A Labile CO₂-Fixing Enzyme Complex in Spinach Chloroplasts

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Activity and activation of ribulose 5-phosphate kinase, ribulose diphosphate carboxylase, phosphoglycerate kinase, and the NADP- and NAD-dependent glyceraldehyde phosphate dehydrogenases from isolated chloroplasts were traced in the course of differential ultracentrifugation. Activation of some of these enzymes was carried out by incubation with NADPH₂ or ATP. Activity of ribulose diphosphate carboxylase could be increased 2,4-fold by incubation with NADPH₂ while phosphoglycerate kinase could not be activated.

The degree of activation of the NADP-specific glyceraldehyde phosphate dehydrogenase and ribulose phosphate kinase was correlated with the velocity of sedimentation. Enzymes, which could be activated, maintained, sedimented more quickly. Two fractions could be obtained after ultracentrifugation: the sedimented fraction could be highly activated while the fraction in the supernatant could not.

It was shown by gel chromatography with Sephadex for the NADP-specific glyceraldehyde phosphate dehydrogenase and ribulose phosphate kinase that the enzyme fractions which could be greatly activated had molecular weights of at least 400,000. In contrast, the enzyme fraction which could not be activated had a molecular weight of 50,000 for the ribulose phosphate kinase and of 240,000 for the glyceraldehyde phosphate dehydrogenases.

Results are discussed in the way that a reversible dissociation of a labile enzyme complex takes place between ribulose phosphate kinase, glyceraldehyde phosphate dehydrogenases, and ribulose diphosphate carboxylase. Enzyme activity changes with the state of aggregation. The dissociated enzymes have higher activity than the complexed ones. Dissociation can be performed by ATP and NADPH₂, which are assumed to be the physiological regulators, and by unphysiological means as dilution and high salt concentrations.

The existence of a CO₂-fixing enzyme complex has been discussed for many years 1-4. Ribulose diphosphate carboxylase is almost always the key enzyme in this complex. It was found in some early experiments 5-8 that ribose phosphate isomerase and phosphoribulokinase are associated with the carboxylase. Yet with massive and specific isolation procedures it was possible to obtain a pure ribulose diphosphate carboxylase preparation free of contaminating enzymes 9. This is no proof against a labile enzyme complex in vivo 3, which may operate only under certain physiological conditions, and which can only be detected only with subtle procedures.

In this paper we report on evidence of a labile CO₂-fixing enzyme complex in vivo, consisting of ribulose diphosphate carboxylase, phosphoribulokinase, and glyceraldehyde 3-phosphate dehydrogenase. It is shown that the state of aggregation between these enzymes is correlated with the state of activity. The possible role of this complex in the regulation of CO₂-fixation is discussed.

Methods

1. Materials

Spinach plants (Spinacia oleracea L.) were harvested from the field and stored in the dark at 4°C for at least 24 hours, if not otherwise indicated.

2. Isolation of chloroplasts

Chloroplasts were isolated in a sucrose medium as described earlier 10. In addition to sucrose and 50 mM tris-(hydroxymethyl)-amino-methane buffer, pH 8.0, the medium contained 3 mM MgSO₄, 3 mM EDTA **, and reduced nicotinamide adenin dinucleotide phosphate, NAD-GAPDH = nitocinamide adenin dinucleotide-specific glyceraldehyde 3-phosphate dehydrogenase, NADP-GAPDH = nicotinamide adenin dinucleotide phosphate-specific glyceraldehyde 3-phosphate dehydrogenase, O.D. = optical density, PGA = phosphoglyceric acid, RuDPC = ribulose 1,5-diphosphate carboxylase, RuP = ribulose 5-phosphate, RuPK = ribulose 5-phosphate kinase, tris = tris-(hydroxymethyl)-aminomethane, V₀ = elution volume, Vₐ = outer volume.

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** Abbreviations used: ADH = alcohol dehydrogenase, ATP = adenosin triphosphate, DTT = dithiotreitol, EDTA = ethylene diamine tetraacetate, GAPDH = glyceraldehyde phosphate dehydrogenase, G-6-PDH = glucose 6-phosphate dehydrogenase, I.U. = international unit, K = catalase, MDH = malate dehydrogenase, m.w. = molecular weight, NAD, NADH₂ = oxidized and reduced nicotinamide adenin dinucleotide, NADP, NADPH₂ = oxidized

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and 0.25 ml of mercapto ethanol per 100 ml. In some cases mercapto ethanol was replaced by 2.5 mM glutathione. The final chloroplast suspension contained 70—90% intact chloroplast as tested with phase contrast microscopy. For further preparations the chloroplasts were destroyed by mild sonication.

3. Determination of protein and chlorophyll

**Protein** was measured by its absorption at 280 nm, with the Folin-Ciocalteau method, and with the biuret reagent.

**Chlorophyll** was determined by the method of **ARNON**.

4. Assay of enzyme activities

**Ribulose diphosphate carboxylase** was measured kinetically in a combined optical test. Ribulose diphosphate and bicarbonate were used as substrates. The product of the carboxylase reaction, 3-phosphoglycerate, was phosphorylated by ATP and phosphoglycerate kinase to diphosphoglycerate, and 3-phosphoglycerate, was phosphorylated by ATP and phosphoglycerate kinase to triosephosphate. The product of the carboxylase was reduced to glyceraldehyde 3-phosphate by glyceraldehyde phosphate dehydrogenase and NADH. Activities of the NAD-specific enzyme with the exception that NADPH was substituted by NADPH, and with the biuret reagent.

For the assay of phosphoglycerate kinase

**NAD-specific glyceraldehyde phosphate dehydrogenase** was assayed in an optical test with 5 mM MgCl$_2$ and glyceraldehyde-phosphate dehydrogenase against 50 mM tris buffer, pH 7.6, containing 5 mM EDTA and 5 mM cysteine. The test contained the following substances: 50 mM tris buffer, pH 7.0, 10 mM MgSO$_4$, 4 mM glutathione, 4 mM ATP, 50 mM NaHCO$_3$, 0.6 mM ribulose 1.5-diphosphate, 0.2 mM NADH, 40 U. of phosphoglycerate kinase, and 8 U. of glyceraldehyde phosphate dehydrogenase. The final pH was 7.6 and the test volume was 1.1 ml. The reaction was started by the addition of ribulose diphosphate. After about 1 min the reaction became linear. Extinction was recorded every 20 sec. Blanks were run without ribulose diphosphate.

**Ribulose 5-phosphate kinase** was measured in a combined optical test at 340 nm via pyruvate kinase and lactate dehydrogenase according to **HURWITZ et al.**. Blanks were run without the addition of ribulose 5-phosphate or ribose 5-phosphate.

**NAD-specific glyceraldehyde 3-phosphate dehydrogenase** was assayed in an optical test in the direction of 1,3-diphosphoglycerate to glyceraldehyde 3-phosphate. The assay contained 20 I.U. of phosphoglycerate kinase in a final volume of 2.5 ml. The kinase was omitted when chloroplast specimens were assayed.

**NADP-specific glyceraldehyde 3-phosphate dehydrogenase** was assayed as the NAD-specific enzyme with the exception that NADH was substituted by NADPH. For the assay of phosphoglycerate kinase a combined optical test was used.

5. Differential ultra-centrifugation

**Step I.** 6 ml of isolated sonicated chloroplasts with about 0.3 mg of chlorophyll/ml were centrifuged for 90 min at 140 000 g and 4 °C. The clear light yellow-greenish supernatant I was pipetted off except for about 1 ml. The dark green pellet and the remaining 1 ml of dark green turbid solution on the bottom were homogenized and termed "sediment I".

**Step II.** Supernatant I was centrifuged for 4 hours at 200 000 g and 4 °C. The resulting supernatant II was colourless. It was pipetted off except for about 0.5 ml. The remaining light greenish 0.5 ml was homogenized with the greenish pellet and termed "sediment II".

6. Activation of enzymes from different materials

**Ribulose diphosphate carboxylase**:

0.1 ml of sediment II was mixed with 0.2 ml of a solution containing 25 mM tris buffer, pH 8.0, 50% glycerol, 1.5 mM EDTA, 1.5 mM glutathione. The activity of the carboxylase was assayed before the addition of 1.2 mg of solid NADPH, and 30 min after the addition. 30 µl of specimen were added to the test.

**Activations of NAD- and NADP-specific glyceraldehyde phosphate dehydrogenases**, ribulose phosphate kinase, and phosphoglycerate kinase were studied in a similar manner:

The **chloroplast homogenate** was diluted 1 : 2 with 50 mM tris buffer, pH 7.6, which contained 3 mM dithiothreitol. Solid NADPH or ATP was added to yield a concentration of 3 or 8 mM respectively. The solution was incubated in a water bath at 22 °C parallel to a control without NADPH or ATP.

**Sediment I** was homogenized in 2 ml of 50 mM tris buffer, pH 7.6, diluted 1 : 2 with tris buffer, and incubated as described above.

**Supernatant II** was incubated as described above without further dilution.

**Sediment II**: 0.2 ml was homogenized with 0.4 ml solution containing 50% glycerol, 25 mM tris buffer, pH 8.0, 1.5 mM glutathione, and 1.5 mM EDTA, and incubated with NADPH or ATP as described above.

**Elution of gel chromatography**: 0.2 ml of fraction number 33 was incubated with 1 mg of NADPH. Activities of the NADP-specific glyceraldehyde phosphate dehydrogenase and ribulose phosphate kinase were assayed before, and 20 min after, addition of NADPH. In the same way, activation of ribulose phosphate kinase was studied in fraction number 43.

**Activation by dilution**: 0.1 ml sediment II was added to 1.0 ml tris buffer pH 7.6 containing 2 mM EDTA and 1 mM DTT. Parallel the same specimen was added to buffer which contained additionally 6 mM ATP. Initial activity was obtained by graphic extrapolation of the activity at 0 °C to zero time.

7. Purification of glyceraldehyde phosphate dehydrogenases

**Step I.** 400 g of deribbed and washed spinach leaves were homogenized in 11 of 0.1 M phosphate buffer, pH 7.1, which contained 20 mM EDTA and 4 ml of mercapto ethanol. The homogenate was filtered...
through two layers of linen and the filtrate was centrifuged 15 min at 20 000 g. The supernatant was de
canted and the pellet discarded.

**Step II.** Solid ammonium sulfate was added to
the supernatant until 50% saturation, pH was adjusted
permanently to 6.6 with 10% or 33% ammonia. Be
cause of the high concentration of ammonium sulfate,
pH was measured only after dilution 1 : 10 with distil
led water. The precipitate was centrifuged 15 min at
20 000 g, and ammonium sulfate was further added
to the resulting supernatant until 70% saturation. The precipitate was centrifuged down, the supernatant
discarded, and the pellet dissolved with 200 ml of
phosphate buffer, pH 7.0, containing 5 mM EDTA and
0.1 ml of thioglycol.

**Step III.** Fractionation again with ammonium
sulfate; here the fraction between 55 and 70% satura
tion was collected.

**Step IV.** About 10 ml of the fraction were put
in a column (radius: 4 cm, length: 95 cm) with sepha
dex G-150. The gel was washed before with 0.1 M
phosphate buffer, pH 7.0, containing 5 mM EDTA and
3 mM cysteine. This buffer was used also for the elu
tion. Fractions containing most of the glyceraldehyde
phosphate dehydrogenase activity were pooled.

**Step V.** The pooled fractions were again treated
with ammonium sulfate. The fraction which precipi
tated between 58 and 68% saturation at pH 8.0 was
collected and dissolved in 3.0 ml of tris buffer, pH 8.0,
with 0.5% NaCl, 1 mM EDTA, and 1 mM glutathione.

A summary of the steps are given in Table I. All
steps were carried out at 4 °C. Long storage of the
purified enzyme was possible without loss of activity
if the same volume of glycerol was added and this
solution kept at —20 °C.

8. Analytical gel chromatography

Chromatography was performed with Sephadex
G-150 superfine in a column with 1 cm diameter and
50 cm length. The outer volume of the gel bed was
11.45 ml as determined with dextran blue. About
0.4 ml fractions were collected. All runs were perfo
rmed at 4 °C. For the analysis of enzyme aggregates
the gel was prewashed with 50 mM tris buffer, pH 7.6,
with 2 mM EDTA, and 2 mM glutathione. For the dis
sociation experiment the solution contained additionally
6 mM neutralized ATP. The run in the presence of
ATP was started only 30 min after addition of the
specimen to the column, in order to avoid a smearing
effect. Solutions for washing and elution were the same.
Chromatographies with and without ATP were perfo
rmed twice. The column was calibrated with the follo
wing enzymes: glyceraldehyde 3-phosphate dehy
rogenase from rabbit muscle, molecular weight 140 000, glucose 6-phosphate dehydrogenase from
yeast, m. w. 104 000, alcohol dehydrogenase from
yeast, m. w. 141 000, pyruvate kinase from rabbit

**Results**

The light-induced activation of the NADP-dependent
glyceraldehyde 3-phosphate dehydrogenase in vivo was investigated in previous publica
tions 17, 18, 19. The kinetics of its activation are very
similar to those for the light-induced activation of
ribulose 5-phosphate kinase 18. Furthermore, both
enzymes can be activated in vitro by ATP and
NADPH 20, 21, 16. In our first experiments we de
monstrated an activation of the ribulose 1,5-dipho
sate carboxylase by NADPH 2 (Table II). So we
assumed a common mechanism of activation for all
3 enzymes.

<table>
<thead>
<tr>
<th>Step</th>
<th>Protein Activity</th>
<th>Total Activity</th>
<th>Specific Activity</th>
<th>Puri-</th>
<th>Yield</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>[mg]</td>
<td>[units]</td>
<td>[units/mg]</td>
<td>fication (%)</td>
<td>(fold)</td>
</tr>
<tr>
<td>I. Extract</td>
<td>7320</td>
<td>2000</td>
<td>0.28</td>
<td>100</td>
<td>1</td>
</tr>
<tr>
<td>II. Ammonium Sulfate 50—70%</td>
<td>575</td>
<td>8590</td>
<td>14.95</td>
<td>417</td>
<td>53</td>
</tr>
<tr>
<td>III. Ammonium Sulfate 55—70%</td>
<td>193</td>
<td>6920</td>
<td>35.8</td>
<td>336</td>
<td>127</td>
</tr>
<tr>
<td>IV. Chromatography</td>
<td>69</td>
<td>4360</td>
<td>68</td>
<td>212</td>
<td>242</td>
</tr>
<tr>
<td>V. Ammonium Sulfate 58—68%</td>
<td>38</td>
<td>3480</td>
<td>91</td>
<td>169</td>
<td>325</td>
</tr>
</tbody>
</table>

* Expressed as μmoles NADPH₂/mg chlorophyll·hour.

Table I. Purification of the NADP-specific glyceraldehyde 3-phosphate dehydrogenase.

<table>
<thead>
<tr>
<th>Activity before Incubation</th>
<th>Activity after Incubation with NADPH₂</th>
<th>Activation</th>
</tr>
</thead>
<tbody>
<tr>
<td>[units/ml]</td>
<td>[units/ml]</td>
<td>(%)</td>
</tr>
<tr>
<td>0.32</td>
<td>0.77</td>
<td>140</td>
</tr>
</tbody>
</table>

Table II. Activation of ribulose diphosphate carboxylase by 5 mM NADPH₂.

**Elution diagram of glyceraldehyde phosphate dehydrogenase**

In the course of the purification of the NADP-
dependent glyceraldehyde phosphate dehydrogenase
a change in the elution pattern was observed. The crude preparations showed two shoulders, one of them at the exclusion volume of the gel bed (molecular weight 400,000) and the other one at a molecular weight of roughly 180,000 (Fig. 1). The first shoulder is correlated with the presence of ribulose diphosphate carboxylase. It is not found with the purified enzyme.

![Elution pattern of the NADP-specific glyceraldehyde phosphate dehydrogenase after gel chromatography with Sephadex G-150 in a short column (length: 30 cm, diameter: 4 cm). V₀: outer volume, V₁: total volume of the gel bed.](image)

Enzyme complex and enzyme activation correlated by centrifugation

In the following we tried to correlate the enzyme complex with the enzyme activation. The intact complex was concentrated by ultracentrifugation. During this course the activity and the activation of the NADP- and NAD-dependent glyceraldehyde phosphate dehydrogenase, ribulose phosphate kinase, phosphoglycerate kinase, and ribulose diphosphate carboxylase were traced.

The NADP-specific glyceraldehyde phosphate dehydrogenase could be activated about 2.2-fold in the starting material, which was a suspension of isolated, sonicated chloroplasts (Table III). After the second centrifugation there was only an activation of 1.1-fold in the supernatant with the lower molecular weight substances, and an activation of 2.3-fold in the light-green sediment. Comparing the total activity of the activated enzyme in the sediment and the supernatant, we obtained an enrichment of about 17-fold. This is also found for the NAD-specific dehydrogenase, which is closely connected with the NADP-dependent enzyme

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Enzyme</th>
<th>Enzyme Activity*</th>
<th>Activation by NADPH₂</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chloroplast</td>
<td>NADP-spec.</td>
<td>400**</td>
<td>862**</td>
</tr>
<tr>
<td>Homogenate</td>
<td>NADP-spec.</td>
<td>369**</td>
<td>2.28</td>
</tr>
<tr>
<td>Sediment I</td>
<td>NADP-spec.</td>
<td>1.38</td>
<td>1.06</td>
</tr>
<tr>
<td>Sediment II</td>
<td>NADP-spec.</td>
<td>7.35</td>
<td>16.8</td>
</tr>
<tr>
<td>super-</td>
<td>NADP-spec.</td>
<td>0.88</td>
<td>1.0</td>
</tr>
<tr>
<td>natant II</td>
<td>NADP-spec.</td>
<td>0.4</td>
<td></td>
</tr>
</tbody>
</table>

* Expressed as units/ml. ** Expressed as μmoles NADPH₂/mg chlorophyll-hour.

Table III. Differential ultracentrifugation of the NADP- and NAD-specific glyceraldehyde phosphate dehydrogenases.

 Very similar results were obtained for ribulose phosphate kinase (Table IV, preparation A). The enzyme in the supernatant of the second centrifugation step could be weakly activated only while the sediment yielded an activation of 3.9-fold. The enrichment by the last step was about 12-fold, which we assume to be not in agreement with a molecular weight of 50,000.

Ribulose phosphate kinase from improperly stored spinach had a high initial activity (Table IV, preparation B). Under these conditions, activation was not possible in either fraction, and the concentration in the sediment was only 1.9-fold.

Activity of phosphoglycerate kinase was traced in the same manner. We could not demonstrate an activation of this enzyme by ATP in either fraction, and the enrichment by the second centrifugation step was only 14% (Table V).
Activity and activation of ribulose diphosphate carboxylase were assayed only in sediment II. Here we found an activation of 2.4-fold (Table II).

Differential centrifugation was performed 3 times and enzyme activities were determined at least twice.

Most of the chloroplast lamellae sedimented during the first centrifugation at 140,000 g. Only a small fraction of 85% for the ribulose phosphate kinase and 83% for the NADP-specific dehydrogenase from the total enzyme activities were found in this pellet. This confirms that the enzymes are not adsorbed to the lamellae, at least not tightly.

Enzyme complex and enzyme activation correlated by gel chromatography

The correlation of enzyme activity and activation with the state of aggregation can be demonstrated directly by gel chromatography in the presence and absence of ATP. For this purpose the sediment II from the differential centrifugation was dissolved and transferred to a column with Sephadex G-150 superfine. Without ATP the molecular weight of glyceraldehyde phosphate dehydrogenases was at least 400,000 (Fig. 2). The same was found for phosphoribulo kinase. Likewise a smaller second peak was present, corresponding to a molecular weight of about 50,000, which agrees well with the reported values 4.

In the presence of 1 mM ATP the peaks for the dehydrogenases and for the kinase are shifted to higher elution volumes (Fig. 3). Identical volumes are obtained for the purified enzymes. Minor peaks are still in the region of the enzyme complex.

The activation of the NADP-dependent dehydrogenase and ribulose phosphate kinase by NADPH2 is possible only with the complexed enzymes (Table VI). The uncomplexed kinase cannot be activated. The same is true for the dehydrogenase.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Fraction No.</th>
<th>Enzyme Activity*</th>
<th>Activation by NADPH2 [%]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ribulose Phosphate Kinase</td>
<td>33</td>
<td>0.64</td>
<td>2.05 220</td>
</tr>
<tr>
<td>NADP-GAPDH</td>
<td>43</td>
<td>0.32</td>
<td>0.30 0</td>
</tr>
<tr>
<td></td>
<td>33</td>
<td>0.50</td>
<td>1.30 160</td>
</tr>
</tbody>
</table>

*Expressed as units/ml.

Table VI. Activation of aggregated NADP-GAPDH and of dissociated and aggregated ribulose phosphate kinase by NADPH2 after separation by gel chromatography.
Ribulose phosphate kinase and glyceraldehyde phosphate dehydrogenases have different affinities to the postulated enzyme complex. While the dehydrogenase dissociated more than 80% under the influence of 6 mM ATP, the kinase dissociated only about 60% (Fig. 3). On the other hand, parallel to a 100% complexed dehydrogenase, the kinase was only 80% aggregated (Fig. 2).

**Degree of activation**

The degree of activation of the NADP-specific dehydrogenase and phosphoribulo kinase is dependent on the initial activity (Table IV). The lower the initial activity the greater the activation, and vice versa. The lowest activity of the NADP-specific dehydrogenase was 200 μmoles NADPH$_2$ per mg of chlorophyll and hour. This activity was found in plants under conditions which favor aggregation:

![Fig. 4. Activation of the NADP-specific glyceraldehyde phosphate dehydrogenase from sediment II by dilution 1 : 11 at different temperatures. Dotted lines: without addition of ATP, straight lines: with 6 mM ATP.](image)

exposure of spinach plants to cold and dark for at least one day. Otherwise, after an exposure of only one or two hours, activity was in the range between 350 and 500 μmoles NADPH$_2$/mg chlorophyll and hour. Irrespective of the initial activity, the final activity was about 1 mmole NADPH$_2$ after activation with light in vivo, or with ATP or NADPH$_2$ in vitro. The activity of ribulose phosphate kinase was even more sensitive to temperatures higher than 4 °C and to light, confirming the less pronounced affinity for the complex.

**Determination of molecular weight**

The molecular weight of the purified glyceraldehyde phosphate dehydrogenases and of the ribulose phosphate kinase was determined by gel chromatography with Sephadex G-150 superfine (see Methods). The molecular weight of the two dehydrogenases was 240 000 (Fig. 5). The highest and lowest values found were 270 000 and 200 000 respectively. The NADP- and NAD-dependent enzymes could not be separated by either procedure employed. This agrees with earlier reports. As the activation behaviors of the two dehydrogenases are distinct, we agree with CARR’s speculation that the two enzymes are tightly bound together.

**Activation by high salt concentrations**

ATP and NADPH$_2$ may be physiological effectors for the regulation of enzyme activity, or for the association and dissociation of the complex. In vitro a labile protein complex can be dissociated by further means. Fractionation with ammonium sulfate results in the separation of the associated complex accompanied by activation (Fig. 1, Tables I and VII). This activation is independent of the fractionation range. We obtained similar results for the NADP-specific dehydrogenase when we precipitated it by adding ammonium sulfate in one step until 90% saturation or when we first separated most of the carboxylase and kinase by initial precipitation until 55% saturation, and then precipitated the dehydrogenase by additional ammonium sulfate until 80% saturation (Table VII).

Starting from a low specific activity of the NADP-dependent glyceraldehyde phosphate dehydrogenase for the enzyme purification a much too high purification factor and yield is feigned by the activation which takes place during fractionation with ammonium sulfate (Table I).
Specific Activity* in Extract | Total Activity** in Precipitate | Precipitation Range [% Satura- | Activation [%] | Specific Activity* in Extract | Total Activity** in Precipitate | Precipitation Range [% Satura- | Activation [%]  
---|---|---|---|---|---|---|---  
200 | 115 | 572 | 55—88 | 400 | 295 | 213 | 705 | 230  
450 | 240 | 560 | 55—80 | 130  
300 | 163 | 675 | 0—90 | 310  

* Expressed as μmoles NADPH₂/mg chlorophyll·hour. ** Expressed as units/total volume.

Table VII. Activation of NADP-GAPDH with different specific activities by high concentrations of ammonium sulfate.

| Specific Activity* in Extract | Total Activity** in Precipitate | Precipitation Range [% Satura- | Activation [%]  
---|---|---|---|---|---|---|---  
295 | 213 | 705 | 230  

* Expressed as μmoles RuP/mg chlorophyll·hour. ** Expressed as units/total volume.

Table VIII. Activation of ribulose phosphate kinase by ammonium sulfate (70% saturation).

Activation by high concentrations of ammonium sulfate was also observed for ribulose phosphate kinase (Table VIII). TROWN reported on a similar effect for ribulose disphosphate carboxylase.

**Activation by dilution**

Dissociation of a labile aggregate should be favored by dilution. We did not directly control the molecular weight of the dissociating complex, but followed the activity of the NADP-specific dehydrogenase. The unactivated enzyme from sediment II was diluted 11-fold and incubated at different temperatures (Fig. 4). The activity increased nearly 3-fold after 35 min at 20 °C (Table IX). Activation was less pronounced at lower temperatures. The same preparation was activated by ATP in order to obtained the maximal possible activation.

This kind of activation was observed in the dilution of only fractionated enzyme, in sediment II after differential ultracentrifugation, or after fractionation with ammonium sulfate. The enzyme was not activated after the dilution of the leaf or chloroplast homogenate.

**Discussion**

Proof of an enzyme complex between ribulose phosphate kinase, ribulose diphosphate carboxylase, and glyceraldehyde phosphate dehydrogenase cannot be complete. The molecular weight of such a complex would be 700 000—800 000. In our experiments with Sephadex G-150 we had a fractionation range below 400 000 only. So the aggregation of the carboxylase with a molecular weight of about 550 000 could not be traced. However, the big change in molecular weight for the kinase and dehydrogenase, and the uniform kind of activation for all 3 enzymes are strong arguments for an enzyme complex.

TROWN assayed the enzymes ribose phosphate isomerase, ribulose phosphate kinase and ribulose diphosphate carboxylase after gel chromatography with Sephadex G-200. The main peaks of the isomerase and kinase were smeared to higher molecular weights and both enzymes had minor second peaks at an elution fraction which also contained aggregated carboxylase. This would confirm some speculations that ribose phosphate isomerase, too, is part of the complex.

PARK and PON and TROWN reported on complexes between the ribulose diphosphate carboxylase itself. This type of complex may be superimposed on an aggregation between different enzymes of the Calvin cycle. Further studies are needed for more information about the details of the complex.

High efficiency is present in an enzyme complex only if all necessary links in the metabolic chain are present and furthermore if the chain has an optimal structure. As the CO₂-fixing metabolic pathway has many branching points, a tight complex in the sense of the enzyme complex for fatty acid synthesis is not possible at all. The regulation of a restricted aggregated state is regarded as a means for adaptation to the specific requirements of synthesis.
The activation of the NADP-specific glyceraldehyde phosphate dehydrogenase in vivo was demonstrated in variety of plants. If this effect is regarded as pars pro toto, the labile CO₂-fixing enzyme complex should be widely distributed in the plant kingdom.

This study was made possible by a grant and financial support from the Deutsche Forschungsgemeinschaft.

**Abbreviations used:** Tricine for (N-(Tris-hydroxymethyl)-methyl)-glycin, MES for 2 (A-morpholino) ethane sulfonic acid, HEPES for A-2-hydroxyethylpiperazine-A-2-ethane sulfonic acid.

The Distribution of Photosynthetic Reactions in the Chloroplast Lamellar System

I. Plastocyanin Content and Reactivity

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(Z. Naturforsch. 27 b, 932—938 [1972]; received May 15, 1972)

Plastocyanin, Chloroplast fragmentation, Photosystem I, Stroma Lamellae, pH-optimum for electron transport

Plastocyanin is released from chloroplasts by sonication, by passing through the French-press, by extraction with detergents, by treatment with organic solvents and by freezing and thawing. No plastocyanin is liberated during osmotic shock of either class I or class II chloroplasts, or by extraction of class II chloroplasts with dilute solutions of EDTA.

Under special conditions it is possible to separate grana and stroma lamellae, which retain plastocyanin. The distribution of plastocyanin between these membrane fractions parallels the distribution of photosystem I.

The different reactivity of plastocyanin with various chloroplast membrane preparations is not always correlated to plastocyanin content. Other parameters, like pH, seem to influence the interaction of plastocyanin with the surface of the chloroplast membrane.

The function of a biological membrane is related to its molecular architecture (membrane topography) as well as to its higher organization (membrane morphology). The possible relation of structure and function in the chloroplast membrane has been discussed in a recent review. With the development of methods to separate distinct regions of the chloroplast lamellar system, it is feasible to study the distribution of activities and components among them. Plastocyanin, an electron transport component...