Fructose 1,6-Bisphosphatase in the Green Alga Selenastrum minutum

I. Evidence for the Presence of Isoenzymes

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ABSTRACT

Two isoforms of fructose 1,6-bisphosphatase are present in the green alga Selenastrum minutum. The isoenzymes can be separated with ionexchange chromatography or acid precipitation. The stability of the two isoenzymes differ largely. The acid insoluble enzyme exhibits properties similar to that of the enzyme from the chloroplasts of higher plants, i.e. an alkaline pH optima in the absence of reductant, a lower affinity for substrate, strong inhibition by phosphate, and a low sensitivity to fructose-2,6-bisphosphate and AMP. The more abundant form of the enzyme exhibits several properties indicative of heterotrophic fructose 1,6-bisphosphatase, i.e. a high affinity for substrate and sensitivity toward fructose-2,6-bisphosphate and AMP. But is absolutely dependent on a reductant for stability and activity. Evidence is provided indicating that previously reported purification protocols cause inactivation of one of the isoenzymes which could lead to the erroneous conclusion that algae have a single fructose 1,6-bisphosphatase isoenzyme.

In contrast to the FBPase from animals and higher plants (for review, 20), very little is known about this enzyme from algae. Heterotrophic systems usually contain only one form of FBPase, while autotrophic tissues of higher plants usually contain two isoenzymes of the enzyme, one in the cytosol and one in the plastids (23, 20 and references therein, 13, 19). The cytosolic isofoms of FBPase in higher plants exhibits the same kinetic properties as the FBPase from animal tissues, i.e. strong inhibition by Fru-2,6-P2 and AMP as well as a neutral pH optima (10, 14, 19).

Although green algae, like higher plants, are autotrophic only one form of FBPase has been reported (1, 2, 11, 16). The properties of the enzyme from Chlamydomonas (11) and Euglena (2) are very similar to that of the plastid FBPase from higher plants. In Scenedesmus obliquus and Chlamydomonas the FBPase activity exhibits an absolute requirement for a reductant (DTT) (11, 16). The FBPase from the cyanobacterium Anacystis nidulans is present as a single isofom and exhibits kinetic properties similar to both the cytosolic and plastid forms of FBPase from higher plants (3). In a very recent paper two forms of FBPase were reported in the marine macro-algae Bryopsis corticulans (22). From the kinetic properties, however, it was concluded that both forms were most probably plastid isofoms of FBPase.

In the present paper, we report the presence of different isofoms of FBPase in a green alga. The solubility, stability, and preliminary kinetic data of the isoenzymes suggest that one form is a typical plastid (autotrophic) FBPase and the other presumably a cytosolic (heterotrophic) FBPase. We also show that previously reported purification procedures for FBPase could have led to the inactivation of one isoenzyme leading to the erroneous conclusion that green algae may have only a single FBPase.

MATERIALS AND METHODS

Material

The green alga Selenastrum minutum (Naeg.) Collins (UTEX 2459) was cultured in chemostats as previously described (8). The medium was buffered to pH 8.0 with 25 mM Na-Hepes. The steady state growth rate of the cells was 1.3 d⁻¹. Fru-2,6-P2 was from the Sigma Chemical Co.; auxiliary enzymes and other cofactors were from Boehringer-Mannheim Biochemicals. Mono-Q-Sepharose was obtained from Pharmacia Fine Chemicals.

Extraction and Partial Purification of Enzymes

All procedures were carried out at 4°C. Approximately 15 g fresh mass of Selenastrum cells were suspended in extraction buffer (100 mM Hepes-NaOH buffer [pH 7.5] containing 10 mM EDTA, 10 mM MgCl₂, 10 mM DTT, and 2 mM PMSF) in a 1:1 ratio and lysed in a French pressure cell (18,000 psi). The extract was centrifuged at 18,000g for 20 min and the resulting supernatant used in all subsequent steps.

For salt precipitation (NH₄)₂SO₄ was slowly added to 35%
saturation. After standing for 30 min the precipitated protein was removed by centrifugation at 12,000g for 15 min. The resulting supernatant was brought to 75% (NH₄)₂SO₄ saturation and centrifuged as described above. The pellet, which contained more than 90% of the total FBPase activity, was dissolved in 20 mM Hepes-NaOH (pH 7.5) containing 10 mM MgCl₂, 10 mM EDTA, and 2 mM DTT. It was dialyzed for 14 h against two changes of the same buffer.

For acid precipitation of FBPase, the pH of the supernatant was adjusted to 4.5 using 2 M formic acid. The precipitate was pelleted by centrifugation at 15,000g for 10 min. The resulting supernatant was neutralized with 2 M KOH. The pellet was resuspended in extraction buffer and the pH adjusted to 7.5. After stirring for 4 h insoluble material was removed by centrifugation at 15,000g for 10 min. FBPase was precipitated from these solutions with (NH₄)₂SO₄ and the residual (NH₄)₂SO₄ removed by dialysis as described above.

Separation of the FBPase isoforms was achieved by ion exchange chromatography. The FBPase extracts were directly applied to a Mono-Q-Sepharose column (1.5 × 20 cm) equilibrated with 20 mM Hepes-NaOH buffer (pH 7.5) containing 2.5 mM EDTA, 2 mM MgCl₂, and 2 mM DTT. The column was washed with one bed volume equilibration buffer and then developed with a linear (0–0.5 M) KCl gradient. The flow rate was 45 mL·cm⁻²·min⁻¹ and 2 mL fractions were collected.

Enzyme Assays
Two different assays were used to measure FBPase activity. All assays were carried out at room temperature. For the measurement of Pi released from Fru-1,6-P₂ a stop assay was used. The final reaction mixture contained, in a final volume of 0.5 mL, 100 mM Bis-Tris propane (pH 7.75 or as indicated), 2 mM DTT, 16 mM MgCl₂, 0.5 mM EDTA, and the enzyme solution. Assays were stopped after 20 min by adding TCA and released Pi was measured colorimetrically (6). In the continuous assay the formation of Fru-6-P from Fru-1,6-P₂ was coupled to NADP⁺ reduction in a total volume of 1 mL. Unless otherwise stated FBPase was assayed in 100 mM Hepes-NaOH (pH 7.75) containing 16 mM MgSO₄, 0.5 mM EDTA,

### Table I. Distribution of FBPase Activity during Acid Precipitation

<table>
<thead>
<tr>
<th>Extract</th>
<th>Total Activity</th>
<th>Treatment</th>
<th>Activity Present in</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>μmol·min⁻¹</td>
<td></td>
<td>Pellet</td>
</tr>
<tr>
<td>Crude + DTT</td>
<td>1.75</td>
<td>Acid precipitation</td>
<td>0.32 (18)</td>
</tr>
<tr>
<td>Acid insoluble activity</td>
<td>0.32</td>
<td>Second acid precipitation</td>
<td>0.29 (91)</td>
</tr>
<tr>
<td>Acid soluble activity</td>
<td>1.45</td>
<td>Second acid precipitation</td>
<td>0.01 (1)</td>
</tr>
<tr>
<td>Crude − DTT</td>
<td>0.71</td>
<td>Acid precipitation</td>
<td>0.46 (65)</td>
</tr>
</tbody>
</table>

*See text for experimental detail. *b Values in parentheses show activity as a percentage of total.
mg⁻¹ protein · min⁻¹) were used. Kinetic constants were determined at the optima pH. Sufficient enzyme extract was added to the reaction mixture to obtain a Vₘₐₓ of approximately 20 nmol·mL⁻¹·min⁻¹. Kinetic data were analyzed using a nonlinear regression program (9). Hill coefficients and IC₅₀ values were determined as described previously (12).

Other

Protein was measured as described by Bradford (4) using BSA as a standard. The KCl concentration was estimated by the conductivity of the fractions eluting from the column. Conductivity was measured with a Radiometer conductivity meter.

RESULTS

Isoforms

The total FBPase activity of Selenastrum minutum was 530 μmol·L⁻¹ Chl⁻¹·min⁻¹ (0.07 μmol·mg⁻¹ protein·min⁻¹). During chromatography on Mono-Q-Sepharose, the FBPase activity eluted as two separate peaks at 0.28 m (peak 1) and 0.36 m (peak 2) KCl, respectively (Fig. 1A). The ratio between these two peaks was approximately 1:4. Inclusion of DTT was an absolute requirement to obtain the high FBPase activity associated with peak 2 (Fig. 1B). When FBPase was extracted, separated on the ionexchange column and assayed in a buffer containing 16 mM MgCl₂ but without any DTT at pH 8.8, only peak 1 activity was detected (Fig. 1B). When the same extract was assayed at pH 8.0 in the presence of 5 mM DTT, the significant peak 2 activity was recovered (Fig. 1C).

During acidification of the total crude extract approximately 20% of the total FBPase activity precipitated while the remaining activity was in the supernatant (Table I). Chromatography of the acid soluble component of the total FBPase revealed only one peak of activity eluting at 0.36 m KCl (peak 2, Fig. 2B). Under the same conditions the FBPase which precipitated during acidification also eluted as a single peak but at 0.28 m KCl (peak 1, Fig. 2C). This demonstrated that the acidification of the extract to pH 4.5 selectively precipi-
tated the FBPase associated with peak 1 (Fig. 2A). When the neutralized supernatant of the first acid precipitation step was subjected to a second acid precipitation less than 1% of the total activity precipitated (Table I). Similarly, if the first acid precipitate was neutralized and then again acidified, more than 90% of the total activity precipitated (Table I) and no activity remained in the supernatant. These results show that the FBPase isoforms are not formed during changes in the pH of the extraction buffer.

**Stability**

The activities of both peak 1 and 2 FBPases were unstable. Following the ionexchange step the loss in activity of peak 1 FBPase was 20% d^{-1} and that of peak 2, 50% d^{-1}. The enzymes were also unstable following the acid precipitation. The acid insoluble FBPase lost 10% activity d^{-1} while the acid soluble form lost 25% of its activity during the same period. After extraction in buffer without any DTT, peak 1 activity could be restored to control levels (extract made in the presence of 5 mM DTT) by a 6 h incubation of the enzyme in 10 mM DTT. However, peak 2 activity could only be partially restored by incubation with DTT. After 24 h in a buffer without any DTT (or during dialysis against a buffer without DTT), only 40% of the activity of peak 2 could be recovered. The two FBPase isoenzyme also have different thermal labilities (Fig. 3). The acid soluble form of FBPase (peak 2) was completely inactivated during a 30 min exposure to 60°C. In contrast, the acid precipitable form (peak 1) lost only 10% of its activity under the same conditions (Fig. 3). When the two enzymes preparations were mixed together in a 1:1 ratio the intermediate rate of inactivation is precisely the theoretical rate. This clearly showed that the observed difference in thermal stability was not a result of the difference in purity of the preparations.

**pH Optima and Substrate Kinetics**

The specific activities of the peak 1 and peak 2 FBPases were 1.2 and 10 μmol·mg^{-1}·protein·min^{-1}, respectively. The pH optimum of the peak 1 FBPase was 8.5 in the absence of DTT and presence of 16 mM Mg^{2+} (Fig. 4A). When DTT was present the pH optima shifted to 8.0. In the absence of any reductant, the peak 2 FBPase exhibited almost no activity, even in the presence of high Mg^{2+} concentrations. In the presence of DTT the pH optima of this isoenzyme was 7.75 (Fig. 4B). Neither enzyme preparation showed significant activity with sedoheptulose 1,7-bisphosphate, F-2,6-P2 or glucose-1,6-bisphosphate as substrate. Similar results were obtained using the stop assay and continuous assay procedure. For the determination of the kinetic constants the continuous assay was used. The peak 2 FBPase exhibited hyperbolic kinetics with a K_{m} for Fru-1,6-P_{2} of 30 ± 4.5 μM (Fig. 5). The peak 1 FBPase had a lower affinity for Fru-1,6-P_{2} (K_{m} = 104 ± 16 μM) and the kinetics were slightly sigmoidal with a Hill coefficient of 1.3 (average of at least three determinations on three separate preparations, data not shown).

**Effectors**

The possible effect of several metabolic intermediates as well as Ca^{2+} on the two FBPase isoenzymes was investigated (Table II). Of these only Pi (K_{i} = 4.8 mm), Ca^{2+} (K_{i} = 0.26 mm) and F-2,6-P_{2} (K_{i} = 33 μM) significantly inhibited the peak 1 FBPase. Peak 2 activity was inhibited by F-2,6-P_{2} (K_{i} = 33 μM).
Table II. Effect of Different Substances on the Activity of the Two FBPase Isoenzymes from S. minutum

Activity was measured at pH 8.0 in the presence of 2 mM DTT, 16 mM MgSO4, and 40 μM fru-1,6-P2.

<table>
<thead>
<tr>
<th>Substance</th>
<th>Concentration</th>
<th>Activity Peak 1</th>
<th>Activity Peak 2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mM</td>
<td>nmol·ml⁻¹·min⁻¹</td>
<td></td>
</tr>
<tr>
<td>None</td>
<td></td>
<td>22</td>
<td>20</td>
</tr>
<tr>
<td>PEP</td>
<td>1</td>
<td>19</td>
<td>17</td>
</tr>
<tr>
<td>3-PGA</td>
<td>1</td>
<td>23</td>
<td>20</td>
</tr>
<tr>
<td>Pi</td>
<td>10</td>
<td>7</td>
<td>10</td>
</tr>
<tr>
<td>Glutamate</td>
<td>2</td>
<td>22</td>
<td>21</td>
</tr>
<tr>
<td>Glutamine</td>
<td>2</td>
<td>21</td>
<td>20</td>
</tr>
<tr>
<td>6-PG</td>
<td>1</td>
<td>20</td>
<td>19</td>
</tr>
<tr>
<td>F2,6P2</td>
<td>0.01</td>
<td>17</td>
<td>8</td>
</tr>
<tr>
<td>AMP</td>
<td>1</td>
<td>21</td>
<td>16</td>
</tr>
<tr>
<td>F2,6P2 + AMP</td>
<td></td>
<td>17</td>
<td>8</td>
</tr>
<tr>
<td>Calcium</td>
<td>1</td>
<td>2</td>
<td>3</td>
</tr>
</tbody>
</table>

= 7 μM, Pi (I0.5 = 10 mM), Ca2+ (I0.5 = 0.22 mM) and AMP (I0.5 = 4 mM).

DISCUSSION

It is evident from the presented data that Selenastrum minutum contains two isoenzymes of FBPase. This is unlike most algal FBPase studies which have reported only one single FBPase isoenzyme (1, 2, 11). The difference may be due to the purification steps used in these studies. When an acid precipitation step is used in the isolation of Selenastrum minutum FBPase, approximately 20% of the total activity precipitates. This acid insoluble form of the enzyme also proves to be relatively heat stable and corresponds to peak 1 of the FBPase activity during ionexchange chromatography. When extracted in a buffer without DTT the acid soluble isoenzymes (peak 2) is largely inactivated.

During the isolation of FBPase from Euglena 20% of the activity was lost during the acid precipitation step. The resulting analysis of the supernatant yielded a single isoenzyme (Fig. 2). In Selenastrum, analysis of the supernatant after acid precipitation also yields one isoenzyme, the second isoenzyme (20% of the total activity) being lost in the acid precipitate (Fig. 2). In Chlamydomonas (11), FBPase was extracted in the absence of reductant. The reported FBPase activity was associated with an acid precipitate. If a similar procedure is carried out on Selenastrum, the lack of reductant leads to a loss of the activity of the acid soluble form. Consequently, acid precipitation of the extract yields a single isoenzyme. In light of these observations previous reports of only one single isoenzyme of FBPase in the algae may need further examination.

The only other alga were two forms of FBPase were previously detected was Bryopsis corticulans (22). In B. corticulans the properties of the two isoforms were so similar that the authors concluded that both isoforms were plastid isoenzymes. In contrast the properties of the two isoenzymes from S. minutum are very distinct.

The affinity of the peak 1 isofrom of Selenastrum FBPase for Fru-6-P (Fig. 5) is similar to that of spinach chloroplast FBPase (13, 23) and the FBPase from Anacystis (3). The pH optima of this isoenzyme in the presence of 16 mM MgCl2 and DTT are the same as that of spinach chloroplast FBPase (17). Both peak 1 (Table II; Fig. 3) and chloroplast FBPase are acid insoluble and heat stable (5, 6, 15, 18), very sensitive to Pi inhibition (7), exhibit a low sensitivity toward Fru-2,6-P2 (18, 19) and almost no sensitivity toward AMP (13).

Collectively these data show that the acid precipitable FBPase activity (peak 1) is a typical plastid FBPase which is generally found in autotrophic organisms. This isoenzyme only represents 20% of the total Selenastrum FBPase activity. The activity of this form (137 nmol·mg⁻¹·Chl⁻¹), however, is sufficient to catalyze the observed steady state rate of starch synthesis present in S. minutum (21).

The cytosolic FBPase of higher plants exhibits a higher affinity for Fru-1,6-P2, a more neutral pH optima and is inhibited by Fru-2,6-P2, a more neutral pH optima and is inhibited by Fru-2,6-P2 and AMP (10, 13, 14, 19). The inclusion of a reductant (mercaptoethanol or DTT) is also a prerequisite for the isolation of this isoenzyme (10, 13, 14). The peak 2 FBPase activity from Selenastrum exhibits all the above mentioned properties (Table II; Figs. 4 and 5) suggesting it may be a cytosolic isoenzyme.

CONCLUSION

Selenastrum minutum contains two isoenzymes of FBPase. The properties of the two isoforms indicate that the one is probably a cytosolic isoenzyme and the other a plastid isoenzyme. The properties of the two isoenzymes furthermore suggest that previous reports of only one form of FBPase in green algae might be a result of the extraction procedures used.

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LITERATURE CITED


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