

Different Forms of Fructose 1,6-Bisphosphatase in *Chlorella*

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In crude extracts of *Chlorella kessleri* two forms of fructosebisphosphatase can be separated by ion exchange chromatography or by acid precipitation. FBPase I is eluted from DEAE cellulose at 200 mM KCl and precipitated at pH 4.5, FBPase II is correspondingly eluted at 310 mM KCl and soluble at pH 4.5. Both enzymes differ in substrate affinity and degree of cooperativity. Based on literature data, FBPase I is assumed to be of cytosolic and FBPase II of chloroplastic origin. The mole mass of FBPase I is identical (51–65 kDa) at pH 6.5 and pH 8.5. That of FBPase II is however, about four times larger at pH 6.5 (257 kDa) than at pH 8.5 (67 kDa). Other pH values have only been tried with crude cell extracts in which still larger mole masses of FBPase resulted at more acidic pH (1349 kDa at pH 6.0). The lower mole mass form of FBPase II shows three times higher catalytic activity. Reducing agents, such as DTT, also increase the activity of FBPase II *in vitro*.

In vivo, alkalization and production of reducing power occurs in the chloroplast stroma during illumination. If the above alterations exist *in vivo*, they would be a means to activate FBPase in the light.

Oligomerization of FBPase II to aggregates with altered catalytic activities and kinetic properties is discussed as result of the action of specific wavelengths and to be responsible for differences in carbohydrate metabolism of *Chlorella* exposed to red or blue light.

Introduction

Specific wavelengths of light affect the overall composition of green algae. Blue light leads to higher protein contents, while red light results in accumulation of carbohydrates [1, 2]. These differences are independent of photosynthesis [3]. They seem to derive from light-mediated alterations of the activity of regulatory enzymes. Influence on the synthesis of enzyme protein [4, 5] and on concentrations of enzyme effectors [6], but also on aggregation and dissoziation processes have been discussed recently. Thus far, wavelength-dependent oligomerizations resulting in alterations in kinetic properties have been described only for the regulatory glycolytic enzymes phosphofructokinase and pyruvate kinase [7–9].

In photosynthetic organisms there is another regulatory enzyme in carbohydrate metabolism: fructose 1,6-bisphosphatase (FBPase). This en-

zyme exists in two forms. One form participates in gluconeogenesis in the cytoplasm, the other form is involved in the regeneration of ribulose 1,5-bisphosphate in the chloroplast [10–12].

For the chloroplastic enzyme, a pH-dependent dimer-tetramer interconversion has been observed *in vitro* [13, 14]. Since there are pH changes in the chloroplast stroma during photosynthesis, association/dissociation processes might occur and be a means of regulation of FBPase *in vivo* as well.

In the following article, an attempt will be made to obtain data on FBPases in *Chlorella* cells and to elucidate ways of their regulation. Interest will be focussed on oligomerization processes. Beside their general importance, this information might help to elucidate the influence of different wavelengths of light on the cells' metabolism.

Materials and Methods

Organisms and growth conditions

The experiments were performed with the unicellular green alga *Chlorella kessleri* Fott et Novákova 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 [15]

Abbreviations: FPLC, fast protein liquid chromatography.

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with iron being supplied as EDTA complex. Culture tubes (4 cm diameter, 45 cm length), aeration with air + 2% CO₂ and the light thermostat used (30 ± 1 °C) have been described by [16].

Light regimes

Cells were grown in white light of equal numbers of warm white (Osram L 36 W/30-1) and cool white (Osram L 36 W/20-1) fluorescent tubes. The light intensity used was 245 µmol·m⁻²·s⁻¹, which corresponds to approximately 10,000 lux.

Preparation of crude cell extracts

Crude cell extracts were prepared as described in [7], except for the use of 100 mM (N-(2-hydroxyethyl)piperazine-N-2-ethanesulfonic acid) buffer (Hepes) pH 7.5 according to [17]. The buffer contained 10 mM EDTA, 10 mM MgCl₂ and dithiothreitol (DTT) in concentrations as indicated in the text.

Acid precipitation

For acid precipitation the pH of the crude cell extracts was slowly lowered to pH 4.5 by adding 1 M acetic acid during continuous cooling on ice [17]. The precipitate was pelleted by centrifugation at 15,000 × *g* for 10 min (4 °C). The resulting supernatant was neutralized by 1 M Hepes buffer before used for experiments. The precipitate was resolved in extraction buffer for 60 min and separated from insoluble material by another centrifugation at 15,000 × *g* (10 min, 4 °C).

Ion exchange chromatography

FBPase isoforms were separated by ion exchange chromatography on DEAE cellulose 23 SN (column 2.2 × 8 cm), using elution buffer according to [17]. After washing the column with one bed volume equilibration buffer, it was developed with a linear KCl gradient (0–0.6 M). 2 ml fractions were collected at a flow rate of 80 ml·h⁻¹. KCl density in the fractions was calculated from the index of refraction, using an Abbe universal refractometer (Schmid and Haensch, Berlin).

Fast protein liquid chromatography

For fast protein liquid chromatography (FPLC) superose 6 and superose 12 have been used as de-

scribed recently [7]. The buffer systems applied for equilibration of the columns and for elution of the proteins are given in the respective figure legends. The superose 6 column was calibrated with the standards given in [7], the superose 12 column with: cytochrome *c* 12.2 kDa, myoglobin 17.4 kDa, chymotrypsinogen 25.0 kDa, hemoglobin 64.5 kDa, albumin 67.0 kDa, catalase 232.0 kDa, and ferritin 440.0 kDa.

Enzyme assay

FBPase activity was measured in a coupled test by following the reduction of NADP⁺. It resulted from oxidation of glucose 6-phosphate produced by phosphoglucose isomerase from fructose 6-phosphate deriving from fructose 1,6-bisphosphate by FBPase action. 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 2 U, glucose 6-phosphate dehydrogenase 1 U, crude extract 25 µl or separated fractions of 200 µl, fructose 1,6-bisphosphate 0.5 mM (start).

Results and Discussion

Two FBPases in Chlorella

In crude extracts of *Chlorella kessleri*, grown autotrophically in white light, FBPase activity of approximately 50 nmol·min⁻¹·mg protein⁻¹ can be measured. Protein separation by ion exchange chromatography of such extracts shows activity in two fractions. FBPase I is eluted with approx. 200 mM KCl, FBPase II with approx. 310 mM KCl (Fig. 1 a), the total activity being enriched by a factor of about ten.

This corresponds to the observation of two isoforms of FBPase in *Selenastrum*, eluted at 280 or 360 mM KCl, respectively [17].

The two enzyme forms differ in their response to reducing agents: Addition of DTT to the extraction and the elution buffer has no influence on the activity of FBPase I; it results, however, in a strong increase in the activity of FBPase II (Fig. 1 b).

The two FBPase forms of *Chlorella* can also be separated by suitable protein precipitation. Acidification of crude extracts to pH 4.5 results in FBPase I activity in the precipitate and in FBPase

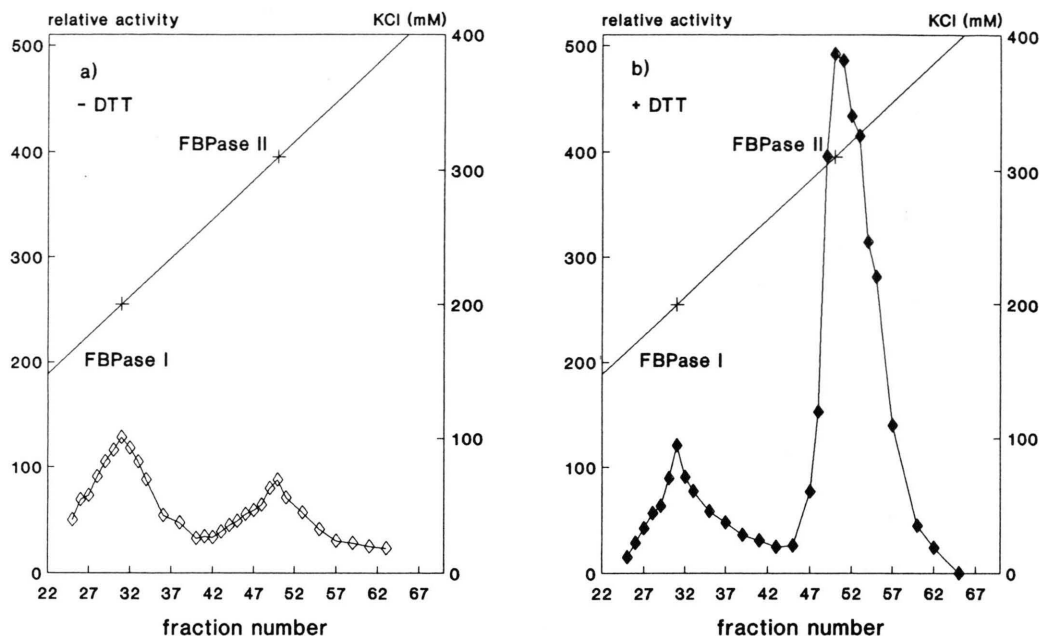
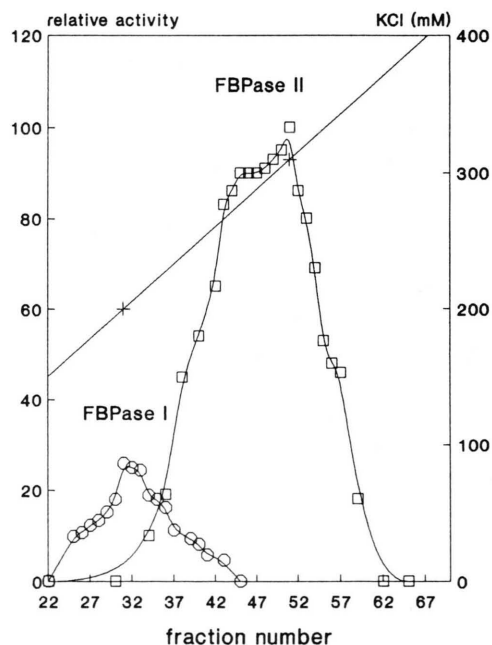


Fig. 1. Distribution of total FBPase activity of crude extracts from *Chlorella kessleri* after ion exchange chromatography on DEAE cellulose, using 20 mM Hepes buffer pH 7.5 containing 2.5 mM EDTA and 2 mM MgCl_2 ; a) without and b) with 5 mM DTT during extraction and elution. Fraction number 31 = $180 \text{ nmol} \times \text{min}^{-1} \times \text{mg protein}^{-1}$, fraction number 50 = $350 \text{ nmol} \times \text{min}^{-1} \times \text{mg protein}^{-1}$.



II activity in the supernatant. Each form can be identified by DEAE chromatography (Fig. 2).

Both enzyme forms differ in substrate affinity and in degree of cooperativity. While for FBPase I an affinity constant of $S_{0.5} = 55 \mu\text{M}$ and a Hill coefficient of $n = 0.5$ have been determined, the respective values for FBPase II are $165 \mu\text{M}$ and $n = 2$.

These different properties suggest different roles of the two FBPases in the metabolism of *Chlorella*.

Localization of FBPase I and FBPase II in *Chlorella*

Since intact organelles cannot be isolated from the *Chlorella* strain used, direct determination of

Fig. 2. Distribution of isolated FBPase I (○-○) and FBPase II (□-□) activity after ion exchange chromatography on DEAE cellulose. FBPase I and II were separated by acid precipitation as indicated in Methods. Elution buffer was 20 mM Hepes buffer pH 7.5 plus 2.5 mM EDTA, 2 mM MgCl_2 and 5 mM DTT.

the distribution of the two FBPases among different cell compartments is impossible. Only comparison with literature data from other organisms can be used as an indirect approach. Isolated chloroplastic isoforms from spinach have been shown to be activated by DTT [12]. In addition, they exhibited positive cooperativities and also lower substrate affinities than cytosolic forms in experiments from refs. [14] and [18]. From this data FBPase II of our *Chlorella* strain would be of chloroplastic origin. Assuming the second form present to be located in the cytosol, as shown for spinach [18, 19], FBPase I would be of cytosolic origin.

Oligomeric forms of FBPase II of *Chlorella*

First information on the existence of various oligomeric forms of FBPase in *Chlorella* came from mol mass determinations by FPLC on superose 6 performed at different pH values. When extraction and FPLC of the enzyme proteins were performed at pH values of 6.0, 6.5, 7.0, 7.5, 8.0 and 8.5 mole masses of 1349, 631, 523, 417, 250 and 88 kDa resulted (Fig. 3).

This observation led to an examination of influences of pH on isolated FBPase I and II. When the pH of both samples obtained by DEAE cellulose chromatography at pH 7.5 was shifted either to pH 6.5 or to pH 8.5 the two isoforms responded differently. Determined by FPLC on superose 12 which led to an additional 5-fold enrichment, FBPase I revealed identical mole masses of 51–65 kDa at both proton concentrations (Fig. 4a). FBPase II, however, showed this low mole mass only at pH 8.5. It had a mole mass of 257 kDa, which is approximately four times larger, at pH 6.5 (Fig. 4b). In addition, alkalization led to an increase in the activity of FBPase II (Fig. 4b). It had no effect on FBPase I activity (Fig. 4a). The latter result indicates regulatory competence of the aggregation/dissociation process. This assumption is supported by further kinetic data. Half maximal activity is reached at 225 μ M with the small form of FBPase II (51–65 kDa), but at 81 μ M with a large one (1349 kDa). The Hill coefficients are 1.8 and 1.1, respectively, indicating positive substrate cooperativity for the small and Menten kinetic for the large enzyme form.

In addition to pH, reducing conditions also produce and/or stabilize a lower molecular mass form with a higher catalytic activity.

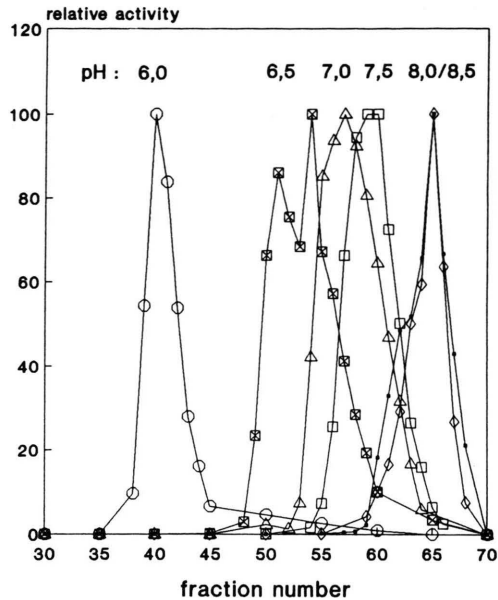


Fig. 3. Influence of pH on the mole mass of FBPase in crude extracts of *Chlorella kessleri*. The figure shows the distribution of enzyme activity after FPLC on superose 6. Preparation of crude cell extracts and FPLC were performed with 50 mM Hepes buffer of pH-values indicated.

In vitro, addition of DTT to a sample of the 1349 kDa form leads to partial dissociation, resulting in an aggregate with a mole mass of approximately 830 kDa (Fig. 5). Different aggregation states of FBPase influenced by reducing agents have also been shown for *Synechococcus leopoliensis* [20, 21]. Whether this is the mechanism of FBPase II activation in *Chlorella* as shown in Fig. 1 remains to be clarified.

Involvement of FBPase oligomerization in carbohydrate metabolism in the chloroplast

While assuming FBPase II to reside in the chloroplast of *Chlorella*, regulatory significance of aggregation/dissociation processes can also be assumed for the enzyme *in vivo*. Being involved in the regeneration of the primary photosynthetic CO₂-acceptor ribulose 1.5-bisphosphate, as stated in Introduction, the enzyme should be activated in the light and inactivated in darkness. Indeed, the activating conditions described above exist in the stroma, the compartment of the enzyme, in the light. The pH value increases because of proton transfer into the inner thylakoid space *via* plastoquinone

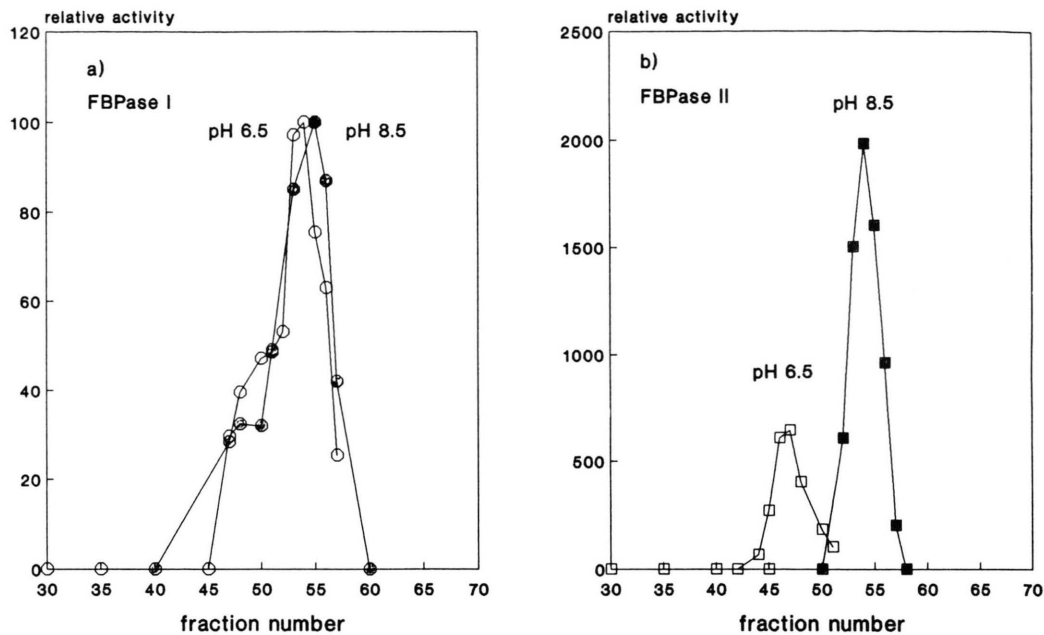
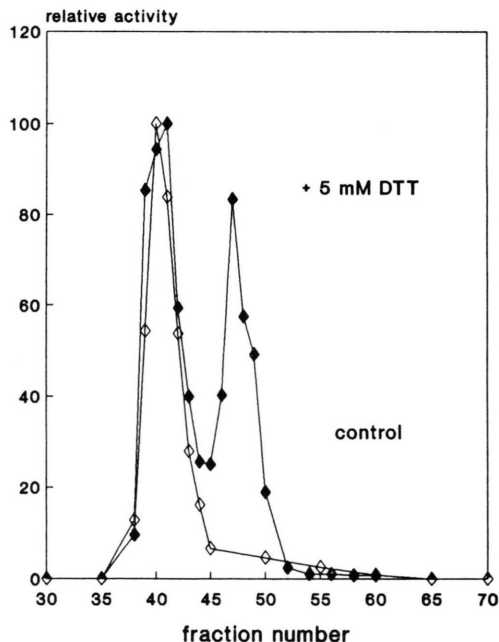


Fig. 4. Influence of pH on the mole masses of FBPases I (a) and II (b). The two isoforms were separated by DEAE cellulose chromatography. Mole mass determinations were performed by FPLC on superose 12. 50 mM Hepes buffer of pH 6.5 or pH 8.5, containing 10 mM EDTA and 5 mM DTT. FBPase II activity in fraction number 47 = $875 \text{ nmol} \times \text{min}^{-1} \times \text{mg protein}^{-1}$, in fraction number 54 = $1650 \text{ nmol} \times \text{min}^{-1} \times \text{mg protein}^{-1}$.



caused by the light-driven electron flow from water to NADP⁺. Applicable reducing power is generated by the ferredoxin-dependent reduction of the thioredoxin system, the electron donor again deriving from light-dependent water splitting. An activating influence of reduced thioredoxin on the enzyme from spinach has been shown by [22] *in vitro*.

Whether there are specific effects of light of different wavelengths on the state of oligomerization of FBPase II cannot yet be answered. First results reveal FBPase II activities two times higher in crude extracts from *Chlorella* cells from red than in those from blue light conditions. If also valid *in vivo*, this could result in comparably less fructose

Fig. 5. Influence of DTT on the mole mass of FBPase II in crude cell extracts of *Chlorella kessleri*. The figure shows the distribution of enzyme activity after FPLC on superose 6. Preparation of crude cell extracts and FPLC were performed with 50 mM Hepes buffer of pH 6.0 without (\diamond - \diamond) or with (\blacklozenge - \blacklozenge) 5 mM DTT.

1,6-bisphosphate in red light, a source for triose-phosphate, the molecule used for the export of photosynthate from the chloroplast. The consequence would be a greater accumulation of carbohydrate inside the chloroplast of red light – than in that of blue light-grown cells. This difference has been reported several times [1, 2]. However, much more information is needed to prove

these conclusions. Corresponding experiments are in progress.

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