters, 1988), support the idea that light may regulate the metabolism via formation or dissociation of oligomeric enzyme forms (Grotjohann *et al.*, 1991). Thereby oligomerization would be an effective mechanism for the fine control of key enzymes.

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