Identification of the Soluble Coupling Factor Transition in Calorimetric Scans of Chloroplast Membranes†

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ABSTRACT: Sensitive differential scanning calorimetry was employed to investigate the structure of spinach chloroplast membranes. In a relatively high ionic strength phosphate-buffered medium, major calorimetric transitions were resolved at 42 °C (A), 56. 5 °C (B), 69.5 °C (C), 76.5 °C (D), 82.7 °C (E), and 88.7 °C (F) (a lipid melting endotherm previously identified at ≈17 °C was not examined in this study). However, as both H^+ and salt concentrations were lowered, the C endotherm was found to split into two component transitions: C_1 at 64.9 °C and C_2 at 69.6 °C. The C_1 transition was then demonstrated by five independent methods to derive from denaturation of the soluble subunit complex of the coupling factor (CF_1). Evidence for this conclusion was as follows: (i) Heat inactivation of CF_1 in situ occurred near the temperature of the C_1 transition. (ii) The endotherm of the isolated coupling factor (64.5 °C) was very similar to that of C_1 (64.9 °C). (iii) ADP and inorganic phosphate (two substrates of CF_1) both stabilized the C_1 and CF_1 denaturation endotherms by approximately the same amount. (iv) The denaturation temperature of the α and β subunits of CF_1 determined in intact chloroplast membranes was identical with the temperature of the C_1 transition, in both the presence and absence of ADP. (v) Elution of the soluble coupling factor from the membranes by three different methods removed the C_1 transition from the membrane scan.

Thylakoid membranes employ a number of proteins and oligomeric protein complexes in transducing light energy to chemical energy. Predominant among the photosynthetic components are the two photosynthetic reaction centers, their associated light-harvesting complexes, a cytochrome b_6/f complex, and a coupling factor comprised of integral (CF_0)[1] and peripheral (CF_1) subunit complexes. Upon gradual heating of thylakoid membranes these components denature, giving rise to endothermic transitions whose characteristic temperatures (T_m), enthalpies (ΔH), and cooperativities are peculiar to the source of each transition. If the cooperativities and ΔH values of the endotherms are high and the transitions do not directly superimpose, these endotherms can be resolved along the temperature axis in a scan of the membranes conducted in a sensitive differential scanning calorimeter. The resulting calorimetric profile thereby constitutes a "molecular fingerprint" of the membrane, displaying a distinct signal corresponding to each major structural component.

Once each calorimetric transition of the chloroplast membrane has been associated with a specific membrane complex, the scan can potentially be useful as a means of obtaining biologically relevant information on membrane components in situ. Thus, the specific site(s) of herbicide perturbation may in some cases be identified in the intact membrane by determining which endotherm(s) is modified upon addition of the herbicide (unpublished observations). Likewise, membrane components that are especially sensitive to environmental stresses such as chilling, drought, elevated salinity, etc., may also be identified by observing which endotherm is altered in the chloroplasts isolated from the stressed plant. By slowly acclimating a plant to a desired environmental stress prior to chloroplast isolation and calorimetry, those membrane structures that can adapt to the external stress can also be identified by analogous procedures (Huner, personal communication). Thus, unlike most other physical techniques, DSC permits the researcher to focus on individual components within a heterogeneous membrane without the need of component isolation or site-specific labeling with reporter groups.

Recent research into the calorimetric properties of spinach chloroplast/thylakoid membranes has revealed the existence of seven resolvable endotherms. The broad, low-temperature, reversible transition centered at ≈17 °C has been shown to derive from bulk polar lipid melting (Low et al., 1984). The A transition, located at ≈42 °C, has been demonstrated to derive from the water-splitting component of photosystem II (Cramer et al., 1981a). Certain properties of the C transition (≈69 °C) have been speculated to be similar to those of CF_1 or ribulose bisphosphate carboxylase (Cramer et al., 1981b), but these conjectures have never been examined in detail. Still, because of the abundance of CF_1 in the chloroplast membrane, the soluble subunit of the coupling factor is expected to yield one of the remaining uncharacterized endotherms. Therefore, the present study was undertaken to identify and characterize the calorimetric transition deriving from CF_1. We demonstrate here that this membrane complex is the source of the C_1 transition, a newly resolved endotherm that merges with another previously obscured transition (C_2) as the salt and H^+ concentrations are raised to yield the largest membrane transition, formerly termed C.

Experimental Procedures

Chloroplast Preparation. Spinach was purchased from a local supermarket or grown in a controlled climate facility as described (Cramer et al., 1981a). Chloroplasts were isolated according to the method of Ort and Izawa (1973), except that

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[1] Abbreviations: DSC, differential scanning calorimetry; CF_1, chloroplast coupling factor; CF_0, membrane spanning portion of chloroplast coupling factor; DTT, dithiothreitol; HEPES, *N*-(2-hydroxyethyl)-piperazine-*N*′-2-ethanesulfonic acid; EDTA, ethylenediaminetetraacetic acid; SDS, sodium dodecyl sulfate.

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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_2PO_4, pH 7.3 (medium I), or 0.2 M sucrose, 10 mM HEPES, pH 7.6 (medium II). Chlorophyll concentration was determined according to the method of Arnon (1949).

Isolation of CF_1. Spinach CF_1 was isolated as described by Younis et al. (1977), except that the concentrated supernatant from the chloroform extraction was passed through a Sephacryl S-300 column instead of Sephadex G-200. CF_1 concentration was determined according to the method of Lowry et al. (1951).

Heat Inactivation of ATPase Activity. The ATPase activity of the chloroplast membrane preparations was determined as a function of the pretreatment temperature. Aliquots of approximately 200 μL of a chloroplast membrane suspension containing 1 mg of chlorophyll/mL were heated in a water bath at 1 °C/min and removed at 2 °C intervals over the desired range of pretreatment temperatures. The samples were then cooled on ice and assayed for ATPase activity according to Lien and Racker (1971). Good results were obtained by using 84 μg of CF_1, based upon the approximation of 0.42 mg of CF_1/mg of chlorophyll (Strotmann et al., 1973). The ATPase was preactivated with DTT without heating unless otherwise mentioned. The reduction of phosphomolybdic acid by ferrous sulfate was monitored by the absorbance at 697 nm.

Treatment of Chloroplasts and CF_1 with ADP and Inorganic Phosphate. Chloroplasts, at a concentration of 0.1 mg of chlorophyll/mL, were treated with up to 4.5 mM ADP in medium II for 30 min in the dark at room temperature. The chloroplasts were then centrifuged at 3000*g* and resuspended at 2–3 mg of chlorophyll/mL for DSC.

The incubation of chloroplasts with phosphate was conducted similarly except that the potassium phosphate concentrations employed ranged from 0 to 15 mM. To ensure that the effects of ADP and phosphate on the calorimetric scans were not a trivial consequence of an increase in ionic strength, control scans were run on membranes suspended in KCl solutions of comparable ionic strength.

Incubation of coupling factor with ADP and KH_2PO_4 was done in a manner similar to that described above, only the incubations proceeded for 45 min instead of 30 min and the CF_1 concentration was 2–3 mg/mL. As before, ionic strength controls were conducted with KCl replacing the KH_2PO_4.

Removal of CF_1. Three independent methods were employed to deplete the chloroplast preparations of CF_1. First, CF_1 was removed by washing the chloroplasts in 300 mM sucrose, 2 mM Tricine, pH 7.8, according to the following adaptation of the method of Strotmann et al. (1973). A portion of chloroplasts containing a total of 5 mg of chlorophyll was suspended in 60 mL of the above sucrose–Tricine buffer and stirred very slowly on ice for 15 min. The chloroplasts were then centrifuged at 3000*g*, washed with the HEPES buffer (medium II), and resuspended in this buffer to give a final chlorophyll concentration of 2–3 mg/mL for calorimetry. Second, CF_1 was removed according to the method of Lien and Racker (1971) by incubation of the chloroplasts in EDTA. Third, the soluble subunit of the coupling factor was eluted from the chloroplasts by treating the suspension with NaBr according to Kamienietzky and Nelson (1975). Basically, chloroplasts were suspended at a concentration of 2 mg of chlorophyll/mL in medium II and then incubated for 30 min on ice with an equal volume of 4 M NaBr. An equal volume of water was then added, and the chloroplasts were centrifuged at 3000*g* for 4 min. The chloroplast pellet was washed twice with the sucrose–HEPES buffer (medium II) and resuspended in this buffer for calorimetry.

Thermal Gel Analysis. In order to estimate the denaturation temperature of the various polypeptides of the chloroplast suspension, thermal gel analysis was conducted essentially as described by Lysko et al. (1981). Aliquots containing approximately 200 μL of chloroplasts at 1 mg of chlorophyll/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 (Laemmli, 1970). These samples were then examined by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) using the Laemmli gel system. The best resolution of the CF_1 α and β subunits was obtained when chloroplast samples containing only 5 μg of chlorophyll were run on 12% acrylamide gels.

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 chloroplasts, 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 chloroplasts 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 chlorophyll concentrations were also kept constant in each series, usually 2–3 mg/mL. Likewise, each series of coupling factor scans was run on the same CF_1 preparation and kept at the same protein concentration, approximately 2–3 mg/mL. Although the results of only a single DSC scan for each study are shown, each experiment was conducted at least three times with similar results.

RESULTS

Figure 1 shows DSC scans of chloroplasts in phosphate buffer (medium I) and in HEPES buffer (medium II). The six transitions, labeled A–F, observed in the phosphate buffer system have been described previously (Cramer et al., 1981a), and these are centered at the following temperatures (°C): A, 42.0; B, 56.5; C, 69.5; D, 76.5; E, 82.7; F, 88.7. The HEPES buffer system resolves the 69.5 °C transition into two endotherms, C_1 and C_2, which have temperatures of 64.9 and 69.6 °C, respectively. The B transition is not clearly seen in this medium due to the superposition of C_1; however, the other transitions are resolved at the following temperatures (°C): A, 42.5; D, 75.8; E, 84.3; F, 88.9. Gradual elevation of the ionic strength or lowering of the pH of the HEPES medium shifts B to lower temperatures and C_1 to higher temperatures, leading to clear visualization of B but also to the merger of C_1 onto C_2. The low ionic strength HEPES buffer system was therefore chosen for this study solely because of its better resolution of C_1 and C_2, the transitions of interest.

In order to estimate the temperature range over which thermal denaturation of the chloroplast coupling factor occurs, the ATPase activity of the chloroplasts was measured as a function of their pretreatment temperature. This was done by heating the suspension at 1 °C/min in a water bath, removing aliquots at ≈1.3 °C intervals, and assaying each aliquot for ATPase activity at 37 °C. The results of this experiment are shown in Figure 2. As reported by others (Farron & Racker, 1970), the effect of a heat treatment on chloroplast ATPase activity is biphasic, comprised of both a heat-activation and heat-denaturation phase. In our buffer system, maximum

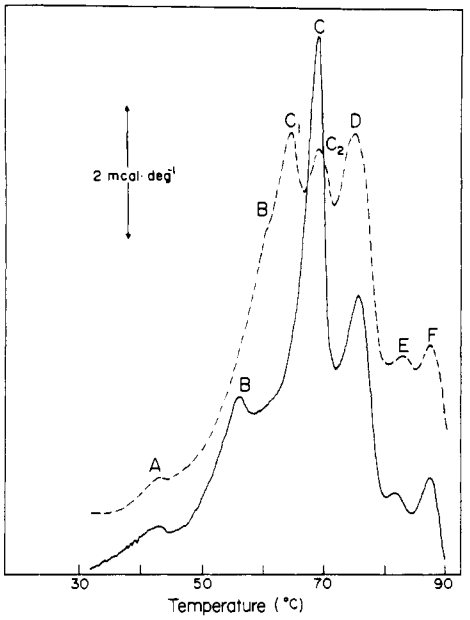

FIGURE 1: Heat capacity of spinach chloroplasts as a function of temperature. Top scan (dotted line): Chloroplasts were suspended in 0.2 M sucrose, 10 mM HEPES, pH 7.6, at a chlorophyll concentration of 2.0 mg/mL. The seven transitions were labeled as follows: A (42.5 °C), B (60.6 °C), C_1 (64.9 °C), C_2 (69.6 °C), D (75.8 °C), E (84.3 °C), F (88.9 °C). Lower scan (solid line): Chloroplasts were suspended in 0.2 M sucrose, 10 mM KCl, 2 mM $MgCl_2$, and 50 mM KH_2PO_4, pH 7.3, at a chlorophyll concentration of 2.0 mg/mL. The six transitions were labelled as follows: A (42.0 °C), B (56.5 °C), C (69.5 °C), D (76.5 °C), E (82.7 °C), F (88.7 °C).

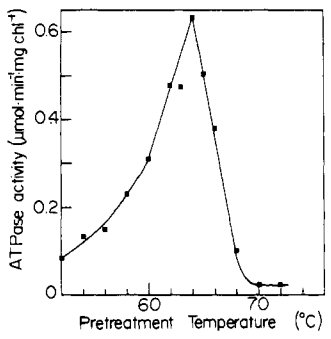

FIGURE 2: ATPase activity of chloroplasts as a function of their pretreatment temperature. Chloroplasts were heated at 1 °C/min in a water bath, and 200-μL aliquots were removed at the indicated temperatures and assayed for ATPase activity. The chlorophyll concentration was 1 mg/mL. See Experimental Procedures.

ATPase activity occurred at 64 °C and the apparent temperature at which half-maximal ATPase activity was lost was 66.5 °C. However, this apparent denaturation temperature (T_d) of the coupling factor must represent an upper estimate of the true T_d, since the well-documented heat activation of CF_1 may have compensated for some heat denaturation occurring at temperatures below 64 °C. Therefore, the membrane endotherm resulting from CF_1 denaturation must occur close to but probably slightly below the apparent heat inactivation midpoint, 66.5 °C. Thus, the most probable candidates for this transition are the C_1 and C_2 transitions or some minor "invisible transition".

One means of distinguishing among the possible CF_1 transitions is to compare the calorimetric properties of the isolated coupling factor with the endotherms of the intact membrane. The calorimetric transition of isolated CF_1 is shown in Figure 3. The T_d observed for this enzyme is 64.5 °C, within the expected 60–66.5 °C range and similar to the T_d of the C_1

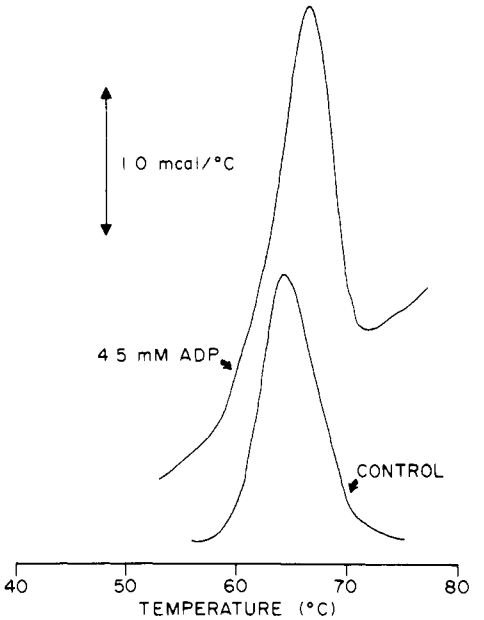

FIGURE 3: Effect of ADP on the calorimetric transition of isolated CF_1. Lower scan: CF_1, at a concentration of 2.8 mg/mL, was incubated in 0.2 M sucrose, 10 mM Hepes, pH 7.6. Top scan: CF_1, at a concentration of 2.2 mg/mL, was incubated in the same buffer containing 4.5 mM ADP. Both incubations proceeded for 45 min at room temperature in the dark before the DSC was conducted.

endotherm of chloroplasts at 64.9 °C. The ΔH of the CF_1 transition is ≈5.6 mcal/mg. On the basis of the estimated abundance of the coupling factor in spinach chloroplasts (≈ 10% of the total membrane protein; Strotmann et al., 1973; Anderson, 1975; Wraight, 1982), it can be argued that the endotherm should be readily visible in a scan of intact membranes, assuming the cooperativity of the transition in situ is similar to that of the isolated component. Thus, one of the major endotherms shown in Figure 1 should derive from CF_1 denaturation.

It has frequently been observed that substrates and cofactors stabilize a protein against thermal denaturation (Donovan & Beardslee, 1975; Chlebowski & Mabrey, 1977; Vickers et al., 1978; Strickland et al., 1981; Chlebowski & Williams, 1983; Manly et al., 1985). Therefore, we looked to see what effect two substrates of CF_1 had on the various transitions of chloroplasts and on the isolated CF_1 endotherm. The ligands chosen for this study were ADP and phosphate. Figure 3 reveals that 4.5 mM ADP promotes a 2.5 °C shift in T_d of the isolated CF_1, from 64.5 to 67.0 °C. Importantly, when chloroplasts were incubated with 4.5 mM ADP, the C_1 transition was similarly shifted from 63.75 to 67.0 °C, a 3.25 °C change (Figure 4). No apparent shift in the C_2 transition was observed in response to this treatment. While other endotherms of the chloroplast membrane were also modified slightly by ADP, these effects did not likely involve CF_1, since the perturbed endotherms were located too far from the T_d of isolated CF_1 to be considered probable candidates. Thus, the parallel response of CF_1 and C_1 to ADP supports the identification of CF_1 as the source of the C_1 transition.

To corroborate the ADP results, the influence of phosphate on the coupling factor and C_1 transitions was also examined (Figure 5). Like ADP, as phosphate concentration was raised, a gradual stabilization of CF_1 was observed; i.e. a change in T_d of 1.9 °C, from 65.5 to 67.4 °C, was produced by 15 mM KH_2PO_4. Similarly, upon incubation of chloroplasts with the same phosphate concentration, the C_1 transition shifted from 64.5 to 66.6 °C, a ΔT_d of 2.1 °C (Figure 6). This similar

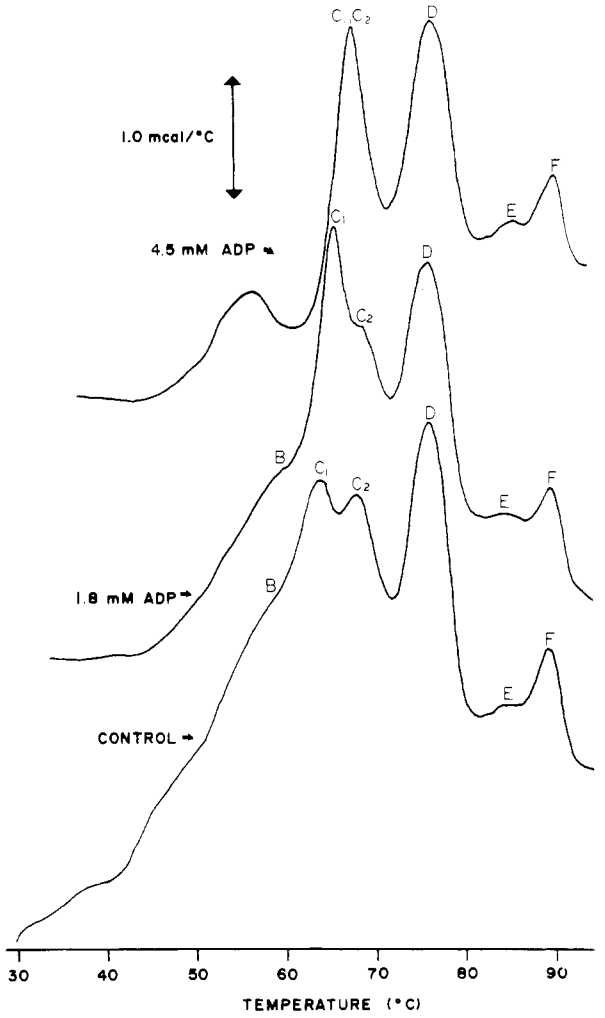

FIGURE 4: Effect of ADP on the calorimetric transitions of chloroplasts. Chloroplasts, at a concentration of 0.10 mg/mL, were incubated with 0 (control), 1.8, or 4.5 mM ADP in 0.2 M sucrose, 10 mM HEPES, pH 7.6. The incubations proceeded for 30 min at room temperature in the dark. The chloroplasts were then centrifuged at 3000*g* and resuspended to a final chlorophyll concentration of 1.47 mg/mL in the same HEPES sucrose buffer containing the indicated amounts of ADP for the DSC.

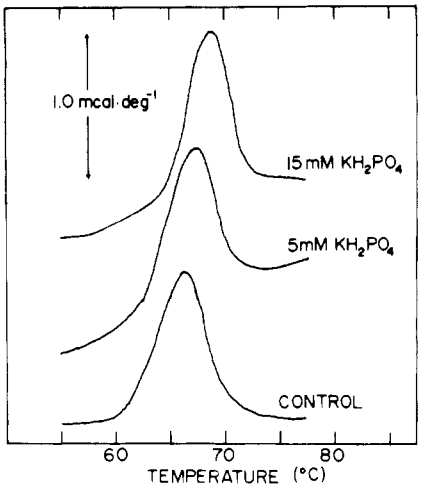

FIGURE 5: Effect of phosphate on the calorimetric transition of isolated CF_1. CF_1, at a concentration of 2.1 mg/mL, was incubated with 0 (control), 5, and 15 mM KH_2PO_4 in 0.2 M sucrose, 10 mM HEPES, pH 7.6. The incubations proceeded for 30 min in the dark at room temperature. CF_1 concentrations of 2.1 mg/mL were used in all three scans.

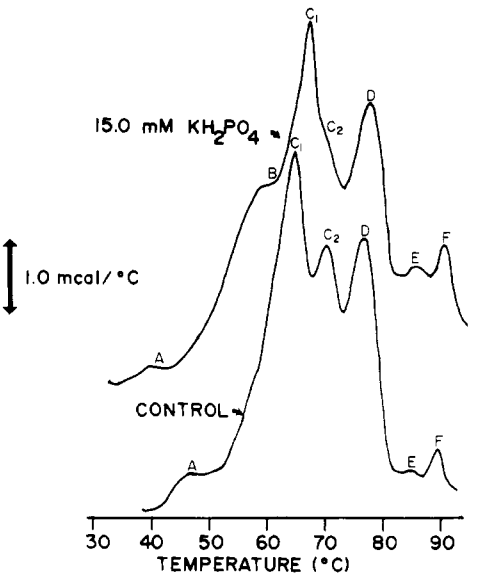

FIGURE 6: Effect of phosphate on the calorimetric transitions of chloroplasts. Chloroplasts, at a chlorophyll concentration of 0.1 mg/mL, were incubated with 0 (control) or 15.0 mM KH_2PO_4 in 0.2 M sucrose, 10 mM HEPES, pH 7.6. The incubations proceeded for 30 min at room temperature in the dark. Chloroplasts were then centrifuged at 3000*g* and resuspended to a final chlorophyll concentration of 2.2 mg/mL in the same buffers prior to calorimetry.

effect of phosphate on the CF_1 and the C_1 endotherms further supports their common source.

Because ionic strength is known to influence the calorimetric profile of chloroplast membranes and since addition of ADP and inorganic phosphate raises the ionic strength of the medium, it is important to establish that the ADP effect is not a trivial consequence of an elevated ionic strength. For this purpose, chloroplast membranes were scanned in the HEPES medium II supplemented with different amounts of KCl. Figure 7 illustrates the effect of a 23.3 mM KCl supplement, a change in ionic strength comparable to the highest ADP concentration examined. Except for a slight destabilization of the B endotherm, no significant changes in the calorimetric profile were observed. Therefore, the ADP effect on C_1 was not due to a change in ionic strength. Treatment of the isolated coupling factor with 31 mM KCl likewise promoted no change in T_d (data not shown).

Thermal gel analysis, a technique that allows the determination of the denaturation temperature of any membrane polypeptide in situ (Lysko et al., 1981), was used to determine whether a subunit of the coupling factor denatures at the temperature of the C_1 endotherm. In this procedure, a suspension of chloroplast membranes is heated at 1 °C/min from room temperature to 100 °C. At 2 °C intervals, an aliquot of the suspension is removed and examined by SDS–polyacrylamide gel electrophoresis, as described in the Experimental Procedures. Membrane proteins that have not yet thermally denatured migrate in their usual manner and appear at the expected positions on the developed gel. However, proteins that have thermally denatured commonly form intermolecular disulfide bridges between sulfhydryl groups which are normally inaccessible in the native protein. These disulfide cross-linked aggregates fail to enter the gel in the absence of an exogenous reducing agent. Thus, the temperature of thermal denaturation of each membrane protein can be determined from the pretreatment temperature that renders the protein impermeable to the SDS–polyacrylamide gel.

Control chloroplasts and those incubated with 4.5 mM ADP were subjected to thermal gel analysis as described above and

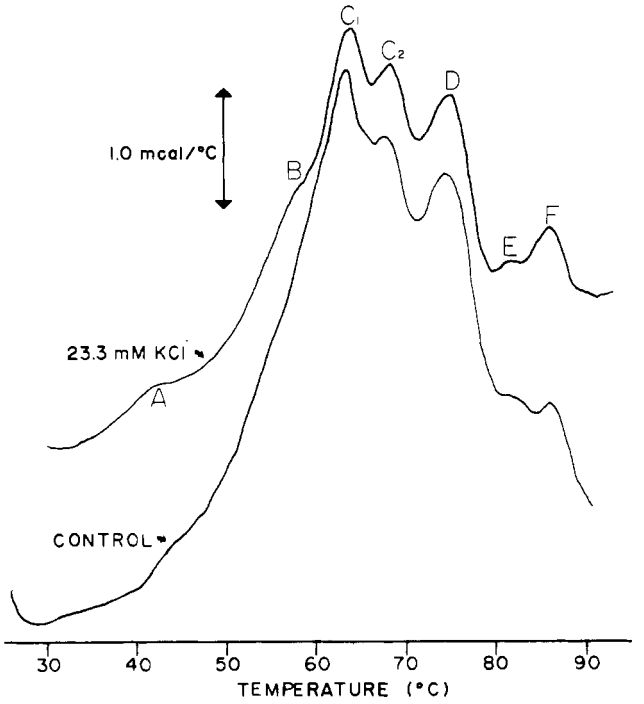

FIGURE 7: Effect of a small increase in ionic strength on the calorimetric transitions of chloroplasts. Chloroplasts, at a chlorophyll concentration of 0.05 mg/mL, were incubated with 0 (control) and 23.3 mM KCl in 0.2 M sucrose, 10 mM HEPES, pH 7.6. The incubations proceeded for 30 min at room temperature in the dark. The chloroplasts were centrifuged at 3000*g* and resuspended to a final chlorophyll concentration of 1.50 mg/mL in the same buffer for calorimetry.

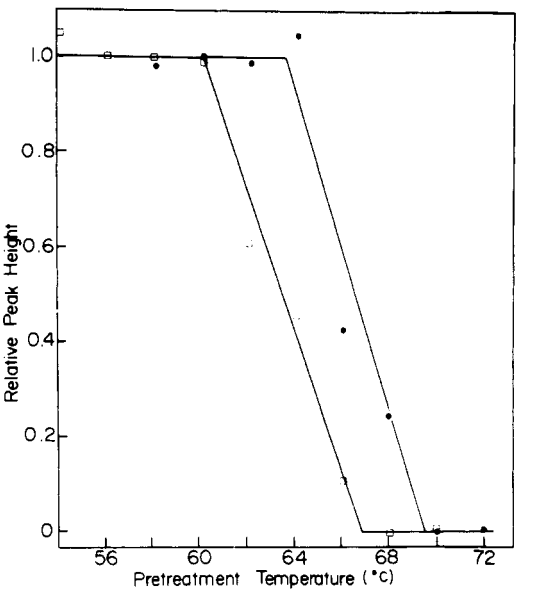

FIGURE 8: Thermal gel analysis of chloroplasts over the temperature range of the C_1 transition. Chloroplasts, at a chlorophyll concentration of 0.10 mg/mL, were incubated with 0 (control) or 4.5 mM ADP in 0.2 M sucrose, 10 mM HEPES, pH 7.6. The incubation proceeded for 30 min in the dark at room temperature. The chloroplasts were then centrifuged at 3000*g* and resuspended to 1 mg of chlorophyll/mL in the sucrose, HEPES buffer containing 0 or 4.5 mM ADP. Aliquots (200 μL) of the chloroplast suspensions were heated to the indicated temperatures at 1 °C/min and then removed and cooled to room temperature. Electrophoresis on 12% acrylamide gels was performed on the samples in the absence of reducing agent, and the Coomassie blue stained gels were scanned at 550 nm in a Gilford UV–visible spectrophotometer. The normalized peak height of the CF_1 β subunit in control chloroplasts (□) and ADP-treated chloroplasts (●) was plotted as a function of temperature. The temperature of half-maximal disappearance of the β subunits was 63.3 °C in control chloroplasts and 66.3 °C in ADP-treated chloroplasts.

examined on 12% acrylamide gels. To determine the denaturation temperature of CF_1 in situ, the gels were scanned and the densitometric intensity of the stained band of the β subunit ($M_r \approx 56$K) of CF_1 was plotted as a function of the pretreatment temperature. Similar to the results above, the β subunit was found to denature at 63.3 °C in control chloroplasts and at 66.3 °C in ADP-treated chloroplasts (Figure 8). Identical plots were likewise obtained when denaturation of the CF_1 α subunit was similarly monitored. This 3 °C difference in the denaturation temperatures between control and ADP-treated chloroplasts paralleled the DSC results in which 4.5 mM ADP produced a 3.25 °C shift in the C_1 transition. Thus, CF_1 denaturation in situ does occur at the temperature of the C_1 transition, in both the presence and absence of ADP.

The soluble coupling factor (CF_1) can be selectively removed from chloroplasts with little perturbation of other membrane components (Strotmann et al., 1973). Thus, a further means of establishing CF_1 as the source of the C_1 endotherm is to selectively elute CF_1 from the membrane and observe whether C_1 also disappears. Three separate methods were employed for this purpose (see Experimental Procedures), and the results of the high sucrose elution procedure are shown in Figure 9. As anticipated, sucrose treatment promotes a large reduction in the C_1 endotherm, corresponding to an 84% decrease in membrane-associated ATPase activity. A DSC scan of the supernatant from the sucrose wash yielded a scan similar to that of the isolated coupling factor in Figure 3 (data not shown). Curiously, the A transition was also inexplicably removed by the sucrose elution procedure, but this effect has not been investigated further. Elution of CF_1 with either EDTA or NaBr also caused the disappearance of C_1 with no effect on A; however, these treatments led to a simultaneous loss of C_2. This loss of C_2 may be explained by the concomitant elution by EDTA and NaBr of membrane-associated carboxylase, an enzyme that denatures near the temperature of C_2. Still, the common removal of solely the C_1 endotherm by all three CF_1 elution procedures demonstrates the role of CF_1 in the C_1 transition.

Discussion

We have presented five independent lines of evidence that the C_1 endotherm of spinach chloroplast membranes derives from denaturation of the soluble subunit (CF_1) of the coupling factor. Heat inactivation of the CF_1 adenosine triphosphatase activity occurs near the temperature of the C_1 transition, the properties of the endotherm of isolated CF_1 are similar to those of C_1, ADP and inorganic phosphate stabilize CF_1 and the component responsible for C_1 similarly, thermal gel analysis reveals the α and β subunits of CF_1 both denature in situ at the temperature of the C_1 transition in both the presence and absence of ADP, and three procedures known to elute CF_1 from chloroplast membranes remove the C_1 transition from the membrane scans. It is thus reasonable to conclude that the thermotropic disruption of CF_1 is responsible for the C_1 endotherm.

From the heat inactivation studies alone it is not possible to determine whether the coupling factor subunits actually unfold or merely dissociate from one another during the C_1 transition. However, two other lines of evidence support the former interpretation. First, thermal gel analysis reveals that both the α and β subunits of CF_1 form nonnative disulfide bonds during the calorimetric endotherm. This clearly implies that protein unfolding occurs as these subunits are heated through the transition. Second, the ΔH of the CF_1 endotherm

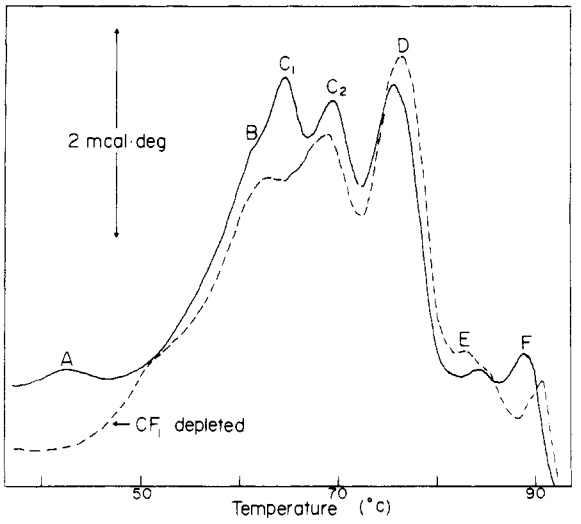

FIGURE 9: Effect of CF_1 removal via sucrose wash on the calorimetric transitions of chloroplasts. Top scan: Chloroplasts were suspended in 0.2 M sucrose, 10 mM HEPES, pH 7.6, at a chlorophyll concentration of 2.5 mg/mL. Lower scan: A portion of chloroplasts containing 5 mg of chlorophyll was washed with 300 mM sucrose, 2 mM tricine as described in Experimental Procedures. After they were washed once with 0.2 M sucrose, 10 mM HEPES, pH 7.6, the chloroplasts were resuspended in the wash buffer at 2.5 mg chlorophyll/mL for calorimetry. Approximately 16% of the original ATPase activity remained associated with the membrane following the above sucrose stripping procedure.

(5.6 mcal/mg) is similar to the enthalpies of protein unfolding of other proteins that denature near 65 °C (Privalov, 1979), but much larger than one would expect for a native subunit dissociation transition. Since the ATPase remains active up to the T_d of C_1, the CF_1 complex must be largely intact up to 64.9 °C, the temperature at which the subunits both dissociate and denature. Although the heat-promoted increase in ATPase activity is significant by 55 °C (Figure 2), there is no major independent endotherm corresponding to this activation event in the DSC scan of the isolated coupling factor (Figure 3). Therefore, the associated structural change, which is believed to involve a dislocation of the ϵ subunit (Nelson et al., 1972; Finel et al., 1984; Richter et al., 1984), must occur with a much smaller ΔH than the gross unfolding of the residual complex.

In an earlier paper, before the C transition was resolved into its C_1 and C_2 components (see Figure 1), it was speculated that the source of the C endotherm might be either the coupling factor or residual membrane-associated ribulose bisphosphate carboxylase (Cramer et al., 1981b). The reason for this conjecture was that removal of either CF_1 or carboxylase reduced the size of the C transition, and a scan of both the isolated CF_1 and carboxylase yielded an endotherm with a T_d near C. Our demonstration that C_1 merges with C_2 to form the larger C transition provides an explanation for the speculation that CF_1 denaturation might contribute to C. Furthermore, if C_2 were to derive from carboxylase denaturation, as suggested by their similar T_m values and elution procedures, the hypothesized involvement of the carboxylase in the C endotherm would also be explained.

Research into the calorimetric properties of the human red blood cell membrane has demonstrated that much biologically relevant information can be obtained from membrane calorimetry once the sources of the endotherms have been identified (Low & Brandts, 1978; Brandts et al., 1978; Snow et al., 1978; Appell & Low, 1981; Appell & Low, 1982; Davio & Low, 1982). The observed sensitivity of the CF_1 transition to ADP and inorganic phosphate, two substrates of the coupling factor, confirm that native protein–ligand interactions can be monitored in chloroplasts by DSC. We suggest it should be similarly possible to identify the specific sites of other membrane perturbations (e.g., chilling injury, drought stress, herbicide modification) by observing which chloroplast transitions are most sensitive to these agents.

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Registry No. ATPase, 9000-83-3; ADP, 58-64-0; PO_4^{3-}, 14265-44-2.

REFERENCES

Anderson, J. M. (1975) *Biochim. Biophys. Acta 416*, 191–235.
Appell, K. C., & Low, P. S. (1981) *J. Biol. Chem. 256*, 11104–11111.
Appell, K. C., & Low, P. S. (1982) *Biochemistry 21*, 2151–2157.
Appell, K. C., Keenan, T. W., & Low, P. S. (1982) *Biochim. Biophys. Acta 690*, 243–250.
Arnon, D. I. (1949) *Plant Physiol. 24*, 1–15.
Brandts, J. F., Taverna, R. D., Sadasivan, E., & Lysko, K. A. (1978) *Biochim. Biophys. Acta 512*, 566–578.
Chlebowski, J. F., & Mabrey, S. (1977) *J. Biol. Chem. 252*, 7042–7052.
Chlebowski, J. F., & Williams, K. (1983) *Biochem. J. 209*, 725–730.
Cramer, W. A., Whitmarsh, J., & Low, P. S. (1981a) *Biochemistry 20*, 157–162.
Cramer, W. A., Low, P. S., Selman, B. R., Whitmarsh, J., & Widger, W. (1981b) *Proc. Int. Congr. Photosynth., 5th* 121–129.
Davio, S. R., & Low, P. S. (1982) *Arch. Biochem. Biophys. 218*, 421–428.
Donovan, J. W., & Beardslee, R. A. (1975) *J. Biol. Chem. 250*, 1966–1971.
Farron, F., & Racker, E. (1970) *Biochemistry 9*, 3829–3836.
Finel, M., Rubenstein, M., & Pick, U. (1984) *FEBS Lett. 166*, 85–89.
Kamienietzky, A., & Nelson, N. (1975) *Plant Physiol. 55*, 282–287.
Laemmli, U. K. (1970) *Nature (London) 227*, 680–685.
Lien, S., & Racker, E. (1971) *Methods Enzymol. 23*, 549–551.
Low, P. S., & Brandts, J. F. (1978) *Arch. Biochem. Biophys. 190*, 640–646.
Low, P. S., Ort, D. R., Cramer, W. A., Whitmarsh, J., & Martin, B. (1984) *Arch. Biochem. Biophys. 231*, 336–344.
Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265–275.
Lysko, K. A., Carlson, R., Taverna, R., Snow, J., & Brandts, J. F. (1981) *Biochemistry 20*, 5570–5576.
Manly, S. P., Matthews, K. S., & Sturtevant, J. M. (1985) *Biochemistry 24*, 3842–3846.
Nelson, N., & Eytan, E. (1979) in *Cation Flux across Biomembranes* (Mukohata, Y., & Packer, L., Eds.) pp 409–415, Academic, New York.
Nelson, N., Nelson, H., & Racker, E. (1972) *J. Biol. Chem. 247*, 7657–7662.
Ort, D. R., & Izawa, S. (1973) *Plant Physiol. 52*, 595–600.
Privalov, P. L. (1979) *Adv. Protein Chem. 33*, 167–241.
Richter, M. L., Patrie, W. J., & McCarthy, R. E. (1984) *J. Biol. Chem. 259*, 7371–7373.
Snow, J. W., Brandts, J. F., & Low, P. S. (1978) *Biochim. Biophys. Acta 512*, 579–591.
Strickland, D. K., Andersen, T. T., Hill, R. L., & Castellino,

F. J. (1981) *Biochemistry 20*, 5294–5297.
Strotmann, H., Hesse, H., & Edelmann, K. (1973) *Biochim. Biophys. Acta 314*, 202–210.
Vickers, L. P., Donovan, J. W., & Schachman, H. K. (1978) *J. Biol. Chem. 253*, 8493–8498.
Wraight, C. A. (1982) in *Photosynthesis: Energy Conversion by Plants and Bacteria* (Govindjee, Ed.) Vol. I, pp 17–61, Academic, New York.
Younis, H. M., Winget, G. D., & Racker E. (1977) *J. Biol. Chem. 252*, 1814–1818.

Covalent Modification of Lysines of the B880 Light-Harvesting Protein of *Rhodospirillum rubrum*†

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ABSTRACT: Dansyl chloride [5-(dimethylamino)-1-napthalenesulfonyl chloride] was used to modify amino acids of proteins covalently in intact, active chromatophores of *Rhodospirillum rubrum* S1. A significant degree of amino acid modification (detected by dansyl fluorescence from proteins) was achieved without significant disruption of the far-red shift of the bacteriochlorophyll absorption spectrum. However, electron transport activity in the chromatophores was severely reduced, to 5–10% of original activity. The α subunit, the organic solvent soluble subunit, of the B880 light-harvesting bacteriochlorophyll protein (which has the composition $\alpha_2\beta_2$) was isolated. It was determined that the single lysine (residue 47 of 52) near the carboxy terminus of the subunit was the only covalently modified residue in that protein chain. A large percentage (up to 70%) of this light-harvesting protein subunit could be modified without disrupting the bacteriochlorophyll binding sites in the $\alpha_2\beta_2$ complex.

The light reactions of bacterial photosynthesis originate in bacteriochlorophyll–protein complexes that are embedded in the cell membrane (Okamura et al., 1982; Feher & Okamura, 1978). These Bchl–protein[1] complexes can be divided into two classes: photoreaction centers and light-harvesting (or antenna) complexes. The main function of the light-harvesting complexes, which bind the bulk of the bacteriochlorophyll, is to capture light energy and to transfer the energy to the reaction centers where photochemistry can occur (Drews, 1985).

The sequences of many Bchl binding proteins from photosynthetic bacteria have been determined over the past several years, and the first crystal structure of an integral Bchl–protein complex, the *Rhodopseudomonas viridis* reaction center, has recently been determined (Deisenhofer et al., 1984). The acquisition of the detailed crystal structure for the *Rps. viridis* reaction center complex will allow for the analysis of the role of particular portions of the polypeptide chains in the various binding sites of the protein complex (Deisenhofer et al., 1984). No crystal structure for a membrane-bound, light-harvesting protein is yet available, although the crystal structure of a water-soluble light-harvesting protein from the green bacterium *Chloropseudomonas ethylica* has been reported (Fenna & Matthews, 1977). Therefore, until crystal structures are available for other Bchl binding membrane proteins, the best evidence for the structure of these proteins in the membrane is still provided by calculating the hydropathy profile for the protein with the amino acid sequence and by probing the surfaces of the proteins with chemical labels and proteases.

The bacterium *Rhodospirillum rubrum* contains a single core light-harvesting protein complex (B880) with an absorption peak about 880 nm in wild-type S1 and about 870 nm in the carotenoidless mutant G-9 (Picorel et al., 1983). This complex from *Rs. rubrum* has been characterized by Picorel et al. (1983) and shown to contain two different polypeptides, α and β, of apparent M_r 7600 and 6400 on SDS–polyacrylamide gels. The minimal oligomer for the B880 complex was found to be $\alpha_2\beta_2$. Bchl and the carotenoid spirilloxanthin were present in a 2:1 mole ratio in the complex from the wild-type strain. Both the α and β polypeptides of the complex have been sequenced (Brunisholz et al., 1981; Gogel et al., 1983; Brunisholz et al., 1984a).

With sequences of the light-harvesting proteins available, it is now possible to conduct structure–function studies to determine the position and roles of specific amino acid side chains in the Bchl binding sites of the proteins. In both the α and β polypeptides of the *Rs. rubrum* B880 complex, a conserved histidine, which has been suggested to serve as a ligand for Bchl (Brunisholz et al., 1981; Theiler & Zuber, 1984), is found near the carboxy-terminal end of a stretch of hydrophobic amino acids in the sequence. Other amino acids in the protein may serve as hydrogen bond donors to Bchl or may form part of salt bridges in the complex. In the α subunit, there is a single lysine residue in the carboxy-terminal portion of the sequence. In order to determine if the ϵ-amino group of this lysine serves some function in Bchl binding, we used dansyl chloride to react with the proteins while the proteins were still membrane bound in chromatophores. Dansyl chloride was chosen because it is a moderately specific reagent that reacts readily with amino groups and because the fluorescent modification products can be identified by chromatography (Woods & Wang, 1967; Hartley, 1970). After covalent modification of the chromatophore membranes with dansyl chloride, the integrity of the binding sites for Bchl in the proteins was monitored with visible absorption spectros-

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[1] Abbreviations: Bchl, bacteriochlorophyll; CM, carboxymethyl; dansyl chloride, 5-(dimethylamino)-1-naphthalenesulfonyl chloride; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate; Tris, tris(hydroxymethyl)aminomethane.

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