Vol. 158, No. 1, 1989 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
January 16, 1989 Pages 11-16

REVERSIBILITY OF THE THERMAL TRANSITIONS OF CHLOROPLAST THYLAKOID MEMBRANES

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Received November 23, 1988

Thylakoid membranes from cucumbers and peas have been examined by high-sensitivity differential scanning calorimetry. Data was collected during both heating and subsequent cooling scans in order to observe reversibility. Cucumber thylakoids exhibited almost no reversibility; a very small reversible exothermic peak was observed at approximately 12°C in cooling scans. However, thylakoids from peas had reversible transitions at 50 and 68°C, as well as other transitions which were visible as shoulders in a second heating scan. When pea grana thylakoids were unstacked, the high temperature transitions were sharpened and their reversibility was enhanced. This is the first report of chloroplast thylakoid membranes exhibiting reversible high temperature transitions. The results indicate that considerable variation can occur in the calorimetric profiles of thylakoids from different plants.

High-sensitivity differential scanning calorimetry (DSC) has been used recently to look for thermal transitions in chloroplast thylakoid membranes which correlate with temperature-induced changes in chloroplast structure and photosynthetic function. A thermal transition at approximately 45°C has been associated with the denaturation of the oxygen evolving complex (1,2), and a transition at 65°C has been assigned to the denaturation of the coupling factor (3). The transitions at 45 and 65°C were reported to be irreversible. In addition a large endotherm at approximately 20°C in tomato thylakoids has been reported (4). Numerous reports have also been made of DSC studies of lipid extracts of whole leaves and chloroplasts, and fractionated lipids (5,6,7).

We report here the first high-sensitivity DSC study of thylakoid membranes combining data collection in both heating and subsequent cooling scans; thus allowing one to look directly at reversibility of the transitions. Cucumbers and

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Abbreviations used: DSC, differential scanning calorimetry; MOPS, 3-(N-morpholino)-propane-sulfonic acid

0006-291X/89 $1.50

peas were chosen because of an interest in thermal-induced changes associated with chilling-sensitivity and chilling-tolerance, respectively. In contrast to spinach (1,2) and cucumber thylakoids, pea thylakoid membranes have several reversible high temperature transitions. We also report the effects of ionic strength and ethylene glycol on the calorimetric profiles.

MATERIALS AND METHODS

Plant material. Pea (Pisum sativum L. cv. Alaska) and cucumber (Cucumis sativus L. cv. Long Green) plants were grown as previously described (8). Pea leaves and cucumber cotyledons were harvested after 10 days of seedling growth.

Thylakoid isolation. Chloroplast thylakoids were isolated as previously described (9) except that they were washed only once in the isolation buffer and then subjected to further washings in media that would preserve grana stacking or would lead to destacking of the grana. For calorimetric runs above $0^{o}C$ the thylakoids were washed as follows: (A) Phosphate wash. Thylakoids were washed twice in 50 mM Sorensen's phosphate buffer (pH 7.4) and 50 mM NaCl with 10 min centrifugations at 9,000 x g. (B) MOPS + Mg^{2+} wash. Thylakoids were washed twice in 5 mM MOPS-NaOH (pH 7.4) and 5 mM $MgCl_2$ with 10 min centrifugations at 9,000 x g. (C) MOPS wash. Thylakoids were washed twice in 5 mM MOPS-NaOH (pH 7.4) with 30 min centrifugations at 45,000 x g. In all cases the thylakoids were finally resuspended in a minimal volume of the respective final wash buffer and chlorophyll determined according to the method of Arnon (10). For calorimetric runs below $0^{o}C$ the thylakoids were washed as in (C) except that the final wash and resuspension buffer was a 1:1 (v/v) mixture of ethylene glycol and two times concentrated buffer.

Calorimetry. Differential scanning calorimetry was performed using a Hart Scientific Model 7707 DSC (Provo, UT). Data collection and control of the calorimeter was done with a WICAT Model 150-6 microcomputer (Orem, UT). Data pairs, temperature and thermopile voltage, were collected at 10 s intervals. Heating and subsequent cooling scans were made at $30^{o}C\ h^{-1}$. Samples were equilibrated at the low temperature limit for 1.0 h, scanned up, held at the high limit for approximately 45 min, scanned down, held at the low limit for 1.5 h, and then the up and down scans were repeated. Consequently, each experiment consisted of two heating and two cooling scans. Samples were thylakoid membrane preparations, consisting of 4 to 12 mg of chlorophyll, sealed in Hastaloy C ampoules with a working volume of 1.5 ml. An equivalent weight of buffer was placed in the reference ampoule.

RESULTS

Fig. 1 illustrates the differences between pea and cucumber thylakoids and the differences between the first and second heating and cooling scans for each. These experiments were performed in moderate ionic strength phosphate buffer (monovalent cation concentration of approximately 140 mM) in which the thylakoids are predominantly stacked (11). Pea thylakoids show a much more complex pattern of thermal transitions than do cucumber thylakoids; however, both have their largest peak between 60 and $70^{o}C$. This peak is at $68^{o}C$ in peas, $62^{o}C$ in cucumber, and at 65 to $68^{o}C$ in spinach (1,3). The most striking difference between pea and cucumber thylakoids is the reversibility seen in heating and cooling scans subsequent to the initial heating scan. Pea thylakoids exhibit a number of reversible thermal transitions in the 30 to $80^{o}C$ temperature range. Prominent peaks occur at 50 and $68^{o}C$ in the second heating scan for peas, whereas cucumber thylakoids have only a single reversible transition at

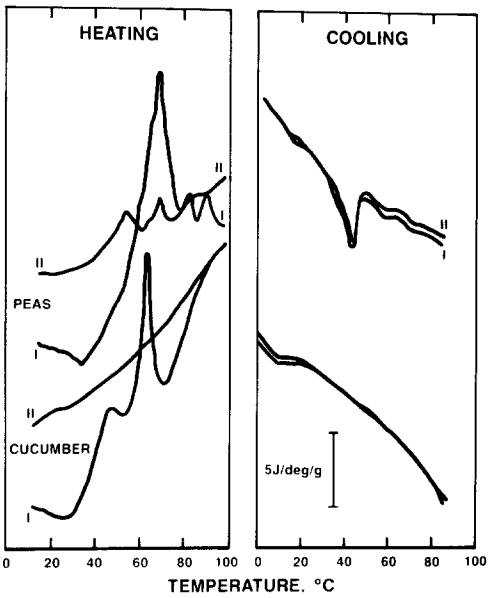

Figure 1. Heat capacity of thylakoid membrane preparations from pea and cucumber seedlings. The membranes were suspended in phosphate buffer (50 mM Sorensen's phosphate (pH 7.4) and 50 mM NaCl). The Roman numerals indicate first and second heating scans, when they are distinguishable. The scale in the right panel pertains to both panels and is in Joules per degree Celsius per gram of chlorophyll. Endothermic transitions cause an upward deflection.

approximately 12°C. This transition is very small [approximately 10 J (g chlorophyll)$^{-1}$], but it was consistently present and visible above the baseline. Also, the initial heating scan for cucumbers is much less complex in terms of overlapping transitions. The large positive change in heat capacity between 70 and 100°C was reproducible and unique to cucumber thylakoids.

Figs. 2 and 3 illustrate the effects of buffer composition on the heat capacity of pea and cucumber thylakoids, respectively. Magnesium ions at concentrations greater than approximately 3 mM maintain stacking of the thylakoids as does phosphate buffer of moderate ionic strength (12). Therefore we expected any effects of stacking on the thermal transitions to be the same in MOPS + Mg^{2+} buffer (Figs. 2 and 3) as in phosphate buffer (Fig. 1). Qualitatively, pea thylakoids in phosphate and MOPS + Mg^{2+} buffers appear very similar (cf. Figs. 1 and 2). Transitions are present at the same temperatures in both the heating and cooling scans, but the relative peak heights and areas vary somewhat. For cucumber thylakoids in phosphate buffer (Fig. 1) the two major transitions were at 47 and 62°C. In MOPS + Mg^{2+} buffer (Fig. 3) the pattern is similar with the transitions at 51 to 52°C and 60°C.

MOPS buffer alone is of sufficiently low ionic strength that the thylakoids would be unstacked. For pea thylakoids in MOPS buffer (Fig. 2) the major peak at 67°C is sharper than with stacked preparations and the reversibility of the transitions is enhanced, as inferred from the larger peak area. Experiments in a

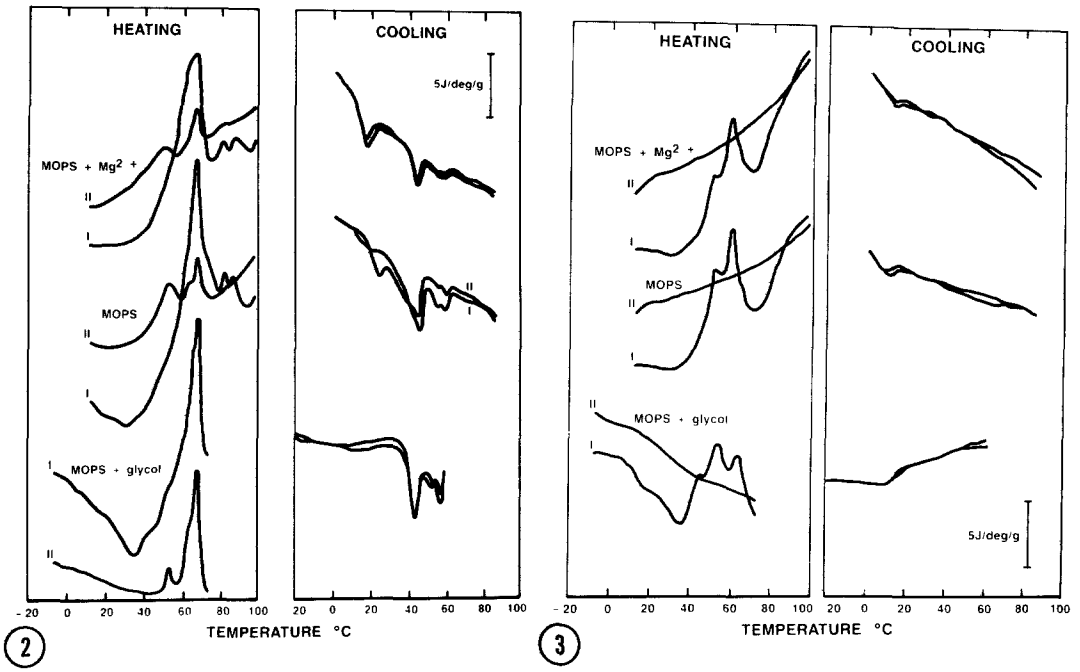

Figure 2. Heat capacity of pea thylakoid membranes. The membranes were suspended in MOPS buffer (5mM MOPS, pH 7.4), MOPS + Mg^{2+} (5 mM MOPS, 5 mM $MgCl_2$, pH 7.4) or MOPS plus glycol (5 mM MOPS, 50% (v/v) ethylene glycol, pH 7.4). Other conditions as in Fig. 1.

Figure 3. Heat capacity of cucumber thylakoid membranes. The same buffers were used as in Fig. 2 and other conditions are as in Fig. 1.

low ionic strength phosphate buffer (2 mM Sorensen's phosphate, pH 7.4, plus 2 mM NaCl), which would also lead to unstacking of the membranes (11), showed a very similar pattern of peaks (data not shown). For cucumbers the relative peak heights are different in MOPS and phosphate buffers (cf. Figs. 1 and 3) , and the peaks occur at the same temperatures in both MOPS and MOPS + Mg^{2+} buffers.

Ethylene glycol was added to the MOPS buffer in order to look at the temperature region from approximately -10 to +10°C. As seen at the bottom of Figs. 2 and 3, the samples were scanned up to about 75°C in order to assess glycol effects on the thermal transitions above 40°C. The presence of ethylene glycol had no major effects on the thermal transitions of pea thylakoids in MOPS buffer (Fig. 2). The same peaks and shoulders were seen in MOPS buffer with and without ethylene glycol. However, the peak areas of the second heating scan and the cooling scans were markedly increased. No additional transitions were observed in the heating or cooling scans between -20 and +20°C. In the case of cucumber thylakoids (Fig. 3), a new peak appears at 44°C in the presence of ethylene glycol but the cooling scans remained featureless except for the exothermic transition at 12°C.

DISCUSSION

Pea and cucumber thylakoids present some of the most dissimilar DSC profiles of chloroplast membranes which have been published (compare for example the species studied in ref. 4). These differences are particularly emphasized by looking at the cooling scans, and point to the utility of this instrumental capability. Whereas cucumber thylakoids show almost no reversibility, the thylakoids from peas have reversible transitions at 50 and 68°C, as well as other transitions which are visible as shoulders in the second heating scan. This is the first report of reversible high temperature transitions of chloroplast thylakoid membranes. Transitions which occur at similar temperatures of thylakoids and photosystem II preparations of spinach are irreversible (1,2) as is the case for cucumbers. The transition which we observe at 68°C is due to coupling factor (H. Hopkins, personal communication) and thus appears to be the same as the C_1 transition of spinach (3).

There was not a clear one-to-one correspondence between small and large exothermic peaks in the first cooling scan and endothermic peaks in the subsequent heating scan of pea thylakoids. Complete reversibility was apparently preserved for these transitions as evidenced by their appearance in the second cooling scans (see Figs. 1 and 2). Also, the sum of the integrated areas of the cooling-scan exothermic peaks was approximately equal to the sum of the areas of the endothermic peaks in the second heating scan. This indicates the absence of any significant slow thermal processes taking place during the high-temperature and low-temperature incubations of the experimental cycle. The exothermic peaks in the cooling scans are found at temperatures 10°C below the peak temperatures in the second heating scan; this is most likely due to the kinetics of the processes that take place during cooling scans being quite slow compared to the scan rate (13). The slower kinetics could also be responsible for the increased detail observed in the cooling scan compared to the second heating scan.

In addition to simple lipid phase transitions and protein denaturation, the thermal response of thylakoid membranes includes a modification of the usual grana structure, a reorganization of intrinsic macromolecular complexes, and the segregation of non-bilayer lipids into other structures, such as cylindrical inverted micelles (14). Thus the reversibility which we see manifested in the cooling scans and the second heating scan of pea thylakoids is reflective of events in a much rearranged membrane.

Evidence has been published which is consistent with a distinguishing transition near 15°C for extracted polar lipids of chilling-sensitive plants (6,7,15). However the molecular basis for this transition is not clear (16) and it was not confirmed by direct calorimetric measurements on thylakoid membranes. Although there is a transition seen at approximately 12°C in the cooling scans of cucumbers, the size of this peak is quite small and is about one-tenth that of the endothermic peak at 20°C reported for chilling-sensitive and chilling-tolerant tomatoes (4). Thus

in agreement with Low et al. (4) we find no large thermal events in the region near freezing which would distinguish chilling-sensitive from chilling-tolerant plants.

In contrast to the results of Smith et al. (3) we do not observe a significant effect of ionic strength on the major endotherm which occurs between 60 and 70°C. Rather our results with pea thylakoids indicate that low ionic strength conditions enhance the reversibility of the transitions as evidenced by the increased detail observed in the cooling scans. Thus our results suggest that upon shifting from high to low ionic strength conditions there is a change in protein-protein and/or protein-lipid interactions such that more of the thermal transitions are reversible. Our results coupled with those of Smith et al. (3) clearly indicate that careful attention must be made to buffer composition and that considerable variation can occur in the calorimetric profiles of thylakoids from different plants, especially with regard to reversibility.

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