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CIRCULAR DICHROISM STUDIES ON THE STRUCTURE AND THE PHOTOCHEMISTRY OF PROTOCHLOROPHYLLIDE AND CHLOROPHYLLIDE HOLOCHROME

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SUMMARY

On the basis of absorption and circular dichroism (CD) spectral measurements, we conclude that the photoreduction of protochlorophyllide to chlorophyllide in homogenates of etiolated bean seedlings (*Phaseolus vulgaris* L.) involves two light steps in series. Before illumination, the active protochlorophyllide occurs in a dimeric form in the holochrome protein. The initial light reaction converts one of the protochlorophyllide molecules and forms a chlorophyllide–protochlorophyllide holochrome intermediate with a weak, characteristic CD spectrum. The second light reaction subsequently converts the second protochlorophyllide in a less efficient reaction that is temperature dependent. This produces a chlorophyllide holochrome which exhibits a strong double CD characteristic of dimers and which is stable below 1°C. At higher temperatures this dimeric chlorophyllide transforms in the dark to a monomeric form with low CD amplitude. Sucrose at high concentrations (2 M) alters the chlorophyllide holochrome CD spectrum and prevents the final dark dissociation step. Analysis of the photochemical kinetics confirms the occurrence of the two-step photoreduction and supports the stoichiometry of two (proto)chlorophyllides per holochrome protein.

INTRODUCTION

The biosynthesis of chloroplast membranes involves several distinct light reactions. In higher plants the best characterized of these reactions is the photoreduction of protochlorophyllide to chlorophyllide by a reaction involving the transfer of two hydrogen atoms at an enzymatic site. Subsequently, chlorophyllide is transformed into chlorophyll by addition of phytol in a dark reaction. Several reviews of this subject have appeared recently[1–3].

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We have focused our attention on the photochemical step and the immediately following dark reactions. Extracts of etiolated leaves can be used to prepare a purified, active component, commonly called protochlorophyllide holochrome[1,4,5]. This pigment–protein complex retains the ability to be photoconverted, and we assume that its basic structure and photochemistry are similar to those occurring in the leaf, even if the spectroscopic properties are not identical. In the leaf, the protochlorophyllide absorbing at 650 nm is photoconverted into chlorophyllide absorbing at 678 nm; in dark steps, involving structural modifications, the chlorophyllide absorption band shifts first to 684 nm and then to 672 nm (Shibata shift)[6]. By contrast, the absorption maximum of the protochlorophyllide holochrome is at 639 nm, and the absorption maximum of the newly formed chlorophyllide shifts directly from 678 nm to shorter wavelengths[4].

Several detailed studies of the kinetics of the photochemical reaction have pointed out that it is not simple first-order[5,7,8]. Both in the leaf and in homogenized material the reaction can be approximated as the sum of two first-order processes having different temperature dependences. The molecular interpretation of these kinetic data has been either that there are two different types of protochlorophyllide molecules, each displaying first-order kinetics[5,8], or that some interaction between two protochlorophyllide molecules leads to a different probability of reaction for the second molecule once the first has been reduced[5].

Several types of experiments indicate that more than one molecule of protochlorophyllide occurs in close proximity in the etioplast subunits. Butler and Briggs[9], in order to explain the absorption shifts observed in leaves, postulated an aggregated state for both the protochlorophyllide and the newly formed chlorophyllide, and concluded that the Shibata shift is the expression of a structural disaggregation. In support of this model, Schultz and Sauer[10] obtained evidence based on CD spectra for interaction between protochlorophyllide molecules and between newly formed chlorophyllides in purified holochrome preparations. This spectroscopic result is in agreement with structural information obtained by Schopfer and Siegelman[11], who found at least two molecules of protochlorophyllide per protein unit. Kahn *et al.*[12], studying the fluorescence properties of partly converted material, concluded that energy transfer occurs among at least four molecules of pigments, both in the leaf and in homogenized material.

Using CD, a technique indicative of short-range intermolecular interaction among chromophores, we have obtained additional information bearing on the structure of the holochrome, on the photochemical steps involved in the pigment transformation and on the subsequent dark reactions. This work has been done using clarified homogenates of etiolated bean leaves. A complementary study on intact leaves will be published elsewhere. The CD data indicate that there are two steps in the photochemical chlorophyllide formation. The first forms a protochlorophyllide–chlorophyllide mixed dimer intermediate; the second transforms this intermediate into a chlorophyllide dimer. The chlorophyllide dimer exists in at least two conformations which can be distinguished on the basis of their CD spectra: a stable one, in the presence of sucrose, and an unstable one, in the absence of sucrose. The latter converts in the dark to a dissociated form, characterized by its short-wavelength absorption (674 nm) and the low magnitude of its CD spectrum.

MATERIAL AND METHODS

Biological material

Red kidney beans (*Phaseolus vulgaris* L.) were grown 11 ± 1 days on vermiculite in the dark at 21 °C (room temperature). The leaves (30 g) were harvested and ground in 80 ml of a solution consisting of 3 vol. of buffer (0.4 M sucrose, 0.1 M Tricine, (pH 8.0)) *plus* 1 vol. of glycerol, in a Waring blendor. Each sample was homogenized during five 90-s intervals, separated by 15 min, with the blendor in a cold room at −12 °C. The homogenate was filtered through 4 layers of cheese-cloth, and the juice was centrifuged 30 min at 16000 × *g*. The supernatant was dialyzed overnight against 2 l of buffer diluted 10 times, and then concentrated 5–10 fold by ultrafiltration against powdered polyethyleneglycol (Baker; mol wt: 6000). After harvesting, all operations were carried out between 0 and 4 °C, under minimum green light. The homogenates were used immediately or stored at −10 °C before use. To prepare a homogenate containing sucrose, we added the desired amount of a 2.7 M sucrose solution in water.

In an alternative preparation, the juice after filtration was centrifuged twice, as above, and the supernatant used directly. In this case the buffer contained 0.1 M sucrose and 0.05 M Tricine (pH 8.0). The results obtained with these preparations were identical in every respect to those obtained with the concentrated material, attesting that the sucrose and the ultrafiltration against polyethyleneglycol have no irreversible effect.

Spectra; illumination

Absorption spectra were measured using a Cary 14 spectrophotometer. Corrections were made for the turbidity of the samples based on measurements on unilluminated material. The absorbance determined in this way is proportional to the concentration of the homogenate, and extraction experiments using acetone–water (4:1, v/v) showed that the absorbance is also proportional to the chlorophyllide concentration.

CD spectra were measured with a Jasco J-20 spectrometer using a bandwidth smaller than 3 nm. For measurements at temperatures lower than room temperature, the cylindrical cuvette was placed in a cooled cell holder in the Cary 14; this cell holder could also be positioned in the CD spectrometer.

Photoconversion was effected by illuminating the sample in white light, with constant agitation. The light from a Sylvania lamp (Triflector DFA; 115 V, 150 W; operated at 70 V) passed through 10 cm of water, followed by a ground glass diffusing plate. Illumination times were between several seconds and several minutes (for complete photoconversion at −12 °C). The sample was thoroughly agitated to ensure homogeneous illumination.

The phototransformation induced by the measuring beam of the CD spectrometer was not negligible and reached a maximum of 5 %. This effect can introduce some alterations in the CD spectra at $\lambda < 650$ nm. This partial photoconversion does not affect the main results reported here, which are concerned mostly with $\lambda > 650$ nm, because the CD spectra were scanned from long toward short wavelengths and the absorption maximum of active protochlorophyllide is at 639 nm. Thus, the photoconversion occurred only after the most relevant spectral region had already been scanned. The absorption spectra were recorded before the corresponding CD spectra.

RESULTS

Spectroscopic properties of homogenates

Homogenates containing a high concentration of sucrose. In the presence of a high concentration of sucrose (approx. 2 M) the protochlorophyllide holochrome is stable and retains its ability to be photoconverted. After photoconversion the chlorophyllide holochrome is also very stable and maintains its spectroscopic properties unchanged for at least one day at room temperature in the dark.

The absorption and CD spectra of homogenates containing protochlorophyllide or chlorophyllide holochrome in 2 M sucrose are given in Fig. 1. The wavelengths of the maximum of absorption are at 639.0 ± 0.5 and 678 ± 0.5 nm for the protochlorophyllide and chlorophyllide forms, respectively. The CD spectra are very similar to those obtained by Schultz and Sauer[10] for purified holochrome, except that there is a general deviation toward negative values with decreasing wavelength in the homogenates. This effect is not clearly understood; it could result from the turbidity of the material or from some additional component present in our unfractionated homogenates.

The CD spectrum of the chlorophyllide homogenate in sucrose is characterized by a large positive peak at 676 ± 1 nm and a small negative peak around 689 ± 2 nm. Some variability is observed in the relative amplitudes of these two peaks; the ratio is

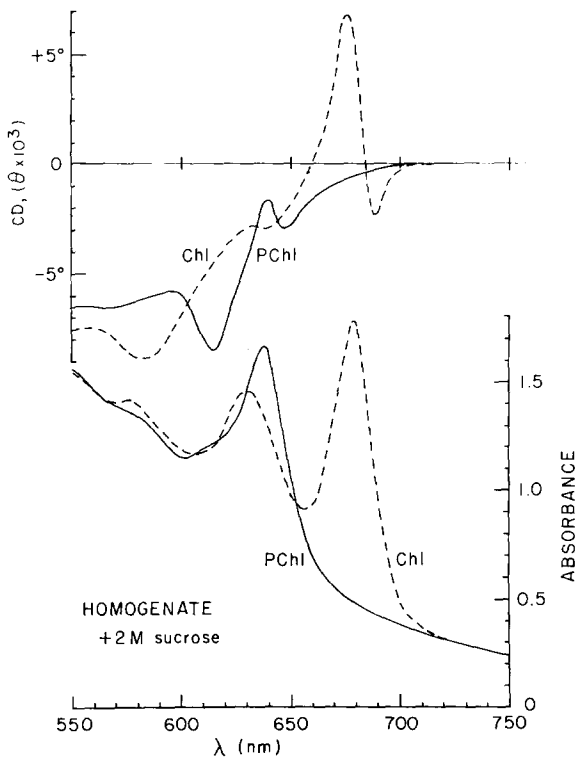

Fig. 1. Absorption and CD spectra of protochlorophyllide homogenate in 2 M sucrose(—) and the same material after complete photoconversion (– – –) at room temperature. Path length, 2 cm.

between 3 and 5 at room temperature. This variation is mostly in the magnitude of the negative peak. Experiments performed at higher sucrose concentration and lower temperature (+1 °C) give a smaller negative peak (*cf* Fig. 1 with Curve 6 of Fig. 5). This difference can be understood in terms of experiments that will be discussed below.

There is some discrepancy between the magnitudes of the CD in our results and in the results of Schultz and Sauer[10], which are expressed as ΔA = difference in absorbance for left and right circularly polarized light. Here we express the CD as the measured ellipticity, θ, in degrees, where: $\Delta A = \theta \times 3 \cdot 10^{-2}$. The discrepancy is attributed to calibration problems in the previous experiments, where different instrumentation was used. It does not affect any part of the interpretation.

Homogenates containing a low concentration of sucrose. In the presence of a low concentration of sucrose (0.04–0.4 M) the spectroscopic properties of the protochlorophyllide holochrome are not distinguishable from those in 2 M sucrose. The CD spectrum of the homogenate in 0.4 M sucrose is given in Fig. 2a (dashed curve). After illumination the chlorophyllide form is not sufficiently stable at room temperature to record the CD spectrum, which requires approx. 30 min. However, the chlorophyllide form is more stable at temperatures below 0 °C. For these measurements we added 20 % glycerol (v/v) to prevent freezing. (At this concentration, glycerol has no apparent effect on the shape of the CD or absorption spectra.) The absorption spectrum is identical to that shown in Fig. 1. The CD spectrum is also similar at wavelengths under 640 nm, but above 640 nm the shape of the CD is significantly different from that in the presence of 2 M sucrose. A negative peak at 685 nm is more prominent in the low sucrose homogenate (Fig. 2a, solid curve). Following measurement of the spectrum at −6 °C, the sample was rapidly warmed to room temperature and two successive

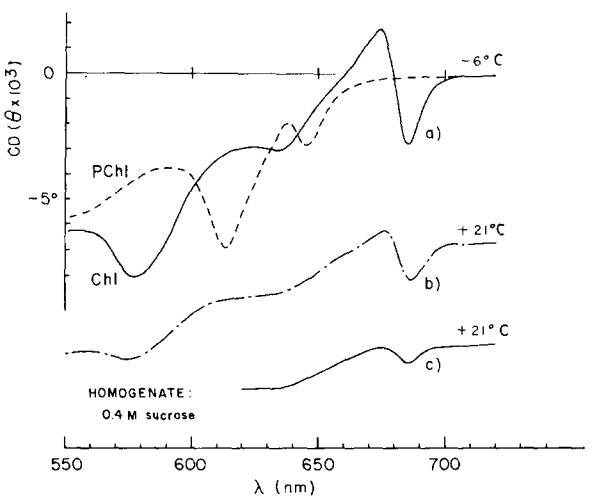

Fig. 2. CD spectra of a homogenate containing 0.4 M sucrose and 20% (v/v) glycerol. Curves a, protochlorophyllide (– – –) and chlorophyllide (—) forms measured at −6 °C; Curves b and c, recorded successively for the chlorophyllide homogenate after warming to 21 °C; path length, 5 cm. Curves b and c artificially displaced downward. Absorption characteristics for chlorophyllide homogenates: (a) absorbance = 1.21 at λ_{max} = 678; $\Delta\lambda_{1/2}$ = 16.2 nm; (b) absorbance = 0.90 at λ_{max} = 677 nm; $\Delta\lambda_{1/2}$ = 19 nm; (c) absorbance = 0.84 at λ_{max} = 674 nm; $\Delta\lambda_{1/2}$ = 19 nm, $\Delta\lambda_{1/2}$ is the width of the long-wavelength absorption at half-maximum height.

spectra (b and c) were recorded. In each of several experiments of this type we observed a progressive decrease in the amplitude of the CD signal leading eventually to its complete disappearance, without noticeable alteration of its shape. This decrease in CD amplitude occurred concomitantly with a shift of the absorption maximum from 678 to 674 nm. We also observed a small decrease in the maximum absorbance, which may result partly from a widening of the absorption band.

Further addition of sucrose, so as to bring its concentration to 2 M, has no effect once the dark shift has occurred. When this addition is made sooner and at 0 °C, it is possible to transform the CD spectrum to a shape very similar to the shape of that in 2 M sucrose (Fig. 7, bottom spectrum). The subsequent dark shift of the absorption spectrum is then blocked.

Effect of dilution of sucrose homogenates with water. Addition of cold water to chlorophyllide homogenates in 2 M sucrose, so as to give a sucrose concentration of approx. 0.4 M, changes the CD to a spectrum typical of homogenates prepared initially in 0.4 M sucrose. Subsequently, it is possible to observe the same dark shift in the absorption spectrum and the progressive disappearance of the CD signal, as with the low initial sucrose homogenate shown in Fig. 2 (b,c). The rate of evolution of the spectra is increased at high dilution of the sucrose and at high temperatures (21 °C).

Photoconversion at various sucrose concentrations. In order to have a more precise understanding of the effect of sucrose, we added various amounts of sucrose to the protochlorophyllide homogenate and recorded the absorption and CD spectra before and after complete photoconversion. These experiments were done at +1 °C, a temperature at which we could run a CD spectrum for all of the photoconverted materials without having any detectable evolution with time. For all sucrose concentrations tested, the absorption and CD spectra before illumination and the absorption spectra after illumination were not detectably affected by the addition of sucrose. After illumination, the CD spectra were also very similar at wavelengths under 640 nm, but at longer wavelengths significant differences appeared at the different final sucrose concentrations (0.04–2.5 M), as depicted in Fig. 3.

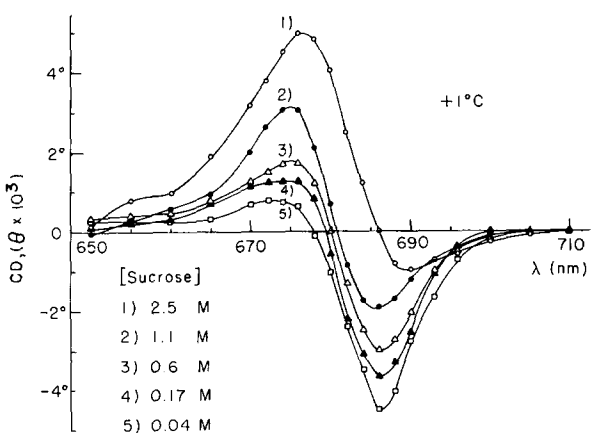

Fig. 3. CD Spectra of chlorophyllide homogenate after complete photoconversion of suspensions containing various amounts of sucrose: (1) 2.5 M; (2) 1.1 M; (3) 0.6 M; (4) 0.17 M; (5) 0.04 M. Temperature, + 1 °C. Path length, 5 cm. The values of the CD have been divided by the absorbance (approx. 0.75) of chlorophyllide at 678.5 nm for each suspension.

The spectra of Fig. 3 show a progressive transformation with increasing concentration of sucrose. A quantitative analysis of this series of spectra using an eigenvalue method[13] indicates that the spectra result from contributions of only two components. In the case of two components we may find isosbestic points in the CD curves. The possible isosbestic points, around 650 and 700 nm, are in regions where the signal is too small to draw any definitive conclusion. The eigenvalue analysis indicates that 99 % of the observed CD can be accounted for without invoking a third component.

Glycerol and polyethyleneglycol have an effect similar to that of sucrose. We did not study these effects quantitatively, however.

Spectroscopic properties of partly photoconverted homogenates

As reported previously[10], the CD spectra of dark and of fully converted material are indicative of strong pigment interaction. Therefore, we sought evidence of some non-linear behavior of the system as a function of progressive chlorophyllide formation: *e.g.* hypo- or hyperchromism, changes in the shape of the absorption or of the CD spectrum.

Material containing a high concentration of sucrose. Progressive illumination of protochlorophyllide homogenates containing 2 M sucrose gives a completely regular evolution of the absorption spectrum, at all temperatures tested (from −40 to +21 °C). The absorption maximum of the chlorophyllide is always at 678.5 nm and the shape of the band does not change throughout the course of the photoconversion (Fig. 4). By extracting the chlorophyllide with a mixture of acetone–water (4:1, v/v), we

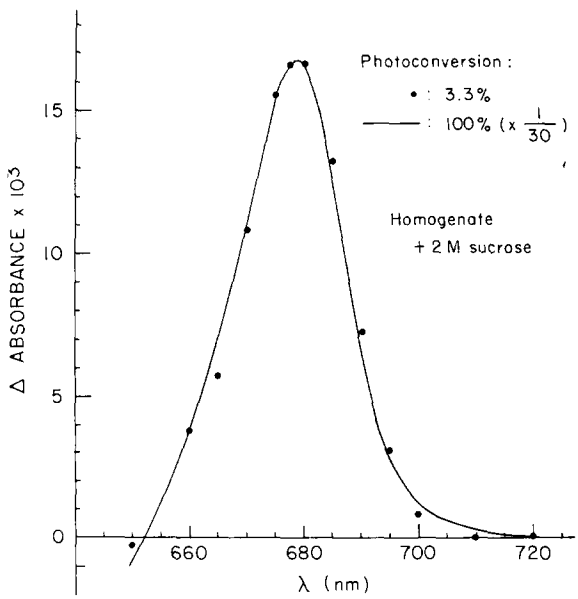

Fig. 4. Differential absorption spectra (the reference cuvette contains unilluminated material) at partial (3.3 %; dots) and complete (line) photoconversion of homogenate in 2 M sucrose. The curves are normalized to the absorbance of the partially converted sample. Room temperature. Path length, 1 cm. At 3.3 % photoconversion the spectrum was measured with the expanded slidewire of the Cary 14 spectrophotometer

found that the absorbance of the homogenates at 678.5 nm was proportional to the chlorophyllide concentration, within ± 5 %. We could not detect any hyper- or hypochromism at partial conversion. The maximum photoconvertibility of the protochlorophyllide in our preparation is approx. 65 %, which is referred to as complete conversion in this article.

By contrast with the absorption spectra, the CD spectra do not evolve linearly during the course of photoconversion. In Fig. 5 the CD spectrum is plotted at different stages of illumination of a single sample. The spectrum at low percentage of photoconversion (<20 %) consists of a single weak positive component centered around 682 nm. Upon further illumination we see the development of the double CD spectrum characteristic of fully converted material (as in Fig. 1). Running such a series of experiments takes around 5 h. At each step of the photoconversion the material is stable over a longer duration in 2 M sucrose.

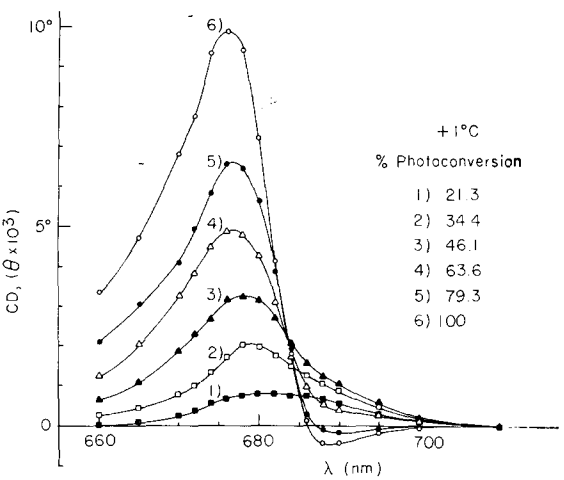

Fig. 5. CD spectra of a homogenate containing 2.3 M sucrose at + 1 °C at different stages of the photoconversion. Path length, 5 cm. Absorbance 1.54 at 678.5 nm, for Curve 6. Percent photoconversion: (1) 21.3; (2) 34.4; (3) 46.1; (4) 63.6; (5) 79.3; (6) 100.

Eigenvalue analysis of this series of spectra (Fig. 5) reveals that they are the result of only two components within ± 1 %. When the CD spectra are normalized to the same amount of chlorophyllide an isosbestic point appears at 681.5 ± 0.5 nm.

The initial positive CD component is difficult to follow quantitatively at high percent conversion. The magnitude of the larger, double CD component can be characterized, approximately, by the difference in CD between 675 and 689 nm. This difference, $\Delta\theta_{675\text{ nm}-689\text{ nm}}$, relative to the maximum value at complete photoconversion is plotted in Fig. 6 for various stages of photoconversion (measured using the absorbance at 678.5 nm relative to that at complete photoconversion). This plot compares several different experiments; each is represented by a different open symbol and includes 3–5 intermediate points. An interpretation of the non-linear behavior of the CD during the photoconversion (Fig. 6) is presented in Discussion.

Material containing a low concentration of sucrose. In order to avoid complications owing to the dark shift following photoconversion, the experiments carried out

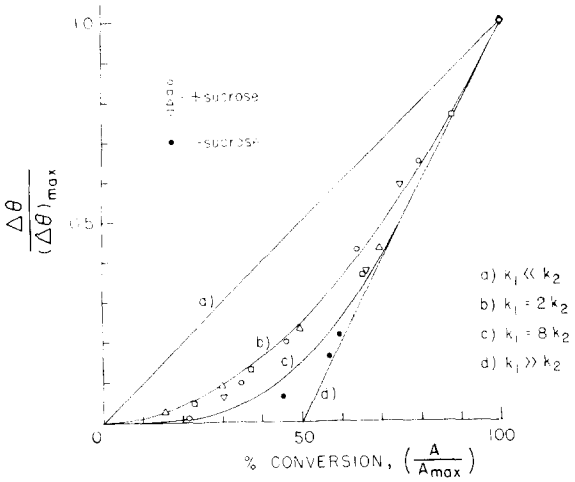

Fig. 6. Plot of the relative CD $(\theta_{675\text{ nm}}-\theta_{689\text{ nm}})/(\theta_{675\text{ nm}}-\theta_{689\text{ nm}})_{max}$ at various percents of photoconversion $(A_{678\text{ nm}}/A_{678\text{ nm, max}})$ for different experiments. Open symbols, homogenate + sucrose (approx. 2 M); each symbol is for a different experiment. Temperature range, + 1 to + 21 °C. Full dots: homogenate + 25 % glycerol, but lower sucrose concentration (approx. 0.04 M). The three points represent three different experiments. Temperature, approx. −10 °C. a, b, c, d are theoretical curves (see text). (a) $k_1 \ll k_2$; (b) $k_1 = 2k_2$; (c) $k_1 = 8k_2$; (d) $k_1 \gg k_2$.

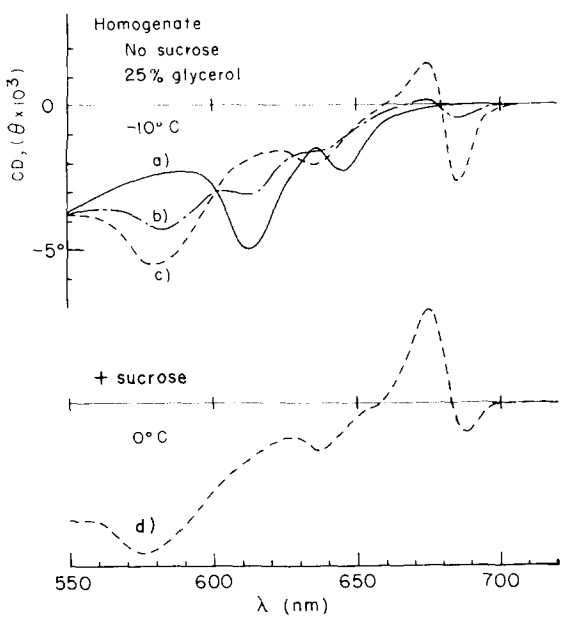

Fig. 7. CD spectra of a protochlorophyllide homogenate (a), and the same material after partia and complete photoconversion (b, c); sucrose 0.05 M; 25 % glycerol; path length, 5 cm; tempera ture −10 °C. (d) + sucrose: sample (c) to which a concentrated solution of sucrose has been added; sucrose 1.4 M; 13 % glycerol; path length, 10 cm; temperature 0 °C. Absorbance at 678.5 nm: (a) 0; (b) 0.52; (c) 0.90; (d) 0.92.

on material suspended in low sucrose were performed at −10 °C, with 25 % glycerol added. Under these conditions the absorption evolves regularly during the photoconversion, but the magnitude of the CD signals is not proportional to the chlorophyll concentration. Fig. 7 (a, b, c) illustrates the evolution of the CD spectrum. At low sucrose concentration there is no evidence for the prominent positive CD signal that occurs in high sucrose (Fig. 7d). Furthermore, the shape of the final CD spectrum is distinct from that obtained with the minimum amount of sucrose (Fig. 3, Curve 5) and is more similar to that obtained with an intermediate sucrose concentration. This difference is attributed to the presence of glycerol, which has an effect somewhat like that of sucrose. In Fig. 6 we report the values for three different experiments in the absence of sucrose, each having only one intermediate point; the non-linear character of the increase in the CD signal is even more accentuated than in the presence of sucrose.

When similar experiments are performed at higher temperatures, a progressive shift from 678.5 to 674 nm in the absorption of this material occurs during measurement, and the CD spectra are not reliable. In 0.3 M sucrose the absorption shift is accomplished in approx. 1 h at room temperature. For partly converted material (4–30 %) the shift occurs only about half as fast. This qualitative observation is of interest in comparison with the behavior of leaves, where the dark shift to shorter wavelength is much faster at low percentage of photoconversion (P. Mathis and K. Sauer, unpublished observation).

Absorption coefficient of protochlorophyllide

Koski and Smith[14] found a value of 34.9 $l \cdot g^{-1} \cdot cm^{-1}$ at 623 nm in acetone for the absorption coefficient of protochlorophyll extracted from etiolated barley leaves. Because etiolated leaves contain far more protochlorophyllide (mol. wt = 613) than protochlorophyll (mol. wt = 891.5), some doubt was expressed by Houssier and Sauer[15] as to the molar absorption coefficient deduced from this specific absorption coefficient. In the experiments described above, where we prepared 80 % acetone extracts of homogenates at different extents of photoconversion, we were able to compare the increase of chlorophyllide absorption with the decrease in that of protochlorophyllide, correcting for the contribution of chlorophyllide at the wavelength of maximum protochlorophyllide absorption in the red. The molar absorption coefficient of chlorophyllide *a* is essentially identical to that of chlorophyll *a* (refs 16–18), which has a value of 81 000 $M^{-1} \cdot cm^{-1}$ at 665 nm in 80 % acetone[19]. On the basis of this value and our experiments, we calculate $\varepsilon_{628\,nm} = 30\,900\ M^{-1} \cdot cm^{-1}$ for protochlorophyllide in the same solvent. Assuming that Koski and Smith[14] did, in fact, isolate phytylated protochlorophyll, their measured absorption coefficient yields $\varepsilon_{623\,nm} = 31\,100$ in 100 % acetone. Both Mackinney[20] and Vernon[19] have shown that the absorption coefficients of chlorophyll *a* differ by only 2 % between 80 and 100 % acetone. If this is also true for protochlorophyll(ide), then our results are in excellent agreement with those of Koski and Smith[14], and the doubts raised by Houssier and Sauer[15] over the authenticity of the protochlorophyll isolated by Koski and Smith[14] appear to be unwarranted. There remains the question of why the molar absorption coefficient of crystalline 4-vinylprotochlorophyll (demonstrated to contain phytol) obtained by Houssier and Sauer[15] from the seed coat of pumpkin seeds is 30 % less than that of protochlorophyll.

DISCUSSION

The interpretation that we propose in order to account for our results is illustrated in Fig. 8. Protochlorophyllide holochrome contains two interacting protochlorophyllide molecules which are photoreduced by two successive photochemical reactions with rates k_1 and k_2, respectively. The chlorophyllide holochrome so produced exists in two forms: the one in the presence of sucrose is stable; the other in the absence of sucrose is not stable and dissociates to give two non-interacting chlorophyllide molecules. We will discuss this interpretation of the structural and photochemical properties in some detail. Our scheme is kinetically simple and does not imply any heterogeneity in the holochrome; however, it includes a number of "states" for the pigment–protein complex, designated P–P, P–P_{suc}, P–C, P–C_{suc}, C–C, C–C_{suc}, C.

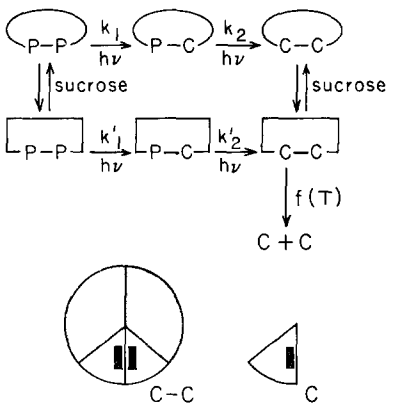

Fig. 8. Schematic representation of pigment–holochrome forms described in this article and their mutual transformations.

Structure

In the presence of high concentrations of sucrose (Figs 1 and 5) we find three different types of CD spectra. Before illumination, the CD spectrum in the bands owing to protochlorophyllide indicates pronounced pigment–pigment interaction, as pointed out previously by Schultz and Sauer[10]. The evidence for this interaction includes the large magnitude of the CD, its multiple character, and the reversal in sign of the long wavelength CD relative to that of monomeric protochlorophyll in diethyl ether[15]. At intermediate photoconversion a second species is formed, characterized by a weak, positive single CD in the chlorophyllide region. At greater extent of photoconversion a double CD (negative at long wavelengths) appears in the region of chlorophyllide absorption. This is indicative of a third constituent, one that exhibits increased aggregation relative to the second[21,22]. The presence of the three constituents and their order of appearance are accounted for by the scheme:

$$\mathrm{P\text{–}P_{suc}} \xrightarrow[k_1]{h\nu} \mathrm{P\text{–}C_{suc}} \xrightarrow[k_2]{h\nu} \mathrm{C\text{–}C_{suc}}$$

In the case of P–C_{suc} the difference in energies of the long wavelength transitions in protochlorophyllide and chlorophyllide is not favorable for exciton interaction. This

intermediate species could also account for the unusually high fluorescence efficiency of the initially formed chlorophyllide[10].

With a low concentration of sucrose and at low temperature a similar pattern:

$$\text{P–P} \xrightarrow{h\nu} \text{P–C} \xrightarrow{h\nu} \text{C–C},$$

accounts for the CD measurements summarized in Fig. 7 (a, b, c). We could not detect any distinctive CD signal attributable to P–C in the 670–700 nm region. Both in the presence and absence of sucrose the chlorophyllide homogenates display a negative CD at 575 nm. We cannot determine whether this results from chlorophyllide or from the remaining inactive protochlorophyllide; however, this peak disappears progressively during the course of the dark shift.

After complete photoconversion, C–C and C–C_{suc} appear to be in different proportions depending on the sucrose concentration (Fig. 3). This observation can explain the variability we found, even at high sucrose concentration, in the negative peak. It is possible that, even at the higher concentrations of sucrose we used, part of the chlorophyllide was in the C–C form (*cf* Fig. 3, Curve 1 with Fig. 5, Curve 6). The transformations between C–C and C–C_{suc} appear to be reversible. The effect of sucrose could result from optical artifacts owing to its own rotatory power, but the absence of such an effect on the CD spectrum of protochlorophyllide holochrome makes this explanation unlikely. The most plausible explanation is that sucrose, by reducing the activity of water, leads to a change in the conformation of the protein moiety, which in turn affects the pigment–pigment interaction. A significant effect of sucrose on protein conformation has been reported by Clement-Metral and Yon[23] for β-lactoglobulin.

The CD spectrum of the mixed protochlorophyllide–chlorophyllide dimer in P–C is also apparently sensitive to the effect of sucrose, and we suggest that the conformational change occurs even before illumination. The CD spectrum of protochlorophyllide holochrome appears not to be sensitive to this conformational change; however, the protochlorophyllide holochrome does exhibit increased stability in the presence of sucrose.

The C–C species changes progressively in a reaction affected by the temperature and perhaps by the viscosity. The product of this evolution has an absorption peak at 674 nm and an undetectable CD (by contrast with the observation of a single, negative CD reported by Schultz and Sauer[10]). We assume this to be indicative of chlorophyllide monomer. A similar dissociation, producing chlorophyllide attached to a small protein, has been reported by Bogorad *et al.*[24]. Butler and Briggs[9] have previously attributed the Shibata shift in leaves to a disaggregation. We have no direct evidence bearing on the state of chlorophyllide after dissociation. It is probably bound to a small part of the protein, but it might be free in solution, as chlorophyllide is somewhat water soluble.

Kahn *et al.*[12] presented experimental evidence based on low temperature fluorescence studies which they interpreted to indicate the presence of at least four molecules of associated pigment in the holochrome. Our results are not necessarily in disagreement with their findings. We may consider the protochlorophyllide and chlorophyllide interactions to occur at several levels, including short-range weak excitation interactions between pigment dimers within the holochrome particles and long-range Förster type interactions[25] among pigments in adjacent holochrome par-

ticles. The observation by Kahn *et al.*[12] of energy transfer leading to fluorescence could result from the long-range Förster transfer. Apparently various dissociating agents can disrupt the aggregates, possibly even at the dimer level, without a dramatic loss of photochemical activity[11,26].

It is remarkable that, apart from the monomeric form C, all chlorophyllide forms mentioned here have the same absorption spectrum. This emphasizes the insensitivity of absorption spectroscopy in such cases. The situation may be different for protochlorophyllide, for which we cannot draw definitive conclusions; it may also be different in the leaf, where complex absorption changes are known to occur during the first stages of greening.

Photochemistry

Previous investigations showed that the photochemical transformation of protochlorophyllide to chlorophyllide is kinetically complex. Boardman[5] was able to fit his results on the isolated pigment–protein using two first-order reactions whose rate constants are affected differently by temperature. Sironval *et al.*[8] obtained similar results with leaves and found, moreover, that the kinetics were different depending on whether the wavelenght of the actinic light was 630 or 647 nm. Our studies confirm the two different rate constants for two photochemical steps in series and demonstrate that these rate constants are altered when the holochrome conformation is changed by added sucrose. The kinetic analysis for two first-order reactions in series is straightforward[27], but it leads to a simple integrated rate expression only if $k_1 = 2k_2$. (The factor of 2 comes from the two protochlorophyllide molecules in the holochrome.) In this case the yield of final product, C–C_{suc}, should be proportional to the square of the total chlorophyllide formed (Fig. 6, Curve b). The other cases represented in Fig. 6 are: (a) $k_1 \ll k_2$, (c) $k_1 = 8k_2$, and (d) $k_1 \gg k_2$. The case where $k_1 \ll k_2$ is excluded by the high quantum yield for the initial photoreduction[28]. In the presence of sucrose and at temperatures between 1 and 21 °C, the points lie slightly below Curve b, indicating approx. $k_1 = 3k_2$. For the experiments performed at −10 °C in the absence of sucrose, the points lie in the region indicating $k_1 > 8k_2$. The fact that all of the experimental points lie to the left of Curve d is consistent with the model where one-half of the initial protochlorophyllide molecules are converted *via* one reaction and the other half react *via* a kinetically distinct mechanism. This provides support for the proposed stoichiometry of two interacting active protochlorophyllides per holochrome.

It is worth noting that, even at 3 % photoconversion, we found no evidence for the pigment absorbing at 668 nm in the leaf, considered by Thorne[29] to be a photochemical intermediate. The intermediate and final forms of chlorophyllide holochrome have indistinguishable absorption spectra and differ only in their CD. Our own results with leaves suggest that the 668 nm form is not an intermediate but is the result of a rapid dark side reaction (P. Mathis and K. Sauer, unpublished results).

CONCLUSION

Both the photochemical analysis and the characteristic shapes of the CD spectra of the components are in agreement with the scheme of Fig. 8. There is a two step photoreduction of dimeric protochlorophyllide in the homogenate. The dimeric pigment–proteins can occur in two different conformations and only the one present

without added sucrose can lead finally to a dissociated chlorophyllide species. The relationship of the pigment molecules to the protein and, in particular, to the reducing site is still unknown. Future studies will be aimed at clarifying this question and relating the observations on homogenates to the analogous process occurring in intact seedlings.

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