A functional five-enzyme complex of chloroplasts involved in the Calvin cycle

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(Received October 15, 1987/January 4, 1988) -- EJB 87 1155

A complex of five different enzymes: ribose-phosphate isomerase, phosphoribulokinase, ribulose-bisphosphate carboxylase/oxygenase, phosphoglycerate kinase and glyceraldehyde-phosphate dehydrogenase, has been purified from spinach chloroplasts. These enzymes catalyse five consecutive reactions of the Calvin cycle, of which the two reactions catalysed by phosphoribulokinase and ribulose-bisphosphate carboxylase/oxygenase are unique to this cycle. The five-enzyme complex has been purified by successive chromatographies on DEAE-Trisacryl, Sephadex G-200 and hydroxyapatite. The homogeneity of the complex has been tested by analytical centrifugation and electrophoresis. Depending on the technique used to estimate its molecular mass, the value obtained varies between 520 kDa and 536 kDa. In addition to the five enzymes mentioned above, the complex contains a 65-kDa polypeptide. The quaternary structure of these enzymes in the complex appears to be different from what has been described for the individual enzymes in the 'noncomplexed state'.

The five-enzyme complex is functional as glyceraldehyde 3-phosphate is formed from ribose 5-phosphate. Preliminary experiments suggest that channelling of reaction intermediates occurs within the complex.

It has been shown in recent years that, besides tightly associated enzymes forming stable complexes, there exist loose or transitory associations of enzymes forming unstable complexes [1, 2]. These loose associations have been described both for proteins of the cytosol [3] and for those of mitochondria [4]. Stable or unstable enzyme complexes may represent a functional advantage for the living cell, as compartmentalization may allow channelling of an intermediate from one active site to another within the same enzyme complex [5, 6]. If these associations are loose they may obviously be disrupted in the course of the purification procedure, thus making it difficult to identify the multi-enzyme complexes. Thus only recently has a large multi-enzyme complex, the so-called 'Krebs cycle metabolon' [4], been shown to occur in mitochondria. No similar complex has ever been reported in the case of enzymes belonging to the Calvin cycle, although some recent reports suggest the possibility of an association between certain enzymes of this cycle [7, 8].

We report in this paper that five enzymes, ribose-phosphate isomerase, phosphoribulokinase, ribulose-bisphosphate carboxylase/oxygenase, phosphoglycerate kinase, glyceraldehyde-3-phosphate dehydrogenase, which catalyse five consecutive reactions of the Calvin cycle, exist as a complex that may be isolated and purified, and which is functionally active. We also discuss some possible biological implications of the existence of this complex.

MATERIALS AND METHODS

Materials

Spinach plants (Spinacea oleracea var. Géant d'Hiver) were grown in a research garden in Aix-Marseille II University. Ribulose 5-phosphate, ribulose 1,5-bisphosphate and yeast ribose-phosphate isomerase were from Sigma, bovine serum albumin from Calbiochem and the other reagents from Boehringer. Trisacryl was obtained from IBF Biotechnics (France), Sephadex G-200 and Sephacryl S-300 from Pharmacia. Deionized water, further purified with a Millipore super Q system, was used throughout.

Purification of the multi-enzyme complex

Intact spinach chloroplasts were isolated by the method of Jensen and Bassham [9]. They were suspended in a buffer (15 mM Tris/HCl, 4 mM EDTA, 10 mM cysteine, pH 7.9) called buffer A, which brings about lysis of these organelles. The whole extract was then centrifuged for 60 min at 48000 × g to remove membranes. The complex was isolated and purified by the supernatant by successive separations on DEAE-Trisacryl, Sephadex G-200 and hydroxyapatite. The chloroplast supernatant was applied first on a DEAE-Trisacryl column (2.5 × 13 cm) equilibrated in buffer A and washed with buffer A containing 50 mM NaCl. Elution of the column was performed with a gradient of 50–300 mM NaCl in buffer A. Fractions containing phosphoribulokinase activity were pooled, concentrated and submitted to molecular sieve chromatography on a Sephadex G-200 (column 2.6 × 95 cm) using another buffer, B, having the following composition: 30 mM Tris/HCl, 1 mM EDTA, 100 mM NaCl, 10 mM cysteine, pH 7.9. The fractions located in the void volume contained ribose-phosphate isomerase, phosphor-
Fig. 1. Copurification of a high-molecular-mass phosphoribulokinase and other enzymes from the Calvin cycle. Elution profiles of Sephadex G-200 and hydroxyapatite. The different enzyme activities were monitored as described in Materials and Methods. Activated phosphoribulokinase corresponds to the enzyme which has been incubated with 50 mM dithiothreitol for 1 h at 30°C before being assayed. (○—○) Phosphoribulokinase, (△—△) ribose-phosphate isomerase, (■—■) glyceraldehyde-3-phosphate dehydrogenase, (●—●) ribulose-bisphosphate carboxylase/oxygenase. (A) Gel filtration on Sephadex G-200. The arrows indicate the predicted positions for elution of the isolated enzymes as follows: (1) ribulose-bisphosphate carboxylase/oxygenase, (2) glyceraldehyde-3-phosphate dehydrogenase, (3) phosphoribulokinase, (4) ribose-phosphate isomerase, (5) phosphoglycerate kinase. Column flow rate was 8 ml/h. The activity ratios, calculated in relation to activated phosphoribulokinase activity, can be considered constant within the experimental error value across the peak eluted in the void volume. Thus, the activity ratios for ribose-phosphate isomerase, ribulose-bisphosphate carboxylase/oxygenase and glyceraldehyde-3-phosphate dehydrogenase were respectively 0.113, 0.064, 0.11 at 125.7 ml; 0.08, 0.055, 0.13 at 151.30 ml; 0.048, 0.042, 0.096 at 164 ml; 0.062, 0.043, 0.091 at 176.80 ml; 0.098, 0.06, 0.085 at 189.50 ml. (B) Chromatography on hydroxyapatite. Column flow rate was 30 ml/h. The line (——) indicates the potassium phosphate concentration. The inset shows the activity ratios calculated in relation to phosphoribulokinase activity for ribose-phosphate isomerase (●—●) and glyceraldehyde-3-phosphate dehydrogenase (△—△) across peaks A and B.

Enzyme assays

Phosphoribulokinase and ribose-phosphate isomerase activities were determined at 30°C by a procedure derived from that described in [10]. These activities were coupled to NADH oxidation via pyruvate kinase and lactate dehydrogenase. When measuring isomerase activity, the assay cuvette contained 50 mM glycyglycine buffer pH 7.7, 10 mM MgCl₂, 0.5 mM EDTA, 2.75 units lactate dehydrogenase, 1 unit pyruvate kinase, 1 mM phosphoenolpyruvate, 5 mM dithiothreitol, 0.15 mM NADH, 1 mM ribose 5-phosphate and 0.5 mM ATP. The same mixture, supplemented with ribose-phosphate isomerase from the yeast Torula, was used for measuring the kinase activity. Glyceraldehyde 3-phosphate dehydrogenase, phosphoglycerate kinase and ribulose-bisphosphate carboxylase/oxygenase activities were determined as described in [11–13]. The complex activity was measured using the ribulose-bisphosphate carboxylase/oxygenase assay, but without the addition of exogenous phosphoribulokinase,
Table 1. Content of enzyme activities in peaks A and B of hydroxyapatite

The enzyme activities were assayed as described in Materials and Methods. The total protein content was 712 µg and 541 µg in peak A and B, respectively.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Peak A</th>
<th>Peak B</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ribulose-bisphosphate carboxylase/oxygenase</td>
<td>8.5</td>
<td>6.7</td>
</tr>
<tr>
<td>Phosphoribulokinase</td>
<td>6</td>
<td>5*</td>
</tr>
<tr>
<td>Ribose-phosphate isomerase</td>
<td>3.7</td>
<td>3*</td>
</tr>
<tr>
<td>Glyceraldehyde-3-phosphate dehydrogenase</td>
<td>0.3</td>
<td>1.4</td>
</tr>
<tr>
<td>Phosphoglycerate kinase</td>
<td>0.09</td>
<td>0.15</td>
</tr>
</tbody>
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* Assays were also done after preincubation with 50 mM dithiothreitol for 1 h. The activities of both enzymes are enhanced from 5 units to 17 units, and from 3 units to 7.5 units.

ribose-phosphate isomerase, glyceraldehyde-phosphate dehydrogenase and phosphoglycerate kinase. Estimation of enzyme activities and absorbance measurements were effected with a six-sample Pye Unicam 8610 spectrophotometer connected to an Apple II microcomputer. A unit of enzyme activity is defined as the amount of enzyme which transforms 1 µmol substrate/min under the conditions of the assay.

Determination of molecular mass and homogeneity of the complex

Molecular mass estimations were effected by molecular sieve chromatography through Sephacryl S-300, by analytical ultracentrifugation and by slab gel electrophoresis. Sephacryl S-300 columns (2.6 × 90 cm) were calibrated with proteins of known relative molecular mass (thyroglobulin 669 kDa, ferritin 449 kDa, catalase 240 kDa, aldolase 147 kDa, bovine serum albumin 68 kDa, peroxidase 50 kDa, cytochrome c 12.5 kDa, aprotinin 6.5 kDa) using buffer B [14]. Analytical ultracentrifugation experiments were performed with a Beckman ultracentrifuge model E equipped with an interferometric system for molecular mass determinations according to the method of Yphantis [15] and ultraviolet or schlieren optics for sedimentation patterns. Slab gel electrophoresis under denaturing conditions was conducted as in [16]. Under non-denaturing conditions the experimental protocol proposed by Pharmacia was followed. Protein concentrations were determined by the dye-binding assay of Bradford [17].

RESULTS

In the course of elution of DEAE-Trisacryl columns, the fractions that contain phosphoribulokinase also contain ribose-phosphate isomerase, ribulose-bisphosphate carboxylase/oxygenase, glyceraldehyde-phosphate dehydrogenase and phosphoglycerate kinase. Despite the large differences of their molecular mass (spanning from 50 kDa to 550 kDa), enzyme activities coelute in the void volume of the Sephadex G-200 column (Fig. 1A). A second peak of phosphoribulokinase activity is obtained later on, during the process of elution, and does not contain the other enzyme activities. The presence of glyceraldehyde-phosphate dehydrogenase and phosphoribulokinase activities in the void volume of the Sephadex G-200 column, is consistent with the previous finding that these enzymes may occur under a state of high molecular mass [11, 18 – 23]. The fractions of the void volume that contain the five enzyme activities generate, upon hydroxyapatite chromatography, two peaks, A and B, each of which contains the same five enzyme activities (Fig. 1B). The ratios of the various enzyme activities remain approximately constant across each of the two activity peaks. Two of these activity ratios are given as an inset in Fig. 1B and some other values are given in the legend of the figure. The phosphoglycerate kinase activity, however, is too low to be determined in every fraction across the elution profile. The reason for this low activity is unknown at the moment but might be due to a poor accessibility of the enzyme's active site within the complex to the bulk substrate.

The results of the hydroxyapatite chromatography are reminiscent of the findings of Nicholson et al. [24], which showed the existence of two fractions of phosphoribulokinase activity extracted from the green alga Scenedesmus obliquus. Table 1 shows the respective amounts of the various enzyme activities present in the fractions of the two peaks A and B.

In order to test the purity of these multienzyme complexes, electrophoresis was performed in polyacrylamide gradient gels (4 – 30%) or under native conditions. As shown in Fig. 2, peak A yields a single band with a molecular mass of 449 kDa, but fractions of peak B contain two molecular entities with molecular mass of 449 kDa and 669 kDa respectively. These results strongly suggest that peak B contains the same multienzyme complex as peak A plus an additional polypeptide. The homogeneity of the multienzyme complex A may be verified by analytical centrifugation. Results of Fig. 3A show that this complex is indeed homogeneous. A schlieren profile of this fraction is also symmetrical thus giving additional support to this conclusion (Fig. 3B).

In order to define their composition, electrophoresis of these protein complexes was performed in 9% SDS/polyacrylamide gels (denaturing conditions). The results obtained are shown in Fig. 4.

Fractions of peak A show the two subunits of ribulose-bisphosphate carboxylase/oxygenase, the subunits of phosphoribulokinase and of glyceraldehyde-phosphate dehydrogenase. The amount of ribose phosphate isomerase and...
phosphoglycerate kinase is such that no bands pertaining to those enzymes are detected. For ribose-phosphate isomerase this is not surprising, since we have found that on polyacrylamide gel, very large amounts (above 30 μg) of this enzyme are needed in order to detect a band by Coomassie blue. Fractions of peak B display basically the same pattern as those of peak A, except that a band of 65 kDa molecular mass is present in the fractions of peak B, but not of the peak A. This 65-kDa protein presents the characteristic of not being stained by silver dyes (data not shown). Although we still do not know the function of this protein, analysis of the literature suggests that it may correspond to a manganese-protein possibly involved in oxygen evolution [25]. It thus appears quite likely that the complex present in the fractions of peak B is also present in the fractions of peak A, but associated with the 65-kDa polypeptide.

If fractions contained in the void volume of Sephadex G-200 filtration are submitted to Sephacryl S-300 chromatography, two peaks are obtained upon elution (Fig. 5). In peak 1 the five enzyme activities are detected as a molecular entity of 536 kDa. Moreover, under these conditions the activity of phosphoribose isomerase, phosphoribulokinase and glyceraldehyde-phosphate dehydrogenase are greatly enhanced by preincubation with dithiothreitol. A second protein peak is also detected on the elution profile that pertains to a molecular mass of 190 kDa and contains ribose-phosphate isomerase and phosphoribulokinase but lacks ribulose-bisphosphate carboxylase/oxygenase, phosphoglycerate kinase and glyceraldehyde-phosphate dehydrogenase activities.

The multienzyme complex of 536 kDa appears homogeneous on analytical centrifugation. By equilibrium sedimentation one finds a molecular mass of 520 kDa, which is consistent with the value of 536 kDa given above. In sedimentation velocity studies the pattern appears symmetrical (Fig. 3B). Analysis of subunit composition of this complex allows one to detect the subunits of ribulose-bisphosphate carboxylase/oxygenase, glyceraldehyde-phosphate dehydrogenase, phosphoribulokinase and the polypeptide of 65 kDa.

Dithiothreitol has been reported to promote the depolymerization of a 'high'-molecular-mass form of phosphoribulokinase [21, 22]. This reductant has, therefore, been used to promote dissociation of ribulose-bisphosphate carboxylase/oxygenase from the rest of the complex and to show whether the enzyme obtained in that way may have a molecular mass different from that of the so-called 'native' enzyme, namely 550 kDa. Upon treatment of the complex with dithiothreitol and molecular sieving through Sephacryl, several fractions are obtained (Fig. 6). The first contains a complex of 10^3 kDa totally devoid of activity. The second protein peak has ribulose-bisphosphate carboxylase activity, but the corresponding molecular mass is 420 kDa instead of 550 kDa. The 65-kDa polypeptide is also detected in the same fraction.

The rather low molecular mass of this form of ribulose-bisphosphate carboxylase/oxygenase strongly suggests that this enzyme may exist with a structure different from the classical A₄B₄ quaternary structure. This 420-kDa form containing the 65-kDa polypeptide resembles, however, another
form of this enzyme isolated from blue-green algae, which shows a molecular mass of 450 kDa [26], and another form from *Rhodopseudomonas palustris* and *sphaeroides* that shows a molecular mass of 360 kDa [27, 28]. The other fractions contain a complex of 130 kDa molecular mass which has phosphoribulokinase activity but has no ribulose-bisphosphate carboxylase/oxygenase. It thus appears probable that the complex present in the first peak is an artefactual molecular aggregate resulting from a thiol—disulfide interchange. The interesting result that emerges from these data is that, after a dithiothreitol treatment, ribulose-bisphosphate carboxylase/oxygenase subunit, was also found. This polypeptide is not represented in the calibration curve because of the poor

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To show that the complex of these five enzymes of the Calvin cycle represents a functional entity, ribose 5-phosphate, the substrate of the first reaction, was mixed with the complex in the presence of ATP, bicarbonate and NADH, substrates required for later steps, and the progress of the final reaction was monitored by measuring the production of NAD⁺. The results (Fig. 7) show that NADH is continuously oxidized by the complex, thus implying that the sequence of the five enzyme reactions was indeed taking place.

Moreover, the steady-state velocity of product appearance at saturating concentrations of ribose phosphate, ATP and NADH was identical, within experimental error, to the rate of oxidation of NADH at saturating glycerate 3-phosphate, coenzyme and ATP concentrations. This result suggests the existence of channelling within this multienzyme complex.

**DISCUSSION**

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cycle multienzyme complex is attached to the inner mitochondrial membrane [4].

Some results presented above are quite consistent with previous proposals. For instance, ribose-bisphosphate carboxylase of pea is associated with ribose-phosphate isomerase and phosphoribulokinase activities [7]. Moreover the forms of phosphoribulokinase and glyceraldehyde-phosphate dehydrogenase of high molecular mass (550 kDa [21, 22] and 600 kDa [18, 20] respectively) that have been called 'regulatory forms' may either represent an aggregated state of the same enzyme molecules or a multienzyme complex similar to the one studied in this report.

The main point of this study is related to the functionality of the multienzyme complex. The existence of a complex of this sort in chloroplasts may represent a functional advantage as it allows channelling of reaction intermediates of the Calvin cycle from one site to another, thus preventing diffusion of these intermediates in the bulk phase. Results presented in this report suggest that this release of intermediates does not occur to a significant extent, as the velocity of the last step of the reaction sequence at saturating substrate concentration is identical to that of the overall process at saturating ribose-phosphate concentration. Although the existence of chan-
nelling cannot yet be taken as proved, it is suggested by these results. Further studies are now under way to investigate it further and to study its dynamics.

The expert technical assistance of Mrs Mireille Rivière and Mr Paul Sauve is deeply appreciated. Thanks are due to Mr A. Ribas for having grown the spinach. This work was supported in part by Biologie Moléculaire Végétale du Centre National de la Recherche Scientifique, Action Thématique Programmée.

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