Calvin cycle multienzyme complexes are bound to chloroplast thylakoid membranes of higher plants in situ
(reductive pentosephosphate cycle/electron transport/enzyme supercomplex/limited proteolysis/immunoelectron microscopy)

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ABSTRACT Further evidence is provided that the Calvin cycle enzymes ribulose-5-phosphate isomerase (EC 5.3.1.6), ribulose-5-phosphate kinase (Ru-5-P-K, EC 2.7.1.19), ribulose-1,5-bisphosphate carboxylase (RuP₂Case, EC 4.1.1.39), glyceraldehyde-3-phosphate dehydrogenase (GAPDH, EC 1.2.1.12), sedoheptulose-1,7-bisphosphatase (Sed-1,7-bPase, EC 3.1.3.37), and electron transport protein ferredoxin-NADP⁺ reductase (FNR, EC 1.18.1.1) are organized into stable CO₂-fixing multienzyme complexes with a molecular mass of >900 kDa. Limited trypsinolysis combined with immunoblotting revealed that all of chloroplast stromal Ru-5-P-K and GAPDH is located in enzyme complexes. The Calvin cycle enzyme complexes remain intact indefinitely at lower ionic strength but dissociate into components at KC₁ concentrations >250 mM. Immunoelectron microscopy showed that Ru-5-P-K, GAPDH, Sed-1,7-bPase, and FNR are bound to stroma-faced thylakoid membranes in situ, whereas RuP₂Case and RuP₂Case activase are randomly distributed throughout chloroplasts. The results indicate that membrane-bound enzyme supercomplexes may play an important role in photosynthesis.

Microcompartmentalization, packing, and interactions of the enzymes catalyzing CO₂ assimilation in the chloroplast matrix are intriguing problems awaiting solution to understand satisfactorily metabolic coupling and regulation of photosynthetic processes. The ease of extractability of stromal proteins by rupture of intact chloroplasts in hypotonic medium has led to the widely accepted assumption of either no or only weak interactions among the components, thus causing their random distribution throughout the higher plant organelle. More recent observations, however, indicate the existence of Calvin cycle enzyme complexes that contain at most five different catalysts, including ribulose-1,5-bisphosphate carboxylase (RuP₂Case, EC 4.1.1.39); metabolite channeling has been claimed to occur within these complexes (1). Furthermore, enzyme pairs of the Calvin cycle have been observed consisting of RuP₂Case and ribulose-5-phosphate kinase (Ru-5-P-K, EC 2.7.1.19) (2, 3), Ru-5-P-K and gerycaldehyde-3-phosphate dehydrogenase (GAPDH, EC 1.2.1.12) (4), and ribose-5-phosphate isomerase (Rib-5-P-I, EC 5.3.1.6) and Ru-5-P-K (5). Phosphoglycerate kinase and fructose bisphosphate aldolase were also found to be complexed proteins in chloroplast stromal extracts (6). Moreover, extraction studies have indicated that several Calvin cycle enzymes may be partially bound to thylakoid membranes (7). However, no convincing evidence was provided that the putative Calvin cycle multienzyme complexes do not represent isolation artifacts and nothing is known about the membrane interactions of Calvin cycle enzymes. Similar problems hold true for other metabolic pathways probably comprising sequentially assembled enzymes (for review, see refs. 8 and 9).

In this study we provide further evidence for the existence of multienzyme complexes containing Calvin cycle components and ferredoxin-NADP⁺ (FNR, EC 1.18.1.1) reductase that reside on chloroplast thylakoid membranes in situ.

MATERIALS AND METHODS

Material. Spinach (Spinacea oleracea var. Matador) was grown in soil culture in a greenhouse.

Immunoelectron Microscopy. Small sections of illuminated spinach leaves were prefixed for 1 hr in 2% (vol/vol) glutaraldehyde/50 mM sodium cacodylate buffer, pH 7.2/1 M sorbitol prior to freezing in liquid propane (--185°C). Cryo-substitution with dry acetone/3% glutaraldehyde was performed overnight at --90°C. The solvent was exchanged against pure acetone at --65°C for 2 hr and then at --35°C for 2 hr. The samples were embedded in LR-White resin (Plannet GmbH, Marburg, F.R.G.) and polymerized chemically at 4°C. Immunogold labeling with 10-nm gold particles-protein A was performed as described (10) and the dried specimens were examined in a TESLA BS 500 transmission electron microscope at 60 kV.

Chloroplast Isolation and Extraction. Intact spinach chloroplasts were isolated according to Jensen and Bassham (11) and suspended to a final concentration of 2 mg of chlorophyll per ml in 25 mM Tris-HCl, pH 8.0/1 mM EDTA, which brings about lysis of the organelles. The suspensions (~30 mg of protein per ml) were centrifuged for 20 min at 20,000 × g prior to the addition of 20% (vol/vol) glycerol to the supernatant.

FPLC of Stromal Protein. Chromatography of stromal extracts (1 ml) was performed on a Pharmacia model FPLC system using a calibrated Sephacryl S-400 column (2.6 × 93 cm) preequilibrated with 25 mM Tris-HCl, pH 8.0/1 mM EDTA/20% (vol/vol) glycerol. Fractions (3 ml) were analyzed for enzyme activities and protein components by NaDodSO₄/urea PAGE following CI₂CCOOH precipitation.

To purify Calvin cycle complexes further, pooled protein fractions were subjected to anion-exchange chromatography on a HiLoad 16/10 Q-Sepharose column preequilibrated with 25 mM Tris-HCl/1 mM EDTA/20% (vol/vol) glycerol. Protein was eluted with a linear 0–1 M KCl gradient (100 ml) at a flow rate of 1 ml/min.

Preparation of Enzymes. RuP₂Case (12) and Ru-5-P-K (13) were isolated according to published methods.

Enzyme Assays. Ru-5-P-K was assayed spectrophotometrically as described by Kagawa (13). One unit of Ru-5-P-K converts 1 μmol of Ru-5-P into RuP₂ at 25°C. Rib-5-P-I activity was measured in a colorimetric assay according to Rutenberg (14). The Rib-5-P-dependent [¹⁴C]CO₂ fixation by isolated Calvin cycle complexes was determined at pH 8.0

Abbreviations: Rib-5-P-I, ribose-5-phosphate isomerase; Ru-5-P-K, ribulose-5-phosphate kinase; RuP₂Case, ribulose-1,5-bisphosphate carboxylase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; Sed-1,7-bPase, sedoheptulose-1,7-bisphosphatase; FNR, ferredoxin-NADP⁺ reductase; Chap 60, chaperonin 60.

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using the RuP2Case assay containing 13 mM MgCl2/8 mM ATP/8 mM dithiothreitol/8 mM NaH[14C]CO3 (0.3 mCi/ mmol; 1 Ci = 37 GBq) without exogenous enzymes added, and 1.1 mM Rib-5-P was used as substrate instead of RuP2 (15). The 3-phosphoglycerate kinase assay was performed as described (16).

**Antibodies to Enzymes and Immunoblot Analysis.** Antisera against spinach RuP2Case, Ru-5-P-K, FNR, and the CF1α subunit of ATP synthase were raised in rabbits (17). Rabbit antisera against the chloroplast proteins of corn sedoheptulose-1,7-bisphosphatase (Sed-1,7-bPase, EC 3.1.3.37) oat GAPDH, and pea chaperonin 60 (Chap60) as well as a mouse antiserum against spinach RuP2Case activase were generous gifts of B. B. Buchanan (Berkeley, CA), R. Cerff (Hannover, F.R.G.), R. J. Ellis (Warwick, U.K.), and A. R. Portis, Jr. (Urbana, IL), respectively. The IgGs were isolated by FPLC on a Mono-Q HR 5/5-column (18). Protein samples were subjected to NaDodSO4/urea PAGE, electrotransferred to nitrocellulose (0.1-μm pore size) using a buffer of 10 mM 3(cyclohexylamino)propanesulfonic acid-hydrochloric acid, pH 11/10% (vol/vol) methanol, and probed with polyclonal antibodies to chloroplast proteins. Detection was by means of donkey anti-rabbit IgG antibodies conjugated to horseradish peroxidase and a chemiluminescent substrate (ECL Western blotting detection system, Amersham) as outlined by the manufacturer.

**Limited Trypsinolysis.** Treatment with L-1-tosylamido-2-phenethyl chloromethyl ketone-treated trypsin of isolated enzymes, enzyme complexes, and Sephadex G-25-filtered or crude stromal extracts at a protease/protein ratio of 1:100 was performed in 25 mM Tris-HCl, pH 8.0/0.5 mM EDTA/20% (vol/vol) glycerol at 25°C. Proteolysis was terminated by the addition of Cl2 CCOOH to a final concentration of 5% (wt/vol). Protein samples were solubilized in 50 mM Tris-HCl, pH 8.0/4% (wt/vol) NaDodSO4/8 M urea/20 mM dithiothreitol/1 mM phenylmethylsulfonyl fluoride.

**Analytical Methods.** NaDodSO4/urea PAGE of protein samples was performed as described (19) and rainbow marker proteins of 2.3–46 kDa (Amersham) were used for molecular mass determination. Protein (20) and chlorophyll (21) concentrations were determined according to published procedures.

**RESULTS**

**Isolation and Properties of Calvin Cycle Multienzyme Complexes.** To study the state of association of Calvin cycle enzymes, a concentrated stromal protein extract from freshly prepared intact spinach chloroplasts was separated by FPLC on a long calibrated Sephacryl S-400 column (Fig. 1A). The column fractions were then analyzed for protein components by NaDodSO4/urea PAGE (Fig. 1B) and assayed for enzyme activities—i.e., Ru-5-P-K and f[14C]CO2 fixation with Rib-5-P as substrate (Fig. 1A)—and several stromal enzymes were detected by immunoblot analysis (Fig. 1C). The Calvin cycle enzymes were detected in the broad main peak (≈1000 kDa to 200 kDa), which also contains the RuP2Case, and in its subsequent shoulder (<200 kDa). A biphasic distribution of Ru-5-P-K was observed with >80% of the total enzyme activity migrating in a sharp peak on the high molecular mass side of the main protein peak (complexed enzyme forms of 900 ± 50 kDa) and the rest being present in the shoulder (free forms of <200 kDa). The activity profile of Rib-5-P-dependent CO2 fixation coincided with the distribution of the high molecular mass form of Ru-5-P-K and required endogeneous Rib-5-P-I activity. Moreover, immunoblotting revealed that GAPDH (42 and 43 kDa), Sed-1,7-bPase (38 kDa), and significant quantities of FNR (37 kDa) cocompartmented with the CO2-fixing activity (Fig. 1C). It should be stressed that the low molecular mass form of Sed-1,7-bPase, probably representing a homodimer of 70 kDa (22), is frequently cleaved into 33-kDa and 18-kDa peptides by an endogeneous protease under these conditions. Chap 60 of ≈800 kDa (23) also coeluted with the complexed Calvin cycle enzymes (Fig. 1C). The elution profile of complexed Calvin cycle enzymes did not change significantly when stromal extracts were chromatographed in the presence of 200 mM KCl, 0.5 mM ATP, 0.5 mM NADP+, or 10 mM 2-mercapto-ethanol. 3-Phosphoglycerate kinase chromatographed as an enzyme of <100 kDa under these conditions.

To further investigate whether the complexed Calvin cycle components obtained from the Sephacryl S-400 column rep-
resent homoaggregates of several enzymes having a similar molecular mass or true heteromeric multienzyme complexes, a subsequent anion-exchange FPLC on Q-Sepharose columns was performed (Fig. 2A). NaDodSO₄/urea PAGE (Fig. 2B) and immunoblot analysis (Fig. 2C) of eluting fractions revealed RuP₂Case (52 and 13.5 kDa), Ru-5-P-K (43 kDa), GAPDH, and poly peptides of 47 kDa and 27 kDa (the latter may be Rib-5-P-I) in the major column peak, which eluted at 0.48 M KCl. The same peak fractions contained Sed-1,7-bPase and FNR (Fig. 2C) and were capable of catalyzing Rib-5-P-dependent CO₂ fixation at rates of about 1 μmol/mg of protein per min. In contrast to isolated RuP₂Case, which coeluted with the putative enzyme complexes at 0.48 M KCl, purified Ru-5-P-K, Rib-5-P-I, and FNR came off of the same column as single components at <0.40 M KCl, and the GAPDH eluted at 0.60 M KCl. This further indicates that the major FPLC peak contains heteromeric enzyme complexes and free RuP₂Case rather than homoaggregated enzymes. However, the distribution of Sed-1,7-bPase and FNR (Fig. 2C) throughout the peak fractions 28–32 suggests that the enzyme complexes may be heterogeneous with respect to the presence of these components. Activity assays and immunoblotting also revealed that ~40% of the Ru-5-P-K and GAPDH applied to the column coeluted at 0.40 and 0.43 M KCl (Fig. 2A and B). This indicates that ion-exchange FPLC causes dissociation at least of a fraction of Calvin cycle complexes into components, thereby also generating stable enzyme pairs. In contrast to the results of Gontero et al. (1), these enzyme complexes did not contain phosphoglycerate kinase and a 65-kDa protein.

**Trypsin Cleavage of Free Versus Complexed Enzymes.** The organization of Calvin cycle enzymes in solution was further investigated by comparing the tryptic peptide patterns of a purified enzyme with the ones obtained from the same component in the isolated CO₂-fixing enzyme complexes and filtered stromal extracts. The method is based on the observation that Lys and Arg residues frequently take part in the formation of protein interfaces on oligomeric proteins (24) and on the assumption that peptide bonds containing these amino acids should become cleavable upon dissociation of protein components. Accordingly, since proteolysis of all samples is performed in the same medium, which in turn should bring about the same conformation of a particular enzyme, any difference in the peptide patterns compared to the purified enzyme should be caused by protein–protein interactions. Fig. 3 shows that within seconds enzymatically active and inactive (not shown) Ru-5-P-K is cleaved into identical peptides by trypsin (Fig. 3, e), whereas at the same protease/substrate ratio the enzyme present in multienzyme complexes (Fig. 3, c) and chloroplast stromal extracts (Fig. 3, a) remained unchanged. As a control, an excess of purified Ru-5-P-K was added to the stromal extract prior to trypsin cleavage (Fig. 3, b). The added enzyme, but not the endogenous one, was rapidly cleaved into peptides like the control enzyme, indicating that only complexed Ru-5-P-K is protease resistant. It should be noted that none of the active Ru-5-P-K preparations was free of degraded enzyme, even when protease inhibitors were used for enzyme purification. More-

![Fig. 2. Anion-exchange FPLC of Calvin cycle enzyme complexes.](image)

![Fig. 3. Trypsinolysis combined with immunoblotting shows binding of Ru-5-P-K to stromal enzyme complexes.](image)
over, addition of active Ru-5-P-K to stoichiometric amounts of RuP2Case, which is not susceptible to trypsin cleavage, did not protect the kinase from cleavage (Fig. 3, d).

GAPDH appears to represent another complexed protein. This is indicated by the finding that GAPDH bound to isolated Calvin cycle complexes is less susceptible to trypsinolysis but becomes rapidly degraded after salt-induced dissociation of protein complexes (see Fig. 4C).

Calvin Cycle Multi-enzyme Complexes Dissociate at High Ionic Strength and Extreme pH Values. Whereas >80% of the Calvin cycle complexes sustain molecular-sieving FPLC at ≤200 mM KCl, higher ionic strength causes their dissociation into components. This can be demonstrated again by limited trypsinolysis combined with immunoblotting. Fig. 4 shows that enzyme complexes appear to dissociate into components at KCl concentrations >250 mM, since fractions of Ru-5-P-K and GAPDH molecules can be degraded proteolytically. However, as compared to the free enzyme (Fig. 3, e), trypsinolysis of the kinase under these conditions generated only a negligible amount of small peptides (<13 kDa), thus indicating that protease cleavage of the Ru-5-P-K fragments cannot further proceed, presumably due to binding to other components.

Tryptic digestion also revealed that the enzyme organization of Calvin cycle complexes is changed at alkaline pH (>9.0), whereas no enzyme dissociation was observed when the pH of protein solutions was reduced to as low as 6.5. A complete dissociation of the Calvin cycle complexes into individual enzymes is achieved by addition of ammonium sulfate (≥1 M) to chloroplast stroma or crude leaf extracts or by adjusting the pH of protein solutions near to the average pI (4.5) of Calvin cycle enzymes.

Immunocytochemical Localization of Chloroplast Proteins. To prevent extraction of soluble proteins and ultrastructural alterations of samples, cryoprepared leaf sections were used for immunoelectron microscopy to investigate the spatial localization of several chloroplast proteins, including RuP2Case, RuP2Case activase, Ru-5-P-K, GAPDH, Sed-1,7-bPase, FNR, and CF1α of ATP synthase (Fig. 5). In agreement with other reports (25, 26), it was found that abundant RuP2Case (Fig. 5a) and the nonabundant RuP2Case activase (Fig. 5b) are evenly distributed throughout chloroplasts and that a fraction of gold particles resides in the vicinity of chloroplast membranes. CF1α is clearly bound to stroma-facing thylakoid membranes (Fig. 5f). Unexpectedly, immunoelectron microscopy of the same leaf sections revealed a predominant association of the nonabundant Calvin cycle enzymes GAPDH, Sed-1,7-bPase, and Ru-5-P-K (Fig. 5c–e) as well as FNR (Fig. 5g) with stroma-faced thylakoid membranes. Only a few, if any, of the 200 gold particles that have been evaluated to spatially localize the enzymes resided on sack-like stroma areas of chloroplasts probably free of thylakoid membranes. Although the density of gold particles was found to be relatively low, immunogold labeling of the minor Calvin cycle enzymes and FNR compared to RuP2Case is assumed to reflect roughly the stoichiometric ratios (about 1:25 to 1:50) of these proteins in chloroplasts.

**DISCUSSION**

Further evidence is provided that in chloroplast stromal extracts the Calvin cycle enzymes Rib-5-P-I, Ru-5-P-K, RuP2Case, GAPDH, and Sed-1,7-bPase are organized into heteromeric CO2-fixing multi-enzyme complexes of ~900 kDa. Almost all of these enzyme complexes appear to sustain molecular-sieving FPLC at low ionic strength. The terminal electron transport enzyme FNR is an intrinsic component of the complexes and its function is thought to provide NADPH for CO2 reduction. Phosphoglycerate kinase and a 65-kDa polypeptide, the latter component previously thought to be a manganese-binding thylakoid protein associated with five Calvin cycle enzyme complexes (1), are absent from the enzyme aggregates described in this paper.

Although evidence has been presented elsewhere for the existence of Calvin cycle enzyme complexes (1–3), it has...
been very difficult to prove that they are not isolation artifacts caused by uncontrolled aggregation of partially unfolded enzymes. Employing limited proteolysis combined with immunoblotting, we have been able to demonstrate conclusively the existence of complexed enzymes after isolation and in stromal extracts. The method is based on the assumption that a purified enzyme, but not the same component organized into enzyme complexes, is cleaved into peptides, if the protease cleavage sites are localized on complementary protein–protein interfaces. Trypsin is suitable for that purpose, because it specifically cleaves Arg and Lys residues, which are predominantly localized on protein interfaces (24). Evidence has been obtained that Ru-5-P-K and GAPDH in chloroplast stromal extracts represent mainly complexed enzymes. Moreover, it has been shown that a fraction of multienzyme complexes, though remaining stable at lower ionic strength, dissociates into enzyme components at higher ionic strength and at extremes of pH. Thus, disintegration by salt of the stromal Calvin cycle complexes is a prerequisite to release constituents into solution in order to allow the isolation of enzymes. This would explain that complexed and free forms of Ru-5-P-K were obtained from chloroplasts and leaf extracts (27, 28) as well as that several pairs of Calvin cycle enzymes have been observed previously (4–6). Limited proteolysis combined with immunoblotting may be applicable to other enzyme systems, in solution and membrane bound, to demonstrate the existence of multienzyme complexes and enzyme pairing in vitro.

Calvin cycle enzymes of higher plants are thought to be randomly distributed throughout the chloroplast matrix because such a distribution was observed for the abundant RuP2Case (25, 26). Our results call into question this assumption. Immunoelectron microscopy of cryoprepared leaf sections showed that the abundant Calvin cycle enzymes Ru-5-P-K, GAPDH, and Sod-1,7-Base and the terminal electron transport enzyme FNR to be localized on thylakoid membranes. A predominant enzyme association with photosynthetic membranes in situ has been also observed in the case of Ru-5-P-K (29, 30), fructose-1,6-bisphosphatease, and thioredoxin (31), but unfortunately cryoprepared material was not used for immunoelectron microscopy. By contrast, RuP2Case and RuP2Case activase are randomly distributed throughout the chloroplast, and a significant number of protein molecules was labeled in the vicinity of thylakoids. The random distribution of the nonabundant RuP2Case activase (32) supports our conclusion that membrane binding of minor Calvin cycle enzymes is unlikely to be an artifact. It is proposed, therefore, that enzymes and FNR are organized into multienzyme complexes, formed through complementary protein interfaces, which are bound to extrinsic proteins on stroma-faced thylakoid membranes in situ. The intermolecular protein bonds appear to be weak, because partial dissociation of Calvin cycle complexes from thylakoid membranes is simply caused by protein dilution upon an osmotic shock of intact chloroplasts (7).

The membrane association of Calvin cycle enzyme complexes may be attributed to enzyme pairing between H+-ATP synthase and RuP2Case as described in vitro (33). The present results indicate that FNR presumably associated with photosystem I subunits (34) may be another important membrane linker of CO2-fixing enzyme complexes. Since FNR and H+-ATP synthase are nearest neighbors at the surface of stroma-exposed thylakoids (35), both enzymes may belong to ATP/NADPH-synthesizing membrane complexes that bind the Calvin cycle enzyme complexes. The formation of photosynthetic enzyme supercomplexes could facilitate concerted enzyme catalysis accompanied by direct channeling of substrates and metabolites. Such a supramolecular organization would also ensure direct access of the CO2-assimilating apparatus to the required cofactors (ATP, NADPH) and may help prevent interference by other metabolic pathways. The presence of GAPDH and FNR in the same enzyme complex is thought to facilitate channeling of NADPH/NADP+ between these catalysts since they are the only proteins involved in NADPH oxidoreduction linked to CO2 fixation. The mitochondrial citric acid cycle very likely represents an analogous metabolic system that appears to be organized close to complex I of the respiratory chain (8). Furthermore, as indicated by this study, the organization of stromal enzymes into stable multienzyme complexes can prevent them from uncontrolled proteolysis and may cause and/or help maintain the constancy of stoichiometric ratios among proteins in chloroplasts.

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