Assembly of the D1 Precursor in Monomeric Photosystem II Reaction Center Precomplexes Precedes Chlorophyll a-Triggered Accumulation of Reaction Center II in Barley Etioplasts

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Assembly of plastid-encoded chlorophyll binding proteins of photosystem II (PSII) was studied in etiolated barley seedlings and isolated etioplasts and either the absence or presence of de novo chlorophyll synthesis. De novo assembly of reaction center complexes in etioplasts was characterized by immunological analysis of protein complexes solubilized from inner etioplast membranes and separated in sucrose density gradients. Previously characterized membrane protein complexes from chloroplasts were utilized as molecular mass standards for sucrose density gradient separation analysis. In etiolated seedlings, induction of chlorophyll a synthesis resulted in the accumulation of D1 in a dimeric PSII reaction center (RCII) complex. In isolated etioplasts, de novo chlorophyll a synthesis directed accumulation of D1 precursor in a monomeric RCII precomplex that also included D2 and cytochrome b559. Chlorophyll a synthesis that was chemically prolonged in darkness neither increased the yield of RCII monomers nor directed assembly of RCII dimers in etioplasts. We therefore conclude that in etioplasts, assembly of the D1 precursor in monomeric RCII precomplexes precedes chlorophyll a-triggered accumulation of reaction center monomers.

INTRODUCTION

In higher plants grown in darkness, formation of the photosynthetic apparatus is contingent on the light-dependent induction of chloroplast development. Light regulates synthesis of the chlorophyll a precursor protochlorophyllide a and is used as a substrate to enable the enzymatic reduction of protochlorophyllide a into chlorophyllide a by NADPH–protochlorophyllide oxidoreductase (POR) (von Wettstein et al., 1995; Fujita, 1996). The subsequent enzymatic esterification of chlorophyllide a to chlorophyll a has been found to be the decisive step for the accumulation of the chlorophyll a binding apoproteins P700A/P700B, CP47, CP43, and D1 of photosystems I and II (PSI and PSII), because binding of chlorophyll a to the apoproteins confers stability against proteolysis (Eichacker et al., 1996a). Similarly, other chlorophyll-associated proteins, including the nuclear-encoded chlorophyll a/b binding light-harvesting proteins (Bennett, 1981; Kuttkat et al., 1998), PSI chlorophyll proteins in Chlamydomonas (Herrin et al., 1992), and bacteriochlorophyll binding proteins of photosynthetic bacteria, are stabilized by chlorophyll (Dierstein, 1983). As shown indirectly by spectroscopy and by photochemical activity measurements, binding of chlorophyll a occurs within the first hour after illumination of etiolated barley (Egnéus et al., 1972; Wellburn and Hampp, 1979; Burkey, 1986; Ohashi et al., 1989; Franck, 1993); however, no direct evidence for chlorophyll a-dependent photosystem assembly has been reported. Chlorophyll a-dependent accumulation of plastid-encoded chlorophyll a binding proteins is regulated at the cotranslational and post-translational levels (Klein and Mullet, 1986; van Wijk and Eichacker, 1996; Edhofer et al., 1998; Mühlbauer and Eichacker, 1998). During the first hour of illumination of etiolated barley, similar amounts of the mRNAs psbA, psbD, psbC, and psaA/psaB, which encode the photosystem chlorophyll apoproteins D1, D2, CP43, and P700A/P700B, respectively, remain associated with polysomes (Klein et al., 1988; Mullet et al., 1990). The abundance of the psbA and psaA mRNAs in their respective translation initiation complexes remains unchanged for up to 16 hr (Kim and Mullet, 1994), indicating that translation initiation is not a control point for the light-induced accumulation of chlorophyll a apoproteins during this stage of chloroplast development. Furthermore, chlorophyll a
synthesis does not alter the extent of ribosome run off from the mRNAs encoding chlorophyll approteins, indicating that translation elongation is not blocked in etioplasts (Kim et al., 1994).

At least 25 different proteins, of which 15 are plastid encoded, are assembled in PSII (Hankamer and Barber, 1997). The reaction center of PSII (RCII) is composed of the D1 and D2 proteins, two cytochrome b$_{59}$ proteins, the psbl gene product, and several small proteins (Tomo et al., 1993; Lorkovic et al., 1995). RCII binds four to six chlorophyll a molecules, two pheophytin a molecules, two carotenoids, two quinones, Fe, and Mn. In higher plants, the D1 protein is synthesized as a precursor (pD1), which has a C-terminal extension that is no longer present in the photoactive PSII complex and is known to be cleaved by a luminal peptidase (Erickson and Rochaix, 1992; Yamamoto and Satoh, 1998). Processing of pD1 might influence assembly of the PSII complex, because work with the LF1 mutant of Scenedesmus obliquus, which contains an inactive processing protease (Trost et al., 1997), indicates that the mutant contains assembled and photoactive PSII core complexes but is unable to evolve oxygen (Diner et al., 1988). Both monomeric and dimeric forms of the RCII are found after separation of the reaction center complex by HPLC size-exclusion analysis (Zheleva et al., 1996). In addition to the RCII proteins, monomeric and dimeric RCII core structures that bind the plastid-encoded antenna proteins CP43 and CP47 have been reported. Reaction center core structures have a calculated molecular mass of ~250 to 300 and 450 to 500 kD, as determined by separation on sucrose gradients (Rögnér et al., 1996; Hankamer et al., 1997). CP43 and CP47 contain six membrane-spanning $\alpha$ helices and bind three to five carotenoids and between nine and 25 chlorophyll a molecules (Tang and Satoh, 1984; Bricker, 1990). An intermediary CP47 RCII complex of 160 kD consisting of CP47, D1, D2, cytochrome b$_{59}$ $\alpha$ and $\beta$ subunits, and possibly the minor PSII proteins psbl, psbK, psbL, psbT, and PsbW, has been resolved, as 8 Å resolution (Zheleva et al., 1996, 1998; Rhee et al., 1998). Finally, dimeric PSII cores and light-harvesting complex II (LHCII) trimers and monomers reportedly form a supercomplex with a calculated molecular mass of 725 kD, which is proposed to represent the in vivo organization of PSII (Hankamer et al., 1997).

In this investigation, the onset of RCII assembly was monitored directly by measuring the chlorophyll a-dependent induction of D1 accumulation. During the binding of chlorophyll a to the de novo-synthesized pD1 protein, the molecular mass increase of the de novo-assembled chlorophyll a-protein complexes was analyzed. Distribution of radiolabeled pD1 and the D1 protein on sucrose gradients and spectroscopic analysis of the de novo-assembled chlorophyll–protein complexes demonstrate that the assembly of pD1 in a monomeric RCII precomplex precedes chlorophyll a-triggered accumulation of reaction center monomers in isolated barley etioplasts.

**RESULTS**

**Molecular Masses of de Novo–Synthesized Chlorophyll a–Protein Complexes in Etioplasts Can Be Derived by Comparison with Protein Complexes Isolated from Chloroplasts**

De novo synthesis of chlorophyll a binding proteins and assembly into chlorophyll–protein complexes can be studied by using etioplasts and chloroplasts; in etioplasts, however, no visible marker protein complexes, such as LHCI or LHCII, or RCI or RCII proteins are present to determine the molecular mass distribution of protein complexes in sucrose gradients (Figure 1, gradient from etioplast). We therefore used membrane protein complexes from chloroplasts to estimate the molecular masses of protein complexes in etioplasts. To characterize the de novo-assembled pigment protein complexes from etioplasts, we radiolabeled etioplasts in organello, solubilized integral membrane proteins, and loaded them onto sucrose gradients (Figures 1C and 2A), which we divided into 10 fractions. The molecular masses of etioplast protein complexes were determined according to the molecular mass standards of the chloroplast gradient (Figure 1). Pigment protein complexes from chloroplast gradients were identified by spectroscopic (Figure 2B) (absorbance, fluorescence, and circular dichroism spectra) methods and by immunological methods coupled to SDS-PAGE (Figures 3A and 3B). To verify the molecular masses and the oligomeric nature of the protein complexes, we analyzed complexes isolated from sucrose gradients by using native (nondenaturing) and blue native polyacrylamide gel electrophoresis (data not shown; see Methods).

In chloroplast gradients, fractions 6, 7, 5, and 2—the LHCII of PSII (fractions 6, 7, and 5) and an LHCI containing the PSII complex (fraction 2)—were readily identified (Figure 2, chloroplast, and Figure 3A, lanes 6, 7, 5, and 2). Fluorescence emission maxima (77K) of these fractions were recorded at 681 nm (fractions 7, 6, and 5) and 738 nm (fraction 2) (excitation at 435 nm), respectively (data not shown). Analysis of protein complexes by native PAGE indicated that LHCII proteins concentrated in fractions 6 and 7 were monomeric (45 kD), whereas trimeric (130 to 150 kD) forms of LHCII protein were found in fraction 5. In addition, immunological analysis clearly revealed that a small amount of pigmented LHCI protein migrated in fractions 6 and 7 (Figure 3A). Fraction 1 from the chloroplast gradients was dominated by PSII core complexes (620 kD), and some PSII-LHCI could be immunodetected and localized in the silver-stained SDS–polyacrylamide gels. In fraction 2, a PSII-LHCI (540 kD) complex was resolved (Figure 3A, lanes 1 and 2). In fraction 3, a second form of a PSII core complex (280 kD) was immunodetected and the RCI complex was identified (Figure 3A, lane 3, and Figure 4A, D2 distribution in gradient from chloroplasts).

In etioplast gradients, NADPH–POR was the most promi-
nent membrane protein (Figure 3B). Two high concentrations of POR were immunologically identified in fractions 7 and 8, with molecular masses of ~75 and 36 kD, respectively. Experiments with blue native PAGE and sucrose gradients indicated that ~50% of the etioplast POR could be present as a dimer in etioplasts (data not shown).

Fractions 1 and 2 in etioplast and chloroplast gradients were enriched with ATPase (coupling factors CF0 and CF1; 450 kD) and contained residual amounts of ribulose-1,5-bisphosphate carboxylase large subunit (plus the small subunit, 540 kD) (Figures 3A and 3B, fractions 1 and 2). Cytochrome bc1 complexes were immunologically identified in fractions 4, 5, and 6 from etioplasts and chloroplasts, with molecular masses of ~250 and 90 kD, respectively (Figures 2A, 3A, and 3B). These findings indicate that the cytochrome bc1 complex is present in a dimeric (fraction 4) and a monomeric (fractions 5 to 6) form, both in etioplasts and chloroplasts. Most proteins of the oxygen-evolving complex (OEC), which had molecular masses of 33, 23, and 16 kD (OEC33, OEC23, and OEC16, respectively), were identified in etioplast and chloroplast gradients in fractions 8 to 10. However, some OEC33 was identified up to fraction 3, indicating that OEC33 remains partly bound to PSII core complexes, whereas OEC23 and OEC16 are stripped from the complex.

When RCII particles isolated according to Nanba and Satoh (1987) were separated by sucrose gradient centrifugation, most of the reaction centers were detected at molecular masses of ~195 to 260 kD in fraction 4 (Figures 1 and 2).
Given the molecular mass of ~110 kD calculated for monomeric reaction center particles, the reaction center particles isolated were mainly in a dimeric form. Furthermore, the identification of two PSII core complexes in fractions 1 and 3 indicated that the high-molecular-mass PSII core complex in fraction 1 might represent a dimeric state of the monomeric PSII core complex seen in fraction 3 (Figure 3A, lanes 1 and 3, and Figure 4A, D2 and α-cytochrome b_{559}, lanes 1 and 3, gradient from chloroplast).

**D2 and Cytochrome b_{559} Assemble into a High Molecular Mass PSII Precomplex in Etioplasts**

In chloroplast gradients, D2 and cytochrome b_{559} were concentrated mainly in fractions 1 and 3, corresponding to dimeric and monomeric PSII core complexes, respectively; however, minor concentrations of both proteins were also detected in fractions 2 and 4, corresponding to a nonstoichiometric presence of core proteins (data for CP43, CP47, and D1 not shown) (Figure 4A, lane C, gradient from chloroplasts).

Etioplasts contain no chlorophyll a, and the chlorophyll a binding PSII proteins D1, CP47, or CP43 also were not immunodetected in etioplasts (data not shown). However, identification of D2 and cytochrome b_{559}, both of which are known to accumulate somewhat in etioplasts, revealed that D2 was localized between fractions 4 and 7, whereas cytochrome b_{559} was mainly distributed between fractions 6 and 9 and in fractions 3 and 4 (Figure 4A, gradient from etioplasts). Hence, in the absence of chlorophyll a, both proteins are present in high-molecular-mass complexes of ~200 kD (fraction 4) and ~90 kD (fraction 6) from etioplasts. Furthermore, only D2 was found in fraction 5, whereas only cytochrome b_{559} was found in a complex of ~280 kD in fraction 3.

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**Figure 3.** Silver Staining of Etioplast and Chloroplast Membrane Proteins after Separation of Protein Complexes by Sucrose Gradient Centrifugation and SDS-PAGE.

(A) Separation and silver staining of chloroplast membrane protein complexes by SDS-PAGE (4 x 10^8 plastids). Plastids were lysed, membranes washed twice in 10 mM Tris-HCl, pH 6.8, 10 mM MgCl_2, and 20 mM KCl (TMK) buffer and solubilized with 1.6% (w/v) DM, and protein complexes were separated by sucrose density gradient centrifugation (Figure 1). Proteins were precipitated with 10% (v/v) trichloroacetic acid, solubilized in SDS, separated by SDS-PAGE, and stained with silver, as described in Methods. Lanes 1 to 10 and P correspond to the fraction numbers and a pellet fraction, respectively. The molecular masses (kD) of prestained SDS-PAGE standard proteins are printed on the silver-stained protein bands.

(B) Separation and silver staining of etioplast membrane protein complexes by SDS-PAGE (4 x 10^8 plastids). Plastids were treated as described in (A).

Proteins identified by protein gel blot analysis are indicated at the left and right in (A) and (B): αβ-CF1, γ-CF1, δ-CF1, and ICF. ATPase subunits; Cyt f, cytochrome f; D2, D1, CP47, and CP43, reaction center and reaction center core proteins of PSII; LHCII and LHCI, light-harvesting complex of PSII and PSI, respectively; OEC33, OEC23, and OEC16, protein subunits of the oxygen-evolving complex OEC; petD, subunit IV of the cytochrome b_{559} complex; POR, NADPH–protochlorophyllide oxidoreductase; and P700, reaction center protein of PSI.
mass PSII precomplexes containing both proteins and either D2 and cytochrome b559, were coimmunoprecipitated with antibodies directed against the α subunit of cytochrome b559 and the D1 protein. Cytochrome b559 was precipitated directly by the α-cytochrome b559 subunit antibody in fraction 6. A 23-kD protein precipitated by the D1 antibody in fraction 6 most likely corresponds to a degradation product of the D1 protein (Figure 4B, fraction 6). In fractions 7 and 8, the D2 anti-body coimmunoprecipitated a subset of proteins identified in fraction 6; however, clearly no cytochrome b559 was detected. In contrast, the cytochrome b559 antibody precipitated the greatest amount of radiolabeled α-cytochrome b559 subunit in fractions 7 and 8.

In addition to D2 or pD1, at least three other proteins were also coimmunoprecipitated with the cytochrome b559 antibody. The pattern of proteins coimmunoprecipitated by the D1 antibody in fractions 7 and 8 was unaltered; however, the intensity of the low molecular mass proteins coimmunoprecipitated by the D1 antibody was greater in fraction 8 than in fraction 7 (Figure 4B, fractions 7 and 8). We conclude from this pattern of protein interaction between D2, cytochrome b559, and D1 that D2 most likely interacts with cytochrome b559 and several unidentified proteins in fraction 6, whereas D1 and its degradation product may be loosely bound to the protein complex of this fraction. The low yield of direct cytochrome b559 precipitation by the α-cytochrome b559 antibody in this fraction may be a result of the large number of proteins interacting with D2 besides cytochrome b559, which may shield the cytochrome b559 epitopes. In fraction 7, the interaction of cytochrome b559 with D1 may be stronger than that with the D2 protein.

We then tested whether the chlorophyll a binding proteins that were directly assembled into the precomplexes were stabilized by degradation product of the D1 protein (Figure 4B, fraction 6). Photometric detection of chlorophyll a bound to isolated protein complexes or extracted from these complexes after 0 min in darkness demonstrated that endogenous protochlorophyllide a was phototransformed to chlorophyllide a (Figure 5A, fraction 8, dotted line at 672 nm). After 80 min in darkness, protochlorophyllide (635 nm) had been resynthesized, and some chlorophyll a was retained in the prolamellar body in fraction 8 (Figure 5A, fraction 8, solid line). In fraction 5, the concentration of chlorophyllide a and chlorophyll a decreased relative to those in fraction 8. Analysis of fraction 3 revealed that chlorophyll a was present only after the seedlings had been incubated in darkness for 80 min (solid line versus dotted line). Compared with fraction 5 or 8, fraction 3 displayed a

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**Figure 4.** Protein Gel Blot Analysis and Coimmunoprecipitation. (A) Protein gel blot analysis of D2 and cytochrome b559 (α-Cyt b559) from etioplasts (E) and chloroplasts (C). Membrane protein complexes from etioplasts and chloroplasts were separated by sucrose gradients (fractions P and 1 to 10), and proteins were separated by SDS-PAGE as described in Methods. Proteins in polyacrylamide gels were transferred onto polyvinylidene difluoride membranes, and proteins were separated by SDS-PAGE as described in Methods. Proteins in polyacrylamide gels were transferred onto polyvinylidene difluoride membranes, and proteins were separated by SDS-PAGE as described in Methods. Proteins in polyacrylamide gels were transferred onto polyvinylidene difluoride membranes, and proteins were separated by SDS-PAGE as described in Methods. Proteins in polyacrylamide gels were transferred onto polyvinylidene difluoride membranes, and proteins were separated by SDS-PAGE as described in Methods. Proteins in polyacrylamide gels were transferred onto polyvinylidene difluoride membranes, and proteins were separated by SDS-PAGE as described in Methods. Proteins in polyacrylamide gels were transferred onto polyvinylidene difluoride membranes, and proteins were separated by SDS-PAGE as described in Methods. Proteins in polyacrylamide gels were transferred onto polyvinylidene difluoride membranes, and proteins were separated by SDS-PAGE as described in Methods. Proteins in polyacrylamide gels were transferred onto polyvinylidene difluoride membranes, and proteins were separated by SDS-PAGE as described in Methods. Proteins in polyacrylamide gels were transferred onto polyvinylidene difluoride membranes, and proteins were separated by SDS-PAGE as described in Methods. Proteins in polyacrylamide gels were transferred onto polyvinylidene difluoride membranes, and proteins were separated by SDS-PAGE as described in Methods. Proteins in polyacrylamide gels were transferred onto polyvinylidene difluoride membranes, and proteins were separated by SDS-PAGE as described in Methods. Proteins in polyacrylamide gels were transferred onto polyvinylidene difluoride membranes, and proteins were separated by SDS-PAGE as described in Methods. Proteins in polyacrylamide gels were transferred onto polyvinylidene difluoride membranes, and proteins were separated by SDS-PAGE as described in Methods. Proteins in polyacrylamide gels were transferred onto polyvinylidene difluoride membranes, and proteins were separated by SDS-PAGE as described in Methods. Proteins in polyacrylamide gels were transferred onto polyvinylidene difluoride membranes, and proteins were separated by SDS-PAGE as described in Methods. Proteins in polyacrylamide gels were transferred onto polyvinylidene difluoride membranes, and proteins were separated by SDS-PAGE as described in Methods. Proteins in polyacrylamide gels were transferred onto polyvinylidene difluoride membranes, and proteins were separated by SDS-PAGE as described in Methods. Proteins in polyacrylamide gels were transferred onto polyvinylidene difluoride membranes, and proteins were separated by SDS-PAGE as described in Methods. Proteins in polyacrylamide gels were transf
bathochromic absorbance shift of 4 nm, indicative of chlorophyll a bound to protein (Figure 5A, cf. fraction 3 and fractions 5 and 8). Subsequent HPLC analysis of sucrose gradient fractions revealed that ~40% of the total de novo-synthesized chlorophyll a was shifted to high molecular mass precomplexes in fractions 3 and 4 (Figure 5B, 280 and 195 kD). The presence of absorbance (676 nm) and 77K fluorescence (686 nm) maxima in fractions 3 and 4 thus indicates that the de novo-assembled chlorophyll proteins resemble PSII complexes. The molecular mass of the complexes also indicates a presence of PSII core and reaction centers, respectively (Figure 5A, absorbance and fluorescence, and Figure 2A). Therefore, we conclude that chlorophyll a-dependent stabilization of reaction center protein D1 or of core proteins CP47 and CP43 triggers accumulation of PSII from PSII precomplexes (Figure 4A, fractions 3 and 4, gradient from etioplasts).

In Isolated Etioplasts, Chlorophyll a Triggers Accumulation of de Novo–Assembled D1 Precursor in Monomeric Reaction Center Precomplexes

Our next set of experiments was designed to determine how chlorophyll a regulates the accumulation of de novo-synthesized proteins into the PSII precomplex (Figure 6). Assembly of the D1 protein was monitored after the etioplasts were radiolabeled for 15 min in the dark, after which plastids were supplemented with lincomycin, geranylgeraniol diphosphate, and light. Hence, polysome runoff of radiolabeled D1 translation intermediates could be followed concurrently with de novo chlorophyll a synthesis, and samples were removed for immunoprecipitation and SDS-PAGE immediately after the onset of chlorophyll a synthesis (time zero) or 80 min thereafter.

At time zero, etioplasts mainly accumulated the pD1 form in fractions 6 and 7, whereas after 80 min, radiolabeled pD1 was processed to the mature form (D1), which was evenly distributed among fractions 5 to 7 (Figure 6, gradient from etioplasts). Hence, radiolabeled pD1 was directly assembled into precomplexes of ~45 to 90 kD in the dark, indicating that D2 and cytochrome b559 play a role during assembly. The induction of chlorophyll a synthesis mainly triggered accumulation of D1 into 45- to 130-kD complexes; however, to our surprise, very little D1 accumulation into the precomplexes in fraction 4 could be detected in organello.

At time zero, chloroplasts isolated from etiolated barley seedlings illuminated for 18 hr incorporated the de novo-synthesized pD1 protein mainly into protein complexes distributed in fractions 4 (200 kD) and 6 (90 kD) and to some extent in fractions 5 (130 kD) and 7 (45 to 75 kD) of the sucrose gradient (Figure 6, gradient from chloroplasts). After 80 min in the presence of chlorophyll synthesis, a large proportion of the de novo-synthesized pD1 was processed, and only D1 accumulated in fractions 1 (550 to 620 kD) and 3 (280 kD) (Figure 6, gradient from chloroplasts). Unpro-
cessed D1 appeared to remain evenly distributed between fractions 4 and 6, with fraction 4 containing roughly equal amounts of pD1 and D1. These data indicate that chloroplasts directly incorporate the pD1 in monomeric (fraction 6) and dimeric (fraction 4) reaction center particles within 15 min, whereas assembly of reaction center core particles (fractions 1 and 3) requires processing of D1 (Figure 6, gradient from chloroplasts, 0 and 80 min).

Comparison of the D1 distribution in etioplasts with that in chloroplast subfractions indicated that a large proportion of the pD1 protein was directly assembled into a monomeric reaction center precomplex (Figure 6, fraction 6, 0 min, gradient from etioplasts). Accumulation of the monomeric reaction center form depended only on de novo synthesis of chlorophyll \(a\); during synthesis, the yield of processed D1 in monomeric RCII particles increased (Figure 6, fractions 5 and 6, 80 min, gradient from etioplasts), whereas in the absence of chlorophyll a synthesis, proteins were rapidly degraded and complexes were lost. Accumulation of higher molecular mass RCII particles was limited to \(\sim 130\) kD (Figure 6, fraction 5, 80 min, gradient from etioplasts), and very little D1 was detected in the dimeric reaction center form (fraction 4), even after prolonged incubation of etioplasts. Therefore, we conclude that in isolated etioplasts, either the amount of chlorophyll a is subsaturating for accumulation of higher mass reaction center complexes, or the formation of dimeric reaction center complexes is limited by the amount of preassembled monomeric reaction centers. Alternatively, a lack of nuclear-encoded factors in the in organello approach could limit assembly.

**Figure 6.** Assembly of D1 Precursor into PSII Precomplexes.

Etioplasts were isolated from 4.5-day-old dark-grown barley seedlings, whereas chloroplasts were prepared from 4.5-day-old etiolated seedlings illuminated for 18 hr in white light (50 \(\mu\)E m\(^{-2}\) sec\(^{-1}\)). D1 and pD1 were radiolabeled during dark incubation of plastids for 15 min. Thereafter, translation reactions were stopped (0 min), or lincomycin (15 \(\mu\)M) and geranylgeraniol diphosphate (12 \(\mu\)M) were added and assays were transferred to the light (50 \(\mu\)E m\(^{-2}\) sec\(^{-1}\)) for 80 min. Radiolabeled protein complexes were separated by sucrose gradient centrifugation, and D1 was concentrated by immunoprecipitation before SDS-PAGE.

**Figure 7.** Assembly of D1 Precursor in RCII Complexes in the Presence of Continuous Chlorophyll a Synthesis.

(A) De novo synthesis of chlorophyll \(a\) in etioplasts. Two different modes were utilized. The synthesis of chlorophyll \(a\) was induced in etioplasts in the presence of geranylgeraniol diphosphate (12 \(\mu\)M) by photoreduction of endogenous protochlorophyllide \(a\) (I) or was induced by addition of exogenous chlorophyllide \(a\) (1.6 \(\mu\)M) in the dark (II), as described in Methods. In the dark, chlorophyllide a plus geranylgeraniol diphosphate were added at time 0 min; thereafter, addition was repeated every 10 min. After 5, 10, 20, 40, and 80 min, the amount of chlorophyll \(a\) synthesized from phototransformed protochlorophyllide \(a\) (closed rhombs) or from exogenous chlorophyllide \(a\) (closed squares) was determined, as described in Methods.

(B) Assembly of D1 into PSII complexes. Etioplasts were radiolabeled for 20 and 80 min in the presence of low (I) and high (II) concentrations of chlorophyll \(a\); assembly of D1 is shown after separation of protein complexes by sucrose density gradient centrifugation and separation of proteins by SDS-PAGE.

**Prolonged High Rate of Chlorophyll a Synthesis Does Not Increase Formation of Dimeric RCII Complexes in Isolated Etioplasts**

In etioplasts, the concentration of protochlorophyllide \(a\) is low (0.1 nmol/10\(^7\) etioplasts), and endogenous chlorophyll \(a\) formed by flash phototransformation is consumed within 10 to 20 min (Figure 7A, closed rhombs). Thereafter, no de novo synthesis of chlorophyll \(a\) occurs in the dark, because of subsaturation of the chlorophyll synthase enzyme with chlorophyllide \(a\). Therefore, we supplemented etioplasts with chlorophyllide \(a\) and geranylgeraniol diphosphate so that the initial high rate of de novo chlorophyll a
synthesis could be maintained for the 80 min tested (Figure 7A, closed squares).

When the assembly of D1 into RCII particles was analyzed under these conditions, the yield of monomeric reaction centers (fraction 6) could not be increased (Figure 7B; cf. gradient II and gradient I). This indicates that the endogenous capacity for de novo synthesis of chlorophyll a in isolated etioplasts (Figure 7A) is sufficient to trigger an accumulation of monomeric reaction center particles. A further increase in the concentrations of monomeric reaction center complexes (fraction 6) apparently was restricted by the limited capacity for de novo synthesis of more pD1 protein. Because this could reflect either a limited capacity of the isolated organelle to reinitiate translation or a lack of nuclear factors, we circumvented the organellar translation situation by radiolabeling D1 in vivo and tested the assembly of reaction centers in the absence or presence of cycloheximide, an inhibitor of cytoplasmic translation.

**Etiolated Barley Seedlings Accumulate de Novo-Synthesized D1 in Dimeric Reaction Center Complexes upon Illumination**

Continued expression of nuclear-encoded proteins is possible if seedlings are incubated in the absence of cycloheximide. In contrast, incubating with cycloheximide allows in vivo analysis of the expression of etioplast-encoded proteins (Figure 8). To monitor the assembly reaction, we immuno-precipitated D1 radiolabeled with 35S-methionine, which was introduced into etiolated barley seedlings that had or had not been pretreated with cycloheximide for 15 min in the dark. Generally, in vivo, less radiolabel is incorporated in plastid proteins in the absence of cycloheximide because the 35S-methionine tracer is metabolized in the cytoplasmic, mitochondrial, and plastid compartments. Under both experimental conditions, the D1 protein accumulated mainly in fraction 4 of the sucrose gradient. This indicates that chlorophyll a synthesis primarily triggers the accumulation of D1 in a dimeric reaction center complex, irrespective of whether nuclear factors were coexpressed during the translation and assembly reaction (Figure 8). Hence, it is not a lack of nuclear gene expression but rather the limited capacity of the in organello system to reinitiate translation that appears to restrict accumulation of dimeric reaction center complexes. Therefore, we conclude that the assembly of monomeric reaction center complexes precedes the formation of dimeric reaction centers and that the monomeric reaction centers are the primary form of reaction center assembly under apoprotein subsaturation.

**DISCUSSION**

In this study, we analyzed the assembly of the photosystem complex in higher plants by using etioplasts from dark-grown barley leaves, which enabled us to analyze translation and chlorophyll synthesis simultaneously. Etioplasts lack chlorophyll and therefore are particularly well suited for studying photosystem assembly, because apoprotein accumulation in etioplasts is strictly regulated by chlorophyll a binding (Eichacker et al., 1990; Kim et al., 1994). Binding of Zn-pheophytin a (metal atom derivative of chlorophyll a) to P700, CP47, CP43, and D1 indicated that individual chlorophyll binding constants of the apoproteins regulate the specific protein yield (Eichacker et al., 1996a, 1996b).

The method for analysis of chlorophyll–protein complexes in sucrose gradients was based on investigations conducted with Chlamydomonas cells (Summer et al., 1997) and with chloroplasts isolated from spinach, maize, and barley (Bassi and Simpson, 1987; Bassi and Dainese, 1992; Anandan et al., 1993; Hobe et al., 1994; Chitinis et al., 1995; van Wijk et al., 1997). By using this method, we were able to characterize in detail on the basis of their molecular mass shift how the pigment protein complexes were assembled de novo in barley etioplasts.

The resolution of sucrose density gradients was particularly well suited to differentiation between monomers and oligomers of protein complexes, for example, LHCII, RCII, PSII core complexes, and cytochrome b6/f, covering a molecular mass range of 30 to 600 kD (Hobe et al., 1994; Breyton et al., 1997; Hankamer et al., 1997).

The strategy for monitoring the chlorophyll a-dependent assembly of de novo-synthesized apoproteins on the basis of changes in molecular mass offered several improvements over previous strategies, namely, the spectroscopic changes or the onset of oxygen evolution observed during primary greening of etiolated plant tissue (Egnéus et al., 1972;
etoplasts. Detection of the protein is already present in 45- to 200-kD complexes in assembly of the RCII. As demonstrated here, the D2 apoprotein could act as a membrane, chlorophyll high-molecular-mass precomplexes, which supports our speculation that the D2 apoprotein would act as a membrane-integrated mold to enable D1 integration into the membrane, chlorophyll a binding to the D1 protein, or assembly of the RCII. As demonstrated here, the D2 apoprotein is already present in 45- to 200-kD complexes in etioplasts. Detection of the α subunit of cytochrome b_{559} and the PsbW gene product (B. Müller, W.P. Schröder, and L.A. Eichacker, unpublished results) in this molecular mass range corroborates the presence of reaction center precomplexes. This may allow immediate accumulation of reaction centers when de novo synthesis of chlorophyll a induced by illumination of etioplasts triggers accumulation of D1. Although no D1 can be detected by protein gel blot analysis within the precomplexes isolated from etiolated barley kept in the dark, radiolabeling of pD1 clearly demonstrated a transient assembly of pD1 into large, 45- to 90-kD precomplexes. Therefore, etioplasts appear to assemble and disassemble pD1 in RCII precomplexes continuously in the absence of chlorophyll a and thereby stringently couple accumulation of photosystem complexes to the presence of chlorophyll a.

Recently, a Chlamydomonas mutant revealed that the α subunit of cytochrome b_{559} is required for assembly of PSII (Morais et al., 1998). Pulse-labeling experiments indicated either that synthesis of PSII subunits (D2, D1, and CP47) was impaired in the psbE null mutant or that subunits were turned over rapidly. The presence of cytochrome b_{559} found in high molecular mass precomplexes in etioplasts may therefore be required to convey proteolytic stability to D2.

In chloroplasts, D2 has been suggested as the sole primary acceptor for the assembly of D1, and other components of RCII—cytochrome b_{559} and the psbL gene product—have been proposed to coordinate after the formation of the D1/D2 heterodimer (van Wijk et al., 1997). According to our measurements with developing etioplasts and chloroplasts, pD1 is directly incorporated into a reaction center precomplex consisting at least of cytochrome b_{559} and D2. This strongly indicates that considering the formation of a D1/D2 heterodimer as the crystallization point for photosystem assembly is an oversimplification. More likely, proteins of the reaction center precomplex may assemble and disassemble in darkness until accumulation of the protein is triggered by the binding of chlorophyll a to the least stable apoprotein component (Figure 9).

Monomeric and dimeric PSII core complexes were formed in the developing chloroplasts, but only in the presence of processed D1, whereas processing of pD1 was not a precondition for the assembly of RCIIls in etioplasts and chloroplasts. Hence, formation of the PSII core complexes may be coupled to the assembly of OEC33 protein into reaction centers by the processing of assembled pD1 (Eisenberg-Domovich et al., 1995). Similarly, a prerequisite of pD1 processing for assembly of the manganese cluster demonstrated by Trost et al. (1997) indicates that a photoactive PSII core complex contains D1 in its processed form only. Whether processing must be completed before assembly of the PSII core complex or whether processing may occur during assembly at an increased enzymatic rate remains to be resolved.

In etioted tissue and isolated etioplasts, the small amount of photoconvertible protochlorophyllide per etioplast and the slow induction of protochlorophyllide resynthesis both limit synthesis of chlorophyll a during the early phase of greening. This chlorophyll a limitation concurrently limits the yield of stabilized chlorophyll a proteins in organello and in vivo. Still, this low chlorophyll a yield allowed the formation of dimeric reaction center complexes in

![Figure 9. Model for Chlorophyll a-Dependent Accumulation of RCII Monomers in Barley Etioplasts.](image)

In the dark and the absence of chlorophyll a, pD1 is continuously assembled in monomeric RCII precomplexes, disassembled, and degraded. During all stages, processing by a luminal processing peptidase (PP) may occur. Upon de novo synthesis of chlorophyll a, pD1 is stabilized against degradation, and accumulation of monomeric reaction center complexes is possible.
illuminated barley seedlings but only assembly of monomeric reaction centers in organello. In isolated etioplasts, the limited capacity for directing several rounds of reinitiation may restrict the formation of dimeric reaction center complexes, although we could circumvent the subsaturation of chlorophyll a by adding a chlorophyll a precursor in organello. Within 80 min, approximately eight rounds of reinitiation occur in vivo, whereas in organello, translation initiation is linearly lost within 60 min (L.A. Eichacker, unpublished results) and the chlorophyll protein yield is less. Hence, dimerization appears to be the result of the higher concentration of reaction center monomers obtained in vivo. We therefore conclude that it is not a lack of constitutively imported or light-induced nuclear factors but rather an embedding of etioplasts in the cellular environment that allows the assembly of dimeric reaction center complexes at a higher yield in vivo than in organello.

METHODS

Plant Material

Barley (Hordeum vulgare var Steffl) seedlings were planted in moist vermiculite and grown for 4.5 days at 25°C in a light-dark growth chamber located in a darkroom. Intact etioplasts were isolated in the darkroom, which was equipped with green safe-light as described (Eichacker et al., 1996a).

In Organello Translation and Chlorophyll Synthesis in Isolated Etioplasts

Chlorophyll a proteins were radiolabeled during translation in organello (1.4 × 10⁸ plastids corresponding to 1.9 μM protochlorophyllide a/75 μL). Reactions were conducted in the dark and in the presence of geranylgeraniol diphosphate (12 μM) dissolved in 50 mM Hepes-KOH, pH 8.0, for 15 min as described (Eichacker et al., 1996b). De novo synthesis of chlorophyll a was induced by light (50 μE m⁻² sec⁻¹) or in the dark by addition of chlorophyllide a (1.9 μM) at 25°C during incubation of etioplasts for 0 or 80 min. Protochlorophyllide, chlorophyllide, and chlorophyll concentrations were determined in samples consisting of 5.6 × 10⁷ etioplasts. Proteins precipitated during the extraction procedure were removed by centrifugation (21,000g for 2 min at 4°C). The amount of pigment was determined from the supernatant according to Helfrich et al. (1994). Translation and chlorophyll a synthesis were stopped on ice. The supernatant was removed by centrifugation (4000g for 1 min at 4°C), and intact plastids were lysed in TMK solution (10 mM Tris-HCl, pH 6.8, 10 mM MgCl₂, and 20 mM KCl). Membranes were concentrated by centrifugation (4000g for 2 min at 4°C) and were washed twice in TMK.

In Vivo Labeling of Etiolated Barley Leaves

Etiolated barley seedlings were cut off 1 cm above the seeds, and primary leaves were collected in ice water. The coleoptile was removed, and leaves were recut with a razor blade and immediately incubated in 50 mM Hepes-KOH, pH 8.0, in the presence or absence of 200 μg/mL cycloheximide at room temperature. Leaves were pre-incubated for 15 min in darkness and thereafter transferred to a reaction tube containing 2 μL ³⁵S-methionine in aqueous solution, as provided by the manufacturer (Amersham Pharmacia Biotech, Braunschweig, Germany). When the methionine was taken up by the seedlings (10 to 15 min), the sample tube was refilled with 50 mM Hepes-KOH, pH 8.0, containing 200 μg/mL cycloheximide or 50 mM Hepes-KOH, pH 8.0. Per assay, five leaves (with or without cycloheximide) were illuminated or kept in darkness for 80 min. Leaves were cut into fine pieces and ground in the presence of sea sand. The mash was washed three times in TMK, and the membranes were solubilized in 400 μL of 2% (w/v) N-dodecyl-β-D-maltoside (DM; Bio-mol, Hamburg, Germany) in TMK for 40 min on ice. Insoluble material was removed by centrifugation (21,000g for 15 min at 4°C), and the supernatant was loaded on sucrose gradients and analyzed as described (see Results).

Separation of Membrane Protein Complexes by Sucrose Gradient Centrifugation

Membranes from 1 × 10⁸ plastids were gently resuspended on ice in 1 volume of TMK and solubilized by the addition of three volumes of 2% (w/v) DM (500 μL final volume) for 40 min in the dark on ice. Solubilized membranes were centrifuged (21,000g for 10 min at 4°C), and the supernatant was loaded on a linear sucrose gradient (10 mL of 0.1 to 1.0 M sucrose in TMK containing 0.06% [w/v] DM). Protein complexes were separated by ultracentrifugation (160,000g for 16.5 hr at 4°C). Afterwards, the sucrose gradient was fractionated into 10 1-mL fractions by aspiration from the bottom of the tube (fraction 1) to the top (fraction 10). The pellet, resuspended in 1 mL of TMK containing 0.06% (w/v) DM, constituted sample P.

Molecular Mass Determination of Membrane Protein Complexes

Membrane proteins were solubilized for separation by sucrose gradients, as described above or in Gall et al. (1998), for separation in blue native PAGE (Schägger and von Jagow, 1991), or they were solubilized according to Allen and Staehelin (1991) for nondenaturing PAGE but in the absence of octylglucoside. A mixture of standard proteins was used for determining molecular masses in simultaneous separations: thyroglobulin; ferritin; catalase; lactate dehydrogenase; BSA (669, 440, 232, 140, and 67 kD, respectively) from Pharmacia or nondenatured marker proteins of urease from jackbean (545 kD [hexamer]); 272 kD [trimer]); BSA (132 kD [dimer]; 66 kD [monomer]); albumin from chicken egg (45 kD); carbonic anhydrase from bovine erythrocytes (29 kD); and α-lactalbumin from bovine milk (14.2 kD) from Sigma, or a cross-linked phosphorylase b as hexamers to monomers (584.4, 487, 389.6, 292, 194.8, and 97.4 kD). The molecular marker protein complexes were used to determine the molecular mass separation range of the sucrose gradient. Values obtained are given as error bars (y axis). For determination of the molecular mass of thylakoid membrane protein complexes, first the highest concentration of one protein of a membrane protein complex was identified by gel blot analysis and silver staining of each fraction obtained by SDS-PAGE. Second, molecular mass values of published complexes (Bassi and Simpson, 1987; Ranty et al., 1990; Hobe et al., 1994; Breyton et al., 1997; Hankamer and Barber, 1997) were checked to determine whether they fell within the molecular mass separation range.
range of the sucrose gradient. Third, the molecular masses of identified protein complexes were determined by comparing their mobility with the standard protein complexes (open triangles). From the molecular masses of these identified protein complexes, an exponential regression graph was calculated (Figure 2A). In fractions 1, 2, 4, and 7, more than one complex was identified per fraction. Here, the molecular masses of the complexes were assigned in parallel after separation in the native electrophoretic systems as 620, 450, 260, and 75 kD (open triangles). Fourth, reaction center complexes of photosystem II (PSII) containing D1, D2, cytochrome b559, and the psbI gene product were isolated essentially according to Nanba and Satoh (1987), with the modifications described in Gall et al. (1998), and used as molecular mass markers in the determinations of newly assembled reaction center particles in the sucrose gradients. Error bars (x axis) correspond to the presence of identified thylakoid membrane complexes in the adjacent fraction of the sucrose gradients (0.5 arbitrary unit < 50% protein overlap ≥ 1.0 arbitrary unit) (Figure 2A).

**Immunoprecipitation of 35S-Methionine-Labeled Proteins and SDS-PAGE**

For immunoprecipitation of proteins, 200 μL of each sucrose gradient fraction was solubilized in 2% (w/v) SDS, boiled for 2 min, and centrifuged in a microcentrifuge (21,000 g at 2 min at room temperature). Proteins in the supernatant were immunoprecipitated as described (Mullet et al., 1990). For SDS-PAGE, 500 μL of each fraction was precipitated with 10% (w/v) trichloroacetic acid for 30 min on ice, proteins were concentrated by centrifugation (21,000 g for 30 min at room temperature), and the pellet was air-dried for 1 hr and afterward resuspended in SDS solubilization buffer (2% (w/v) SDS, 20% (w/v) sucrose, 66 mM DTT, and 66 mM Na2CO3). Samples were separated by 12.5% (w/v) SDS-PAGE containing 4 M urea as described (Eichacker et al., 1996a). Proteins of the protein gel were transferred onto polyvinylidene difluoride membrane according to Towbin et al. (1979). Proteins of interest were detected by using enhanced chemiluminescence (Amersham Pharmacia Biotech).

**UV/Visible Spectroscopy and Fluorescence Spectroscopy**

UV/visible (Lambda 2; Perkin-Elmer, Norwalk, CT), 77K fluorescence (SPEX-Flurolog 221; Yobin Yvon, Longjumeau, France), and circular dichroism (Dichrograph CD6; Yobin Yvon) spectra of each fraction were taken directly after fractionation of the gradient.

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    Three-dimensional structure of the plant photosystem II reaction


Assembly of the D1 Precursor in Monomeric Photosystem II Reaction Center Precomplexes Precedes Chlorophyll a−Triggered Accumulation of Reaction Center II in Barley Etioplasts

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