In Vitro Processing of Precursors of Thylakoid Membrane Proteins of Chlamydomonas reinhardtii y-1

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ABSTRACT

Studies of in vitro processing of precursors of the major chlorophyll a/b-binding polypeptides of Chlamydomonas reinhardtii y-1 were undertaken to define the precursor-product relationships. Analysis of translatates, prepared from C. reinhardtii poly(A)-rich RNA in a rabbit reticulocyte lysate system, which were incubated with the soluble fraction from C. reinhardtii cells, showed that the 31,500 relative molecular mass (Mr) precursor was converted to the M, 29,500 thylakoid membrane polypeptide whereas the M, 30,000 precursor was converted to the M, 26,000 product. Furthermore, the M, 31,500 polypeptide, when bound to antibodies, was not processed to the mature polypeptide of M, 29,500, although the presence of antibodies did not prevent the precursor of M, 30,000 from being converted to the mature M, 26,000 polypeptide. The mature fraction of Mr, 26,000, was separated into two bands corresponding to polypeptides 16 and 17 in the electrophoretic system of Chua and Bennoun (1975 Proc Natl Acad Sci USA 72: 2175–2179).

Processing activity was present in the soluble fraction obtained from cells grown in the light or in the dark. Therefore, processing of the precursor polypeptides does not appear to be involved in the regulation by light of the accumulation of these polypeptides in thylakoid membranes.

Defining the precursor-product relationships for the pLHCP and the mature membrane polypeptides is complicated by a number of factors. The three mature polypeptides and the two precursors are similar in mol wt and, thus, in electrophoretic mobility. Slight changes in gel conditions cause differences in migration of the polypeptides relative to each other (7, 18). In addition, these polypeptides show immunological cross-reactivity, e.g. antibodies raised against polypeptide 11 react with all of the mature polypeptides and the two precursors (17). This feature presumably reflects the structural similarities in the mature polypeptides (18, 21, 23).

Additional problems arise when partial proteolytic digests of in vitro generated precursors and in vivo mature products are compared in order to determine precursor-product relationships. For instance, in vivo labeling of the mature proteins is limited because of the restricted uptake of amino acids by the algal cells (15, 19), and also the precursors contain an additional sequence at the N-terminus known as the transit sequence. These problems and their implications have been discussed by Schmidt et al. (21) and by Shepherd et al. (23). Schmidt et al. (21) comment that many of the partial digestion products of the two precursors and the two mature membrane proteins of the pea comigrate, and Shepherd et al. (23) state that “making unequivocal assignments between in vitro and in vivo products is difficult.”

Rather than using intact chloroplasts for processing of pLHCP, we decided to use a fully in vitro system, obtaining the processing enzymes in the soluble fraction of the cell. This system allowed quantitative analyses of the conversion of the pLHCP to their products. During these studies of the in vitro cleavage of the pLHCP, we found that the M, 31,500 precursor, when bound to antibodies was not cleaved by the processing enzyme. However, the M, 30,000 precursor was still cleaved, and only the M, 26,000 mature polypeptide was produced. This finding and the results of quantitative studies indicate that the M, 31,500 and 30,000 precursors are converted to M, 29,500 and 26,000 products, respectively.

MATERIALS AND METHODS

Cells. Green cells of C. reinhardtii y-1 were obtained by growth at 25°C as previously described (19, 20). Yellow cells were obtained after transfer of green cells to the dark and subsequent growth at 25°C for 3 d.

Preparation and Translation of Poly(A)-Rich RNA. Poly(A)-rich RNA was extracted from algal cells by treatment with chloroform:phenol, isolated on oligo(dT)-cellulose columns, and translated in a rabbit reticulocyte lysate system (Bethesda Research Laboratories). [35S]Methionine (Amersham) was used to label the translated proteins. Details of these procedures were described previously (17).

Immunoprecipitation. The translation mixtures were either directly treated with antibody to polypeptide 11 or first precipitated with (NH4)2SO4 and then treated with antibody as described.
previously (17). Antigen-antibody complexes were recovered by adsorption onto Staphylococcus aureus cells (IgGsorb, The Enzyme Center). The antibodies to the small subunit of ribulose bisphosphate carboxylase were kindly supplied by Dr. Maurice M. Margulies (The Radiation Biology Laboratory, Smithsonian Institution, Rockville, MD).

Preparation of Cell Extracts. Cells grown in the light or dark, as described above, were harvested and suspended in one packed-cell volume of 25 mM Tris-HCl (pH 7.8). The cells were broken by passage through a French pressure cell at 5,000 p.s.i. to obtain the crude homogenate. Homogenates were centrifuged at 1,000g for 10 min and the supernatant fluid was then centrifuged at 100,000g for 1 h. Processing activity in this final supernatant solution, designated the soluble fraction, was stable for at least 1 month when stored at -70°C.

Treatment of in Vitro Translated Polypeptides with Cell Extract. Pellets obtained from the translation mixtures by precipitation with (NH₄)₂SO₄ (17) were treated at room temperature with crude homogenates, prepared from green or yellow cells as described above. Treatment of translates or immunoprecipitates with the soluble fraction (see above) was performed at room temperature with additional conditions as described in legends to Figures. Reactions were terminated by bringing the solution to a final concentration of 2% SDS and heating in boiling water for 2 min. This treatment also solubilized polypeptides in immunoprecipitates, which then were separated from Staphylococcus aureus cells by centrifugation.

Electrophoresis and Autoradiography. Two systems were used for electrophoresis. Processed translates and immunoprecipitates were routinely separated on 10 to 20% polyacrylamide gradient gels (0.15% SDS) and autoradiography of the labeled polypeptide bands was performed as described previously (17). In this system, polypeptide 11 migrates more rapidly than the Mᵣ 30,000 precursor, but polypeptides 16 and 17 are not resolved. The method of Chua (6) was utilized for electrophoresis on 7.5 to 15% gels (0.10% SDS) to separate membrane polypeptides 16 and 17. However, in this system, polypeptide 11 migrates more slowly than the Mᵣ 30,000 precursor.

Autoradiograms were scanned with a Gilford spectrophotometer and the relative amount of radioactivity in various protein fractions was determined by the area under a peak.

RESULTS

In Vitro Cleavage of pLHCP. Precursors of the LHCP were prepared by translation of C. reinhardtii poly(A)-rich RNA. The translates were then treated with fractions obtained by centrifugation of broken cells. When crude homogenates from either green or yellow cells were used, conversion of pLHCP to the mature forms was obscured by general proteolysis, which affected most of the proteins in the translate (data not shown).

Clear evidence for processing was obtained only after cell homogenates were centrifuged sufficiently to sediment all membrane material. Additional bands corresponding to the Mᵣ 29,500 and 26,000 membrane polypeptides were present on gels after electrophoresis of total translates treated with the soluble fraction of cell homogenates (Fig. 1A). To ensure that these new bands that appeared in the translate contained the membrane polypeptides, translates were first incubated with the soluble fraction and then treated with antibodies to polypeptide 11. The immunoreactive polypeptides subsequently were subjected to electrophoresis. Antibodies to polypeptide 11 (Mᵣ 29,500) were previously shown to bind not only 11 but also the structurally related 16 and 17 (Mᵣ 26,000) and the precursors of these polypeptides (17). Four bands were now present on the gel (Fig. 1B, lane 5, and Fig. 2, lane 3), two corresponding in Mᵣ to the precursor and two to the mature polypeptides. Immunoprecipitates from undigested translates contained only the precursors (Fig. 1B, lane 2).

The data shown in Figure 2 indicate that in vitro cleavage of the precursors occurs at the same site as in vivo. The processed polypeptides, which remain antigenic, migrated at rates very similar to the mature, membrane polypeptides. Slight differences in migration might be expected, since modifications, particularly N-terminal blocking, occur in vivo (18). This N-terminal block has thus far precluded determination of the amino acid sequence at this end of the mature polypeptides. However, since extended incubation with the processing activity did not alter the polypeptide pattern shown in Figures 1B and 2, we conclude that the two polypeptides that appeared during the incubation were indeed the Mᵣ 29,500 and 26,000 membrane polypeptides.

Conversion of the precursor (pS) of the small subunit of ribulose-1,5-bisphosphate carboxylase [3-phospho-D-glycerate carboxy-lyase (dimerizing), EC 4.1.1.39] to the mature form (S) was also observed (Fig. 1B, lanes 3 and 4). The general electrophoretic pattern of the total translate did not change significantly when the incubation was as long as 2 h, which indicated that other proteolytic activities were low in the soluble fraction.

Processing of both pS and pLHCP to their mature forms was detected in the pH range from 7 to 9. Although extensive processing of pS could be achieved under certain conditions (Fig. 1B, lane 4), processing of pLHCP was always incomplete (see below). Addition of EDTA (0.25 mM), DTT (4 mM), Mg⁴⁺ ions (1 to 3 mM), or Triton X-100 (2.5%), or pretreatment of the precursors with urea (5 M) did not significantly change the extent of conversion. Heating the translate (5 min at 50°C) prior to treatment with the cell extract prevented conversion. Processing also was inhibited by SDS (0.5%).

Effect of Prior Immunoprecipitation on Processing of pLHCP. When we treated the translates first with antibodies and then incubated the immunoprecipitates with the soluble fraction, we found only the Mᵣ 30,000 precursor was cleaved. As shown in Figure 3, lane 3, three bands, rather than four, appeared on gels under these conditions. These three bands corresponded to the two precursors and the Mᵣ 26,000 LHCP. The LHCP of Mᵣ 29,500 did not appear during the incubation period nor did the Mᵣ 31,500 precursor diminish significantly in amount. This finding suggested that the Mᵣ 30,000 polypeptide is the precursor of the thylakoid membrane polypeptides of Mᵣ 26,000.

Precursor-Product Relationships. To further define the quantitative precursor-product relationships, aliquots of the immunoprecipitated precursors of Mᵣ 31,500 and 30,000 were treated with increasing amounts of the soluble fraction. Under these conditions (Fig. 4A), the Mᵣ 31,500 precursor remained relatively constant in quantity and the mature polypeptide of Mᵣ 29,500 was not produced. However, as the amount of processing activity increased, the progressive decrease in the Mᵣ 30,000 fraction was complemented by a corresponding increase in the Mᵣ 26,000 polypeptide. Thus, a precursor-product relationship was established between these two fractions.

In order to show that the Mᵣ 31,500 polypeptide was the precursor of the Mᵣ 29,500 polypeptide, aliquots of the total translate were treated first with increasing amounts of cell extract and then immunoprecipitated. In this case, both the Mᵣ 31,500 and the Mᵣ 30,000 precursors progressively decreased in quantity as the Mᵣ 29,500 and the Mᵣ 26,000 fractions increased (Fig. 4B). The decrease in the Mᵣ 31,500 precursor was equivalent to the increase in the Mᵣ 29,500 polypeptide, while the decrease in the Mᵣ 30,000 precursor corresponded to the increase in the Mᵣ 26,000 polypeptide.

The quantitation of recovery of radioactivity after processing (Fig. 4) eliminates the possibility that the Mᵣ 31,500 precursor is converted to the Mᵣ 26,000 membrane polypeptide. For example, in the set of data points obtained with 120 µg protein in the processing fraction, the amount of label in the Mᵣ 31,500 polypeptide decreased from about 35 to 18% of the total radioactivity.

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in these polypeptides, whereas the label in the $M_r$ 26,000 polypeptide increased from 0 to 32% of the total. Therefore, the amount of the $M_r$ 31,500 precursor that was processed was too small to account for the amount of the $M_r$ 26,000 polypeptide that was produced. However, the appearance of the $M_r$ 29,500 polypeptide accounted for about 17% of the total, almost precisely as expected if the $M_r$ 31,500 precursor was converted to the $M_r$ 29,500 polypeptide. (The gain in radioactivity in the product should be equal to or less than the loss of radioactivity from the precursor, depending on the amount of labeled methionine in the portion of the polypeptide that is lost during the cleavage process.) Furthermore, as the $M_r$ 30,000 precursor decreased from about 66 to 31%, the $M_r$ 26,000 membrane polypeptide increased from 0 to 33% of the total radioactivity. Along with the results in Figure 3, which showed that the $M_r$ 30,000 polypeptide is the precursor of the $M_r$ 26,000 polypeptides, the quantitative relationships shown in Figure 4 indicate that the $M_r$ 31,500 component is the precursor of membrane polypeptide 11 ($M_r$ 29,500).

Only about 60% of each pLHCP fraction was cleaved to its product in these experiments. The extent of processing corresponded to the amount of soluble protein added to the translates. Increasing the time of incubation did not cause further processing of the pLHCP, nor did the pretreatment of the precursors with heat, urea, or Triton X-100. Although processing was incomplete, the assignment of precursor-product relationships was supported by the observation in all experiments that the ratio of the amounts of the products was similar to the ratio of the initial amounts of their respective precursors. Furthermore, the amount of the $M_r$ 31,500 precursor that was processed was always too small to account for the amount of the $M_r$ 26,000 membrane polypeptide that was produced. In addition, with the exception of the $M_r$ 30,000 precursor, the recovery of precursors and products did not decrease significantly with increases in the amount of soluble protein in the incubation mixture, which indicated a low level of general protease activity.

**Separation of Polypeptides 16 and 17.** Polypeptides 16 and 17 were not resolved by the electrophoretic system on 10 to 20% polyacrylamide gels used for the experiments described above. However, this system was chosen because it clearly separated membrane polypeptide 11 from the more slowly migrating precursors (pLHCP). To determine whether polypeptides 16 and 17 were both produced by *in vitro* processing, another electrophoretic system on 7.5 to 15% polyacrylamide gels was used (6) which separated 16 and 17, but in which polypeptide 11 migrated between the two precursors. To avoid ambiguity, immunoprecipitates of pLHCP, in which the $M_r$ 31,500 precursor was not cleaved to its $M_r$ 29,500 product (polypeptide 11), were treated with the soluble fraction and then subjected to electrophoresis. As shown in Figure 5, under these conditions, the processed fraction previously designated $M_r$ 26,000 separated into two bands (lane 2), which migrated at rates corresponding to mature membrane polypeptides 16 and 17 (lane 3). The recovery of 16 and 17 was low in this electrophoretic system, although when the same sample as shown in Figure 5 was run on 10 to 20% gels with the conditions used in Figures 1 to 3, the amount of the...
radioactivity in the $M$, 26,000 polypeptide (16 and 17) was approximately equal to that in its precursor fraction ($M$, 30,000) (data not shown).

From calculations of the areas under the peaks corresponding to polypeptides 16 and 17 in Figure 5, the amount of radioactivity in 16 was determined to be approximately three times that in 17. In contrast, with isolated thylakoid membranes, the amount of polypeptide 16 (as determined from the amount of stain in the gel bands) was approximately equivalent to that of polypeptide 17 (Fig. 5, lane 3). Because these two polypeptides have very similar amino acid compositions (18), these results indicate that

*in vitro* processing produces less polypeptide 17 than is produced *in vivo*.

Because polypeptide 17 has a blocked N-terminus, while 16 does not (18), we tested whether postprocessing acetylation might be required for the generation of polypeptide 17. Oxaloacetate and citrate synthetase were added to the processing system in order to remove endogenous acetyl coenzyme A. Under these conditions, polypeptide 16 was the predominant product with only a trace of 17 appearing (data not shown). These experiments suggested that blocking acetylation prevented appearance of polypeptide 17. However, when acetyl coenzyme A was added to the processing system, the amount of 17 relative to that of 16 did not increase above that shown in Figure 5, lane 2. Perhaps acetyl coenzyme A is not a limiting factor in this *in vitro* system.

**Presence of Processing Activity in Cells Subjected to Different Conditions.** When translates were incubated with soluble fractions from cells grown in the light or dark, about equal processing activity was observed in each instance (data not shown).

**DISCUSSION**

Many proteins that function in the chloroplast are synthesized on cytoplasmic ribosomes (9, 11, 16). These proteins include the small subunit of the most abundant enzyme in nature—ribulose-1,5-bisphosphate carboxylase—and the major components of the LHCP in the thylakoid membrane system. Such proteins are synthesized as precursors larger than the mature forms found in the chloroplast. When incubated with isolated chloroplasts, the precursors enter the organelle and can be reisolated as the mature form. Processing of the precursors is rapid and apparently occurs immediately following entry into the plastid (10, 14, 21).

We found that precursors of the major thylakoid membrane proteins from *C. reinhardtii* were processed *in vitro* by an enzyme
activity present in the soluble fraction of the cell (Figs. 1 and 2). We also observed processing of the precursor of the small subunit of ribulose-1,5-bisphosphate carboxylase by the same cell fraction (Fig. 1), as reported by others [Dobberstein et al. (13) and Schmidt et al. (22)]. This processing enzyme apparently resides within the chloroplast (24). When Chlamydomonas cells are broken, the chloroplasts also are ruptured, which releases the processing activity into the soluble fraction.

Only the Mr, 30,000 polypeptide precursor was cleaved by the processing enzyme while complexed with antibodies (Fig. 3). Since the antibodies were raised against polypeptide 11, possibly more antibodies were bound to various antigenic sites on its precursor (Mr, 31,500), including regions near the site of cleavage. Thus, processing to produce polypeptide 11 did not occur in the presence of antibodies. Polypeptides 16 and 17 react much more weakly with this antibody preparation (17 and unpublished data), and, therefore, the cleavage site on the precursor of these polypeptides remained exposed to the protease in the complex with the antibodies. This unexpected finding facilitated determination of the precursor-product relationships. A quantitative analysis of the processing provided evidence that the Mr, 31,500 and 30,000 precursors were converted to the Mr, 29,500 and 26,000 polypeptides, respectively (Fig. 4). Although cleavage of the precursor forms was incomplete, for reasons that are not known, both were converted to products to the same extent (Fig. 4B). These direct, complementary relationships between the precursors and products also provide strong support for our assignments. Our attempts to increase processing in vitro by treatment of the precursors with heat, urea, etc. were unsuccessful. The efficiency of processing in vivo possibly is facilitated by association of precursors with membranes which, of course, are not present in our in vitro system. It is interesting that the precursor of the small subunit of ribulose-1,5-bisphosphate carboxylase, a soluble chloroplast protein, can be cleaved completely to products in the in vitro system.

The apparent size of the transit sequence—the additional amino acids attached to the N-terminus of chloroplast proteins while they reside in the cytosol (11, 22)—is about 2,000 D in the case of the larger (Mr, 29,500) thylakoid membrane polypeptide, but 4,000 D for the smaller (Mr, 26,000) membrane polypeptides. The size of the transit sequence for the Mr, 26,000 membrane polypeptides of C. reinhardtii corresponds to that for the Chl a/b binding polypeptide 15 of pea as determined by gene sequence studies (5). However, the transit sequence for the Mr, 29,500 membrane polypeptide of C. reinhardtii is considerably smaller than those for other cytoplasmically synthesized chloroplast proteins (5, 22).

Based on a comparison of the peptides produced by digestion of the thylakoid membrane protein precursors of C. reinhardtii with the Staphylococcus aureus V-8 protease, Shepherd et al. (23) proposed different precursor-product relationships than we have found. However, they realized the equivocal nature of their analysis, which was limited to comparisons of only a small number of radioactive digestion products. Because of limitations...
in the ability to label the proteins both in vitro and in vivo, it is not possible to take advantage of the actual large number of fragments produced by digestion of the mature membrane proteins with the V-8 protease (18). Although the conclusions of Shepherd et al. (23) may be consistent with their observations, our results with a different technique have produced less ambiguous results and indicate a different relationship, i.e. between the larger precursor and product, and the smaller precursor and product, respectively. Schmidt et al. (21) and Broglio et al. (4) have reported that, with the analogous proteins of the pea, the relationships are similar to those we have found in Chlamydomonas. Although the actual mol wt of the pea proteins are different from those of Chlamydomonas, the larger precursor is converted to the larger product, etc.

In our studies, the processing enzyme obviously was present in crude cell homogenates, but its action was obscured by a generalized proteolytic activity which was associated with the particulate material of the cell. Only after the membranous fractions had been removed from cell homogenates was processing of the thylakoid membrane polypeptide precursors observed. Since general proteolytic activity in the soluble fraction is low, this proteolytic activity associated with membrane fractions may be responsible for the degradation of these polypeptides that occurs when they are unable to assemble into functional complexes (17). This finding supports the suggestion of Bennett (3) and Bellemare et al. (2) that degradation of LHC polypeptides occurs after association with membranes.

Since membrane polypeptides 16 and 17 are very similar both structurally and immunologically (17, 18), it is possible that they are produced by posttranslational modifications of the same or a very similar precursor. We have found that both 16 and 17 are produced by in vitro processing. The differences between these two polypeptides are not fully established. They have the same mobility in 10 to 20% polyacrylamide gradient gels (Figs. 1–3), but, under slightly different conditions, they separate in 7.5 to 15% gels (Fig. 5). Polypeptide 17 has a blocked N-terminus, not found on 16, which may affect its mobility (18). Moreover, polypeptide 17, but not 16, is cleaved when membranes are treated with thermolysin (8), and polypeptide 16, but not 17, is phosphorylated in illuminated cells (26). In this study, we have found that although removal of acetyl-CoA from the processing system decreased the amount of polypeptide 17 produced, addition of acetyl-CoA did not increase it (Fig. 5). Therefore, we were not able to unequivocally establish whether acetylation is a mode of posttranslational modification.

The presence of processing activity both in light and dark grown cells suggests that the amount of the processing enzyme is not regulated by these conditions. The changes in the amount of membrane protein produced in vivo under these two conditions must, therefore, be due to regulation of other steps between the synthesis on cytoplasmic ribosomes of the mRNA for the precursors and the insertion of the mature proteins into thylakoid membranes within the chloroplast.

LITERATURE CITED


