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TRANSITORY PIGMENT-PROTEIN COMPLEXES SIMILAR TO PHOTOSYNTHESIS ACTIVE CENTRES DURING PROTOCHLOROPHYLL(IDE) PHOTOREDUCTION

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SUMMARY

By illuminating etiolated bean leaves (*Phaseolus vulgaris*, var. Commodore) at temperatures around 178 K, and by freezing them in liquid nitrogen, hitherto unknown intermediates in the reduction of protochlorophyll(ide) to chlorophyll(ide) have been trapped.

These intermediates appear to have red absorption bands located at wavelengths longer than 680 nm, up to 750 nm. They consist of pigment-protein complexes with a very short life time at room temperature. They do not emit any appreciable fluorescence at 77 K. When heated to temperatures above 178 K, they are transformed in the dark into a chlorophyll(ide)-protein complex: the $P_{688-678}$ form already described.

Energy is transferred from short wavelength absorbing pigments to the intermediate long wavelength pigments. The significance of the intermediates described, especially their similarity to photosynthetic reaction centres, are discussed briefly.

INTRODUCTION

By plunging immediately into liquid nitrogen an etiolated bean leaf which has received an intense flash of 1 msec, Sironval et al. [1] have trapped a transitory complex containing chlorophyll(ide). This complex is present in the 30 msec following the flash [2]. It has been described by Sironval and Kuiper [3] as an entity — the energy transfer unit — containing a mixture of varying proportions of protochlorophyll(ide) and chlorophyll(ide) bound to proteins in the membranes of the etioplast.

Sironval and Kuiper concluded from their experiments that this entity originated from an unknown intermediate X. The high rates of the reactions in the fresh leaf have so far prevented this intermediate from being studied.

We found that it is possible to slow down the reactions by freeze-drying the leaves. The photoreduction of protochlorophyll(ide) takes place in such leaves as it does in fresh leaves, but with certain specific characteristics [5,6]. In particular, in the freeze-dried leaves, the pigment-protein complexes are remarkably stable. It is possible to make a series of sudden transitions from 77 to 300 K, and back again, without changing their observed properties; this is far from being the case in the fresh leaf [5]. The etiolated freeze-dried leaf (like the fresh leaf) contains three distinct complexes between protochlorophyll(ide) and a protein: a nonphotoreducible complex $P_{633-628}$; and two photoreducible complexes, $P_{657-650}$ and $P_{645-637}$ [5] (the first subscript refers to the approximate position of the main fluorescence emission band and the second to the approximate position of the main red absorption band).

$P_{645-637}$ transfers the light energy which it absorbs to $P_{657-650}$ [4,7,8]. At the end of the illumination of a freeze-dried etiolated leaf, a complex which is stable at normal temperature ($P_{688-678}$) is found [5]. Hence the following may be written:

$$\left.\begin{matrix} P_{657-650} \\ \\ P_{645-637} \end{matrix}\right\} \xrightarrow[+\ 2H]{h\nu} P_{688-678} \qquad \text{(reaction 1)}$$

It is not known whether the initial two complexes are transformed along parallel paths or whether one of them forms the other before being transformed into $P_{688-678}$.

We will now show that: (1) as predicted by Sironval and Kuiper [3], there exist intermediate states of the pigment-lipoprotein complexes between the substrates of reaction (1) ($P_{657-650}$ and $P_{645-637}$) and their common product ($P_{688-678}$); (2) Some of these states have properties which resemble those of the active centres of photosynthesis.

MATERIALS AND METHODS

Bean leaves (*Phaseolus vulgaris*, var. Commodore) are freeze-dried according to the method already described [5]. To permit handling at the required temperatures, the leaves are surrounded with a plastic film which protects them from humidity without interfering with the experiment; the film also prevents the formation of cracks during freezing and/or thawing. The leaf is placed on a special flat carrier held at a given height just before a transparent window above the level of the liquid nitrogen in a Dewar; an alcohol thermometer measures the ambient temperature. The light is directed onto the leaf inside the Dewar.

RESULTS

The emission (F) and absorption (A) spectra of a freeze-dried etiolated leaf which has never received light, as recorded at 77 K, are shown in Fig. 1 a and b, spectra (0). If, after heating the leaf to 293 K, it is illuminated at that temperature by an intense photographic white flash and then plunged again into liquid nitrogen, the spectra (2), Fig. 1, are recorded.

Before illumination, the red absorption and fluorescence emission show the presence of $P_{657-650}$ (fluorescence emission and absorption peaks seen respectively at 657 and 648 nm) and $P_{645-637}$ (fluorescence emission quenched by energy transfer to $P_{657-650}$; absorption peak seen at 635 nm). After illumination a pigment complex absorbing at 678 nm and emitting at 688 nm ($P_{688-678}$) is found. This spectral change illustrates reaction (1).

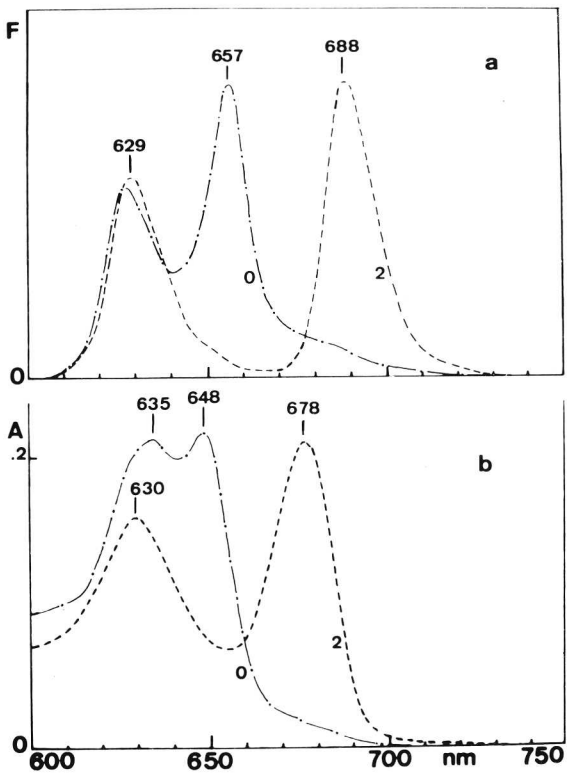


Fig. 1. 77 K Fluorescence emission (a) and absorption (b) spectra of an etiolated lyophilized bean leaf (curves 0), and of the same leaf which has been warmed up from 77 to 293 K, then illuminated by a 1 msec polychromatic photographic flash (Multiblitz Report Porba, electric energy 125 J, colour temperature 5800 K) at a distance of 4 cm, and finally frozen in liquid nitrogen (curves 2). Several transitions from 77 to 293 K do not alter the spectral properties and photoactivity of the pigment-protein complexes of the lyophilized leaves [8,20].

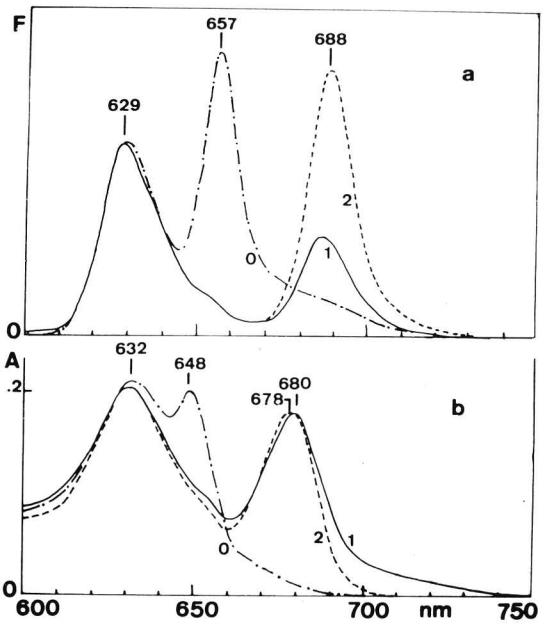


Fig. 2. 77 K Fluorescence emission (a) and absorption (b) spectra of an etiolated lyophilized leaf (curves 0). The leaf has been heated to 253 K and has been illuminated at 253 K with a 1 msec polychromatic photographic flash (as described under Fig. 1) before being plunged in liquid nitrogen again (curves 1). Then the leaf has been heated to 293 K for 1 min and plunged for a third time in liquid nitrogen (curves 2). The fluorescence emission spectra 0, 1 and 2 were arbitrarily adjusted at the same value at 629 nm. The absorption spectra 1 and 2 were adjusted at the same value at 680 nm.

If the same experiment is carried out at 253 K, the following situations are recorded: before the flash, the 77 K absorption (A) and emission (F) spectra are as in Fig. 2 a and b, spectra (0), similar to spectra (0), Fig. 1 a and b; after the flash at 253 K, followed by immediate freezing at 77 K, the spectra are as shown in Fig. 2 a and b, spectra (1). These spectra indicate that the flash at 253 K has caused the disappearance of most of the $P_{657-650}$, whereas a pigment absorbing around 680 nm has appeared; one observes also the formation of a certain quantity of pigment absorbing at the long wavelength side of the spectrum, as demonstrated by the long absorption tail extending to 750 nm. The emission of fluorescence by the etiolated leaf at 657 nm disappeared after the flash, but only a weak fluorescence appeared in the 688 nm region. Obviously, pigment complexes have been produced with a rather poor fluorescence emission around 688 nm at 77 K.

The transitory, intermediate character of these complexes is demonstrated as follows: the leaf is heated up to 293 K in the dark, then kept in darkness at 293 K for 1 min; it is then frozen at 77 K and the spectra are recorded again. The dark heating greatly increases the intensity of the emission of fluorescence

at 688 nm, whereas the excess absorbance between 690 and 750 nm disappears; the position of the principal absorption band shifts slightly towards shorter wavelengths, from 680 to 678 nm (Fig. 2, spectra (2)).

The differences (absorption before - absorption after dark heating) and (emission before - emission after dark heating) — not shown in Fig. 2 — indicate that pigments produced by the flash at 253 K and absorbing between 690 and 750 nm are converted into $P_{688-678}$, the product of reaction (1): these are intermediate complexes in this reaction.

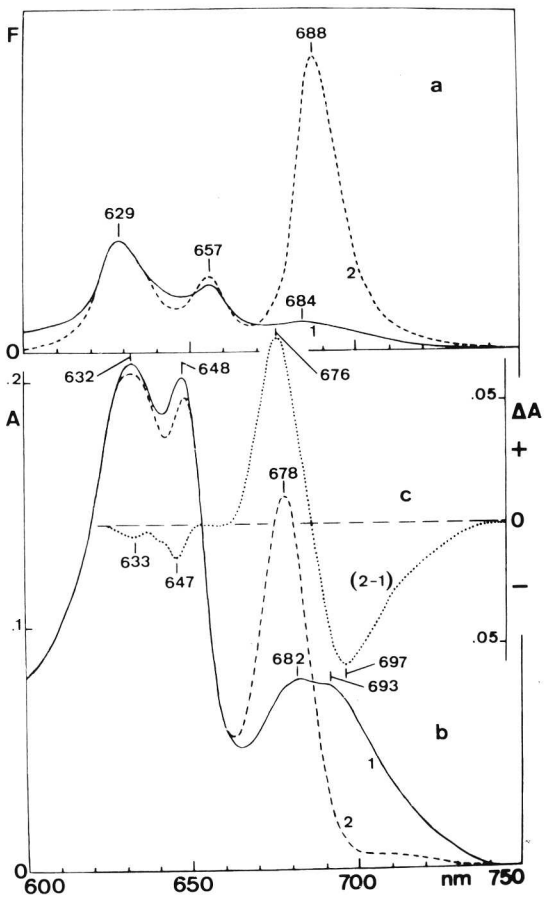


Fig. 3. 77 K Fluorescence emission (a) and absorption (b) spectra of an etiolated lyophilized leaf which has been illuminated at 168 K for 1.5 min with a white 500 W projector lamp, the light being focalised by adequate optics on the sample surface (curves 1). The same leaf was warmed up to 253 K, in the measuring Dewar inside the Cary spectrophotometer and it stayed at this temperature for 10 min. Liquid nitrogen was then added, without moving the sample, for registering the absorption spectrum again (curve 2,b). The fluorescence emission spectra registered before (curve 1,a) and after heating (curve 2,a) were adjusted at the same value at 629 nm.

The illumination of freeze-dried leaves at a temperature of 165—180 K enables a substantial quantity of intermediate complexes to be accumulated. An etiolated freeze-dried leaf is illuminated for 1.5 min at 168 K and then frozen at 77 K. Before the illumination, the spectra are as shown in Figs. 1 and 2, spectra (0). Illumination at 165—180 K makes the fluorescence emission of the complex $P_{657-650}$ at 657 nm disappear, but it does not make any emission appear at 688 nm; a very weak emission is found at about 684 nm

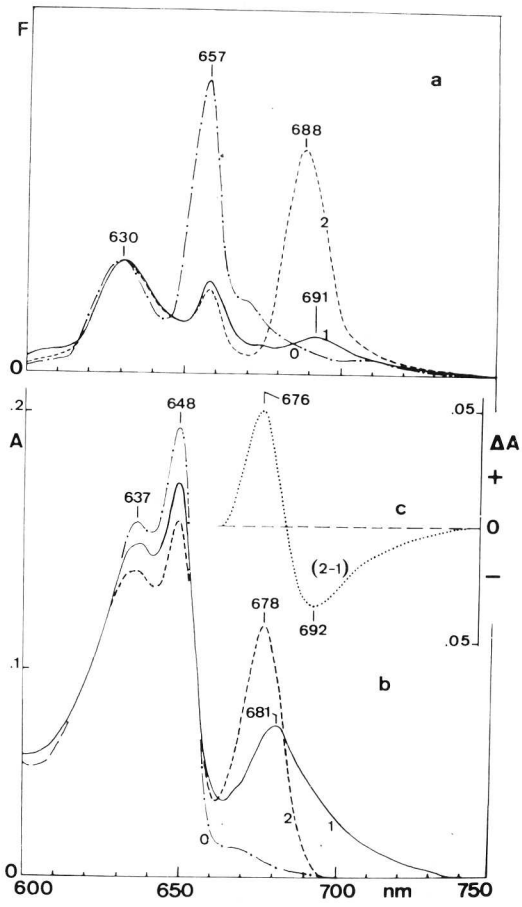


Fig. 4. 77 K Fluorescence emission (a) and absorption (b) spectra of an etiolated, fresh bean leaf (curves 0). The same leaf has been heated to 178 K, then illuminated during 60 sec with the polychromatic light of a 500 W projector source as in Fig. 3, and finally plunged into liquid nitrogen for registering the fluorescence and absorption spectra (curves 1, a and b). Thereafter, the sample has been heated in the measuring Dewar inside the Cary spectrophotometer to a temperature of about 250 K. It remained at that temperature for 1 min. Then, without moving the sample, liquid nitrogen was poured inside the Dewar, for registering the absorption spectrum (curve 2,b). The fluorescence emission spectrum (curve 2,a) was registered just after registering of curve 2b. The fluorescence emission spectra were adjusted to the same value at 630 nm.

(Fig. 3a, curve (1)). The absorption spectrum shows that a part of the complexes $P_{657-650}$ and $P_{645-637}$ has disappeared during the illumination while new absorption bands have appeared centred at 682 and 693 nm respectively (Fig. 3b, curve (1)); pigments absorbing between 700 and 750 nm have also been formed.

If the leaf is heated in darkness from 77 to 253 K, and kept at 253 K for 10 min, a further recording of the spectra at 77 K (Fig. 3, curves (2)) proves that during the heating in darkness:

(1) the absorption between 690 and 750 nm disappears, while a rather narrow band centred on 678 nm is formed;

(2) the absorbances between 630 and 650 nm are slightly diminished;

(3) the emission of fluorescence at 688 nm appears.

It is clear that the product $P_{688-678}$ is formed in darkness from intermediates in reaction (1).

These intermediates emit only very weak fluorescence, even at 77 K, whereas their absorption — as shown by the difference spectrum in Fig. 3c — is characterized by a maximum around 697 nm, extending with gradually decreasing intensity as far as 750 nm.

Demonstration of the presence of intermediate complexes in fresh leaves follows the same procedure as in lyophylized leaves, but (1) heating above 273 K must be avoided in this case and (2) the temperature must be kept at about 180 K during illumination.

During an illumination at sufficiently low temperature the fresh leaves accumulate a certain quantity of a pigment-protein complex which is apparent, after reheating in darkness, from the increase or development of fluorescence emission at 688 nm [3,9,10].

Fig. 4 a and b, spectra (1), concerns a fresh etiolated leaf which has formed, in light at 178 K, a certain quantity of intermediate complexes, the presence of which is sufficient to quench the 77 K fluorescence emission at 688 nm. These intermediate complexes absorb from 680 to 750 nm. Reheating in darkness up to 250 K increases the emission intensity at 688 nm while the absorption grows up at 678 nm and slows down above 690 nm (Fig. 4 a and b, spectra (2)). The same experiment carried out with extracts of etiolated fresh leaves in a suitable buffer [11] gives the same result.

DISCUSSION AND CONCLUSIONS

The observed intermediates correspond to hitherto unknown steps in reaction (1). They possess the following properties:

(1) They are formed during illumination from the pigment-protein complexes $P_{657-650}$ and/or $P_{645-637}$ of the etiolated leaf.

(2) Their formation may take place at 180 K (and below); their life-time is temperature dependent and is very brief at room temperature.

(3) They give rise to the complex $P_{688-678}$ when the temperature is raised above 77 K (probably mainly from about 150 K); it follows from (1) and (3) that they are themselves pigment-protein complexes.

(4) They are stable at a temperature of 77 K.

(5) 178 ± 5 K seems an optimum temperature for intermediate accumulation.

(6) They form a family of pigment-lipoprotein complexes absorbing up to 750 nm with a principal absorption band around 695 nm.

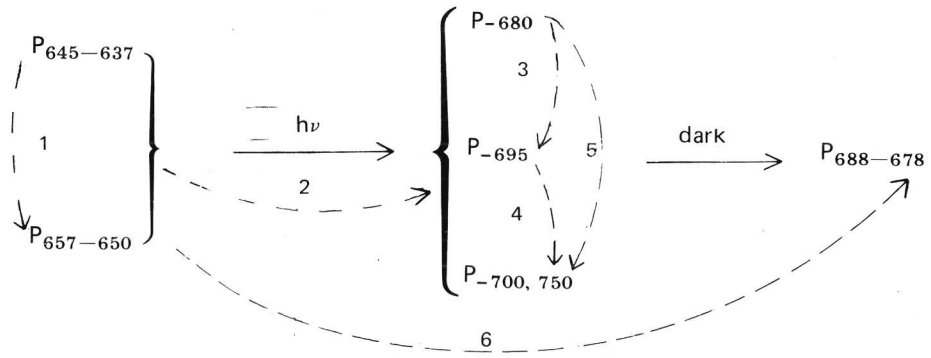
(7) They emit only very weak fluorescence at 77 K, if any.

Because of their spectral properties, we propose designating the intermediate complexes by the following formulae: P_{-680}, P_{-695}, $P_{-700,\,750}$, in which only the approximate position of the principal absorption band in the red is given as a subscript. In particular P_{-680} designates a state of the pigment-protein complex whose red absorption band appears around 680 nm when illuminating at a low temperature and which emits a feeble fluorescence in the 680—690 region at 77 K (Figs. 3 and 4).

When warming up in darkness a leaf which has been illuminated at 178 K the formation of $P_{688-678}$ from P_{-680} reflects a change from a situation in which the pigment absorbing at 680 nm is thought to transfer its excitation energy to P_{-695} and to $P_{-700,\,750}$, to another situation in which the transfer becomes impossible because of the disappearance of the long wavelength absorbing forms. On the other hand, it must be admitted that P_{-695} and $P_{-700,\,750}$ are transformed into $P_{688-678}$ since the latter is the end product of reaction (1). Consequently, when the intermediates are warmed up, a transient situation is encountered during which on the one hand the P_{-680} complex formed at low temperature simultaneously with P_{-695} and $P_{-700,\,750}$ emits little fluorescence, while on the other hand the complex $P_{688-678}$ formed by warming the intermediates emits fluorescence at 688 nm.

The absorption and fluorescence properties of P_{-695} and $P_{-700,\,750}$ suggest that these intermediates contain pigment aggregates of various sizes [12,13]. Their main red absorption bands at 695 and 680 are reminiscent of those of the active centres P_{700} and P_{680} of photosynthesis in green plants. Their role as a trap for the energy absorbed by neighbouring pigment-lipoprotein complexes also likens them to these centres. On the other hand, recent data to be published elsewhere [14,15] demonstrate that some complexes appearing at the first illumination of etiolated leaves, are endowed with photobiochemical activity, while a theoretical study of the kinetics of the photoreduction of protochlorophyll(ide) in which we are currently engaged leads to the conclusion that during the photoreduction of protochlorophyllide transitory intermediate pigment complexes should be capable of producing a reducing power in the light (in course of preparation [16]). In this respect, the weakness of the 77 K fluorescence emission should be stressed because it is at variance to the behaviour attributed to photoactive centres in the green leaf which may emit a fluorescence at 77 K. It should also be stressed that the long wavelength intermediates are likely to be present, although at a low concentration, as long as chlorophyll is formed in a leaf, a process which also occurs in the fully green leaf [17,18].

At all events, reaction (1) is undoubtedly complex. It must be replaced by the following chart.



In this chart, the broken arrow lines indicate possible energy transfers. Transfers 1 and 6 were already known [4,7,8].

It is crucial to notice that long wavelength absorbing chlorophyllproteins (above 690 nm) which were thought until now to appear at the end of greening (after some hours illumination [19]) are produced within a very short time in etiolated leaves in which the pigment concentration is low.

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