

Temperature-induced Changes in Hill Activity of Chloroplasts Isolated from Chilling-sensitive and Chilling-resistant Plants

Received for publication June 15, 1976 and in revised form February 15, 1977

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ABSTRACT

The effect of temperature on Hill activity has been compared in chilling-sensitive and chilling-resistant plants. The Arrhenius activation energy (E_a) for the photoreduction of 2,6-dichlorophenolindophenol by chloroplasts isolated from two chilling-sensitive plants, mung bean (*Vigna radiata* L. var. Mungo) and maize (*Zea mays* L. cv. PX 616), increased at low temperatures, below 17 C for mung bean and below 11 C for maize. However, the E_a for this reaction in pea (*Pisum sativum* L. cv. Massay Gem), a chilling-resistant plant, likewise increased at temperatures below 14 C. A second change in E_a occurred at higher temperatures. The E_a decreased above about 28 C for mung bean, 30 C for maize, and 25 C for pea. At temperatures approaching 40 C, thermal inactivation of Hill activity occurred. These results, when taken together with previous results obtained with the chilling-resistant plant barley, indicate that chloroplasts from both chilling-sensitive and chilling-resistant plants can undergo a change in chloroplast membrane activity at low temperatures above freezing and that the presence of such a change in chloroplast membranes is not necessarily correlated with chilling sensitivity.

tion of either DCIP or ferricyanide increased at temperatures below 9 C. The change was reversible and additional changes in E_a took place at 20 and 29 C. Thus, there were at least four different values of E_a as the temperature was varied within the physiological temperature range for growth of barley. The alterations in E_a of Hill activity by the temperature became apparent only when a rate-limiting reaction in coupled barley chloroplasts was circumvented by the addition of uncouplers or by other means.

Since the studies with barley indicated that changes in membrane properties and function induced by low temperatures were, at least for chloroplasts, not confined to chilling-sensitive plants, the effect of temperature on Hill activity was compared in two chilling-sensitive plants, mung bean and maize, and another chilling-resistant plant, pea. Chloroplasts isolated from all of these plants showed a distinct increase in the E_a for Hill activity at low temperatures, as well as another change in E_a at temperatures above 25 C. These results, together with those previously obtained with barley (12), showed that the E_a for Hill activity was not constant as the temperature was varied throughout the physiological temperature range for growth. While the temperatures causing changes in the E_a were characteristic for different plant species it was not possible on this basis to distinguish chilling-sensitive from chilling-resistant plants.

MATERIALS AND METHODS

Plant Material. Plants of mung bean (*Vigna radiata* L. var. Mungo), maize (*Zea mays* L. cv. PX 616), and pea (*Pisum sativum* L. cv. Massey Gem) were grown in vermiculite in a constant temperature room at 22 C. Grow-lux fluorescent tubes and incandescent bulbs provided light at 2,200 lux for 16 hr/day. Leaves were harvested after 9 to 17, 12 to 20, and 9 to 18 days of growth of mung bean, maize, and pea seedlings, respectively.

Chloroplast Isolation. Chloroplasts were isolated (at 0-4 C) in a medium of 0.05 M Sørensen's phosphate buffer (pH 7.5), 0.05 M NaCl, and 0.5% (w/v) BSA. Dithiothreitol at 5 mM and 2% (w/v) Polyclar AT were also included in the medium used for isolating mung bean and maize chloroplasts. Leaf segments were blended in a Sorvall Omni-Mixer operated at 0.75 of line voltage for four periods of 5 sec each. The homogenate was filtered through one layer of Miracloth and the filtrate centrifuged at 200g for 90 sec. Chloroplasts were isolated from the supernatant by centrifuging for 10 min at 1,000g. The chloroplasts were then washed twice in the blending medium (dithiothreitol and Polyclar omitted) with 15-min centrifugations at 12,000g and resuspended in the same medium.

Assays. The photoreduction of DCIP was measured in an Aminco-Chance dual wavelength spectrophotometer equipped with a temperature-controlled cuvette compartment as previously described (12, 18). The wavelengths used were 575 nm minus 550 nm. The cuvette compartment was continuously

The growth of plants indigenous to lowland tropical and subtropical climates is adversely affected by temperatures below about 12 C and damage (chilling injury) can result from exposure to such chilling temperatures (6, throughout 13). It has been reported that a fundamental difference in the response to these low temperatures exists between mitochondrial and other cellular membranes of chilling-sensitive plants and membranes of plants which are resistant to chilling temperatures (7, 13, 14). The E_a of membrane-bound respiratory enzymes in mitochondria of chilling-sensitive plants increased below a critical chilling temperature, typically between 10 C to 15 C (7), and this change was correlated with a temperature-induced change in the molecular ordering of the mitochondrial lipids as detected with the aid of spin-labeled compounds (16). The membrane lipids of chloroplasts from chilling-sensitive plants also exhibited similar changes below the chilling temperature (14, 15) and there was an increase in E_a for photoreduction of NADP⁺ although not of other Hill oxidants (18). Neither mitochondria nor chloroplasts isolated from chilling-resistant plants showed any of the temperature-dependent changes in membrane properties at temperatures close to or within the chilling-temperature range (7, 13).

Recently, we have investigated the effect of temperature on the Hill activity of chloroplasts isolated from barley (12). Although barley is a chilling-resistant plant, the E_a for photoreduc-

¹ Abbreviations: DCIP: 2,6-dichlorophenolindophenol; E_a : Arrhenius activation energy.

flushed with dry N_2 gas to prevent condensation. The basic reaction mixture (0.75 ml) consisted of 45 mM Sørensen's phosphate buffer (pH 7.5), 45 mM NaCl, 21 μ M DCIP, 0.045% (w/v) BSA, and 3 μ g Chl. The uncouplers when included in the reaction mixture were either 74 mM methylamine (pH 7.6) or 4 μ g ml^{-1} gramicidin D. The reaction mixture (*minus* chloroplasts) was preequilibrated to the required temperature. The chloroplasts, stored at 0 C, were added in a small volume (10 μ l) to the reaction mixture. The cuvette containing the reaction mixture was then placed in the cell compartment and after a further equilibration period of 2 min, the chloroplasts were assayed for photochemical activity. The temperature of the reaction mixture was measured with a calibrated thermocouple just before and immediately after the completion of the photochemical assay. The variation between the two temperature readings was small (± 0.2 C or less) and the average value was used in the calculations.

The Hill reaction activities in μ mol DCIP reduced (mg Chl) $^{-1}$ hr $^{-1}$ were calculated from steady-state rates. In practice, each reaction was allowed to run long enough to allow an accurate rate to be determined from the recorder trace, usually for 2 to 4 min after illuminating the reaction mixture, depending upon the temperature. In any experiment if the activity of the chloroplast preparation stored at 0 C had decreased by more than 10% by the end of the experiment, then the activities measured at various temperatures were corrected for the loss of activity. The results are presented as an Arrhenius plot, that is, the logarithm of the activity is plotted as a function of the reciprocal of the absolute temperature. Determinations of lines of best fit through the data points and correlation coefficient and *t* test analyses were performed as described by Raison and Chapman (15). The statistical significance for changes in slope and intercept for the straight lines was significant at $P < 0.005$ unless otherwise stated.

Chl was determined according to the method of Arnon (2).

RESULTS

Effect of Temperature on the Hill Activity of Chloroplasts from Two Chilling-sensitive Plants. Figure 1 shows the effect of temperature on the Hill activity of chloroplasts isolated from different plants. The Arrhenius plot of the Hill activity of mung bean chloroplasts, measured in the presence of methylamine (Fig. 1A), showed a straight line for temperatures just above 0 C to about 17 C but above this temperature the slope changed to a lower value. There was a second change to a still lower slope above 28 C. The E_a for the different temperature ranges is shown in Table I. Above 35 C the Hill activity of mung bean chloroplasts declined. In the figures, a straight line has been drawn through the data points at temperatures exceeding that at which inactivation first becomes apparent; however, a gradual curve may also be suitable (12).

Figure 1B shows an Arrhenius plot of the Hill activity of isolated mung bean chloroplasts measured in the absence of an uncoupler of photophosphorylation. The change in E_a at 17 C is not seen although a change in E_a occurred at 27 C. However, the value of the E_a above 27 C was greater than that found in the presence of uncoupler (Table I).

Figure 1, C and D shows data obtained from several experiments with isolated mesophyll chloroplasts of maize whose photosynthesis is inhibited by chilling temperatures (20). The Arrhenius plot of Hill activity measured with gramicidin present in the reaction mixtures again showed changes in E_a at two temperatures (Fig. 1C and Table I). One change occurred around 11 C, the other at about 29 C. The changes in maize were similar to those previously found in barley (12) except that a change corresponding to the one found at 20 C in barley was not detected in maize. In the absence of gramicidin, only the change

at the higher temperature was evident in maize chloroplasts, at about 29 C in the experiment shown in Figure 1D.

Effect of Temperature on the Hill Activity of Chloroplasts from a Chilling-resistant Plant. Figure 1E shows an Arrhenius plot of the photoreduction of DCIP in the presence of methylamine for chloroplasts isolated from pea, a chilling-resistant plant. Again, two changes in E_a are seen, one at around 14 C and the other at 25 C. The E_a values are given in Table I. Thus, the data for pea confirm the experiments carried out with barley (12) which demonstrated the existence of temperature-induced changes in E_a of Hill activity of chloroplasts isolated from a chilling-resistant plant.

In the absence of methylamine, changes in E_a at 14 C and 25 C were not observed (Fig. 1F) and a constant E_a was found up to 37 C (Table I).

Thermal Inactivation of the Hill Reaction. At temperatures approaching 40 C, Hill activity declined in the chloroplast preparations from the three plants utilized for this study (Table II). Maize chloroplasts were a little more resistant to heat than chloroplasts from the other two plants or from barley, but the various preparations did not show marked differences in heat sensitivity. The temperature span between maximal activity and complete thermal inactivation was fairly narrow, usually between 5 and 6 degrees.

The chloroplasts appeared to be more resistant to high temperatures in the absence than in the presence of an uncoupler, but this may not necessarily reflect a real difference in the heat sensitivity of coupled and uncoupled chloroplasts. Assuming that the reaction which limits the rate of DCIP photoreduction in coupled chloroplasts is not inactivated at these temperatures, then a thermal inactivation of DCIP photoreduction would not become apparent in coupled chloroplasts until the rate of the temperature-sensitive step of electron transport activity had declined to below that of the original rate-limiting step.

Reversibility of Temperature-induced Changes in Mung Bean. Figure 2 shows the results of an experiment to ascertain whether temperature-induced irreversible changes occurred during the 2-min incubation period before assays were performed. Mung bean chloroplasts were incubated at selected temperatures and the temperature was lowered before assay. The changes at 17 and 28 C shown by mung bean chloroplasts were reversible at least after a 2-min treatment, irrespective of the presence or absence of uncoupler. At 42 C part of the activity was irreversibly lost and after 2 min at 46 C at least 70% of the activity was lost.

DISCUSSION

Our studies show that chloroplasts isolated from both chilling-sensitive and chilling-resistant plants can undergo at least two temperature-induced changes in Hill activity (Fig. 1 and Table I). In barley these reversible changes are thought to result from temperature-induced changes in molecular ordering of membrane components (12). Shneyour *et al.* (18) reported that chloroplasts isolated from chilling-sensitive plants showed a temperature-induced change in E_a at approximately 12 C for NADP $^+$ photoreduction but not for DCIP or diquat reduction. Chilling-resistant plants had a constant E_a over the temperature range studied (approximately 4–25 C) for all three Hill reagents. We have confirmed the results of Shneyour *et al.* (18) in that no change was found in E_a between 2 and 25 C for DCIP reduction by coupled chloroplasts isolated from each of the four plants studied (Fig. 1, B, D, F, and Table I). A similar result has recently been reported for chloroplasts from lettuce (10). However, using uncoupled chloroplasts isolated from these same four plants, at least one change in E_a occurred in this temperature range (Fig. 1, A, C, E, and Table I). Also, at least one additional change was found at temperatures above 25 C. The

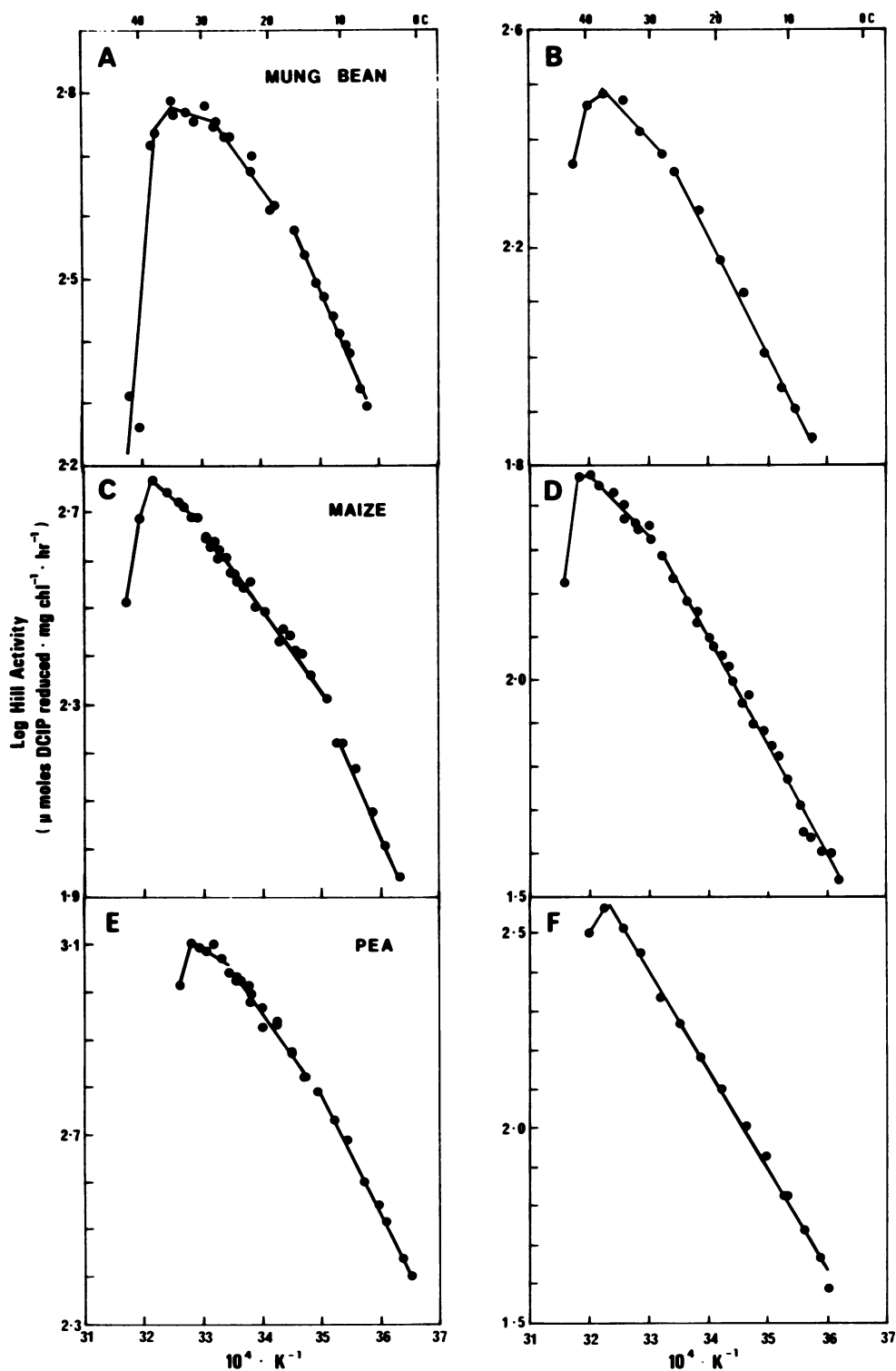


FIG. 1. Arrhenius plots of DCIP photoreduction by chloroplasts isolated from different plants. A: mung bean chloroplasts in the presence of methylamine. Results from two chloroplast preparations have been normalized. The change in slope at 28 C was significant at $P < 0.1$. B: mung bean chloroplasts in the absence of an uncoupler; C: maize chloroplasts in the presence of gramicidin. Results from three experiments have been normalized. D: maize chloroplasts in the absence of an uncoupler. Results from three experiments have been normalized. Activities have been corrected for loss of activity during the experiment (see text). The change in slope at 29 C was significant at $P < 0.025$. E: pea chloroplasts in the presence of methylamine. Results from two experiments have been normalized. Activities have been corrected for loss of activity during the experiment (see text). The change in slope at 25 C was significant at $P < 0.01$. F: pea chloroplasts in the absence of an uncoupler.

probable explanation for this is that the addition of an uncoupler removes a rate-limiting reaction with a constant E_a which masks the effect of temperature in coupled chloroplasts.

The observation that maize and mung bean chloroplasts, even

in the absence of uncoupler, still showed the upper temperature-induced change whereas pea did not, may be due to a partial uncoupling during isolation of the maize and mung bean chloroplasts. This was supported by the fact that addition of an uncou-

pler to maize or mung bean chloroplasts rarely stimulated the rate of electron transport by more than 2- to 3-fold, whereas for peas and barley the stimulation was 5- to 6-fold or greater. Presumably, if the maize and mung bean chloroplasts became progressively more uncoupled, the temperature-induced changes in the vicinity of 12 C would also eventually become evident. Thus, unless fully uncoupled chloroplasts are used, experimental results on the presence or absence of temperature-induced effects on electron transport activity could be misleading depending upon the degree of uncoupling in the isolated chloroplasts.

Murata *et al.* (10) have reported that the photosynthetic membranes of *Anacystis* show temperature-induced changes in structure and function (at temperatures above 0 C) but that the membranes of chloroplasts isolated from higher plants (spinach and lettuce) do not. This difference in behavior between *Anacystis* and higher plant lamellae was attributed to their different lipid compositions. It was suggested that phase changes in mem-

Table I. E_a for Hill Activity in Various Temperature Ranges

The values for E_a were calculated from the data given in Fig. 1, except for barley where the data are taken from ref. 12. All values are for the photoreduction of DCIP.

Plant	Uncoupler present	Temperature range C	E_a kJoules·mol ⁻¹
Mung bean	Methylamine	Below 17	43.5
		17 - 28	26.1
		28 - 35	5.7
Maize	Gramicidin D	Below 27	42.8
		27 - 37	22.8
		Below 11	53.9
Pea	Methylamine	11 - 30	29.7
		30 - 38	19.7
		Below 29	48.2
Barley	Methylamine	29 - 41	26.8
		Below 9	63.7
		9 - 20	40.4
Maize	None	20 - 29	20.2
		29 - 36
		Below 38	48.1

Table II. Temperature Ranges for Thermal Inactivation of Hill Activity

Chloroplasts were incubated at a given temperature for 2 min in the dark and then illuminated and rate of photoreduction of DCIP measured at the same temperature, as described in Materials and Methods. The lower temperature shown in the Table for each condition refers to the minimum temperature causing obvious thermal inactivation of Hill activity and corresponds to the temperature shown in Fig. 1 after which activity begins to decrease with a further increase in temperature. The higher temperature is the apparent minimum temperatures giving complete inactivation of Hill activity within 2 min of the sample being illuminated. The values for barley are taken from ref. (12).

Plant	Uncoupler	Temp. range for inactivation of Hill activity C
Mung bean	None	37 - 46
	Methylamine	35 - 40
Maize	None	41 - 46
	Gramicidin D	38 - 44
Pea	None	37 - 43
	Methylamine	32 - 37
Barley	None	38 - 42
	Methylamine	36 - 40

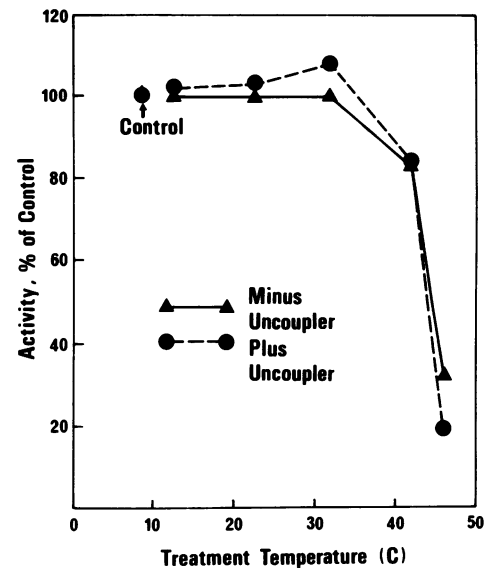


Fig. 2. Reversibility of temperature-induced effects on DCIP photoreduction by mung bean chloroplasts. Chloroplasts were added to a cuvette containing the basic reaction mixture *minus* DCIP and incubated for 2 min at the various treatment temperatures. The preparation was then brought to the assay temperature (8.5 C) by placing the cuvette in a water bath for 3 min, DCIP was added, and activity immediately assayed. Controls were preparations which were processed similarly except that the treatment temperature was the same as the assay temperature.

brane lipids above 0 C might be expected in *Anacystis* whose photosynthetic membranes contain no polyunsaturated fatty acids (1), whereas such a change above 0 C would not be expected for chloroplast membranes from higher plants because of their high content of polyunsaturated fatty acids. In spinach, *e.g.*, at least 75% of the fatty acids of the chloroplast lamellae are polyunsaturated (1). The assumption that temperature-induced membrane changes above 0 C would not be expected in polyunsaturated chloroplast membranes appears to be negated by a subsequent report (9) of temperature effects in *Euglena* resembling those found in *Anacystis*. As much as 60% of the chloroplast's lamellar fatty acids of *Euglena* are polyunsaturated (17).

There are several reports of temperature-induced effects on chloroplast lamellae of higher plants (4, 5, 8, 21-23) that are likely to be related to those reported here, and Nobel (11) has demonstrated, by the reflection coefficient method, temperature-induced changes in the chloroplast envelope which also contains a relatively large amount of unsaturated fatty acids (3). Thus, the evidence indicates that changes in temperature can affect the structure and function of the chloroplast membranes of higher plants, but that the existence of such changes need not be related to the degree of polyunsaturation of the chloroplast fatty acids.

Chilling sensitivity of plants has also been correlated with a change in the physical structure and enzymic activity of membranes below a critical chilling temperature while chilling resistance has been characterized by the apparent absence of such changes (6, 13, 15). While our data obtained with the chilling-sensitive species maize and mung bean are consistent with this, the data obtained with peas and barley, both chilling-resistant plants, are not, insofar as the existence of a low-temperature-induced change in chloroplast membranes does not necessarily render the plant chilling-sensitive. In presenting the data we have drawn straight lines through the data points and have presented previously statistical evidence in support of this (12). While alternative arguments might be put forth about establishment of the best fit of lines or curves through the data points, the

important conclusion remains, namely that chloroplasts isolated from the chilling-resistant plant pea, and also barley (12), show the same apparent changes in E_a for Hill activity with changing temperature as do chloroplasts from the two chilling-sensitive plants. The temperatures at which changes occur are characteristic for each plant, but do not appear to be correlated with chilling sensitivity.

In barley, however, the membrane changes clearly correlate with temperature-induced changes in growth and development of the leaf (12, 19). Similar experiments are being carried out with chilling-sensitive plants since, while temperature-induced changes in membrane properties may affect growth and other physiological processes in both types of plants, it may be the magnitude of the changes in the temperature coefficients of growth processes, rather than the temperature at which they occur, that largely distinguishes chilling-sensitive plants from chilling-resistant plants.

Acknowledgment—The authors wish to thank C. Turner for technical assistance.

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