Identification and Partial Characterization of the Denaturation Transition of the Photosystem II Reaction Center of Spinach Chloroplast Membranes

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ABSTRACT
Sensitive differential scanning calorimetry was employed to investigate thylakoid membrane structure. Calorimetric scans of chloroplast membranes suspended in a low ionic strength Heps-buffered medium revealed endothermic transitions centered at the following temperatures (°C): A (42.5), B (80.6), C1 (64.9), C2 (69.8), D (75.8), E (84.3), and F (88.9). The B transition was demonstrated by several different methods to originate from denaturation of the photosystem II reaction center complex. Evidence for this conclusion is as follows: (a) the isolated reaction center complex denatures near the temperature of the B transition; (b) inorganic phosphate destabilizes the isolated reaction center complex and the B endotherm to a similar extent; (c) heat inactivation of the photosystem II-mediated 1,5-diphenylcarbazide → dichloroindophenol photoreaction occurs at the temperature of the B transition and is influenced in a manner similar to B by the presence of phosphate; (d) thermal gel analysis indicates that the 43 and 47 kilodalton polypeptides of the photosystem reaction center complex denature at the temperature of the B transition, both in the presence and absence of phosphate; (e) low temperature (77 Kelvin) fluorescence reveals that a change in photosystem II emission at 695 nanometers occurs during the B transition; and (f) ioxynil, a specific inhibitor of photosystem II, selectively stabilizes the B endotherm. With the identification of the B transition established, the origins of six of the eight major transitions of the chloroplast membrane have now been determined.

DSC\(^2\) measures the excess heat capacity of a system as a function of temperature and has recently been applied to the analysis of such complex systems as biological membranes (1, 4–8, 10, 12, 13, 21–24). After identification and characterization of a membrane’s major calorimetric transitions, DSC can be used to study the properties of individual membrane components in situ without having to chemically label these components or separate them from their native environment (6–8, 23). In our laboratory, DSC has been used most recently to investigate the structure of the chloroplast membrane with the ultimate intent of using it to evaluate the action of herbicides and environmental stresses on specific membrane components.

DSC of chloroplast membranes reveals seven endothermic transitions between 40 and 90°C, labeled as follows (°C): A (42.5); B (60.6); C1 (64.9); C2 (69.8); D (75.8); E (84.3); and F (88.9). Each of these transitions originates from the denaturation of proteins or protein complexes within the thylakoid membrane. (A broad, reversible endotherm is also observed around 17°C and has been shown to arise from the melting of polar thylakoid lipids.) The sources of four of the seven major protein denaturational transitions have already been identified. The A transition, occurring at 42.5°C, has been attributed to the disruption of the oxygen evolving complex of PSII (6, 7). This transition appears to be extremely sensitive to the oxidation state of Cyt \(b_{599}\) (23). The 64.9°C (C1) transition has been shown to originate from denaturation of the soluble subunit complex of the CF1 (21). Predictably, this endotherm is sensitive to the concentration of adenine nucleotides in the suspension. In the preceding paper (22), the 75.8°C (D) transition was shown to arise from the LHC-II. Finally, much evidence indicates that residual ribulose 1,5 biphosphate carboxylase on the membrane is the source of the C3 transition (6; our unpublished results). Since the 60.6°C (B) transition has not yet been identified, the objective of our current work has been to identify and characterize its origin.

Because of much previous work, the number of possible sources of the 60.6°C B transition can be significantly narrowed. If it is assumed that most major membrane components yield only a single cooperative endotherm upon denaturation, the membrane complexes responsible for the previously identified A, C1, C2, and D transitions can be immediately removed from primary consideration. Further, when it is recognized that only major membrane components are present in sufficient quantities to yield resolvable endotherms, the many minor membrane constituents can also largely be eliminated as candidates. The components/complexes still warranting scrutiny, e.g. Cyt \(b/f\) complex, ferredoxins, PSI, the integral membrane subunit of the coupling factor (CF0), and the RCII, can be even further prioritized based on thermal inactivation data available from the literature. Thus, there is good evidence to suggest that electron transport components in PSII lose their transport functions in the temperature range of the B transition (19, 23, 26). In this paper, we present both catalytic and structural data to

1 This research was supported by Monsanto Agricultural Products.
2 Abbreviations: DSC, differential scanning calorimetry; RCII, reaction center of PSII; D-1 and D-2, 32 and 34 kD reaction center proteins of PSII; LHC-II, light harvesting complex associated with PSII; CF1, chloroplast coupling factor 1; DPC, 1,5-diphenylcarbazide; DOX, deoxycholate; ioxynil, 3,5-diido-4-hydroxybenzonitrile; \(T_m\), midpoint temperature of thermal denaturation; DCIP, dichloroindophenol.
demonstrate that the B endotherm derives from thermal denaturation of the RCII.

**MATERIALS AND METHODS**

**Chloroplast Preparation**

Spinach (*Spinacia oleracea*) was purchased from a local supermarket or grown in a controlled climate facility as described (7). Thylakoids were isolated according to the method of Ort and Izawa (20), except that the washing medium was the same as the suspension medium. The suspension medium used for calorimetry was either 0.2 M sucrose, 10 mM KCl, 2 mM MgCl$_2$, and 50 mM KH$_2$PO$_4$ (pH 7.3), or 0.2 M sucrose, 10 mM Hepes (pH 7.6). Chl concentration was determined according to the method of Arnon (2).

**Isolation of the PSII Core Complex**

The PSII core complex was isolated from spinach according to the procedure of Ghanotakis and Yocum (9). An NaCl concentration of 0.5 M was used to precipitate the LHC-II, and the supernatant following the 90 min centrifugation step was desalted by about 3 h dialysis at 4°C against a solution containing 0.2 M sucrose, 10 mM Hepes (pH 7.6).

**Incubation of Chloroplast Membranes and the PSII Reaction Center Complex at Various Ionic Strengths and with Inorganic Phosphate**

Chloroplast membranes were incubated in the same manner as described previously (22), except in 0.2 M sucrose, 10 mM Hepes (pH 7.6), containing 0 to 15 mM KH$_2$PO$_4$ or 34.4 mM KCl. Isolated PSII core complexes were suspended in the same buffers by diluting about 12 mg protein of the PSII preparation into 35 to 40 mL of the desired solution and incubating for 30 min in the dark on ice. After centrifuging at 40,000g for 90 min, the PSII pellet was resuspended in the same buffer at a concentration of about 5 mg/mL for DSC.

**Heat Inactivation of PSII Photochemical Activity**

The photochemical activity of PSII in chloroplast membrane preparations was determined as a function of their pretreatment temperature. Aliquots containing 200 µL of membranes at 1.4 mg Chl/mL were heated in a water bath at 1°C/min and removed at 2°C intervals over the desired temperature range. The samples were cooled on ice and washed with 16 mL of 0.4 M Tris (pH 8.0) to destroy the water splitting activity. The PSII activity of the samples was then measured by monitoring the photoreduction of DCIP according to Vernon and Shaw (26), with the exception that the reaction mixture was illuminated with a 100 W light bulb and the photoreduction of DCIP was monitored by following the absorbance at 600 nm.

**Thermal Gel Analysis**

In order to estimate the denaturation temperature of the various polypeptides of the chloroplast membrane, thermal
the solution conditions of the suspending medium. Thus, changes in ionic strength, pH and divalent cation concentrations shift the various membrane transitions independently along the temperature axis, allowing the investigator to choose conditions where the endotherm under study can be most effectively resolved or sensitively modified. We have chosen 0.2 M sucrose, 10 mM Hepes (pH 7.6), for identification and characterization of the B transition, since it allowed visible displacement of the B transition to lower temperatures upon increasing ionic strength and because the majority of our other calorimetric data on chloroplast membranes has already been obtained in this buffer.

The possibility that a component of PSII might be responsible for the B (61°C) transition was suggested by two previous observations. First, calorimetric data on isolated PSII particles demonstrated that two of five PSII endotherms were located near 61°C (23). Second, as shown by other laboratories (19, 26), heat inactivation of RCII photochemical function (ability to mediate the DPC → DCIP photoreaction) occurs near this temperature. In order to evaluate whether the heat inactivation of RCII might yield a major endotherm with the temperature and magnitude of B, we have isolated RCII and scanned it in the calorimeter, as shown in Figure 1 (see control scan). The derived transition, centered at 65.0°C, was found to coincide with the temperature of the C1 transition previously shown to derive from denaturation of the proton ATPase (CF1). However, the endotherm was also sufficiently near the B transition (61°C) that we decided to investigate their possible common identity further. The decision to study the thermal denaturation of RCII in greater detail seemed especially justified, since the thermostability of a membrane component is often altered upon removal from its native membrane environment, and since the adjacent C1 and C2 endotherms had been previously shown to derive from the denaturation of the coupling factor (CF1) (21) and ribulose 1,5 biphosphate carboxylase (6), respectively.

To test the possibility that RCII denaturation might fortuitously occur near the temperature of the B transition but not be the principal source of this transition, solution conditions were sought that might shift the isolated RCII transition in order to determine whether they might similarly alter B. It has been previously observed (21, 22) that the B transition migrates to lower temperatures when thylakoids are transferred from the Hepes/sucrose medium to the phosphate-buffered medium. This indicates that some variable in the latter buffer, possibly the presence of phosphate or the increase in ionic strength must promote a significant destabilization of B. Figure 2 shows DSC scans of these membranes incubated in the presence and absence of 15 mM KH2PO4. The B transition occurs at 61.4°C in the control thylakoids and at 56.5°C in the phosphate-treated samples. At intermediate phosphate concentrations the destabilization of B was also intermediate. (The B transition is more clearly seen in the scans of the phosphate-treated membranes due in part to its shift to lower temperature and in part to the simultaneous migration of C1 to higher temperatures.)

Treatment of the isolated RCII core complex with the same phosphate concentrations yielded a response similar to that observed for the B transition. As shown in Figure 1, incuba-

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**Figure 3.** Thermal gel analysis of chloroplast membranes over the temperature range of the B transition. Thylakoids were incubated in the Hepes/sucrose buffer containing 0 or 15 mM KH2PO4, as described in Figure 2. Aliquots containing 500 µL of membranes at 1.6 mg Chl/mL were heated to the indicated temperatures at 1°C/min and then removed and cooled to room temperature. Electrophoresis on 12% acrylamide gels (11) was then performed in the absence of reducing agent and the Coomassie blue stained gels were scanned at 550 nm in an EC densitometer. The normalized peak height of the 47 KD polypeptide of PSII in control thylakoids (●) and phosphate-treated thylakoids (○) was plotted as a function of temperature. The temperature of half-maximal disappearance of the 47 KD polypeptide was 61.0°C in control thylakoids and 57.5°C in phosphate-treated membranes.

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**Low Temperature Fluorescence Emission Spectra**

Emission spectra of thylakoid membranes at 77K fluorescence were obtained on a Perkin-Elmer 650-40 fluorescence spectrophotometer. Excitation was at 440 nm through 4 nm slits. Corrected emission spectra were collected through 4 nm slits. The data were plotted using Perkin-Elmer supplied software. Thylakoids were suspended to a final concentration of 5 to 10 µg/mL in the Hepes/sucrose buffer system and 50% (v/v) glycerol.

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**Differential Scanning Calorimetry**

The calorimetry of chloroplast membranes has been described previously (22). Each series of PSII core complex scans was run on the same PSII core complex preparation and kept at the same protein concentration, approximately 4 to 5 mg/mL. Although the results of only a single DSC scan for each study are shown, each experiment was conducted at least two to three times with similar results.

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**RESULTS**

One peculiar feature of the calorimetric transitions of the chloroplast membrane is that they are extremely sensitive to
tion of the core complex with 15 mM KH$_2$PO$_4$ resulted in a 3.7°C destabilization from 65.0 to 61.3°C. Although the absolute temperature of the RCII transition and the B endotherm differed, the similar magnitude and direction of the phosphate effect on the two transitions suggests a common source.

Because addition of phosphate increases the ionic strength of the medium, and because ionic strength is known to influence the calorimetric profile of chloroplast membranes, modifying in particular the B transition, we decided to establish what fraction of the phosphate effect was phosphate specific and what fraction was due to ionic strength alone. For this purpose, the above comparative study was repeated, except that the KH$_2$PO$_4$ was replaced by an equal ionic strength of KCl; *i.e.* 34.4 mM KCl (Fig. 2). The apparent phosphate effect on B is partly but not entirely accounted for by the increase in ionic strength. Of the total 4.9°C displacement, 3.2°C was mimicked by the isotonic KCl. A similar ratio of phosphate-specific to ionic strength effect was obtained in an analogous experiment on the isolated PSII reaction center complex (data not shown). Hence, phosphate appears to have both a direct and indirect (ionic strength) influence on the stability of the PSII core, each effect contributing roughly equally to the net destabilization of the complex.

Thermal gel analysis (13, 22) was also employed to determine whether the polypeptides of the PSII core complex denature *in situ* at the temperature of the B endotherm. The denaturation of PSII was monitored by following the disappearance from the gels of the major 47 kD PSII Chl-binding protein because the 32 and 34 kD reaction center polypeptides were partially obscured in our 12% acrylamide gels by other thylakoid polypeptides of similar mol wt. In Figure 3, the densitometric intensity of the Coomassie blue-stained 47 kD band is plotted as a function of pretreatment temperature for thylakoid membranes incubated in the presence and absence of 15 mM KH$_2$PO$_4$. In agreement with the calorimetric results on whole chloroplast membranes, the 47 kD PSII polypeptide was found to denature at 61.0°C (*i.e.* the temperature of the B transition) in the Hepes/sucrose medium. This result confirms that the complex is indeed less stable *in situ* than after detergent isolation. Furthermore, addition of phosphate caused the anticipated destabilization of the complex, resulting in a displacement of the unfolding temperature of the 47 kD band to 57.5°C. Similar behavior was also observed for the 43 kD polypeptide of PSII (not shown). Thus, the denaturation of PSII polypeptides *in situ* does occur at the temperature of the B transition, both in the presence and absence of phosphate.

The temperature of heat inactivation of PSII *in situ* was also determined by monitoring the DPC → DCIP catalyzed photoreaction of thylakoids as a function of the temperature of preheating. This was accomplished by suspending the chloroplasts in the Hepes/sucrose medium in the presence or absence of 15 mM KH$_2$PO$_4$, heating the suspensions at 1°C/min in a water bath, removing aliquots at 2°C intervals, and assaying each aliquot for its ability to mediate the DPC → DCIP photoreaction at room temperature. The results of this experiment are shown in Figure 4. In general agreement with the calorimetric and thermal gel results, phosphate treatment lowers the temperature of thermal inactivation of the PSII core complex by about 3°C. However, unlike the results from the above two gross denaturation studies, loss of catalytic function occurred with midpoint temperatures of 54.6 and 57.5°C in the presence and absence of phosphate, respectively. Importantly, such loss of function prior to total loss of structure is not uncommon for complex membrane components (8, 18). By analogy to these other systems, we would suggest that the dissociation of electron transport components might precede thermal denaturation of the same polypeptides by a few degrees.

The PSII core complex has been reported to exhibit a low temperature (77K) fluorescence maximum at 695 nm which is thought to originate from the 47 kD Chl-protein (16, 25). In order to provide further support for the involvement of the PSII core complex in the B transition, it was decided to
evaluate whether heating of chloroplast membranes in the temperature range of the B transition would have an effect on the 695 nm emission. Figure 5 shows the 77K fluorescence spectra of membranes heated to various temperatures between 52 and 74°C. As seen in these spectra, the 695 nm emission maximum weakens and disappears over the 54 to 68°C temperature range. By 70°C this fluorescence maximum is no longer visible. The disappearance of this PSII-specific fluorescence centered near 62°C provides additional evidence for the involvement of the PSII core in the B transition.

Finally, there are numerous herbicides known to inhibit plant growth by directly modifying the structural/functional properties of the PSII core complex. Although a detailed investigation of the effects of several herbicides on the DSC of chloroplast membranes will be the subject of a future study, it is still instructive to examine the effects of at least one PSII herbicide on the DSC of our thylakoids as a means of confirming the identity of the B transition. We have chosen the nitrophenol type herbicide, ioxynil, for this purpose. Figure 6 shows the effects of increasing ioxynil concentrations over the concentration range where ioxynil perturbs several chloroplast functions, including inhibition of DCIP photoreduction (14, 17). First, ioxynil causes a significant stabilization of the B transition. In fact, Figure 6B it can be seen that the B endotherm is the only transition that is consistently displaced from its normal Tm. This observation confirms the involvement of RCII components in B. Second, a gradual diminution

**Figure 5.** Low temperature (77K) fluorescence emission spectra of thylakoids over the temperature range of the B transition. Chloroplast membranes were incubated in 0.2 M sucrose, 10 mM Hepes (pH 7.6). The incubation and heating was carried out as described in the legend to Figure 3, except that the Chl concentration during heating was 1.0 mg/mL. The samples were mixed thoroughly with three drops glycerol and stored at 193K until use. The membranes were prepared for fluorescence by suspending them at a final Chl concentration of 5 to 10 μg/mL in Hepes/sucrose buffer containing 50% (v/v) glycerol. The fluorescence emission spectra were obtained with a Perkin-Elmer 650-40 fluorescence spectrophotometer. Excitation was at 440 nm through 4 nm slits and corrected emission spectra were collected through 4 nm slits. This figure shows fluorescence emission spectra of thylakoids heated between 52 and 74°C.

**Figure 6.** A, Effect of ioxynil on the calorimetric transitions of chloroplast membranes. Membranes, at a Chl concentration of 0.10 mg/mL were incubated in 0.2 M sucrose, 10 mM KCl, 2 mM MgCl2, 50 mM KH2PO4 (pH 7.3) containing 0 to 1.0 mM ioxynil. The incubation proceeded for 20 min at room temperature. The membranes were then centrifuged at 3000 g and resuspended to final Chl concentrations of 2.8 mg/mL. B, Effect of ioxynil on the calorimetric transitions of chloroplast membranes. The temperature shifts induced in the transitions are plotted as a function of ioxynil concentration. The symbols for the transitions are as follows: A (†), B (●), C (▼), D (○), E (□), and F (▲).
of the $C_3/C_4$ composite endotherm is also seen (Fig. 6A). This indicates that ioxynil is not totally specific in its modification of RCII but has additional disruptive effects on other chloroplast components, i.e. in this case either CF$_1$, or ribulose bisphosphate carboxylase. As will be reported later, other herbicides also exhibit second site perturbations.

**DISCUSSION**

Six independent lines of evidence have been presented to demonstrate that the PSII core complex is the source of the B transition: (a) the isolated PSII core complex denatures near the temperature of the B transition; (b) inorganic phosphate displaces both the isolated RCII and B transitions in the same direction and to similar extents; (c) heat inactivation of the PSII mediated DPC $\rightarrow$ DCIP photoreaction occurs near the B transition temperature and is similarly affected by the presence of phosphate; (d) thermal gel analysis indicates that the 43 and 47 kD polypeptides of RCII denature at the temperature of the B transition, both in the presence and absence of phosphate; (e) low temperature (77K) fluorescence reveals that a change in RCII emission at 695 nm occurs during the B transition; and (f) ioxynil, an inhibitor of PSII, selectively stabilizes the B endotherm.

Our calorimetric results on RCII in intact thylakoids appear to differ from those of Thompson et al. (23) on isolated PSII particles. These workers attributed separate transitions to the denaturation of the 43 and 47 kD polypeptides, whereas our data demonstrated that both polypeptides denature at the same temperature, i.e. the B transition. Thus, not only did the 43 and 47 kD polypeptides unfold at the same temperature during thermal gel analysis, but also the isolated RCII complex (which contains both proteins) yielded only a single endotherm in the DSC. With regard to this discrepancy, it should be noted that Thompson et al. speculated their PSII particles in solutions buffered at pH 6, while all of our data were collected at pH 7.6. When we incubated our PSII core complex at lower pH (pH 6.6), two transitions appeared, a minor broad component at 53.4°C and a major transition at 68.6°C (data not shown). It is possible that lowering the pH of the suspension detaches the 47 kD protein from the rest of the PSII core, enabling it to denature independently and yield a separate transition.

The destabilizing effect of inorganic phosphate on the RCII transition suggests that phosphate may modulate the structure or function of this complex in vivo. In fact, phosphate has been reported to dampen oscillations in the oxygen evolving capacity and Chl $a$ fluorescence of chloroplasts upon illumination, but no mechanism for this effect has been proposed (15). The phosphate-promoted destabilization of RCII indicates that phosphate favors a more dissociated or unfolded form of the complex. However, since the denaturation transition even at high phosphate concentrations occurs at temperatures greater than those experienced in nature, it is very unlikely that complete phosphate-induced complex disruption ever occurs in vivo. More likely, the observed destabilization derives from a minor conformational change, suggesting a possible role for phosphate in modulating the distribution of RCII complexes between different structural forms. In this respect, it should be mentioned that bicarbonate, an anion known to be essential for the efficient transfer of electrons from Q$_0$ to Q$_1$ and on to the plastoquinone pool (3), causes a destabilization of the B transition similar to that seen for phosphate (data not shown).

The effect of ioxynil on the calorimetric profile of the chloroplast membrane demonstrates the usefulness of DSC in studying the behavior of individual components in complex biological systems. Without conducting electron transport assays or labeling the samples with photoaffinity analogs of ioxynil, it was possible to conclude that the herbicide perturbs both RCII and CF$_1$, or ribulose bisphosphate carboxylase. Evaluation of which of the latter two components of the C transition is a secondary site of ioxynil binding requires only a scan of ioxynil-treated chloroplasts in the Hepes/sucrose medium where the C$_1$ and C$_2$ transitions are readily resolved. While ioxynil has long been known to interact with RCII, its perturbation of a C transition component is revealed only by DSC. Thus, as a supplementary technique, DSC should be able to provide useful information on the sites of perturbation of numerous chemical and environmental stresses.

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**LITERATURE CITED**