Identification and Partial Characterization of the Denaturation Transition of the Light Harvesting Complex II of Spinach Chloroplast Membranes

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ABSTRACT

Differential scanning calorimetry was employed to investigate the structure of spinach (Spinacia oleracea) chloroplast membranes. In a low ionic strength Hepes-buffered medium, major calorimetric transitions were resolved at 42.5°C (A), 60.8°C (B), 64.9°C (C1), 69.6°C (C2), 75.8°C (D), 84.3°C (E), and 88.8°C (F). A lipid melting transition was also commonly seen at 17°C in scans starting at lower temperatures. The D transition was demonstrated by four independent methods to derive from denaturation of the light harvesting complex associated with photosystem II (LHC-II). Evidence for this conclusion was as follows: (a) the endotherm of the isolated LHC-II (74.0°C) was very similar to that of D (75.8°C); (b) the denaturation temperature of the 27 kilodalton LHC-II polypeptide determined in intact chloroplast membranes by thermal gel analysis was identical to the temperature of the D transition at pH 7.6 and after destabilization by shifting the pH to 6.6 or by addition of Mg²⁺; (c) analysis of the stability of the LHC-II complex by electrophoresis in native gels demonstrated that the complex dissociates during the D transition, both at pH 7.6 and 6.6; and (d) the 77 Kelvin fluorescence maximum of LHC-II in chloroplasts was seen to shift to lower wavelengths (indicating gross denaturation of LHC-II), at the temperature of the D transition when examined at either of the above pHs. With this identification, five of the eight major endotherms of the chloroplast membrane have now been assigned.

Differential scanning calorimetry has been employed by several laboratories recently to obtain information on the structure of the chloroplast membrane (4–6, 12, 16). Calorimetric scans of spinach thylakoids reveal eight endothermic transitions between 15 and 90°C which correspond to lipid melting or denaturation of major proteins or protein complexes within the thylakoid membrane. The major protein complexes of thylakoids include the reaction centers of PSI and PSII, their light harvesting complexes, the Cyt b₆f complex, and the coupling factor complexes (CF₁-CF₄). Because of their abundances in the membrane, it is expected that denaturation of each of these complexes should give rise to endotherms large enough to be detected by DSC. Thus, it has been our goal to identify the source of each calorimetric transition and once each transition is identified, to use the calorimetric profile as a means of determining the sites of perturbation in the chloroplast membrane of chemical (e.g., herbicides, growth regulators), dietary (e.g., mineral deficiency), or physical (e.g., cold, heat, drought, light, salinity) stresses. By comparing the scan of unmodified chloroplasts with the scan of chloroplasts isolated from a stressed plant, the stress-sensitive component(s) of the chloroplast membrane can often be identified by observing which transition(s) is perturbed.

Previous work has established the origins of four of the eight major membrane endotherms and some characteristics of several other transitions have been at least partially determined. A broad, reversible transition occurring around 17°C has been shown to derive from bulk, polar lipid melting (12). The A transition, centered around 42°C, originates from denaturation of the oxygen evolving system associated with PSII (4, 5, 16). The stability of this center apparently depends upon the redox state of the associated Cyt b₅₅₉ (17). The C₁ transition, located at 65°C, derives from the soluble subunit complex of the coupling factor (CF₁) (15). Finally, the 69.6°C endotherm, referred to as the C₂ transition, has been shown to originate from ribulose 1,5-bisphosphate carboxylase denaturation (5; our unpublished results).

Work by Thompson et al. (16, 17) on PSII fragments has shown the existence of five transitions centered at the following temperatures (°C): 47.2, 48.2, 53.7, 59.9, and 66.3. While they proposed the involvement of LHC-II in the two transitions in the 59 to 67°C range largely on the basis of size comparisons, little more was done to test this hypothesis. For example, in no case was LHC-II purified and compared calorimetrically with the properties of the five different endotherms.  

2 Abbreviations: CF₁ and CF₄, chloroplast coupling factor subunits 0 and 1; DSC, differential scanning calorimetry; LHC-II, light harvesting complex associated with photosystem II; DOX, deoxycholate; Tₘ, midpoint temperature of thermal denaturation; F-684, Chl a fluorescence emission maximum at 684 nm.

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dothers of the multicomponent PSII particle, nor were comparisons made between scans of the PSII particles and those of intact thylakoids. Further, because their studies were conducted under significantly different solution conditions, a direct comparison of their DSC data with ours is not possible since solution components often alter macromolecular stability. One clear example of the impact of membrane and solution environment of DSC is that their 59.9 and 66.3° LHC-II transitions lie within the temperature range of the chloroplast C1 and C2 transitions (63–70°C), which have already been shown by us to derive from denaturation of CF1 and ribulose 1,5-bisphosphate carboxylase, respectively.

The purpose of this study is to identify the source of the D transition, centered at 76°C in whole chloroplast membranes. We present several lines of evidence to demonstrate that this transition derives from the thermal denaturation of LHC-II in the intact thylakoid.

MATERIALS AND METHODS

Chloroplast Membrane Preparation and Isolation of LHC-II

Spinach (Spinacia oleracea) was purchased from a local supermarket or grown in a controlled climate facility as described (4). Thylakoid membranes were isolated according to the method of Ort and Iwata (14), except that the washing medium was the same as the suspension medium. The suspension medium used for calorimetry was 0.2 M sucrose, 10 mM Hepes (pH 7.6 or 6.6, where indicated). Chl concentration was determined according to the method of Arnon (3). Spinach LHC-II was isolated exactly as described by Krupa et al. (8).

Incubation of Thylakoids and LHC-II at pH 6.6, 7.6 (control), and in the Presence of Mg^{2+}

Thylakoids, at a concentration of 0.1 mg Chl/mL were incubated in 0.2 M sucrose, 10 mM Hepes (pH 7.6 or 6.6) for 30 min in the dark on ice. The thylakoids were then centrifuged at 3000g and resuspended at 2 to 3 mg Chl/mL for DSC.

The incubation of thylakoids with Mg^{2+} was conducted similarly, but the Hepes/sucrose buffer was always kept at pH 7.6. The Mg^{2+} concentrations ranged from 0 to 3 mM.

Incubation of isolated LHC-II was done by dialyzing the complex against 2 L of Hepes/sucrose buffer at pH 6.6 or pH 7.6 containing 0 to 5 mM MgCl2. The dialysis proceeded for 20 to 24 h in the dark at 4°C.

Thermal Gel Analysis (Denaturing)

To estimate the denaturation temperature of the various polypeptides of the chloroplast membrane, thermal gel analysis was conducted essentially as described by Lysko et al. (13). Aliquots containing 200 µL of membranes at 1 mg Chl/mL were heated at 1°C/min in a water bath. Samples were removed at 2°C intervals over the desired temperature range, cooled to room temperature, and solubilized in an equal volume of Laemmli sample buffer without β-mercaptoethanol (10). Samples containing approximately 15 µg Chl were then examined by SDS-PAGE in 12% Laemmli gels. The resulting gels were stained with Coomassie blue, scanned, and quantitated by densitometry.

Thermal Gel Analysis (Nondenatured)

Thermal gel analysis was also done in native gels in order to determine the dissociation temperature of the native Chl-protein complexes. One mL aliquots of thylakoid preparations containing 1 mg Chl were heated at 1°C/min in a water bath. Samples were removed at 2°C intervals over the desired temperature range, cooled to room temperature, mixed thoroughly with four drops of glycerol, and frozen at 193 K until use.

The chloroplast membrane samples were later thawed on ice and prepared for electrophoresis according to Huner et al. (6). In this procedure the samples were washed once in cold, deionized water, once in cold 1 mM EDTA (pH 8.0), and then twice in cold 50 mM Tricine (pH 8.0). The samples were then resuspended in 13% (v/v) glycerol, 1% (w/v) SDS (SDS:Chl = 10:1), 0.3 M Tris (pH 8.8), and then 2% (w/v) deoxycholate (DOX) in 0.3 M Tris (pH 8.8) was immediately added to give a final DOX:SDS:Chl ratio of 20:10:1. Thirty µL portions of the solubilized membranes were subjected to SDS-PAGE at 4°C in the dark according to the method of Waldron and Anderson (18). Densitometric scans of the gels were obtained at 652 nm on a Kontron UVIKON 810 spectrophotometer. Absorption spectra of the Chl-protein bands were obtained on an IBM 9420 UV-visible spectrophotometer in order to confirm the identities of the various Chl-containing species in the gel, as described (6).

Low Temperature Fluorescence Emission Spectra

Emission spectra of thylakoid membranes of 77 K fluorescence were obtained on a Perkin-Elmer 650-40 fluorescence spectrophotometer. Excitation was at 400 nm through 4 nm slits and corrected emission spectra were collected through 4 nm slits. The data were plotted using Perkin Elmer supplied software. Thylakoids were subjected to a final concentration of 5 to 10 µg Chl/mL in the Hepes/sucrose buffer and 50% (v/v) glycerol.

Differential Scanning Calorimetry

Heat capacity measurements were obtained on a Microcal 1 differential scanning calorimeter (Amherst, MA) at a scanning rate of 1°C/min. Approximately 1 mL of thylakoids, after equilibration in the desired buffer, was loaded into the sample cell, and an equal volume of buffer was placed in the reference cell. The membranes for each series of comparative scans were prepared on the same day from the same batch of spinach, since minor variations in the calorimetric profiles could often be observed among different batches of spinach, presumably due to differences in growth and storage conditions. The Chl concentrations were also kept constant in each series, usually 2 to 3 mg/mL. Likewise, each series of light harvesting complex scans was run on the same LHC-II preparation and kept at the same protein concentration, approxi-
 Results

Figure 1 shows a typical DSC scan of thylakoids suspended in the low ionic strength Hepes sucrose buffer. The seven endothermic transitions, labeled A to F, are centered at the following temperatures (°C): A (42.5), B (60.6), C (64.9), C₂ (69.6), D (75.8), E (84.3), and F (88.9). Several of these transitions have been described more extensively in previous papers (4, 5).

To approximate the temperature where LHC-II denatures, a calorimetric scan of the isolated LHC-II was conducted and this scan is shown in Figure 2. The LHC-II preparation used in this scan appeared to be homogeneous, with the anticipated LHC-II polypeptides in the 25.5 to 27.9 kD range. The T_m observed for this asymmetric transition is 74.0°C, similar to the T_m of the D transition of chloroplast membranes, which occurs at 75.8°C. Because of this similarity, and since isolation of the complex from its membrane environment could easily account for the minor difference in T_m values, the possible identity of the D endotherm as the denaturation transition of the light-harvesting complex was investigated further.

For comparison of the properties of LHC-II denaturation with the chloroplast membrane D transition, it seemed useful to find solution conditions which would alter the D transition and then observe whether the stability of LHC-II in situ was similarly modified. Two conditions were found that caused a large temperature shift in the D endotherm. As shown in Figure 3, lowering the pH of the thylakoid suspension medium from pH 7.6 to 6.6 induced at 4.4°C displacement of D to lower temperatures, in this case from 75.0 to 70.6°C. Second, addition of Mg^{2+} to the membrane suspension caused a similar migration of D to lower temperatures (Fig. 4). Magnesium concentrations as low as 0.1 mM produced a 2.3° shift in D (data not shown), whereas higher Mg^{2+} concentrations displaced D sufficiently that it became obscured by the C₂ transition (Fig. 4). In the experiments described below, these two sensitivities have been exploited to confirm the proposed denaturation of LHC-II during the D endotherm.

Thermal gel analysis allows the denaturation temperature of membrane polypeptides to be evaluated in situ (17) and was used in this study to determine whether the major LHC-II polypeptide denatures at the temperature of the D transition in intact thylakoids. In this procedure, a suspension of chloroplast membranes was heated at 1°C/min from room temperature to 90°C. At each 2°C interval, an aliquot of the suspension was removed and examined by SDS-PAGE as described in “Materials and Methods.” Membrane proteins that were not yet thermally denatured when each thylakoid aliquot was removed migrated in their usual manner and appeared at the expected positions on the gel. However, proteins that had already thermally denatured tended to cross-link via intermolecular disulfide bonds, forming aggregates too large to enter the gel in the absence of an exogenous reducing agent. Therefore, the temperature of thermal denaturation of each membrane protein was determined from the pretreatment temperature that rendered the protein impermeable to the SDS gel.

Thermal gel analyses of the major 27 kD LHC-II polypeptide are shown in Figures 5 and 6 as function of pH and Mg^{2+} concentration. In these studies, the gels were scanned at 550 nm and the densitometric intensity of the Coomassie blue-stained 27 kD band was plotted versus pretreatment temperature. Curiously, for both pH 7.6 and pH 6.6-treated chloroplast membranes, a somewhat biphasic denaturation transi-
Thylakoids, at a temperature of 0.10 mg/mL were incubated in 0.2 M sucrose, 10 mM Hepes at pH 7.6 or 6.6 for 30 min on ice in the dark. The membranes were then centrifuged at 3000g and resuspended to a final Chl concentration of 2.4 mg/mL in the same buffer at the indicated pH for the DSC.

Figure 3. Effect of pH on the calorimetric transitions of thylakoids. Thylakoids, at a Chl concentration of 0.10 mg/mL were incubated in 0.2 M sucrose, 10 mM Hepes at pH 7.6 or 6.6 for 30 min on ice in the dark. The membranes were then centrifuged at 3000g and resuspended to a final Chl concentration of 2.4 mg/mL in the same buffer at the indicated pH for the DSC.

Figure 4. Effect of Mg$^{2+}$ on the calorimetric transitions of chloroplast membranes. Membranes, at a Chl concentration of 0.10 mg/mL were incubated with 0 to 3.0 mM MgCl$_2$ in 0.2 M sucrose, 10 mM Hepes (pH 7.6). The incubations proceeded for 30 min at room temperature in the dark. The samples were then centrifuged at 3000g and resuspended to a final Chl concentration of 2.0 mg/mL in the same buffer containing the indicated amounts of MgCl$_2$ for the DSC.

Mg$^{2+}$-treated membranes, respectively. These temperatures and the Mg$^{2+}$-induced 6.8°C shift to lower temperature correspond very well with the midpoint temperatures and displacement of the D transition under similar solution conditions (Fig. 4).

Because LHC-II migrates as an intact complex in non-denaturing gels, its denaturation temperature could also be determined by evaluating the pretreatment temperatures which led to the disappearance of the intact LHC-II band from native gels. For this experiment, thylakoids were slowly heated and sampled as described above, after which each sample was dissolved in non-denaturing detergents and separated electrophoretically. The Chl-protein bands corresponding to LHC-II were identified on the basis of their spectral properties and relative abundances and positions in the gel banding profile (data not shown). In the gel scans shown in Figure 7B, the LHC-II oligomer was assigned on this basis to band 3. Comparison of gel scans conducted at 56, 72, and 82°C clearly demonstrates the disruption of the oligomeric complex over this temperature range. A more detailed plot of the densitometric intensity of the LHC-II band (monitored at 652 nm) versus pretreatment temperature is shown in Figure 7A. Consistent with the data shown in Figures 5 and 6, the plot is also biphasic. The more stable component, again representing roughly two-thirds of the complexes, denatured...
with midpoint temperatures of 75.0 and 72.0°C at pH 7.6 and 6.6, respectively. The close correspondence between the temperature of the D transition and the temperature of LHC-II disruption under similar solution conditions confirms the mutual identity of these two events. Furthermore, the observation that the native LHC-II complex disintegrates in two stages is consistent with the contention that LHC-II exists in two forms or environments in the membrane. This observation also eliminates the possibility that the biphasic plots of Figures 5 and 6 are due to the fortuitous denaturation in this temperature range of two distinct proteins that comigrate in the gel.

It has been reported that LHC-II in thylakoids exhibits a low temperature (77 K) fluorescence maximum around 684 to 685 nm (6, 7). To firmly establish the proposed denaturation of LHC-II in the D transition, it was desirable to see whether heating thylakoids to near 75°C might have an influence on this fluorescence. Indeed, a shift in the LHC-II fluorescence maximum from 684 nm (characteristic of the native complex) to 677 nm (representative of the thermally disrupted complex) is observed in Figure 8A where fluorescence emission spectra are displayed for control thylakoids heated to various temperatures. Similarly, the pH 6.6-treated membranes underwent a fluorescence shift from 682 to 677 nm (spectra not shown). The wavelength of the LHC-II fluorescence maximum at 77 K is plotted as a function of the pretreatment temperature in Figure 8B. As can be seen, this fluorescence transition occurs with a midpoint temperature near 77°C in the control chloroplast membranes (pH 7.6) and 72°C for the pH 6.6-treated samples. Thus again, within experimental error, a reasonable agreement was obtained between the temperature of the D transition and the temperature of LHC-II denaturation.

**DISCUSSION**

We have presented evidence that the D transition in the calorimetric scans of chloroplast membranes originates from the denaturation of the light harvesting complex associated with PSII. This evidence was obtained from four separate studies comparing the denaturation properties of LHC-II in situ with the behavior of the D transition under similar conditions of varying pH and Mg²⁺ concentration. The results from these studies collectively demonstrate that the LHC-II complex not only dissociates but its component subunits also denature during the D transition. The endotherm thus qualifies as the complex's gross denaturational transition.

During the course of the above experiments, we observed an unusual characteristic of LHC-II which merits further comment, namely that it proceeds in two distinct phases. This biphasic response was observed in thermal gels where we monitored both the denaturation of the 27 kD LHC-II poly-peptide (Figs. 5 and 6) and the disruption of the intact LHC-II oligomer (Fig. 7A). Further evidence for the heterogeneous nature of LHC-II denaturation could be detected in the asym-
Figure 7. A, Nondenaturing thermal gel analysis of chloroplast membranes over the temperature range of the D transition. Conditions are the same as described in the legend to Figure 5, with modifications as described in the "Experimental Procedures." Electrophoresis was performed on 8% acrylamide gels according to Waldron and Anderson (18) and the Chl-protein bands were scanned at 652 nm in a Kontron UVikon 810 spectrophotometer. The normalized peak height of the oligomeric LHC-proteins in control () and pH 6.6-treated chloroplast membranes () was plotted as a function of temperature. The temperature of half-maximal disappearance of the LHC oligomer was 75.0°C in control and 72°C in pH 6.6-treated chloroplasts. B, Gel scans of Chl-protein complexes of chloroplast membranes. 1, CP1a (PSI core complex with associated LHC-I); 2, CP1 (PSI core complex); 3, LHC-II, (oligomer); 4, LHC-II, (dimer); 5, CPa (PSII core complex); 6, LHC-II (monomer); 7, FP (free pigment. Arrow denotes origin of gel.

metry of the DSC scans of isolated LHC-II, where a shoulder was found to precede the main denaturational transition (Fig. 2). Data from other sources also seem to indicate such multicomponent character. Kyle et al. (9) concluded on the basis of pulse-chase experiments that two populations of LHC-II exist in the thylakoid membrane, one of which is presumably tightly associated with the PSI core and does not move upon phosphorylation, while the other is only loosely connected with PSII and migrates freely from the grana to the stroma lamellae in changes in phosphorylation. Larsson and Andersson (11) have also suggested the existence of two LHC-II complexes based on two-dimensional electrophoresis of grana and stroma lamellae vesicles. They have further proposed that the mobile and immobile LHC-II fractions have different polypeptide compositions. Moreover, the calorimetric study of PSII by Thompson et al. (16) implied the presence of two LHC-II populations: one influenced by Mg²⁺ and involved in Mg²⁺-induced structural transitions, and the other unaffected by Mg²⁺. In this respect, it is interesting to note that only the more stable population of LHC-II, in our experiments, appeared to shift to lower temperatures upon addition of Mg²⁺ (Fig. 6).

We had originally anticipated that the change in the D transition induced by lowering the pH or adding low amounts of Mg²⁺ might be due to the dissociation of LHC-II into its constituent monomers, as reported by Argyroudi-Akoyunoglou and Akoyunoglou (1, 2). However, when our pH 6.6-treated thylakoids were analyzed on deoxycholate gels, the oligomer was still found to be the predominant form present in the membrane (data not shown). A possible reason for this apparent discrepancy might be that different buffer systems were used in the two studies. Argyroudi-Akoyunoglou and Akoyunoglou (1) have noted that the nature of the buffer itself can affect the organizational state of the Chl-protein complexes.

With the identification of the D transition completed, the sources of five of the eight chloroplast transitions have now been firmly established. The identification of the remaining three transitions is currently in progress, and once the calorimetric transitions have been identified, the effects of herbi-
cides and environmental stresses on chloroplast membrane structure will be investigated via DSC. Differential scanning calorimetry could prove to be an extremely useful tool for studies such as these, because it permits the analysis of membrane proteins in their natural environment and allows the investigator to evaluate the sensitivities of membrane components to perturbations without having to first isolate the components. For example, Huner et al. (6) recently observed changes in the D transition among rye thylakoids isolated from plants grown at high and low temperatures and concluded that growth temperature alters the organization of LHC-II.

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