

Fructose 1,6-Bisphosphatase in *Chlorella kessleri* Grown in Red or Blue Light

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Total activity of FBPase of *Chlorella kessleri* grown either in blue or in red light of equal fluence rates differs (26.8 and $61.0 \text{ nmol} \cdot \text{min}^{-1} \cdot \text{mg protein}^{-1}$) in crude cell extracts prepared at pH 8.5. The difference largely depends on FBPase II, an enzyme species assumed to be located in the chloroplast. FBPase II is strongly influenced by reducing agents. Addition of DTT, however, does not compensate for the difference in activities. Although it leads to a higher increase (197%) in the activity of the enzyme from blue than in that of the enzyme from red light conditions, the final activities (79.7 and $133.1 \text{ nmol} \cdot \text{min}^{-1} \cdot \text{mg protein}^{-1}$) still differ by a factor of 1.67.

In contrast to alkaline conditions the activity is approx. equal when crude cell extracts are prepared at pH 6.0. It is $62.7 \text{ nmol} \cdot \text{min}^{-1} \cdot \text{mg protein}^{-1}$ in blue light and 70.7 in red light. Under these conditions DTT enhances both activities equally (189 and $201 \text{ nmol} \cdot \text{min}^{-1} \cdot \text{mg protein}^{-1}$). These results indicate that the observed difference in enzyme activity at pH 8.5 does not result from different enzyme quantities.

At pH 8.5 the mole mass of FBPase II is 88 kDa, while it is 1349 kDa at pH 6.0 under both light conditions. The smaller mole mass represents the monomeric form of the enzyme. The previously assumed pH-dependent oligomerization/dissociation [8], which is generally proven, is therefore not responsible for the different activities of the enzyme from cells grown in red or in blue light. However, the monomeric form seems to possess a higher stability when prepared from cells from red light conditions.

The different stability and activity of FBPase II under alkaline conditions are discussed in context with an altered carbohydrate metabolism in *Chlorella kessleri* grown autotrophically in different wavelengths of light.

Introduction

Carbohydrate metabolism in green algae is largely influenced by specific wavelengths of light [1–2]. These light effects are independent of photosynthesis [3]. They are realized by alterations in the activity, and/or the kinetic properties of key enzymes [4, 5]. It has recently been reported that such alterations also result from the aggregation/dissociation of such enzymes in autotrophically grown *Chlorella kessleri* [6–8]. One of these is FBPase II. This enzyme is thought to be located inside the chloroplast, where it plays an important role in the regeneration of ribulose 1,5-bisphosphate. Assuming that it is involved in the accumu-

lation of reserve carbohydrates in this organelle during growth in red light, FBPase II with a high activity would be needed. This might be a form with a low mole mass, the formation of which has been achieved *in vitro* by increasing the pH value [8–11].

In this paper we report on efforts to identify forms of different mole masses of FBPase II in crude cell extracts of *Chlorella kessleri* grown autotrophically either in red or in blue light of equal fluence rates.

Materials and Methods

Organism and growth conditions

The experiments were performed with the unicellular green alga *Chlorella kessleri* Fott et Novákova no. 211-11 h of the Culture Collection of Algae of the Inst. for Plant Physiology at Göttingen University, Germany. The algae were grown in an

Abbreviations: FPLC, fast protein liquid chromatography; DTT, dithiothreitol; DEAE, diethylaminoethyl.

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inorganic medium according to [12] with iron added as an 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 [13].

Light regimes

Cells were grown in red or in blue light leading to an equal dry matter production. The light intensity used was $33.2 \mu\text{mol} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$ for both light qualities. Red and blue light were produced by passing either the radiation of Osram-L 36 W/36 natura fluorescent tubes through a 3 mm red plexiglass sheet or that of Osram-L 11 W/20 cool white fluorescent tubes through a 3 mm blue plexiglass sheet (Röhms GmbH, Darmstadt, Germany).

Preparation of crude cell extracts

Crude cell extracts were prepared as described in [6], except for the use of 50 mM Hepes-NaOH buffer pH as indicated in the text.

Ion exchange chromatography

FBPase I and FBPase II were separated by ion exchange chromatography on DEAE cellulose 23 SN (column 2.2×8 cm) using 50 mM Hepes buffer (containing 5 mM DTT) for elution at pH 7.5 or 6.0, respectively. The column was washed with two bed volumes of this buffer and developed with a linear KCl gradient (0–0.6 M). Two ml fractions were collected at a flow rate of 80 ml/h as described recently [8]. 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 has been used as described in [6]. The buffer systems applied for the equilibration of the columns and for the elution of the proteins are given in the respective figure legends.

Enzyme assay

FBPase activity was measured in a coupled test by following the reduction of NADP⁺ by glucose-6-phosphate dehydrogenase deriving from fruc-

tose 6-phosphate, which is produced by FBPase from fructose 1,6-bisphosphate.

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-phosphatedehydrogenase 1 U, crude extract 25 μl or separated fractions 200 μl and fructose 1,6-bisphosphate 0.5 mM (start).

Determination of half life

The semilogarithmic plotting of FBPase activity against the incubation time at 45 °C results in a straight line, which indicates first order kinetics for the temperature dependent inactivation. Therefore, inactivation can be described by the equation $N_t = N_0 \cdot e^{-kt}$ (N_t and N_0 : activities at time t and $t = 0$; t : time; k : first order rate constant). Half life $t_{1/2}$ is calculated by the equation $t_{1/2} = \ln 2/k$.

Results and Discussion

FBPase of *Chlorella kessleri* at acidic pH

The total activities of FBPase in crude cell extracts of *Chlorella kessleri* grown autotrophically in blue or in red light of equal fluence rates are identical. Cell extracts prepared at pH 6.0 and tested at optimized conditions – including the presence of DTT – yield FBPase-activities of $189 \text{ nmol} \cdot \text{min}^{-1} \cdot \text{mg protein}^{-1}$ and of $201 \text{ nmol} \cdot \text{min}^{-1} \cdot \text{mg protein}^{-1}$, respectively. The major part of this activity derives from FBPase II. This can be shown by ion exchange chromatography. It reveals two activity peaks, one of which is only very low and represents FBPase I in the cytosol. FBPase II however, is probably located in the chloroplast. Henceforth, data for the total FBPase in crude cell extracts represent mainly activities of FBPase II. FPLC on superose 6 reveals identical mole masses for FBPase of cells from both light conditions. There is only one peak with FBPase activity at 1349 kDa, (Fig. 1).

Chromatography of the proteins on DEAE also shows no difference in the pattern of separation: both cell extracts reveal the low activity of FBPase I and the high activity of FBPase II (Fig. 2), as recently reported for cells from white light conditions [8].

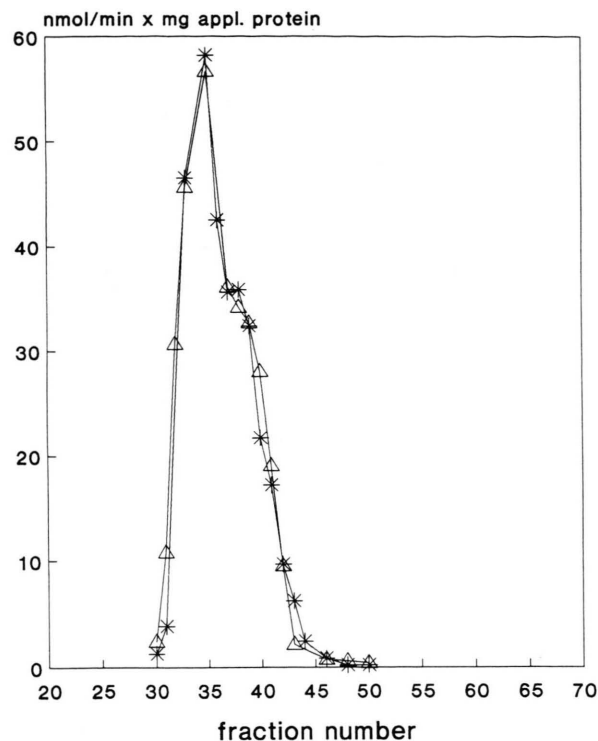


Fig. 1. Distribution of total FBPase activity in crude extracts from *Chlorella kessleri* grown in blue (Δ - Δ) or in red light ($*$ - $*$) after FPLC on superose 6. Extraction and elution were performed with 50 mM Hepes buffer at pH 6.0. Equal fluence rates: $33 \mu\text{mol} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$.

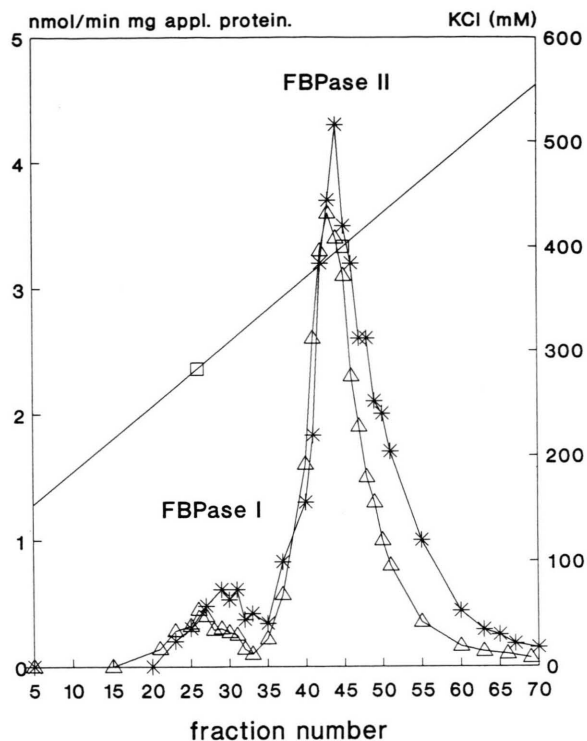


Fig. 2. Distribution of FBPase I and FBPase II activity of crude extracts from *Chlorella kessleri*, grown in blue (Δ - Δ) or in red light ($*$ - $*$) after ion exchange chromatography on DEAE cellulose. Extraction and elution were performed with 50 mM Hepes buffer at pH 6.0. Equal fluence rates: $33 \mu\text{mol} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$.

FBPase of *Chlorella kessleri* at alkaline pH

In attempts to verify the reported (l.c.) influence of pH on the enzyme's state of oligomerization, we examined crude cell extracts prepared at pH 8.5 instead of at pH 6.0. The previous results were confirmed: FPLC on superose 6 resulted in a FBPase activity in protein fractions of the smaller mole mass of 88 kDa (Fig. 3). This was identical for the enzyme from red and from blue light conditions. There was, however, one significant difference: the FBPase activity in extracts of cells from blue light conditions was considerably lower, ($79 \text{ nmol} \cdot \text{min}^{-1} \cdot \text{mg protein}^{-1}$), than that of extracts from cells from red light conditions ($133 \text{ nmol} \cdot \text{min}^{-1} \cdot \text{mg protein}^{-1}$). DEAE chromatography at this pH indicated that the difference in activity is mainly a result of higher FBPase II ac-

tivity in extracts of cells from red light conditions (Fig. 4).

Influence of reductant on FBPase

Upon considering the recently observed influence of reducing agents on FBPase II, [8] and interpreting the above data as an indication of lower stability of the enzyme from blue light conditions than of that from red light conditions, we examined the influence of DTT. A pronounced influence of DTT was found: when DTT is omitted in the extraction buffer, but later added to the enzyme assay, it leads to an increase in activity in all cases tested, as could be expected from [8, 14]. In preparations at pH 6.0, the identical initial activity for cells from red and from blue light conditions of approx. $65 \text{ nmol} \cdot \text{min}^{-1} \cdot \text{mg protein}^{-1}$ could both

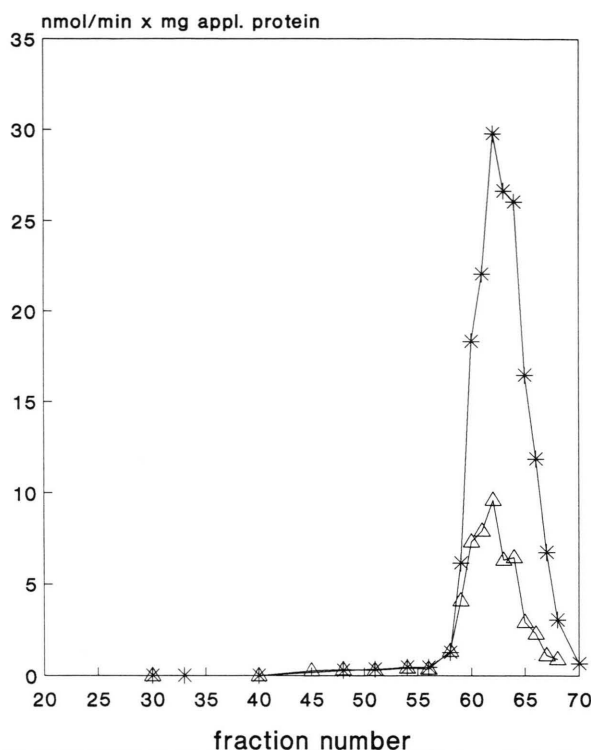


Fig. 3. Distribution of total FBPase activity in crude extracts from *Chlorella kessleri* grown in blue ($\Delta-\Delta$) or in red light ($*-*$) after FPLC on superose 6. Extraction and elution were performed with 50 mM Hepes buffer at pH 8.5. Equal fluence rates: $33 \mu\text{mol} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$.

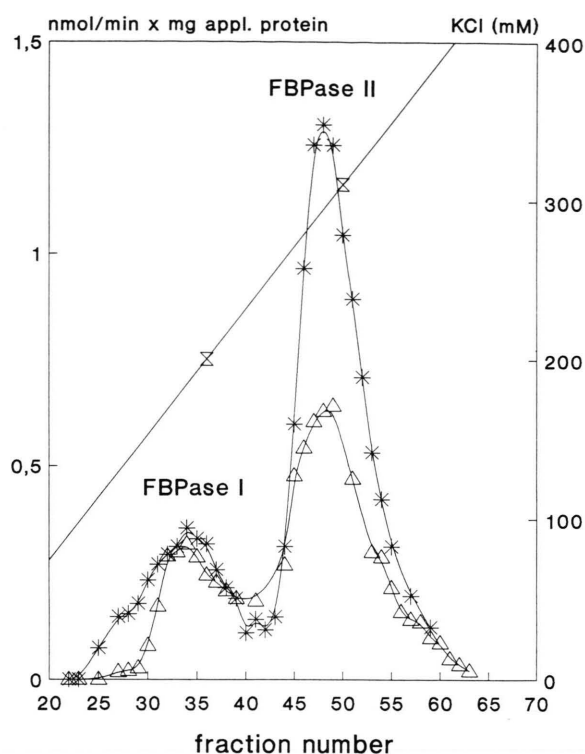


Fig. 4. Distribution of FBPase I and FBPase II activity of crude extracts from *Chlorella kessleri*, grown in blue ($\Delta-\Delta$) or in red light ($*-*$) after ion exchange chromatography on DEAE cellulose. Extraction and elution were performed with 50 mM Hepes buffer at pH 7.5. Equal fluence rate: $33 \mu\text{mol} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$.

be enhanced to about $195 \text{ nmol} \cdot \text{min}^{-1} \cdot \text{mg protein}^{-1}$ (Fig. 5a). In preparations at pH 8.5, the different initial activity of cells from both light conditions could be enhanced by approx. 200% in preparations from blue light, but only by approx. 100% in preparations from red light conditions. However, these differing effects did not lead to equal maximum activities, which were $79 \text{ nmol} \cdot \text{min}^{-1} \cdot \text{mg protein}^{-1}$ for cells growing in blue light and $133 \text{ nmol} \cdot \text{min}^{-1} \cdot \text{mg protein}^{-1}$ for red light-grown cells. This is still approx. 80% higher activity in red light than in blue light (Fig. 5b).

To find out whether these results depended partly on different reducing powers in the various extracts, FBPase II of cells from both light conditions was separated by FPLC on superose 6 and subsequently tested for DTT activation. In gener-

al, there was the same result obtained as with crude extracts. Preparations at pH 6.0 revealed identical activations of FBPase for both light qualities (Fig. 6a). Preparations at pH 8.5, however, showed increases in FBPase activity by approx. 200% for blue light-grown cells, but only by approx. 100% for those grown in red light. This resulted in different final activities. Therefore, the protein containing FBPase II activity might have a different conformation at this pH in both preparations.

Influence of enhanced temperature on FBPase

The proposed difference in FBPase stability at pH 6.0 and 8.5 could also be demonstrated by its inactivation through higher temperatures. When exposed to 45°C , the half life was 180–240 min at

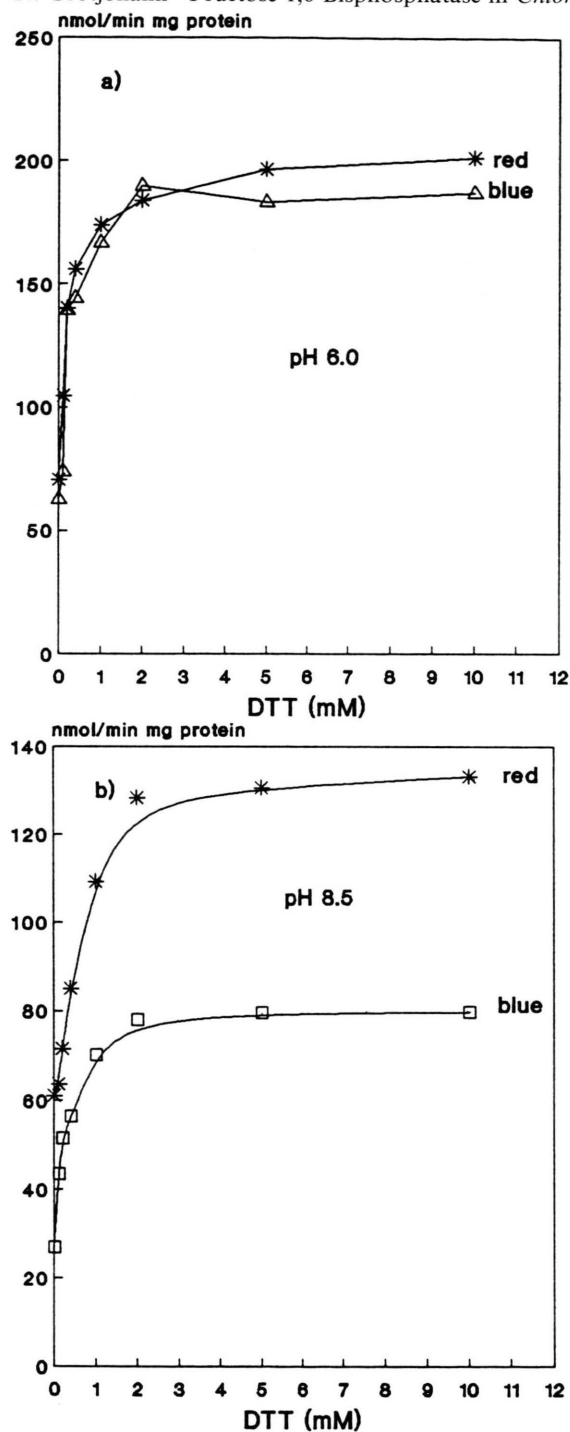


Fig. 5. Influence of DTT on FBPase II activity in crude extracts of *Chlorella kessleri* grown in blue ($\Delta-\Delta$) or in red light ($*-*$) of equal fluence rates ($33 \mu\text{mol} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$). a) preparation of cell extracts with 50 mM Hepes buffer at pH 6.0, b) identical preparation at pH 8.5.

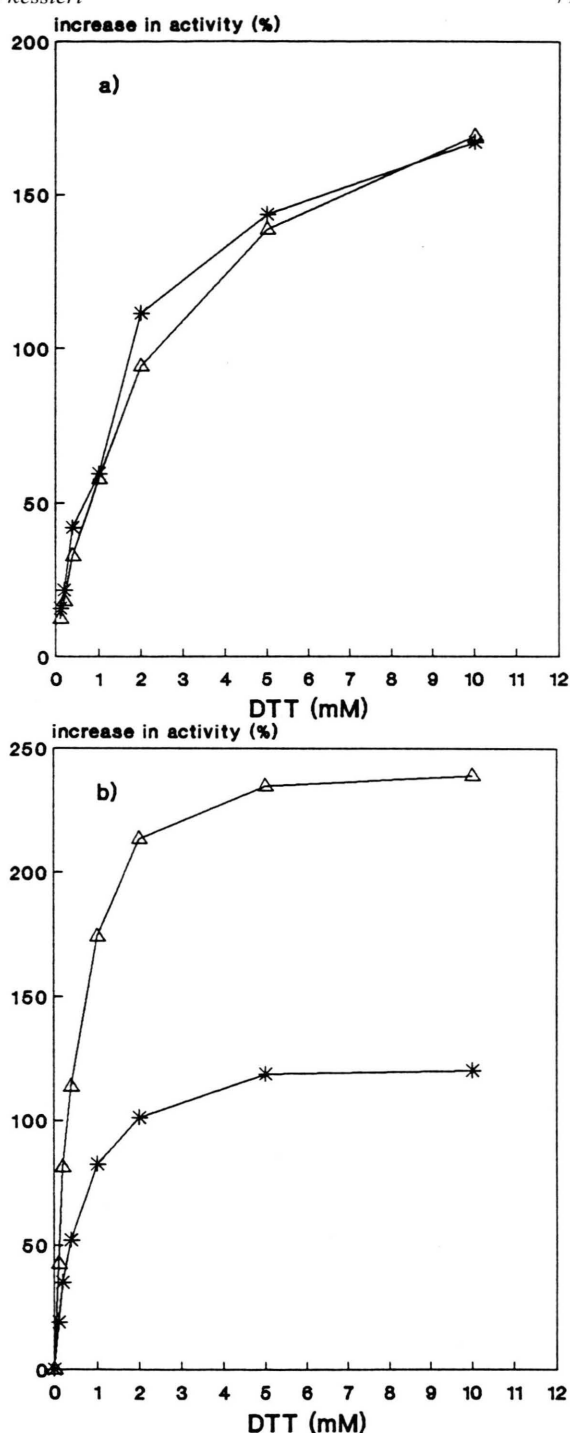


Fig. 6. Increase in FBPase II activity on additional DTT in the enzyme assay after separation of crude extracts by superose 6 FPLC. a) using pH 6.0 and b) using pH 8.5 for extraction and elution with 50 mM Hepes buffer. Cells were grown in blue light ($\Delta-\Delta$) or red light ($*-*$) of equal fluence rates ($33 \mu\text{mol} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$).

acidic, but 30–40 min at alkaline pH. No significant differences were found for extracts from blue or red light.

Conclusion

The intention of the above investigation was to clarify firstly, whether fructosebisphosphatase activity is altered in *Chlorella* cells grown in red or in blue light, and secondly, whether oligomerization/dissociation processes of this enzyme are involved as a regulatory mechanism in this alteration of FBPase activity. As a result, further information about the role of FBPase in the well-documented accumulation of reserve carbohydrates in *Chlorella* cells in red light [1, 2] was expected.

Although the results obtained do not answer both questions with finality, they allow several conclusions which might stimulate further research on the problem.

The much higher FBPase activity in crude extracts at pH 8.5 from cells grown autotrophically under red light, compared to that in extracts from cells grown under blue light conditions, supports the hypothesis that the enzyme may be involved in the differing accumulation of carbohydrate reserves in the chloroplasts under both light conditions. FBPase dephosphorylizes fructose 1,6-bisphosphate to fructose 6-phosphate, a precursor for starch biosynthesis. While operating at high activity, the enzyme would deliver these substances in larger amounts and thereby drain off the pool of fructose 1,6-bisphosphate. This might cause a drop in the pool of triosephosphates, which would otherwise be exported into the cytosol.

FBPase II activity exists in fractions of both 1349 kDa (pH 6.0) and 88 kDa (pH 8.5) derived

from blue and red light adapted cells respectively. Thus enhanced carbohydrate accumulation does not depend on dissociation of FBPase aggregates. Therefore, the focus has rather to be laid on the different total activities in extracts from blue and from red light-grown cells. This obviously does not result from different total amounts of the enzyme under the two light conditions; the identical high activity in both cell extracts at pH 6.0 excludes this possibility.

There is also no dependence on different grades of reduction. There is however, a pronounced influence of DTT on the enzyme (during the extraction and activity test), yet this artificial reductant does not compensate for the difference in activities of the enzyme produced under both light conditions. Forthcoming research will have to deal with alterations of the enzyme protein itself, as in modifying its ability to react with regulatory compounds. The influence of cofactors, especially those produced under specific light control, would be of particular interest. Preliminary results of Ruyters [15, 16] from ^{31}P NMR spectroscopy have shown an enhancing level of sugarphosphates in extracts of *Scenedesmus* and *Chlorella* mutant cells illuminated with blue light.

Further purification of the enzyme protein itself as well as a closer analysis of the components of the crude cell extracts are necessary.

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