

# Energy Transfer from NADPH to Protochlorophyllide in Isolated Protochlorophyllide Holochrome as Determined by UV-Fluorescence Excitation Spectroscopy at 77 K

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The low-temperature fluorescence excitation analysis of different protochlorophyllide (PChlide) forms has been extended to the UV part of the spectrum. A new band at about 360 nm was detected in excitation spectra of active PChlide forms bound to isolated protochlorophyllide holochrome. This band is very similar to the absorbance of NADPH in this region and its intensity depends on the redox state of the surrounding medium. On illumination at low temperature the intensity of the band decreases considerably without any corresponding changes in the redox state of “free” NADPH in the surrounding medium. A new intermediate state exhibiting a mixed excitation spectrum between PChlide and chlorophyllide (Chlide) was detected in the course of PChlide photoconversion.

## Introduction

The NADPH requirement for the conversion of protochlorophyllide (PChlide) to chlorophyllide (Chlide) a phototransformation was shown by Griffiths, but the exact mechanism of the reaction is still not clear [1–3]. In the present study we tried to detect the NADPH absorption band in the fluorescence excitation spectrum of PChlide attached to protochlorophyllide holochrome (PCH). If NADPH donates electrons and hydrogen directly to the pigment it should be located close enough to the PChlide that energy transfer can also take place.

## Materials and Methods

PCH was isolated in complete darkness from leaves of 14-day-old etiolated seedlings of *Phaseolus vulgaris* L. cv. Commodore by the method of Schopfer and Siegelman [4]. All the manipulations were performed under dim green light of nonactinic intensity.

**Abbreviations:** Chlide *a*, chlorophyllide *a*; PChlide, protochlorophyllide *a*; PCH, protochlorophyllide holochrome.

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The stock solution of the crude enzyme preparation was dissolved to the appropriate concentration, subdivided into equal aliquots and kept in darkness at 0 °C until use. NADPH, NADP<sup>+</sup> or solid dithionite were added to sample in the dark. Illumination at room temperature with a polychromatic 1 ms light flash was performed with the samples spreaded on a plexiglass sample holder. Immediately after the flash the holder was dropped into liquid nitrogen. Illumination at low temperature was done with samples on the same holder maintained above the surface of liquid nitrogen in a quartz Dewar vessel. In both cases an uncondensed 1 ms flash was used, or continuous white light of energy  $20 \text{ J} \times \text{m}^{-2}$  and  $100 \text{ W} \times \text{m}^{-2}$ , respectively, in combination with an appropriate glass filter transmitting visible light only.

The low-temperature fluorescence excitation and emission spectra were recorded with the home-made equipment, described elsewhere [5].

## Results and Discussion

The low-temperature fluorescence spectrum of photoactive PChlide in isolated PCH consists of two main emission bands at 646 and 654 nm with a shoulder at about 630 nm (Fig. 1 a). The UV part of the excitation spectrum of the two main photoactive PChlide bands exhibits a broad UV excitation band at about 360 nm (Fig. 2 a, curve 1).



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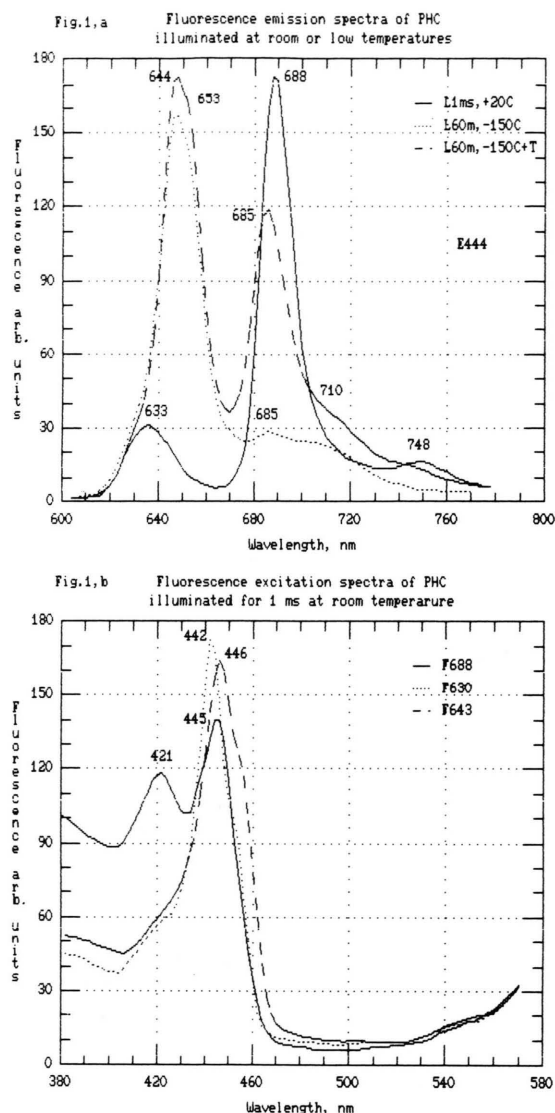


Fig. 1. Fluorescence emission (a) spectra of isolated protochlorophyllide holochrome (PCH), illuminated for 1 ms at 20 °C (.....), or for 60 min at -150 °C without (-----) or with (—) subsequent heating up to -80 °C for 10 min in the dark. Excitation wavelength = 444 nm. Fluorescence excitation (b) spectra of different pigment forms in isolated PCH, illuminated for 1 ms at 20 °C. Detection wavelengths indicated inside the figure.

This band was absent from either the excitation spectrum of the 630 nm fluorescence or the excitation spectrum of isolated PChlide. However the same band is seen in excitation spectrum of NADPH fluorescence measured at 460 nm (Fig. 2a, curve 5).

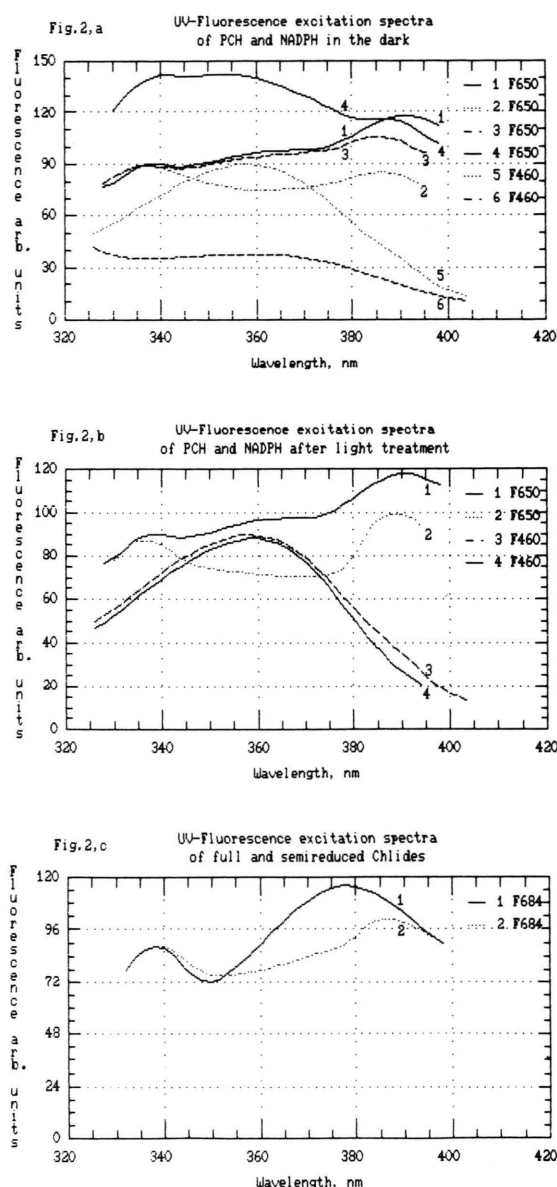


Fig. 2. UV-Fluorescence excitation spectra of isolated protochlorophyllide holochrome (PCH) (a) Dark sample. PChlide fluorescence (detection at 650 nm): a,1 - nontreated, a,2 - after addition of NADP, a,3 - after addition of NADP and sodium dithionite, a,4 - after addition of NADPH. NADPH/NADP fluorescence (detection at 460 nm): a,5 - nontreated, a,6 - after addition of NADP. (b) Sample illuminated for 60 min at -150 °C and then heated up to -80 °C for 10 min in the dark. PChlide fluorescence (detection at 650 nm): b,1 - before illumination, b,2 - after illumination. NADPH/NADP fluorescence (detection at 460 nm): b,3 - before illumination, b,4 - after illumination. (c) Chlide fluorescence of the illuminated sample (detection at 684 nm): c,1 - illumination for 1 ms at 20 °C, c,2 - illumination for 60 min at -150 °C and subsequent heating up to -80 °C for 10 min in the dark.

By adding excess NADP<sup>+</sup> to a solution of isolated PCH in the dark before freezing, the intensity of the 360 nm band in the fluorescence excitation spectra of both PChlide and of NADPH decreased considerably (Fig. 2a, curves 2, 6). Addition of dithionite restored the original spectra (Fig. 2a, curve 3). Addition of NADPH to the initial solution increased the intensity of the 360 nm band in fluorescence excitation spectrum of PChlide (Fig. 2a, curve 4). Thus we conclude, that the 360 nm band in the excitation spectrum of PCH does not belong to the pigment, but to bound NADPH.

Illumination of the sample with a 1 ms flash at room temperature led to a complete transformation of both the 646 and 654 nm emission bands into a new one at 688 nm (Fig. 1a). The excitation spectrum of this emission exhibits rather intense bands at 380 and 420 nm, which are also observed in the absorption spectrum of Chlide *a* (Fig. 1b, 2c, curve 2). The Soret excitation maximum of both pigments is at 445 nm and does not change on illumination. But the intensity of 360 nm band seems to be lower in excitation spectrum for 688 nm fluorescence band.

When a similar sample was illuminated with a 1 ms flash and incubated in the dark for 10 min at room temperature before freezing the main emission band shifted to 675 nm. This new emission band has an excitation spectrum shifted by 5 nm to the blue, which seems to indicate as being due to the "free" Chlide *a* [1, 6].

It has already been shown that illumination of etiolated leaves at low temperature leads to the formation of a nonfluorescent intermediate, which might consist of either a semireduced pigment form or reduced pigment form in a semireduced dimer [7, 8]. Our experiments also show that illumination of frozen dark-adapted isolated PCH at -150 °C leads to about 50% reduction in the original intensity of both main emission bands (Fig. 1a). The Soret part of the excitation spectra with 644 and 653 nm bands, seen after such treatment, was the same as observed before illumination, apart from a pronounced decrease in the in-

tensity of 360 nm band in the UV indicating oxidation of NADPH bonded to PChlide (Fig. 2b, curve 2). On the other hand, the excitation spectrum of free NADPH fluorescence at 460 nm did not change after illumination, indicating no change in the redox state of NADPH in the surrounding medium after PChlide transformation (Fig. 2b, curves 3, 4).

After heating the low-temperature illuminated sample up to about -80 °C in the dark, a new emission band at 685 nm was observed (Fig. 1a). The excitation spectrum of this band in the Soret region was similar to that of Chlide *a*, which could be formed after a 1 ms flash at room temperature, but in its UV part neither a broad 380 nm band (as should be expected for Chlide *a*) nor the broad 360 nm band (belonging to NADPH) was seen (Fig. 2c). Thus we conclude, that the 685 nm fluorescence band belongs to a pigment only partly transformed from PChlide to Chlide *a* [6], but which is not due to a reduced monomeric pigment in a dimer.

Thus, the results presented here show a close association of NADPH and PChlide in a photoactive form of PCH which could be visualized by UV-fluorescence excitation spectroscopy. The pigment photoreduction at low temperature is accompanied by the oxidation of the bound NADPH without any pronounced change of the NADPH redox state in the surrounding medium. The Chlide formation proceeds through an intermediate step with a fluorescence emission maximum at 685 nm, which could be a monomeric intermediate without a second hydrogen in ring IV.

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