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Isolation and characterization of a thylakoid membrane module showing partial light and dark reactions

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Abstract

A functional thylakoid membrane module of photosynthesis was isolated from cell free extracts of *Anacystis nidulans* by stepwise sequential ultracentrifugation. The thylakoid membrane fractions sedimenting at 40,000×*g*, followed by 90,000×*g* and finally at 150,000×*g* were collected. These fractions had all the components of electron transport chain, ATP synthase, phycobiliproteins, ferredoxin–NADP reductase but no ferredoxin. Five sequential enzymes of Calvin cycle viz phosphoriboisomerase, phosphoribulokinase, RuBP carboxylase, 3-PGA kinase and glyceraldehyde-3-phosphate dehydrogenase were found to be associated with thylakoid membranes. Among the three different thylakoid fractions, the 150,000×*g* fraction showed highest activities of these enzymes and also higher rate of whole chain electron transport activity on chlorophyll basis. An important finding was that the 150,000×*g* fraction showed appreciably higher rate of R-5-P+ADP+Pi dependent CO_2 fixation in light compared to the other two fractions, indicating the efficiency of this fraction in utilizing ATP for Calvin cycle. This thylakoid membrane fraction represents a fully functional module exhibiting a synchronized system of light and dark reactions of photosynthesis. Most of the components of this module remained together even after sucrose density gradient centrifugation. This is the first report on the isolation of a photosynthetic module involving membrane and soluble proteins.

Keywords: Calvin cycle enzymes; Cyanobacteria; Electron transport system; Photophosphorylation; Thylakoid membrane

1. Introduction

In the current era of functional genomics and proteomics, it is being increasingly realized that the ensembles of proteins have to be organized as supramolecular complexes. In water-limited environment in vivo, it is very unlikely that sequential enzymes of the metabolic pathway would be randomly distributed and could communicate with each other only through the diffusion of metabolites. This realization has led to the establishment of the concept of system biology [1], which deals with the identification of different components of a system and understanding their functional networking. Advances in high through-put technologies have resulted in developing the procedures like yeast two-hybrid arrays and Tandem Affinity Purification (TAP) which have identified functional interactomes of proteins mainly in the eukaryotic model system of yeast [2–5].

Such metabolic networks have to exist in several other systems including the photosynthetic autotrophs. Significant progress has been made to unravel the structure–function of isolated pigment–protein complexes in photosynthetic prokaryotes and eukaryotes [6–9]. Mass spectrometry was employed to identify the lumenal and peripheral thylakoid proteins [10]. Many groups have also reported interactions among Calvin cycle enzymes viz phosphoriboisomerase (RPI), phosphoribulokinase (RPK), RuBP carboxylase (Rubisco), phosphoglycerate kinase (PGK), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and sedoheptulose-1,7-bisphosphatase (SBPase) [11–14]. Two non-sequential enzymes of Calvin cycle, RPK and GAPDH, were shown to oligomerize with a non-enzymatic peptide CP12 to form a reversible complex [15]. All these observations suggested that the soluble enzymes of Calvin cycle have a

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tendency to associate with each other and may form functionally significant interactomes in vivo. In the protein-crowded environment of stroma, photosynthetic carbon reduction cycle operates along with other metabolic pathways, which utilize ATP and reducing power generated by light reactions in thylakoid membranes. Cryo immunoelectron microscopy revealed that many enzymes of the Calvin cycle are located along the thylakoid membranes in vivo [14,16–18]. A crucial role of thylakoid membranes in supramolecular organization of Calvin cycle enzymes was suggested by use of differentially permeabilized cells of *Anacystis nidulans* [19]. These investigations were further extended, and the present work reports isolation of a fully functional thylakoid membrane fraction from *A. nidulans* showing association with five sequential enzymes of Calvin cycle.

2. Materials and methods

2.1. Cell culture

A. nidulans (BD1) cells were grown in BG-11 medium [20] with continuous white light, 30 W/m^2, without aeration. Cells in exponential growth phase were harvested (O.D. 660 nm, ml^{-1}=~0.3).

2.2. Isolation of thylakoid membranes

Cells were washed with membrane isolation buffer (MIB) composed of 10 mM Tris–HCl pH 7.8; 10 mM $MgCl_2$; 50 mM $NaHCO_3$; 1 mM EDTA; 12 mM β-mercaptoethanol; and 10% sucrose [21]. The cell pellet was plunged in liquid nitrogen and stored at −70°C. Cells were resuspended in 20 ml of MIB (0.5–1×10^7 cells/ml) and broken either by French press (100,000 psi, single passage) or by sonication for 30 min in pulse mode. The cell extract was centrifuged at 10,000 rpm for 10 min, to remove unbroken cells and large cell debris. The supernatant was subjected to stepwise sequential ultra-centrifugation, first round at 40,000×*g* for 1 h, successively followed by second and third rounds, at 90,000×*g* and 150,000×*g* respectively, for 1 h each. The pellet after each round was collected and the supernatant was further subjected to centrifugation at a higher '*g*'. The pellets were resuspended in 0.5–1 ml of MIB and referred as 40 K (obtained at 40,000×*g*), 90 K (obtained at 90,000×*g*) and 150 K (obtained at 150,000×*g*) fractions. The supernatant after third round of centrifugation was used as soluble fraction (150 K sup).

2.3. Assay of photochemical activities in thylakoid membrane fractions

PSII and PSI activities were measured according to Ono and Murata [22] and Izawa [23]. FNR activity was measured by the method of Davis and San Pietro [24]. Whole chain electron transport activity (water to methyl viologen), excluding ferredoxin–NADP reductase, was measured as described by Hind [25].

2.4. Assay of Calvin cycle enzymes

The activities of the sequential enzymes of Calvin cycle were monitored as CO_2 fixation using various intermediates of this pathway as described by Sainis et al. [19]. Supernatant and freshly isolated membrane fractions resuspended in MIB were used for these assays. GAPDH activity was measured in these fractions, essentially according to Udvardy et al. [26] without adding PGK. R-5-P dependent GAPDH activity was measured using the same procedure except that 2 mM R-5-P was used instead of 3-PGA as substrate and 20 mM $NaHCO_3$ and 10 mM DTT were added in the above reaction mixture.

2.5. Photophosphorylation dependent CO_2 fixation

The procedure described by Ono and Murata [22] to monitor the photophosphorylation in thylakoid membranes of *A. nidulans* was adapted to measure R-5-P+ADP+Pi dependent CO_2 fixation. The reaction mixture (500 μl) contained thylakoid membrane fraction (10 μg chlorophyll); 50 mM Tricine–NaOH pH 7.5; 600 mM sucrose; 10 mM NaCl; 5 mM $MgCl_2$; 5 mM K_2HPO_4; 3 mM ADP; 10 mM DTT; and 20 mM $NaH^{14}CO_3$ (specific activity 0.5 mCi/mmol). The reaction was started after preincubation for 10 min, by adding 2 mM R-5-P and 2 mM methyl viologen. This will involve whole chain electron transport from water to methyl viologen, which includes PSII and PSI coupled to ATP synthesis. The same reaction mixture along with 0.026 mM DCPIP and 20 mM sodium ascorbate was used for the assay involving only PSI. The assay was performed under white light of intensity 21 W/m^2, at room temperature. CO_2 fixation will involve the sequential activities of phosphoriboisomerase, phosphoribulokinase and RuBP carboxylase. The acid stable reaction product of CO_2 fixation was measured as described earlier [19]. This activity was best in freshly isolated membrane fractions.

2.6. LDS PAGE, Western blotting and chlorophyll estimation

LDS PAGE for membrane and supernatant samples was carried out according to Guikema and Sherman [27]. Samples were solubilized at 0 °C for 15 min in sampling buffer and proteins were resolved on 7–20% gradient LDS polyacrylamide gel or 12% SDS PAGE. Western blotting was done using antisera against large subunit of Rubisco, GAPDH, α and β subunit of CF_1 (ATP synthase) from spinach and CP12 from *Synechocystis*. The bands were detected using chemiluminescence method with goat anti-rabbit antibody conjugated to horseradish peroxidase as secondary antibody. Chlorophyll from thylakoid membrane fractions was measured according to Tandeau de Marsac and Houmard [28].

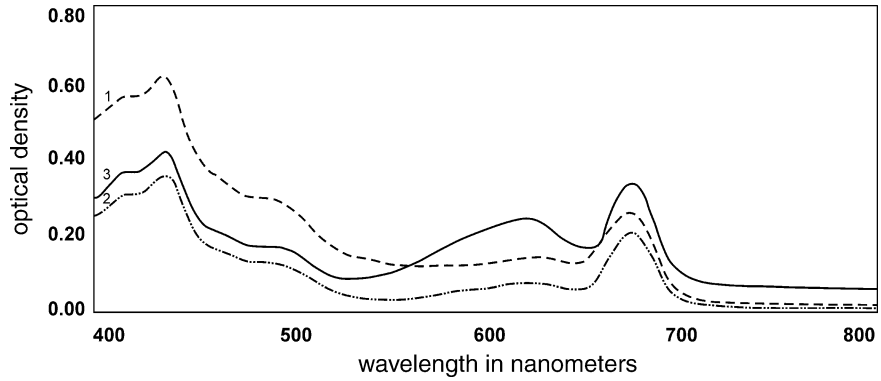

Fig. 1. Absorption spectra of thylakoid fractions: 1 (– – –): 40 K, 2 (·–· ·–·): 90 K, and 3 (—): 150 K fractions. Membranes were resuspended in MIB containing 10% sucrose. The concentration of chlorophyll in all the samples was 5 μg ml^{-1}.

2.7. Sucrose density gradient centrifugation

The 150 K thylakoid membrane fraction (200 μl) was subjected to density gradient centrifugation on 4 ml sucrose gradient (15–60%) at 150,000×*g* for 4 h using SW55Ti rotor of Beckman. The fractions (~200 μl) were collected and analyzed. Absorbance at 678 nm and 620 nm was measured to detect chlorophyll and phycobiliproteins respectively. CF_1 was detected using dot blots. The fractions were also checked for RuBP dependent and R-5-P+ATP dependent CO_2 fixation activities. The coupled assay of photophosphorylation dependent CO_2 fixation was carried out for selected pooled fractions using DCPIP and ascorbate as electron donors. The procedure followed was essentially same as described earlier except that Ru-5-P was used as the substrate instead of R-5-P.

3. Results

Thylakoid membranes are generally isolated from cyanobacterial cells after disrupting the cells by sonication or by French press, followed by ultracentrifugation, and are further treated with detergents to purify PSII, PSI and other components of electron transport. The aim of the present work was to examine native thylakoid membranes for their association with soluble Calvin cycle enzymes. Previously, Murata et al. [29] had done differential sedimentation of native thylakoid fractions at different '*g*' values. Therefore, the cell free extract of *A. nidulans* was subjected to successive ultracentrifugation. The 40 K, 90 K and 150 K thylakoid fractions and 150 K supernatant were characterized.

3.1. Detecting the components of light and dark reactions in the thylakoid membranes

The absorption spectra of all the thylakoid membrane fractions (40 K, 90 K and 150 K) showed the presence of carotenoids and chlorophyll (Fig. 1). In addition to these, the 150 K fraction had a prominent broad peak between 570 nm and 620 nm indicating the presence of phycocyanin and allophycocyanin. The room temperature fluorescence of this fraction showed a single prominent peak at 645 nm when excited at 530 nm (Fig. 2). This suggested that the 150 K fraction had retained the functionally intact, soluble phycobiliproteins.

Chlorophyll distribution among the thylakoid membrane fractions was nearly consistent in all the batches of

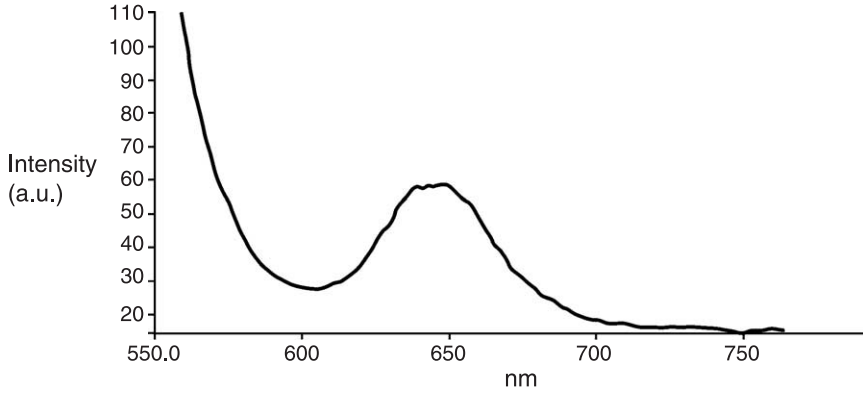

Fig. 2. Room temperature fluorescence spectrum of 150 K thylakoid fraction. Sample containing 5 μg chlorophyll ml^{-1} in MIB was used. Excitation wavelength was 530 nm.

membrane preparation. On an average (n=5) the 40 K fraction had about 76±2% whereas the 90 K and 150 K fractions contained 18±2% and 6±1% respectively of total chlorophyll present in membrane fractions. The protein profile of thylakoid membrane fractions and supernatant was observed by LDS PAGE (data not given). The three thylakoid membrane fractions showed many bands in common, however the relative intensities of some of the bands were different. The 90 K and 150 K membrane fractions showed additional bands which were common with the supernatant.

The thylakoid membrane fractions were analyzed for activities of individual components of light reactions as well as for enzymes of Calvin cycle. Table 1a shows the activities on chlorophyll basis and Table 1b shows the percentage distribution of the activities among three thylakoid fractions. The activities of PSII and PSI on chlorophyll basis were similar in the 40 K and 90 K fractions. The 40 K fraction of thylakoid membranes contained the highest proportion of total PSII and PSI activity. The 150 K fraction contained only 4% of total PSII and PSI activities while the specific activities were reduced to half. Ferredoxins could not be detected in these fractions but FNR activity was found in all the thylakoid fractions (Tables 1a and 1b). Almost 40% of total FNR activity was found associated with thylakoid membranes. Interestingly, among the thylakoid fractions, the 150 K fraction exhibited the highest specific activity of FNR on chlorophyll basis.

In view of the fact that most of the components of electron transport chain were present in thylakoid fractions, whole chain electron transport activity from water to methyl viologen was monitored (Fig. 3). This activity involves oxygen-evolving complex, PSII, cytochromes, PSI and the mobile electron carriers like quinones and plastocyanin/cytochrome c but excludes ferredoxin and FNR. Nearly 74% of total activity was found in 40 K, 16% in 90 K and only 10% in 150 K. However, the 150 K fraction showed the highest activity on chlorophyll basis.

RuBP and R-5-P+ATP dependent CO_2 fixation activities could be detected in the isolated thylakoid membranes (Tables 1a and 1b). R-5-P+ATP dependent CO_2 fixation activity confirmed the presence of phosphoribulokinase and Rubisco in these fractions. In all the batches of membrane extractions, about 30% of total RuBP carboxylase and 20% of R-5-P+ATP dependent CO_2 fixation activity were found in the thylakoid fractions, irrespective of the cell breakage by sonic oscillation or French press. Consistency in percentage distribution of activities ruled out the possibility of these associations being an artifact of cell breakage and membrane isolation procedures. The activities of these Calvin cycle enzymes were differentially distributed among the fractions. The 150 K fraction showed the highest percentage as well as the highest specific activity of RuBP and R-5-P+ATP dependent CO_2 fixation. Though the 40 K fraction had the major portion of thylakoid, it exhibited the lowest percentage of activity and low specific activity of these enzymes. We also measured 3-phosphoglyceric acid, glyceraldehyde-3-phosphate, fructose-1,6-diphosphate and sedoheptulose-1,7-diphosphate dependent CO_2 fixation activities which would involve linked activities of other Calvin cycle enzymes to generate RuBP [19]. However, these activities could not be detected in the isolated thylakoid membrane fractions (data not given). Some of these enzymes may not be directly bound or loosely bound to the thylakoid membranes and hence could have been dissociated from membranes during isolation.

Gontero et al. [12] had isolated a complex of five sequential enzymes of Calvin cycle viz RPI, RPK, Rubisco, PGK and GAPDH. Of these, RPI, RPK and Rubisco were

Table 1a
Activities of electron transport components and Calvin cycle enzymes on chlorophyll basis

Thylakoid fractions	PSII	PSI	FNR	Rubisco	RPI, RPK and Rubisco	PGK and GAPDH
40 K	26.17	480.49	109.50	2.15	1.87	2.01
90 K	30.43	499.75	239.55	25.97	19.43	14.12
150 K	18.18	236.13	463.20	86.97	78.88	56.19

Specific activity—PSII: μmol of ferricyanide reduced, mg chl^{-1} h^{-1}; PSI: μmol of O_2 consumed, mg chl^{-1} h^{-1}; FNR: μmol of DCPIP reduced, mg chl^{-1} h^{-1}; Rubisco: μmol of CO_2 fixed, mg chl^{-1} h^{-1}; RPI, RPK and Rubisco: μmol of CO_2 fixed, mg chl^{-1} h^{-1} (measured as R-5-P+ATP dependent CO_2 fixation); PGK and GAPDH: μmol of NADPH oxidized, mg chl^{-1} h^{-1} (measured as 3PGA dependent GAPDH activity).

Table 1b
Percent distribution of activities among thylakoid fractions

Thylakoid fractions	PSII	PSI	FNR	Rubisco	RPI, RPK and Rubisco	PGK and GAPDH
40 K	76.51	77.81	55.87	15.69	12.28	14.59
90 K	19.07	17.38	24.43	34.47	27.78	34.97
150 K	4.40	4.79	19.69	49.84	59.94	50.42

Percentage values in this table indicate the distribution of activities only among thylakoid fractions. About 60% of total FNR activity, 70% of RuBP dependent and 80% of R-5-P+ATP dependent CO_2 fixation activity and 85% of GAPDH activity were found in 150 K supernatant.

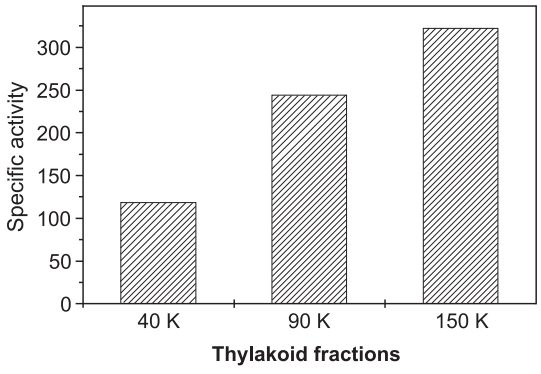

Fig. 3. Whole chain electron transport activity in thylakoid fractions. Whole chain electron transport activity (water to methyl viologen) excluding ferredoxin–NADP reductase was monitored. Oxygen consumption was measured in light and dark using oxygraph. Specific activity is expressed as μmol of O_2 consumed mg chl^{-1} h^{-1}. The distribution of activity among the thylakoid fractions was 74% in 40 K, 16% in 90 K and 10% in 150 K.

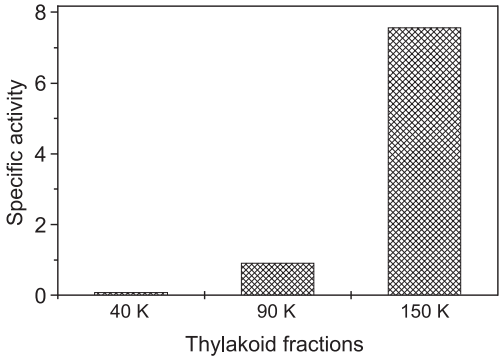

Fig. 4. R-5-P+ATP dependent GAPDH activity in thylakoid fractions. Specific activity is expressed as μmol of NADPH oxidized mg chl^{-1} h^{-1}. This assay involved the linked activity of five sequential enzymes viz. RPI, RPK, Rubisco, PGK and GAPDH. The substrates in this linked assay were R-5-P, ATP and $NaHCO_3$. The oxidation of NADPH was monitored at 340 nm. The distribution of activity, when compared only among thylakoid fractions, was 15% in 40 K, 31% in 90 K and 54% in 150 K.

detected in thylakoid fractions by R-5-P+ATP dependent CO_2 fixation assay. 3-PGA dependent NADPH oxidation measured without the addition of kinase in the reaction mixture detected the presence of PGK and GAPDH in the membrane fractions (Table 1a). Nearly 85% of GAPDH activity was detected in the supernatant, while the remaining 15% was membrane bound (Table 1b). Among the membrane fractions, the 40 K fraction showed the least activity of GAPDH as compared to the 90 K and 150 K fractions.

The activity of GAPDH was also observed using R-5-P, ATP and $NaHCO_3$ as substrates mainly in the 90 K and 150 K fractions (Fig. 4). This suggested the linked action of the five consecutive enzymes of Calvin cycle viz RPI, RPK, Rubisco, PGK and GAPDH. Interestingly, the rate of reaction on chlorophyll basis was comparatively high in the 150 K fraction, indicating better synchronized function of the five sequential enzymes in this fraction. Besides, the distribution of this activity showed that the 150 K fraction had the highest percentage of activity.

Table 2a
RuBP carboxylase activity in thylakoid fractions with and without triton treatment

Thylakoid fraction	Specific activity	
	Untreated	Treated
40 K	2.34	2.21
90 K	8.46	8.78
150 K	62.02	58.92

Specific activity: μmol of CO_2 fixed, mg chl^{-1} h^{-1}. Thylakoid membranes were treated with 1% triton on ice for 10 min followed by assay for RuBP dependent CO_2 fixation activity.

The presence of Rubisco and GAPDH in thylakoid membrane and supernatant fractions was confirmed by Western blotting using antibodies against these enzymes (Fig. 5A, B). Both the enzymes were detected mainly in the supernatant and in the 90 K and 150 K fractions of thylakoid membranes. On SDS PAGE, the cross-reacting bands showed molecular weights of 55 kDa for Rubisco and 45 kDa for GAPDH. CP12, a nonenzymatic protein involved in the oligomerization of RPK and GAPDH [15], could be detected in thylakoid membrane fractions as well as in supernatant on Western blots. The supernatant showed the presence of three CP12 antibody cross-reacting bands, whereas only one cross-reacting band was present in the membrane fractions (Fig. 5C). On SDS PAGE, the molecular weights of these three bands were 45 kDa, 54 kDa, 62 kDa.

CF_1 was detected in all the thylakoid membrane fractions using Western blots but could not be detected in the supernatant, indicating a complete removal of thylakoid membranes from soluble fraction (Fig. 5D). The molecular weight of the cross-reacting band on SDS PAGE was around 56 kDa.

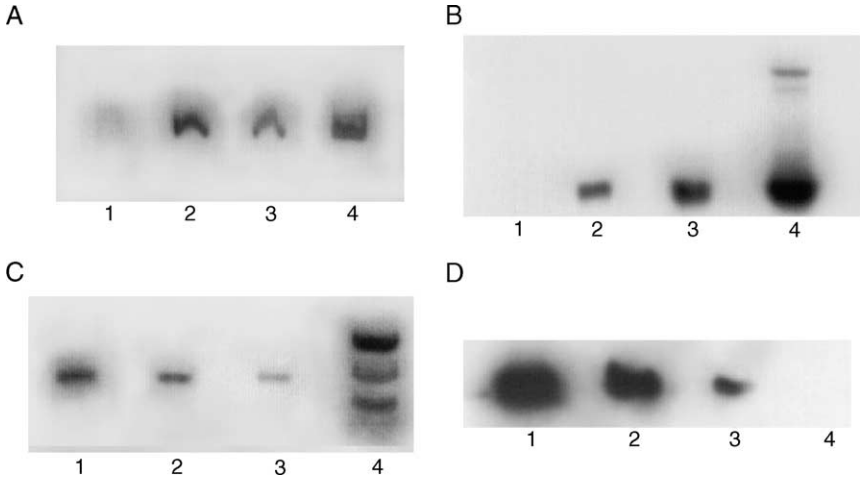

Fig. 5. Western blots. Samples were loaded on equal protein basis on LDS PAGE and transferred to PVDF membranes which were probed with (A) anti-Rubisco antibody, (B) anti-GAPDH antibody, (C) anti-CP12 antibody, and (D) anti-CF_1 antibody. Lane 1: 40 K; Lane 2: 90 K; Lane 3: 150 K thylakoid membrane fraction; and Lane 4: 150 K supernatant.

Table 2b
RuBP carboxylase activity in thylakoid fractions after treatment with anti Rubisco antibody

Thylakoid fraction	Activity remaining in supernatant	
	Untreated	Treated
40 K	0.042	0
90 K	0.11	0
150 K	0.256	0.002

Activity: μmol of CO_2 fixed, h^{-1}. The thylakoid membrane fractions were incubated with appropriate dilution of rabbit anti-Rubisco antibody for overnight at 4 °C. Goat-anti-rabbit antibody was added to the mixture and was allowed to stand at 4 °C for another 5 h. The antigen–antibody complexes were removed by centrifugation (12,000×*g*) and the supernatant was checked for RuBP carboxylase activity.

Thus the results showed that isolated thylakoid membranes had most of the components of electron transport chain, ATP synthase but no ferredoxin. The 150 K fraction of thylakoid was showing the highest association with soluble proteins such as phycobilisomes, FNR and five sequential enzymes of Calvin cycle viz phosphoriboisomerase, phosphoribulokinase, Rubisco, 3-phosphoglycerate kinase and glyceraldehyde-3-phosphate dehydrogenase.

3.2. Investigating the possibility of the formation of membrane vesicles

There was a possibility that membrane vesicles might be formed during sonication, which would entrap the soluble Calvin cycle enzymes. In this case, treating the membrane fractions with triton would increase the activity of enzymes. The incubation of membranes with 1% triton did not result in any increase in the activity of RuBP carboxylase (Table 2a). Another possibility was the formation of vesicles, which would be permeable to small molecules but impermeable to proteins. Antibody against spinach RuBP carboxylase was used to investigate this possibility. The thylakoid membrane suspensions were treated with anti-Rubisco antibody followed by incubation with secondary antibody. This treatment could precipitate all the Rubisco from the suspension of thylakoid membranes and therefore, no activity could be detected in the supernatant (Table 2b). The results on triton treatment suggested that the soluble enzymes were not entrapped in thylakoid membrane vesicles and the immuno-precipitation of Rubisco indicated that the enzyme was exposed and thus accessible to antibody. The possibility of a formation of inside-out vesicles was also checked by a simple quantitative test. Almost 97% of phycocyanin could be removed by repeated washing of the 150 K fraction with low ionic strength buffer without sucrose, indicating that phycocyanins were not trapped in the vesicles.

3.3. Sucrose density gradient centrifugation

As the soluble enzymes were exposed and not entrapped in membrane vesicles, it raised another possibility that the soluble proteins could have just co-sedimented along with the thylakoid membranes. The

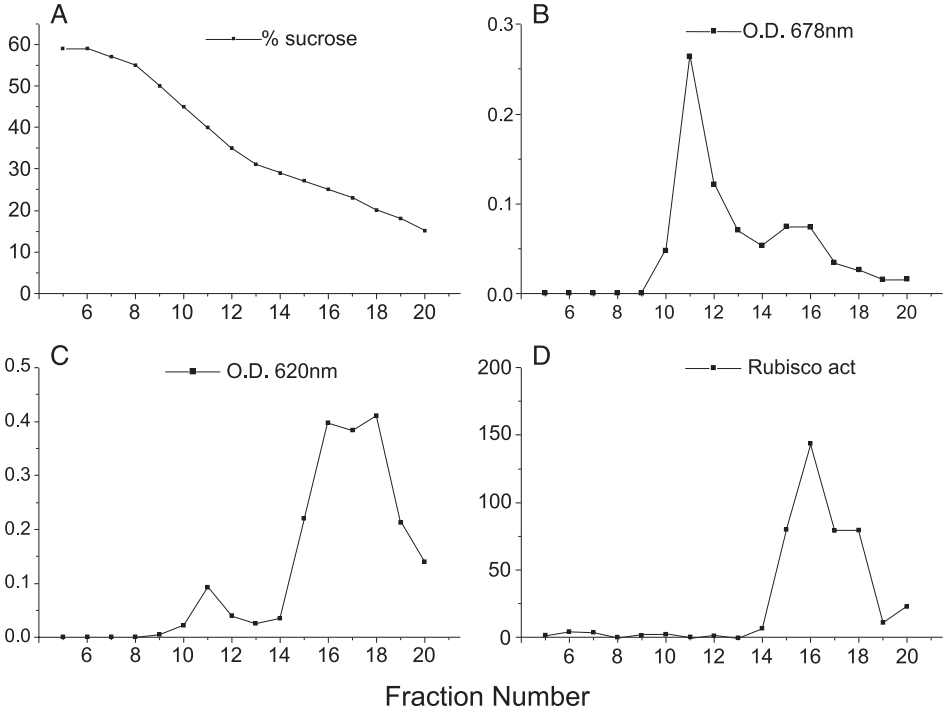

Fig. 6. Sucrose density gradient. The 150 K thylakoid membrane fraction was subjected to density gradient centrifugation on 4 ml sucrose gradient (15–60%) at 150,000×*g* for 4 h. The fractions were collected and analyzed for (A) percent of sucrose; (B) O.D. at 678 nm; (C) O.D. at 620 nm; and (D) Rubisco activity (nmol h^{-1} fraction).

Table 3
Photophosphorylation dependent CO_2 fixation activity in thylakoid fractions

Thylakoid fraction	Normal light				Low light	
	Control-ADP	Control-DTT	PSII+PSI	PSI	PSII+PSI	PSI
40 K	ND	ND	ND	ND	ND	ND
90 K	1.84	0.44	23.04	21.54	0.01	5.85
150 K	1.36	0.96	103.83	98.25	46.08	34.26

Photophosphorylation dependent CO_2 fixation activity in thylakoid fractions was measured using R-5-P+ADP+Pi as substrates. Assay time was 1 min. The values reported in the table represent specific activity in μmol h^{-1} mg^{-1} of chlorophyll. The control samples did not contain ADP or DTT. Reaction involving PSII+PSI was carried out using water as the electron donor. Reaction involving PSI alone was done using reduced DCPIP as electron donor. Light intensity: normal—21 W/m^2, low—less than 10 W/m^2; ND: not detectable.

150 K fraction, which showed the highest association with soluble proteins, was therefore subjected to sucrose density gradient centrifugation. The fractions were analyzed for the presence of thylakoids and also for soluble pigments and enzymes (Fig. 6). Two peaks of thylakoid membranes (O.D. at 678 nm) were obtained, a major peak between sucrose concentration of 30% and 50% (fraction nos. 10–13) and a minor peak between 20% and 27% sucrose concentration (fraction nos. 14–18). The peaks for phycobiliproteins (O.D. 620) and Rubisco activity overlapped with the minor peak of thylakoid membranes. R-5-P+ATP dependent activity could be detected in these fractions but the rates were much reduced in comparison to RuBP dependent activity. CF_1 was detected in fractions 11–19 using dot blots. Thus the components of light and dark reactions in the 150 K fractions remained associated on sucrose gradients. Pure Rubisco was also loaded on a similar gradient for which the peak was found at 18–20% sucrose.

3.4. Exploring the link between light reactions and activities of Calvin cycle enzymes

Since the isolated thylakoid membranes showed electron transport activity, association with Calvin cycle enzymes and the presence of CF_1, the link between light and dark reactions was explored. CO_2 fixation in light was measured using R-5-P+ADP+Pi as substrates. The reaction will involve whole chain electron transport in light, formation of ATP and subsequent utilization of ATP by Calvin cycle enzymes. Linked activities of RPI and RPK will generate RuBP, the substrate for CO_2 fixation. R-5-P+ADP+Pi dependent CO_2 fixation was observed only in the 90 K and 150 K fractions. The 150 K fraction showed the highest specific activity (Table 3). No activity could be detected in the 40 K fraction. The rate of CO_2 fixation in this assay was similar irrespective of whether only PSI or PSII–PSI both were involved in electron transport. The controls included the reaction mixtures where ADP or DTT were not added. In the absence of ADP, the activity was negligible, indicating that no ATP was present in the preparation. The dependence of reaction on DTT indicated that kinase activation was required for this assay. The rate of reaction was also reduced in low light intensity, indicating the light dependency of this reaction. When the cells were pre-incubated in the dark for 30 min before the extraction of thylakoid membranes, the rate of R-5-P+ADP+Pi dependent CO_2 fixation was reduced by about 60%.

Interestingly, the rate of reaction with R-5-P+ADP+Pi mixture was very high for the initial 30 s (Fig. 7). This is in agreement with the results obtained on photophosphorylation by isolated thylakoids of *A. nidulans*, which was shown to occur most efficiently during first 30 s of reaction [22]. When external ATP was used, a lag of 30 s was observed for R-5-P+ATP dependent CO_2 fixation activity, indicating that ATP produced in situ was more efficiently utilized by the phosphoribulokinase than the externally supplied one.

Photophosphorylation dependent CO_2 fixation activity was also measured in the fractions obtained after sucrose density gradient centrifugation (fractions 14–18, Fig. 6) forming the minor peak of thylakoid. These fractions were pooled and RuBP, R-5-P+ATP dependent CO_2 fixation activities were measured (Table 4). Since R-5-P+ATP dependent CO_2 fixation activity was low in these fractions, Ru-5-P was used as substrate and Ru-5-P+ATP and Ru-5-P+ADP+Pi dependent CO_2 fixation was measured. Phosphoribulokinase and Rubisco were the two enzymes that contributed to CO_2 fixation. The pooled fraction showed RuBP, R-5-P+ATP, Ru-5-P+ATP as well as Ru-5-P+ADP+Pi dependent CO_2 fixation in light (Table 4). The presence of Ru-5-P+ADP+Pi dependent CO_2 fixation in light suggested that the phosphoribulokinase in the fraction could use ATP, freshly generated from ADP+Pi.

The above observations suggested that the soluble enzymes of Calvin cycle had not just co-sedimented with

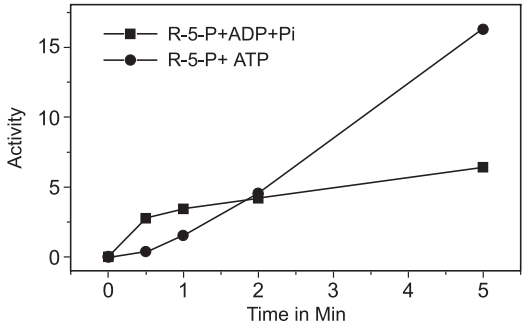

Fig. 7. Time course of R-5-P+ADP+Pi and R-5-P+ATP dependent CO_2 fixation by 150 K fraction. The rate of R-5-P+ADP+Pi and R-5-P+ATP dependent CO_2 fixation was measured using labeled bicarbonate. The aliquots were removed at different times and the acid stable reaction product was counted in a liquid scintillation counter. The activities are expressed as μmol of CO_2 fixed mg chl^{-1} using ATP generated by photophosphorylation (R-5-P+ADP+Pi) and externally supplied ATP (R-5-P+ATP).

Table 4
CO_2 fixation activity in pooled fractions of sucrose density gradient centrifugation

Substrates used for carboxylation	Total activity
RuBP	302.318
R-5-P+ATP	2.84
Ru-5-P+ATP	178.4
Ru-5-P+ADP+Pi	43.85

Total activity: nmol of CO_2 fixed, h^{-1}. Fraction nos. 14–18 (Fig. 6) from sucrose density gradient were pooled. CO_2 fixation was measured with RuBP, R-5-P+ATP and Ru-5-P+ATP as substrates. Ru-5-P+ADP+Pi dependent CO_2 fixation was measured in light.

thylakoid membranes in the 150 K fraction, but there may be a functional significance to this association.

4. Discussion

The avalanche of information from genomics and proteomics is shifting the focus of research from "molecular to modular biology" [30]. Though easy to conceptualize in silico, it has been difficult to map the interactomes unequivocally, or isolate such modules and study them in vitro. During photosynthesis, electron transport and photophosphorylation occur in the thylakoid membranes while photosynthetic carbon reduction takes place in the stroma. Despite the need to understand the organizational relationship between the components of light and dark reactions in vivo, the research in photosynthesis has been concentrated on studying these components individually. The supermolecular organization of soluble proteins and enzymes around the thylakoid membranes remains to be investigated.

We are interested in investigating the involvement of thylakoid membranes in the organization of Calvin cycle enzymes [19]. In the present investigations, three fractions of native thylakoid membranes sedimenting at 40,000×*g*, 90,000×*g* and 150,000×*g* were isolated. Rubisco activity was measured initially in these fractions, followed by linked activities of five sequential Calvin cycle enzymes viz RPI, RPK, Rubisco, PGK and GAPDH. Surprisingly, the 150 K fraction of thylakoid membranes showed higher activities of Calvin cycle enzymes as compared to the 90 K and 40 K fractions. The higher association of Calvin cycle enzymes with the lighter 150 K fraction was counterintuitive as an association with soluble enzymes should have made the fraction heavier. The three thylakoid membrane fractions were further characterized to study the distribution of pigments and proteins, and were subjected to Western blot analysis with antibodies against CF_1, CP12, Rubisco and GAPDH. The results indicated that the amounts of soluble proteins in the different thylakoid fractions were not in proportion with thylakoid content. Experiments were done to detect the components of electron transport chain individually and also collectively by measuring whole chain electron transport. Interestingly, among the thylakoids, the 150 K fraction showed the highest efficiency for whole chain electron transport on chlorophyll basis, though the activities of individual components were comparatively less.

The presence of whole chain electron transport, CF_1 and five sequential Calvin cycle enzymes requiring ATP and NADPH enthused us to investigate R-5-P+ADP+Pi dependent CO_2 fixation activity in the three-thylakoid fractions. Different controls and various possible conditions used for the assay suggested that the reaction was light dependent and ATP produced in situ was preferentially used. The results showed that there was a functional synchronization of the activities of electron transport, ATP synthesis and CO_2 fixation, which was possibly supported by the supermolecular organization in the 150 K fraction. This fraction, therefore, represented a functional photosynthetic module. Such synchronization of light and dark reactions was not observed in 40 K, the major fraction of thylakoid, though this fraction contained most of the required components. This suggested a functional heterogeneity among the thylakoid fractions.

The soluble enzymes associated with the 150 K thylakoid fraction were exposed, not entrapped in membrane vesicles. The removal of phycocyanins by washing the 150 K fraction with low ionic strength buffer indicated the absence of inside-out vesicles in membrane preparation. Hence, the isolated membranes could either be simple membrane fragments or right side out vesicles or a mixture of two. ATP formation in this fraction could be due to proton gradient in these vesicles. In case of simple membrane fragments, the ATP synthesis could be supported by localized sequestered proton domains [31]. The 150 K fraction of thylakoid membranes is probably similar to the native stroma lamellae [32].

The possibility that these associations could be a simple contamination of the thylakoid preparations by soluble proteins was ruled out by the fact that these components of light and dark reactions remained associated even after sucrose density gradient centrifugation. Besides, differential distribution of soluble proteins like CP12 was observed in supernatant and membrane fractions. The coupled photophosphorylation dependent CO_2 fixation also argues against the possibility of any non-specific associations. The results indicated that the 150 K fraction was a very small piece of membrane that did not sediment until 150,000×*g*.

Though only 15–30% of the total activities of Calvin cycle enzymes were detected with membrane fraction, this does not undermine the significance of the association. Probably, at a given time, only a fraction of Calvin cycle enzymes may be actively involved in photosynthesis. It has been suggested that an entire population of a given enzyme may not have the same fate, but can be divided into subpopulations depending on the reactions that they participate actively at any given time. Besides, many proteins may have moonlighting functions due to conformational diversity

[33]. The evidence from anti-sense transgenics [34] has also shown that ambient photosynthesis remains unaffected even after significant reduction in the expression of most of the key regulatory Calvin cycle enzymes.

Previously, several researchers had unintentionally detected associations among sequential as well as non-sequential components of light and dark reactions of photosynthesis. Many of these associations were labeled as contaminations during the purification of a specific component and were, therefore, ignored. A graphical representation of such repeatedly but unintentionally or intentionally discovered interactions is presented as a figure in the online supporting information (Dani-Sainis online supporting information). Such observations, if considered in isolation, may not convey any meaning. However, when compiled together, they suggest a 3-dimensional functional module, comprising components of light and dark reactions. The 150 K fraction isolated in the present study encompasses most of these interactions involving all the components of electron transport chain, except ferredoxin and five sequential enzymes from RPI to GAPDH. Thus, it represents a functional module of thylakoid membranes. In vivo, there may be many such modules that co-exist and participate in different metabolic pathways. The flexibility in their composition and organization will be vital for fine regulation of the metabolic activities. Functionally improved modules either due to the incorporation of more active enzymes or an advanced spatial arrangement of their components may explain the environmental adaptation and also the evolution. Investigations on the significance of modular organization will be interesting in the context of regulation of photosynthesis and thylakoid membrane biogenesis, assembly and repair.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbamem.2005.01.001.

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