Ribulose-1,5-bisphosphate Carboxylase-Binding Chloroplast Thylakoid Membrane Proteins. *In vitro* Evidence that H^+-ATP Synthase may Serve as a Membrane Receptor

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Chloroplast, Ribulose Bisphosphate Carboxylase, H^+-ATP Synthase, Enzyme Interaction, Affinity Binding

Affinity binding to Sepharose 4B-immobilized ribulose-1,5-bisphosphate carboxylase (Rubisco) and its isolated small (SSU) and large (LSU) subunits of Nonidet P40-solubilized chloroplast thylakoid proteins has been performed to search for Rubisco membrane receptors *in vitro*. It was found that Rubisco and both of its isolated subunit types specifically bind the same proteins including H^+-ATP synthase, cytochrome f/b_6 complex, ferredoxin-$NADP^+$ reductase (FNR) and components of photosystem (PS) II which presumably form multienzyme complexes. PS I was not recognized by Rubisco components. Of the Rubisco-binding thylakoid proteins, at least coupling factor 1 (CF_1) is shown to form specific complexes with the enzyme as revealed by affinity chromatography and protein hybridization on nitrocellulose filters. Employing the latter approach, the SSU and LSU of Rubisco were observed to bind independently to isolated α and β, but not to γ, δ and ε subunits of CF_1. The results indicate that H^+-ATP synthase may serve as a Rubisco membrane receptor at least *in vitro* and suggest that the irreversible inhibition of photophosphorylation by Rubisco [R. T. Furbank *et al.*, Biochim. Biophys. Acta **852,** 46–54 (1986)] is mainly due to its binding to the α/β subunit pairs of CF_1.

Introduction

The structure-function relationship between energy-transducing protein complexes of the thylakoid membrane and the various stroma enzymes in chloroplasts is largely unknown. Although previously thought to be free to move and hence randomly distributed throughout the chloroplast matrix, some enzymes of the Calvin cycle and related processes including Rubisco [1], phosphoribulokinase [2], glucose-6-phosphate dehydrogenase [3], phosphofructokinase [4] and fructose-1,6-bisphosphatase [5] have now been reported as being partially bound to thylakoid membranes, presumably by means of electrostatic attraction. About 40% of fructose-1,6-bisphosphatase [5, 6] and 30% of Rubisco [7] were found to be membrane-associated, thus indicating that membrane attachment may play a pivotal role for the spatial organization and regulation of enzymes catalyzing CO_2 fixation in photosynthesizing chloroplasts. The light-dependent activation of Rubisco, which correlates with the formation of a transmembranal pH gradient [8], and of other Calvin enzymes that is known to be mediated by the ferredoxin-thioredoxin reductase system [9] perhaps takes place at or close to the thylakoid membrane. The idea that extrinsic subunits of membrane proteins at the stroma-facing surface of thylakoids serve as receptors for particular stroma enzymes has prompted us to search, by means of protein affinity binding, for Rubisco-binding thylakoid proteins *in vitro*.

Abbreviations: Rubisco, Ribulose-1,5-bisphosphate carboxylase; SSU and LSU, small and large subunits of Rubisco; CF_1, catalytic portion of H^+-ATP synthase; NP 40, Nonidet P40; FNR, ferredoxin-$NADP^+$ reductase; PS, photosystem, LHC-II, light-harvesting chlorophyll *a*/*b*-protein complex of PS II.

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Materials and Methods

Isolation and fractionation of chloroplasts

Chloroplasts from field bean (*Vicia faba*) leaves were isolated and fractionated into thylakoid membranes and stroma protein as described [10]. Thylakoid membranes were dissolved at 1 mg of chlorophyll/ml in 10 mM Tricine-NaOH, pH 8.0, containing 2% (w/v) NP 40 and 0.5 mM phenylmethyl sulfonylfluoride, centrifuged at 20,000 × *g* for 10 min and the supernatant was used immediately for affinity chromatography.

Isolation and immobilization of enzymes

CF_1 [11] and Rubisco [12] were isolated from unlabeled and $^{14}CO_2$-radiolabeled plants [10] by established methods. The SSU and LSU of Rubisco were isolated by gel chromatography on a Biogel A-0.5 m column in the presence of 8 M urea [12] and dialyzed against 100 mM $NaHCO_3$. CF_1, Rubisco and its subunits dissolved in the same buffer were covalently coupled to CNBr-Sepharose-4 B at 20 °C yielding about 2 mg of each protein immobilized per ml of gel. Sepharose-4 B-immobilized glycine and bovine serum albumin (BSA) served as control matrices for affinity chromatography.

Affinity chromatography

Affinity chromatography was performed at 20 °C and one protein binding-elution cycle was completed within 40 min. Nonabsorbed stroma or thylakoid protein was washed away with 15 column volumes of 10 mM Tricine-NaOH, pH 8.0, without or with 0.5% NP 40, respectively. Protein was eluted either with 8 ml each of the wash buffer containing 50, 100, 200, 500 and 1000 mM NaCl or a continuous 0–1 M NaCl gradient (50 ml) and precipitated with 2% (w/v) TCA. Protein samples containing NP 40 were washed with 2% TCA in 70% (v/v) aqueous ethanol to remove the detergent.

Solid support hybridization of proteins

Hybridization of purified proteins has been carried out on nitrocellulose filters similarly as described previously [13]. Either nonradiolabeled CF_1, Rubisco or isolated subunits of these proteins were dialyzed against 10 mM Tricine-NaOH, pH 8.0, containing 1 mM ATP and different quantities (1 to 20 µg) dotted onto nitrocellulose filters prior to incubation at 36 °C for 2 h. The filters were soaked 2 h with the same buffer containing additionally 3% BSA to saturate free nitro groups and nonspecific binding sites on bound enzymes. Protein hybridization was performed by incubating the filters for 30 min in 20 mM Tricine-NaOH, pH 8.0, 1% BSA and 0.05% (v/v) Tween 20 containing 0.1 mg/ml of either ^{14}C-radioactive CF_1 or Rubisco (*ca.* 300,000 cpm). After three washes, 5 min each, with the same buffer omitting proteins, filters were dried on air and autoradiographed [13].

Analytical methods

Chlorophyll [14] and protein [15] concentration were determined and protein samples analyzed by SDS/urea PAGE [16] according to established methods.

FNR, cytochrome *f* and LHC-II were detected immunoenzymatically using specific antibodies [11, 16, 17] after SDS/urea PAGE and electrotransfer onto a nitrocellulose filter [18]. Antibody-antigen complexes were labeled with a peroxidase-conjugated goat anti-rabbit IgG as the secondary antibody and stained with 3 mM chloronaphthol and 0.006% H_2O_2 in 50 mM Tris-HCl, pH 8.0.

Results

Affinity chromatography has been employed as an approach to isolate Rubisco-binding thylakoid proteins *in vitro*. Pure Rubisco and its isolated small (SSU) and large (LSU) subunits immobilized on CNBr-Sepharose-4 B were used as matrices for chromatographing NP 40-solubilized thylakoid proteins in the presence of 10 mM Tricine-NaOH, pH 8.0, and 0.5% NP 40. After protein loading and extensive washing, only small quantities of chlorophyll-bearing material remained bound to the columns. Using 0–1 M NaCl gradients for protein elution, about 80% of the bound components emerged in a distinct brownish fraction from the columns at 50 mM NaCl, while the rest eluted in the range up to 200 mM NaCl (Fig. 1). Only negligible amounts, if any, of these proteins bound to CNBr-Sepharose-4 B-coupled glycine and BSA thus indicating that protein recognition by immobilized Rubisco or its subunits was specific rather than afforded by a nonspecific ion exchange reaction in these conditions. This conclusion is further supported by the observations that addition of 0.2% (w/v) BSA to thylakoid extracts yielded only small quantities of that protein bound to Rubisco-columns as well as that Rubisco-associated thylakoid proteins were not replaced by BSA at low ionic strength. Whereas the void column fractions were largely enriched in proteins of PS II and PS I, SDS/urea PAGE of different Rubisco-binding protein fractions revealed essentially the same major thylakoid polypeptides among which subunits of H^+-ATP synthase predominate (Fig. 1 and 2). Other components have been identified immunoenzymatically after transfer onto nitrocellulose

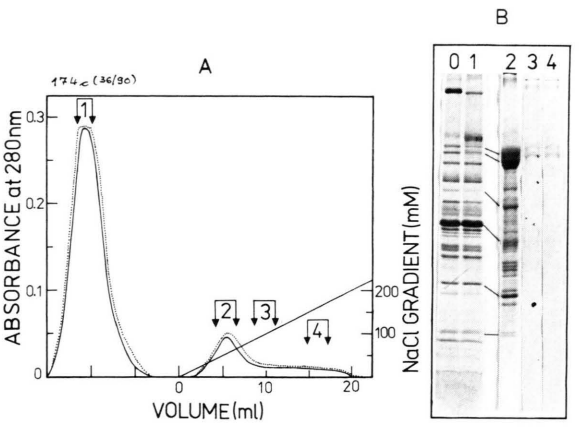

Fig. 1. Affinity chromatography of NP 40-solubilized thylakoid membrane proteins on Sepharose-4 B-coupled Rubisco (solid line) and its isolated large subunits (dashed line) (A). Each 2 ml of detergent-solubilized thylakoid protein equivalent to 1 mg of chlorophyll/ml were chromatographed on small affinity columns containing 2 ml of gel as detailed in Methods. The same results were obtained with immobilized small subunits of Rubisco.
B: SDS/urea PAGE of the NP 40-thylakoid membrane extract (lane 0) which was used for chromatography and of the eluate fractions 1 to 4 from the Rubisco-column in A (lanes 1 to 4, respectively).

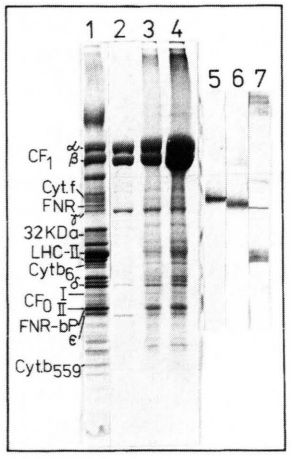

Fig. 2. Identification of thylakoid proteins which bound to immobilized large and small subunits of Rubisco. Thylakoid membrane proteins (lane 1), purified CF_1 (lane 2) and the thylakoid protein fraction which was eluted at 50 mM NaCl from LSU-Sepharose-4 B (lane 3) and SSU-Sepharose-4 B (lane 4) columns were separated by SDS/urea PAGE. Lanes 5 to 7 show the immunoenzymatic detection of cytochrome *f*, ferredoxin-NADP⁺ reductase and light-harvesting chlorophyll *a*/*b*-protein complex II, respectively, by Western blot analysis of proteins as separated in lane 4. Other details are described under Materials and Methods.

filters to be ferredoxin-NADP⁺reductase, cytochrome *f* and light-harvesting chlorophyll *a*/*b* protein complex II (Fig. 2). The presence of 32 kDa reaction center proteins and Cyt. b_{559} of PS II as well as of Cyt. b_6 is also indicated based on previous work [10]. However, the 47 kDa and 43 kDa chlorophyll a-proteins of PS II and polypeptides of PS I could not be detected in these column fractions.

That the Rubisco-binding thylakoid proteins form stable complexes even after membrane solubilization is indicated by the observations that (i) all thylakoid protein fractions that were eluted from Rubisco-columns contained the same polypeptides and (ii) that most of these components migrated in a peak of high M_r during rechromatography on a Biogel A-5 m-column (unpublished results).

Since the occurrence of such enzyme aggregates did not allow identification of the true Rubisco-binding protein(s), but the catalytic portion, CF_1, of H⁺-ATP synthase was expected to be a potential candidate for binding due to its location on the outer surface of thylakoids [19, 20], partially purified CF_1 has been chromatographed on columns of immobilized Rubisco and its isolated subunits. Notably, the five-subunit (α, β, γ, δ, ε) and three-subunit (α, β, γ) CF_1 bound to all of these columns and could be eluted quantitatively with 50 mM NaCl buffer in the presence of 1 mM ATP thereby yielding a higher purity of the enzyme (not shown). Therefore, the subunits δ and ε seem not to be involved in the formation of complexes with Rubisco components.

To further prove these observations, protein hybridization on nitrocellulose filters has been performed in the presence of an excess of BSA and Tween 20. Fig. 3 shows that immobilized CF_1 binds soluble ^{14}C-radioactive Rubisco very specifically and *vice versa*. Moreover, it is seen that α and β subunits of CF_1 as isolated in [13] still bind radioactive Rubisco and both the SSU and LSU of Rubisco independently bind radioactive CF_1 as well. No association could be observed between the immobilized γ-subunit of CF_1 and Rubisco. The protein hybrid complexes were dissolved or did not form in the presence of 0.3 M NaCl thus indicating that complementary ionic binding sites must exist between both large subunits of CF_1 and SSU and LSU of Rubisco at low ionic strength.

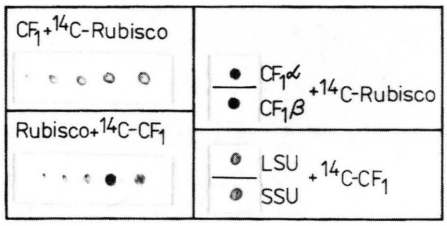

Fig. 3. Hybridization of Rubisco and CF_1 components on nitrocellulose filters. Nonradioactive CF_1 and its isolated α and β subunits as well as Rubisco and its isolated LSU and SSU were bound to nitrocellulose filters prior to hybridization with soluble ^{14}C-radiolabeled Rubisco or CF_1 and detection of protein hybrid complexes as detailed in Materials and Methods.

Additional experiments have been performed to check which chloroplast stroma proteins bind to CF_1-Sepharose-4B-columns. Fig. 4 shows that several major stroma components were recognized by immobilized CF_1 and could be eluted in the range between 50 mM (80% of the protein bound) and 1 M NaCl. SDS/urea PAGE revealed the same

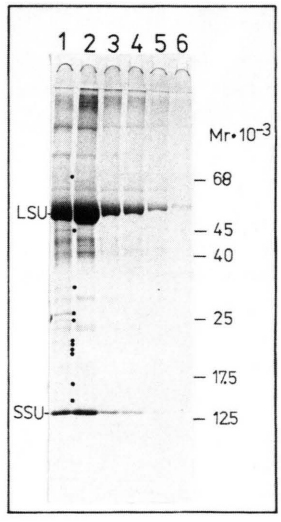

Fig. 4. Affinity chromatography of chloroplast stroma protein on CF_1-Sepharose-4B. A stromal extract (2 ml) containing 4 mg of protein/ml (lane 1) in 10 mM Tricine-NaOH, pH 8.0, and 0.1 mM phenylmethyl sulfonylflouride was loaded on a 2 ml CF_1-affinity column prior to washing with the same buffer. Absorbed protein was eluted consecutively with equilibration buffer containing 50 mM (lane 2), 100 mM (lane 3), 200 mM (lane 4), 500 mM (lane 5) and 1 M NaCl (lane 6) and analyzed by SDS/urea PAGE. The stroma polypeptides labeled by points righthand of lane 1 did not bind to the affinity column.

polypeptide composition of the different CF_1-binding stroma protein fractions analyzed despite the fact that Rubisco was the major component. Since distinct stroma proteins as well as proteins of a yeast extract and rabbit blood plasma did not bind to the column, the observed associations between CF_1 and stroma components were considered to be specific. However, it cannot be excluded at present that the CF_1-binding stroma proteins are components of multienzyme complexes and their binding to CF_1 is afforded by Rubisco alone or a few other components only.

Discussion

The present data indicate the existence of complementary ionic binding interactions between α and β subunits of H^+-ATP-synthase and SSU and LSU of Rubisco at low ionic strength *in vitro*. Accordingly, the action of pure Rubisco as an irreversible energy-transfer inhibitor of photophosphorylation in washed chloroplasts [21] is likely due to its binding to catalytically active α/β subunit pairs of CF_1 thus preventing substrate binding and/or essential conformational changes during ATP synthesis. Although evidence is still lacking, the random distribution of Rubisco throughout the chloroplast matrix [22] and an association of this enzyme with low-salt washed thylakoid membranes [7] suggest that CF_1-ATPases at the stromal surface of nonappressed stroma thylakoids [19, 20] may serve as Rubisco receptors *in vivo*, too. Such an enzyme interaction would be facilitated if the effective ionic strenght of the stroma is low due to the binding of metabolites and ions to macromolecular structures [23]. However, if this is the case, Rubisco bound to H^+-ATP synthase must differ considerably in its properties from the noncomplexed form of the enzyme which irreversibly inhibits ATP synthesis *in vitro* [21]. That this may be accomplished by other stroma enzymes linked to membrane-bound Rubisco is indicated by the observation that inhibition of photophosphorylation in isolated thylakoids by Rubisco of crude stroma extracts is transient rather than irreversible and depends on membrane energization by light [21]. Different Rubisco forms may therefore be required to keep photosynthesis active. Notably, at least two Rubisco forms of the configuration LSU_8SSU_8 and LSU_4SSU_4 have been de-

scribed previously in higher plants, the latter one being in association with other Calvin enzymes and partially bound to thylakoids [24, 25, K.-H. S., in preparation]. The thylakoid-bound form representing about 10 weight% of all Rubisco (to be published) would be sufficient to form binary complexes with H^+-ATP synthases as receptors. Accumulation of soluble, noncomplexed Rubisco forms within pyrenoids of algae chloroplasts and of cyanelles [26, 27] may be an alternative strategy to preserve ATP synthesis from irreversible inhibition by the enzyme.

The present results also support the existence of thylakoid multienzyme complexes accomodating at least H^+-ATP synthase, cytochrome f/b_6 complex, ferredoxin-$NADP^+$ reductase-receptor complex and components of PS II. This has been infered from the fact that these proteins specifically bind to and coelute from Rubisco-columns, remain associated during rechromatography on Bio-gel A-5 m-columns and can be immunoprecipitated with CF_1 antisera from Triton X-100 extracts of thylakoids at low ionic strength (unpublished results). The possibility that these membrane proteins exist in tight association also *in situ* is indicated by immunoelectronmicroscopical investigations [20, 28] which revealed populations of the same oligomeric proteins to be nearest neighboured. Perhaps these multienzyme complexes provide the structural basis for localized proton gradient coupling mechanisms between H^+-ATP synthase and electron transport chains in the stroma thylakoids and stroma-exposed margin region of grana stacks as extensively discussed in [29, 30].

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