In Vitro Synthesis, Assembly and Function of a Photosynthetic Membrane Protein

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ABSTRACT

Cell-free translation of Chlamydomonas reinhardtii RNA in the presence of photosynthetic membranes resulted in association of the herbicide binding (Qb) protein with membranes. Incubation of recovered membranes with high salt did not extract the polypeptide from membranes. Tryptic digestion of in vitro labeled membranes or membranes recovered from in vitro translation mixtures showed that Qb had similar orientation. In vitro translation in the presence of chloroplast membranes from cells exposed to high light intensity restored the membrane associated kinase activity lost by photo-inhibition. Thus, in vitro synthesis resulted in functional integration of the Qb protein within the photosynthetic membrane.

There is increasing evidence that at least some chloroplast thylakoid membrane proteins are synthesized by polyribosomes bound to photosynthetic membranes (5, 17). We and others have shown that thylakoid-bound ribosomes of higher plants and algae produce a number of photosynthetic membrane proteins (3, 6–8, 10, 11, 13–15). The 32 kDa Qb is one of the major products of such ribosomes. This polypeptide is synthesized, inserted, and processed to the mature form in vitro on the membrane of Chlamydomonas (10). In this report we show the synthesis and cotranslational insertion of Qb polypeptide in a heterologous system consisting of poly(A)⁺ RNA and chloroplast membranes (which are unable to stimulate protein synthesis alone, i.e. essentially polyribosome free) from Chlamydomonas, and a reticulocyte extract. Our results with trypsin digestion of membranes indicate that Qb, synthesized in vitro, achieves the same orientation in the membrane as when it is synthesized in vivo. The in vitro synthesized Qb also is functional since it restores the endogenous membrane protein kinase activity, which is lost in chloroplasts of photo-inhibited cells (19, 24).

MATERIALS AND METHODS

Chlamydomonas reinhardtii 137⁺ (wild type) cultures were grown photoautotrophically under continuous light to obtain asynchronous cells and under a 12:12 light:dark cycle for synchronous cells (8). Thylakoids were isolated according to published methods (10, 11, 17) washed with 25 mM Hepes, 10 mM EDTA (pH 7.8), and resuspended in translation buffer (80 mM KCH₃COO, 0.65 mM Mg(CH₃COO)₂, 20 mM Hepes [pH 7.5]). The EDTA washed membranes were depleted of polyribosomes bound to thylakoids.

Total cellular and poly(A) lacking RNA was prepared as described (9). Translation of RNA in the presence or absence of thylakoids was accomplished in a reticulocyte extract (New England Nuclear) for 1 h as specified by the supplier. Cycloheximide (10 µg/mL) was added to all in vitro translation reactions and thylakoids were added to those reactions not already containing them. Incubation was continued for an additional 0.5 h after which thylakoids were recovered from all samples, washed by centrifugation with 25 mM Tricine (pH 7.5), and resuspended in electrophoresis sample buffer. Washed thylakoids, recovered from translation mixtures, were also resuspended in buffer with and without 1 M LiCl, incubated at 0°C for 0.5 h, washed by centrifugation with 25 mM Tricine (pH 7.5), and resuspended in electrophoresis sample buffer. Trypsin treatment of recovered thylakoids was according to published methods (16, 18).

Photo-inhibition and PAGE analysis of the light dependent membrane kinase activity using [α-³²P]ATP as substrate was performed as described (7, 21). Thylakoids from photoinhibited cells were either used immediately after isolation or stored at −80°C (19). Stored thylakoids used within 1 week of isolation did not show light dependent phosphorylation. However, the thylakoids did exhibit light independent phosphorylation in the presence of duroquinol (data not shown) (24). Addition of synthetic electron donors to PSII (e.g. diphenylcarbazide) did not increase light dependent phosphorylation in control or reconstituted reactions.

RESULTS AND DISCUSSION

Photosynthetic membranes without bound ribosomes were isolated from C. reinhardtii grown photoautotrophically (11). The membranes did not stimulate in vitro translation without addition of exogenous RNA. This is consistent with our previous results that showed antibiotic stabilization of polyribosomes on thylakoids (17). Without antibiotic pretreatment of cells, we could not detect bound polyribosomes in isolated thylakoids. However, we washed the isolated thylakoids with EDTA to ensure polysome-free membrane preparations. Electrophoretic analysis of products from translation reactions containing membranes without exogenous RNA showed no radioactive translation products (data not shown). When thylakoids were recovered from the complete translation mixture (with RNA) after a period of incubation in the presence of...
[35S]methionine, washed, and analyzed by electrophoresis and fluorography, it was apparent that a polypeptide with same $M_r$ as Qb had become associated with the washed thylakoids (Fig. 1A; co, lanes 2 and 3). The same polypeptide was not associated with thylakoids if membranes were added to the incubation mixture after translation had been ongoing for 1 h and incubated for an additional 0.5 h in the presence of cycloheximide to prevent further translation (Fig. 1A; post, lanes 4 and 5).

We have used two properties of Qb to characterize the in vitro product recovered with thylakoids. First, it is known that the Qb is an integral membrane protein and cannot be removed from membrane by high salt concentrations (23). Second, tryptic digestion of the membrane form of the Qb yields two well defined polypeptides which remain with the membrane (1, 18). In our experiments, as shown in Figure 1B, the polypeptide associated with membranes recovered from in vitro translation was resistant to extraction by 1 M LiCl. When the Qb protein was synthesized in the absence of membranes, digestion of the total reaction mixture resulted in a radioactive band of 16 kD (Fig. 1C, lanes 3 and 4). However, Qb synthesized in vivo and in vitro in the presence of membranes was digested by trypsin to the characteristic 19 and 17 kD tryptic fragments associated with the membrane (Fig. 1C lanes 1, 2, 5, and 6). These analyses support the conclusion that the in vitro synthesized polypeptide is oriented similarly to the in vivo labeled Qb.

To determine whether the Qb protein translated in vitro was functional we added as receptor, during in vitro translation, membranes that were isolated from photoinhibited cells. Such cells, in which PSII activity is inhibited by exposure to high light intensity, are characterized by high PSII fluorescence, no DCMU/ atrazine induced increase in PSII fluorescence occurs (21) and DCMU/ atrazine binding to photosynthetic membranes cannot be observed (19). Coincident with these changes is the loss of intact Qb protein in membranes of irradiated algae or higher plants (19, 21). Loss of the Qb protein results in a dramatic decline in endogenous kinase activity when chloroplast membranes from photoinhibited cells are tested for their ability to phosphorylate proteins (20,

![Figure 1. Cell-free reconstitution of synthesis and assembly of herbicide binding protein. (A) Nonpolyadenylated RNA was translated in a reticulocyte lysate in the presence of thylakoid membranes (0.5 mg/ml Chl). The total incorporation of [35S]methionine into hot TCA insoluble polypeptides was not affected by membranes (e.g. endogenous incorporation without membranes was 5050 cpm/µL reaction, and 4950 cpm/µL with membranes; while exogenous RNA resulted in a 5- to 10-fold stimulation of cpm). Thylakoids from synchronous cells at the middle of the light period (lanes 3 and 5) and asynchronous cells (lanes 2 and 4) were used as acceptors for the in vitro products of translation. Lane 1 contained thylakoid membranes from cells labeled in vivo in the presence of cycloheximide (6). In lanes 4 and 5, thylakoids were added after translation was ongoing for one hour, cycloheximide (10 µg/mL) was added to all samples and incubation continued for an additional 0.5 h to monitor post-translational insertion of Qb. The arrow points to an endogenous reticulocyte product which has affinity for thylakoid membranes. LS notes the 55 kD subunit of ribulose 1,5-bisP carboxylase. (B) Membranes, as recovered from reaction mixtures described in A, lane 2, were incubated with 1 M LiCl for 0.5 h at 0°C. Lane 1 contained membranes recovered after translation and then incubated with buffer; lane 2, membranes recovered after translation and then incubated with buffer containing LiCl. (C) Trypsin digestion of membranes. Lanes 1 and 2 show the thylakoid polypeptides synthesized in vivo in the presence of cycloheximide, lanes 3 and 4 show total translation products of poly(A)+ RNA and lanes 5 and 6 show membrane-associated translation products (as in A, lane 2). However, thylakoids analyzed in lanes 2, 4, and 6 were digested with trypsin (50 µg/mL) for 0.5 h at 0°C. The radioactive label and membrane concentration was the same as in A.

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this heterologous system is small compared to the amount made by *Chlamydomonas* membranes isolated with bound polyriboosomes or in *vitro* with isolated RNA (10, 15). A chloroplast derived (homologous) translation system may be more efficient to form membrane-bound polyriboosomes necessary for protein synthesis and insertion. However, the results we have shown here indicate that we are now able to study membrane synthesis *in vitro* starting from mRNA. Our results indicate that the polypeptide modified by photoinhibition has been restored to the membrane *in vitro* and is functional. Recent reports have shown the possibility of using: (a) protein translated *in vitro* by RNA transcribed from cloned cDNA of a nuclear gene and isolated chloroplasts in reconstitution studies (4, 12); or (b) cloned chloroplast genes in a total *in vitro* transcription/translation system (2). These new data will ultimately allow identification of the nucleotide domain necessary for specific membrane interaction and function.

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**LITERATURE CITED**


