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KINETICS OF PHOTOCONVERSION OF PROTOCHLOROPHYLLIDE 649 TO CHLOROPHYLLIDE 676 AT LOW TEMPERATURE IN ETIOLATED COTYLEDONS OF *PHARBITIS NIL*

MASAKATA OGAWA and MICHIO KONISHI

Laboratory of Applied Botany, Faculty of Agriculture, Kyoto University, Kyoto 606 (Japan)

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Summary

The kinetics of the photoconversion of protochlorophyllide 649 to chlorophyllide 676 were studied spectrophotometrically over the temperature range of −15 — −80°C under light-saturating conditions in etiolated cotyledons of *Pharbitis nil*. Photoconversion obeyed the sum of two first-order kinetics over this low temperature range. Activation energies obtained from the rate constants were about 5000 cal; this suggests that these two processes may be physical processes not chemical reactions. The results indicate that photoconversion involves two main steps. One is the step dependent on both light intensity and temperature that has been well studied. The other, which is concerned in this study, is the step dependent on temperature only, which may be the requisite for photoconversion. This latter step seems to be related to the binding mode of protochlorophyllide to a holochrome protein or to conformational changes in the protochlorophyllide-holochrome.

Introduction

Since Smith and Benitez [1] first studied the photoconversion of protochlorophyll to chlorophyll in etiolated barley leaves, there have been many investigations of this reaction in protochlorophyllide-holochrome and in leaves [2—7].

Smith and Benitez [1] found that the kinetics of transformation were con-

Abbreviations: PChld, protochlorophyllide; Chld, chlorophyllide. Pigment forms are represented by the wavelength position of their main absorption peaks in the red region.

sistent with a second-order reaction, which indicated that the reaction is bimolecular with respect to protochlorophyll. This was affirmed by Virgin [2]. Boardman [3] concluded that the active protochlorophyll molecules were bound to the protein in two different ways and that both forms were transformed to chlorophyll *a* by first-order reactions, but at different rates. Sironval et al. [4] and Sironval and Brouers [5] found that the kinetics of the photoconversion of protochlorophyllide 647 (PChld 647) to chlorophyllide 676 (Chld 676) were of the first-order when 647-nm photons were used, but the sum of two first-order reactions when 630-nm photons were used. Thorne and Boardman [6] noted the energy transfer from PChld to Chld in macromolecular units, and found that the true kinetics of photoconversion were first-order. Nielsen and Kahn [7] also came to the same conclusion; they proposed a dynamic model describing photoconversion in macromolecular units that can be summarized as follows:

$$\text{PChld 649} \underset{k_3}{\overset{k_1}{\rightleftharpoons}} \text{PChld}^* \text{ 649} \xrightarrow{k_2} \text{Chld 676}$$

where k_1, k_2 and k_3 are rate constants, and PChld* is excited PChld.

Except for a few reports [1,3,5], most experiments were done at room temperature. In this study we investigated the photoconversion of PChld 649 to Chld 676 over the temperature range of −15 — −80°C under light intensity-saturating conditions. We found that there was a temperature-dependent step that differed from well-studied reaction that depended on both light intensity and temperature.

Materials and Methods

Plant materials. Etiolated cotyledons of *Pharbitis nil* strain 'Violet' were used. Seeds were treated and sown as described previously [8]. The germinated seeds were planted 1—1.5 cm deep in vermiculite irrigated with Hyponex solution (1 g/l) in plastic boxes, and were kept in darkness at 25°C. Cotyledons usually sprouted after two days; they were grown in the dark for two more days, at which time the photoconversion activities were maximum [9].

Preparations of sample cotyledons for spectral measurements. Cotyledons were harvested then stuck on a transparent acrylic plate with a piece of transparent cellophan tape; a piece of opal tape was used as the blank control to balance the diffusion effect of the cotyledon. The copper sample holder, to which the acrylic plate was attached, was set on the cap for the special Dewar vessel (Hitachi Crynogenic cell holder) (Fig. 1). These procedures were done under dim-green, safe light.

Temperature measurements. The temperature of the surface of the sample cotyledon was measured with a copper-constantan thermocouple and a self-registering microvoltmeter. The junction of the thermocouple was fixed on the cotyledon surface, which made continuous temperature measurements possible (Fig. 1). This device never affected spectral measurements.

Setting the temperature of a sample. A sample on the holder was immersed on liquid nitrogen, then transferred as quickly as possible to the Dewar vessel, in which the base of the holder was immersed in liquid nitrogen. Irradiation

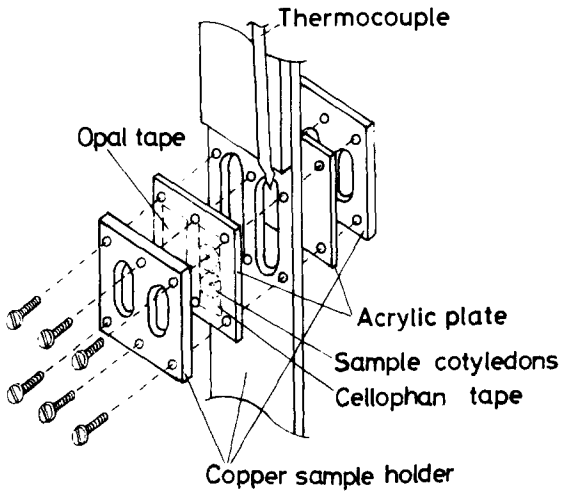

Fig. 1. Copper holder used to attach a sample cotyledon.

and spectral measurements were made in this apparatus to avoid injuries to the sample due to gradual freezing. This procedure was done under dim-green, safe light. About 15 min later, the temperature of the sample reached a sub-equilibrium state. Various sub-equilibrium states could be produced by changing the immersion time in liquid nitrogen and the amount of liquid nitrogen in the Dewar vessel. Temperature between −15°C and −196°C was used in our experiments. A sub-equilibrium state continued for at least 10 min, which was enough time for spectral measurements. An intended temperature, however, could not always be obtained with this method, and difficulty in making precise repetitions occurred. But the temperature for each spectral measurement could be recorded accurately. Consequently, statistical treatments were not made; individual values were used in our results. Spectral measurements were made continuously at the temperature of irradiation.

Spectral measurements. Absorbance was measured with a Hitachi 356 two-wavelength double-beam spectrophotometer. At the temperatures used in our experiments, Chld 676 showed no further spectral changes, and no photobleaching of Chld 676 was detected, because the sample was under anaerobic conditions in the Dewar vessel filled with nitrogen gas. Consequently, an absorption spectrum from 800 nm to 600 nm was measured before irradiation, then the time course of absorbance at 676 nm under the influence of red light (654 nm) irradiation was measured.

Irradiation. A sample cotyledon in the Dewar vessel was irradiated intermittently, with several-second intercepts inserted for monitoring the absorbance measurements. This irradiation method, however, can be considered continuous irradiation. A combination of a slide projector and a mirror system attached to the Dewar vessel (Fig. 2) was used for irradiation. To gain different actinic light intensities (2700, 3000, 5400 and 7000 ergs $\cdot$ cm^{-2} $\cdot$ s^{-1}), several projector lamps (Kondo Sylvania Limited, Japan, 100, 150, 300 and 500 W) were used in the slide projector. For red monochromatic light, an interference

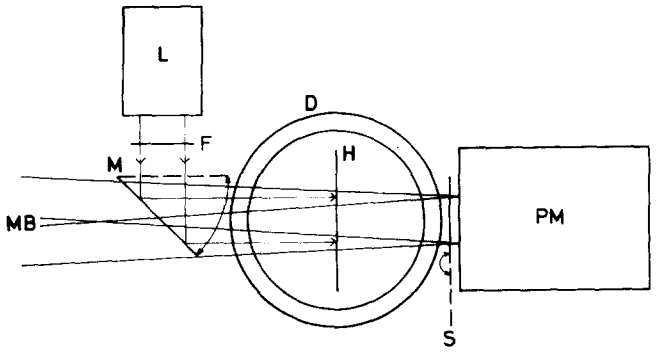

Fig. 2. Devices used for irradiation and absorbance measurements of a sample. L, light source for irradiation (slide projector); F, interference filter; M, mirror; MB, measurement beam of spectrophotometer; D, Dewar vessel; H, sample holder; PM, photomultiplier; S, shutter.

filter (Toshiba KL-65, λ max 654 nm, Hw 13.2 nm) was used. Light intensities were estimated with a Kipp and Zonnen thermopile in front of the Dewar vessel because the thermopile was too large to be set in the Dewar vessel.

Calculation of the percentage photoconversion. The percentage photoconversion $P(\%)$ was calculated at each time for each sample as follows:

$$P(\%) = \frac{A_{676(t)} - A_{676(D)}}{A_{676(\infty)} - A_{676(D)}} \times 100 \quad (1)$$

where $A_{676(D)}$ was absorbance at 676 nm before irradiation, and $A_{676(t)}$ the absorbance at 676 nm at time t after the onset of irradiation, and where $A_{676(\infty)}$ was the absorbance at 676 nm at an infinite time after the onset of irradiation.

Results

PChld 649 was photoconverted to Chld 676 at −105°C with etiolated cotyledons of *P. nil*, but at a temperature lower than −105°C no photoconversion was observed. Kinetic studies, however, were done over the temperature range of −15 – −80°C. One result of the photoconversion measurements is shown as log vs. linear plots in Fig. 3. The photoconversion follows not a first-order law, but the sum of two first-order reactions. Almost all the results of our experiments were consistent with this result.

One can calculate two different rate constants from the log vs. linear plots shown in Fig. 3, and read the ratio of two-component reactions from the intercepts of two straight lines. From these calculations the PChld 649 → Chld 676 photoconversion at low temperature can be expressed as follows;

$$P(\%) = 100 - A \exp(-k_1 t) - (100 - A) \exp(-k_2 t) \quad (2)$$

This equation also has been obtained by Boardman [3] and by Sironval and Brouers [4]. A in Eqn. 2 is the ratio of one of the component reactions. A is 54.6% and $(100 - A)$ is 44.6% as averaged from the calculations. Thus, A can

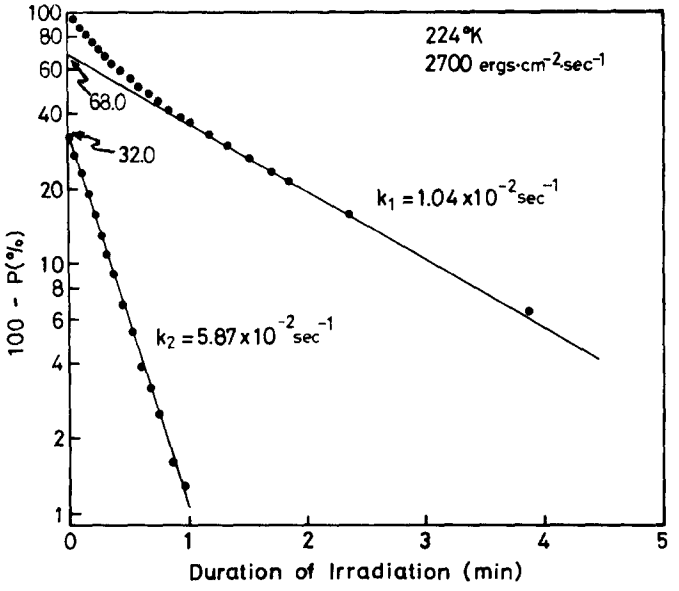

Fig. 3. Kinetics for photoconversion of PChld 649 to Chld 676 when irradiated with 654-nm photons of 2700 ergs · cm^{-2} · s^{-1}, at −49°C. $P(\%)$ was estimated by Eqn. 1. Each point was measured in the same sample. Two straight lines were calculated from Eqn. 2. k_1 and k_2 were the two first-order rate constants.

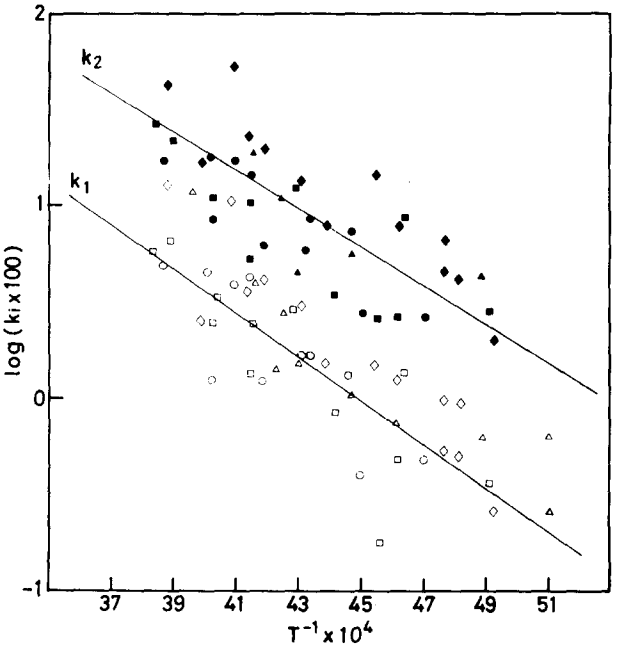

Fig. 4. Temperature dependence of the two rate constants, k_1 and k_2, calculated as in Fig. 3. Each point was an individual value. White mark, k_1; black mark, k_2; △, 2700; □, 3000; ○, 5400, and ◇, 7000 ergs · cm^{-2} · s^{-1}.

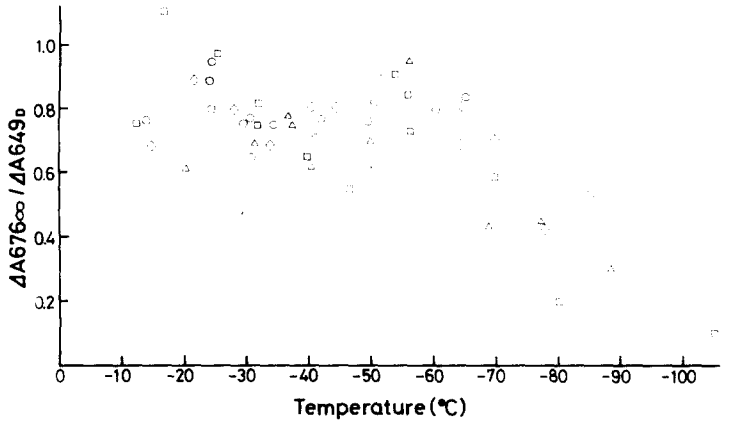

Fig. 5. Effect of temperature on the extent of photoconversion of PChld 649 to Chld 676. [$\Delta A_{676\infty}/\Delta A_{649D} = (A_{676\infty} - A_{676D})/(A_{649D} - A_{800D})$], where $A_{676\infty}$ is the absorbance at 676 nm at maximum photoconversion. A_{676D}, the absorbance at 676 nm before photoconversion. A_{649D} and A_{800D}, absorbances at 649 nm and 800 nm, respectively, before photoconversion. Each point was an individual value. Marks for light intensities are the same as in Fig. 4.

be equal to $(100 - A)$, resulting in 50%. Eqn. 2 now can be rewritten as

$$P(\%) = 100 - 50\exp(-k_1 t) - 50\exp(-k_2 t) \quad (3)$$

At various light intensities the values of $\log(k_i \times 100)$ were plotted as the function of the inverse of absolute temperature, k_i being the two first-order rate constants ($i = 1, 2$) (Fig. 4). This shows that at low temperature, so far as our experiments could determine, there is no effect of light intensity on photoconversion, because light intensities saturate in the experiments. The correlation between the rate constants, k_1 and k_2, and temperature (from Fig. 4) can be shown by the Arrhenius equation:

$$k_1 = 1200\exp(-5180/RT)$$

$$k_2 = 2000\exp(-4590/RT)$$

where T is the absolute temperature and R is the gas constant, 1.987 cal/mol per degree.

The activation energies of the two reactions are 5180 and 4590 cal, respectively. These two values are very close and somewhat low when compared to those usually found for an enzymatically activated collision process; this shows the existence of two similar physical processes.

The extent of photoconversion is affected by temperature but not by the light intensities used (Fig. 5). It increases with temperature, especially below −50°C. This is consistent with the results of Smith and Benitez [1], but we did not find, as they did, that the progress of photoconversion was related to the total incident energy (data not shown), perhaps because of differences in experimental conditions (especially light intensity).

Discussion

Many reports on the photoconversion of PChld 649 to Chld 676 show a rather complicated situation in kinetic studies. This may be due, at least

partially, to the likelihood that photoconversion involves at least two steps, one that is only temperature-dependent and one that is light-dependent and probably temperature-dependent. In this study high intensity light was used to saturate the light-dependent step; a light-independent step that is rate-limiting at low temperature was found.

The characteristics of this step are:

(1) It involves two similar reactions because two rate constants exist; these reactions obey first-order kinetics and have almost the same activation energy (about 5000 cal).

(2) These two rate constants have a ratio of one to one (see Eqn. 3).

(3) In terms of activation energy the reactions probably are simple physical processes rather than chemical reactions.

No photoconversion of PChld 649 to Chld 676 occurred at temperatures below −105°C, although energy transfer from photoactivated PChld to non-photoactivated PChld and/or Chld has been reported, even at −196°C [6,10, 11]. This fact suggests that the temperature-dependent step must be inhibited. Boardman [3] reported that protein-denaturing agents inhibited photoconversion. When PChld is not attached to a holochrome protein it does not photoconvert to Chld [13]; thus, the temperature-dependent step may be related to the way PChld binds to a holochrome protein and may include conformational changes in the holochrome protein. A special binding mode is necessary for the photoreduction of PChld. Nevertheless, other possibilities which would account for this process (for example, the hydrogen donor) cannot be denied from our results alone. The existence of two physical processes (a ratio of one to one) with almost the same values for activation energy indicates that there may be two binding modes of PChld to a holochrome protein. This is evidence to support the hypothesis that a basic unit of PChld holochrome contains two PChld pigments [14,15].

The correlation between this temperature-dependent step and the well-studied, light-dependent step must be considered. We here propose a model of the photoconversion of PChld 649 to Chld 676 to explain the correlation between the two steps (Fig. 6). In Fig. 6 we call the temperature-dependent step Reaction I, and the light-dependent step includes Reactions II—IV. Reaction II is the photoactivation of PChld, and Reaction III includes several processes (de-excitation of excited PChld, and energy transfer between excited PChld and Chld and/or PChld) [7]. Reaction IV is the reduction of excited PChld [3,7].

At room temperature in the dark, a holochrome protein is the form **H.P.**, which is required for photoconversion. On irradiation PChld turns to PChld*, then reduced to Chld. Under these conditions, the rate-limiting step must be Reaction II (photoactivation of PChld) when light intensity is low, and Reaction IV (reduction of PChld*) when light intensity saturates.

At low temperature where no photoconversion is observed, a holochrome protein is the form H.P., which inhibits photoconversion. It changes into the form **H.P.** as the temperature rises. This is the Reaction I. Under light-saturating conditions, PChld exists as PChld*. In our experimental conditions, the rate-limiting step consists of two physical processes whose activation energies are about 5000 cal. As Reaction IV is not a physical process but a chemical reaction, Reaction I must be rate limiting. This means that Reaction I consists

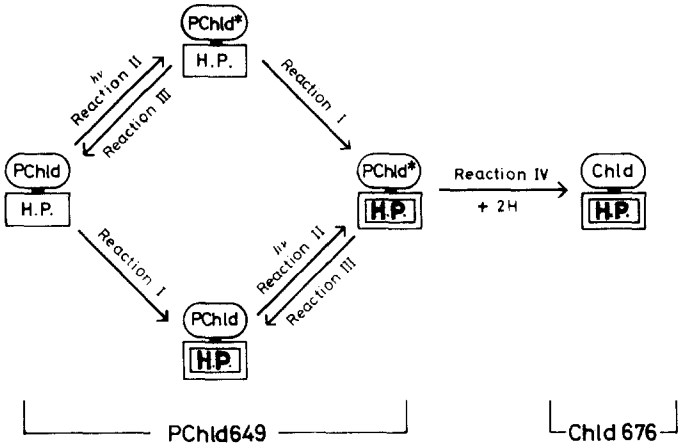

Fig. 6. Model explaining the photoconversion of PChld 649 to Chld 676. H.P. is the form of holochrome protein that inhibits photoconversion. **H.P.** is the form of holochrome protein required for photoconversion. PChld* is an excited state of PChld.

of two similar physical processes; two types of H.P. change into the form(s) **H.P.**.

Only a few investigators [1,3,5] have conducted experiments at low temperatures. Sironval and Brouers [5] calculated rate constants at low temperatures and postulated that the kinetics of photoconversion might obey the first-order law, based on their results at room temperature which indicated first-order kinetics. This postulate does not seem valid. Smith and Benitez [1] calculated an activation energy similar to ours, although they used lower light intensities than we did, and their kinetics differ from ours.

The suggestion by Boardman [3] that active PChld molecules are bound to protein in two different ways, and that both forms are transformed to chlorophyll *a* by first-order reactions but at different rates, seems to fit our findings better than it does his activation energies (5900 and 10700 cal) calculated between −35°C and −55°C. The latter energy is higher than that of physical processes. As the light intensities they used (0.63—8.5 $\mu W \cdot cm^{-2}$) were much lower than ours, Reaction II in Fig. 6 may be rate-limiting in contrast to ours.

Acknowledgement

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