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PHOTOTRANSFORMATION OF PHYTOCHROME IN THE DARK

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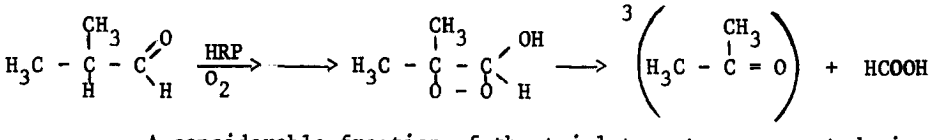
SUMMARY

Enzymatically generated triplet acetone transfers its energy to the ground state phytochrome and promotes to some extent, in the dark, the conversion of P_r into P_{fr} and of P_{fr} into P_r. This is the first report of inverse dark reversion "in vitro".

INTRODUCTION

The enzymatic (peroxidase-oxidase) generation of electronically excited species, in addition to those which occur in bioluminescence, have been firmly established by Cilento et al. (1-3). The system which has been studied in the greatest detail is the oxidation of IBAL by HRP (4-5).



A considerable fraction of the triplet acetone generated is shielded from collisional quenching by O_2. The energy can be transferred from the enzymatically generated species to external acceptors, including natural acceptors such as flavins (6). This finding has opened the possibility of photobiology in the dark (1,7). Photochemical-like transformation of DNA (8,9) and chloropromazine (10) have already been achieved.

Abbreviations: IBAL, isobutyraldehyde; HRP, horseradish peroxidase; P_r, phytochrome in the red absorbing form; P_{fr}, phytochrome in the far-red absorbing form.

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It is well known that the photomorphogenic receptor, phytochrome, exerts its function through the reversible conversion mediated by light (11):

$$P_r \underset{\text{far red light}}{\overset{\text{red light}}{\rightleftharpoons}} P_{fr}$$

Regarding this system, there are some poorly understood or contradictory facts such as the dark reversion (11,12) and inverse dark reversion (13). It was therefore of interest to verify whether phytochrome could be phototransformed in the dark.

In this work, we present evidence that enzymatically generated triplet acetone can promote, in the dark, the conversion of P_r into P_{fr} and of P_{fr} into P_r.

MATERIALS AND METHODS

HRP, type VI was obtained from Sigma. IBAL from Eastman was purified by distillation under nitrogen. Large molecular weight phytochrome from oat seedlings was isolated as described by Pratt (11,14). The results presented here were obtained with a preparation having an absorbance ratio (A_{667}/A_{280}) after far-red irradiation of 0.18 and which was non-fluorescent at room temperature (15); however, essentially identical results were obtained with less pure preparations and small molecular weight phytochrome. The phytochrome concentration was determined spectrophotometrically using $\varepsilon_{667} = 7 \times 10^4$ 1/mol/cm (16).

Absorption spectra were recorded on a Cary 118 Spectrophotometer using 1 cm cells. The chemiluminescence intensity was measured with a Beckman LS-250 Liquid Scintillation Counter. The centrifugation of the reaction mixture was performed at 4°C in a clinical centrifuge.

The experiments were performed at room temperature. The standard reaction mixture consisted of 0.4M phosphate buffer, pH 7.4, 2.0×10^{-6}M HRP, 1.0×10^{-5}M EDTA, 7.0×10^{-2}M IBAL, 0.5% ethanol and 1% glycerol, the last two reagents were employed to solubilize the IBAL. The final volume after addition of phytochrome was 1.3 ml.

In order to demonstrate the characteristic photoreversibility of phytochrome, $P_r \rightleftarrows P_{fr}$, the difference spectra of P_{fr} vs. P_r after irradiation at 660 nm and 720 nm were obtained as described by Jung and Song (17).

The difference spectra without irradiation, using the enzymatic system as the energy donor, were obtained in the following way. The samples were prepared by adding IBAL solution to the mixture of enzyme and phytochrome, while the blanks were prepared by adding phyto-

chrome to the mixture of enzyme and IBAL solutions which had been incubated for 30 minutes. Thus, in the blanks, the phytochrome molecules were subjected to a much lower level of triplet acetone, controlled by diffusion of O_2 into the solution (cf. (5) and Fig. 1). Before taking spectra, the samples and the controls were incubated for ca. 10 minutes in the dark and then centrifuged to remove any turbidity.

The difference spectra of the enzymatic reaction system alone were taken in the same way as described above except that a buffer, which had been used for the preparation of phytochrome solution, was added instead of phytochrome. All experiments were performed under a dim green safe light.

RESULTS AND DISCUSSION

The energy transfer from triplet acetone to phytochrome is demonstrated in Figure 1, showing that the chemiluminescence generated via the triplet acetone is substantially quenched by energy transfer. It is possible that other quenching modes such as reactions between the triplet acetone and phytochrome contribute to the quenching data shown in the figure. However, such chemical quenching is expected to be of minor importance during the initial phase of chemiluminescence, considering the extremely low concentration of the triplet acetone and phytochrome. Thus, energy transfer from triplet acetone to phytochrome accounts for the major fraction of the quenching shown (Fig. 1).

Figures 2 and 3 verify energy transfer from triplet acetone to phytochrome. Curve 1 in Figure 2 shows the difference spectrum of phytochrome (P_{fr} vs. P_r). Curve 2 is the difference spectrum obtained from P_r incubated in the dark with the triplet acetone-generating system vs. the P_r added to the "resting" enzymatic system (cf. Materials and Methods). The absorption band at 727 nm, which is characteristic of P_{fr}, is almost completely bleached by far-red irradiation, and the magnitude of this bleaching is matched by nearly the same magnitude of increase in absorbance at 667 nm characteristic of P_r (Curve 2 → 3). This is consistent with partial conversion of P_r to P_{fr} in the enzymatic system. The same system should also trigger the reversion ($P_{fr} \rightarrow P_r$), since the spectral overlap between the acetone triplet and the phytochrome singlet is favorable. The results indeed confirm this expectation

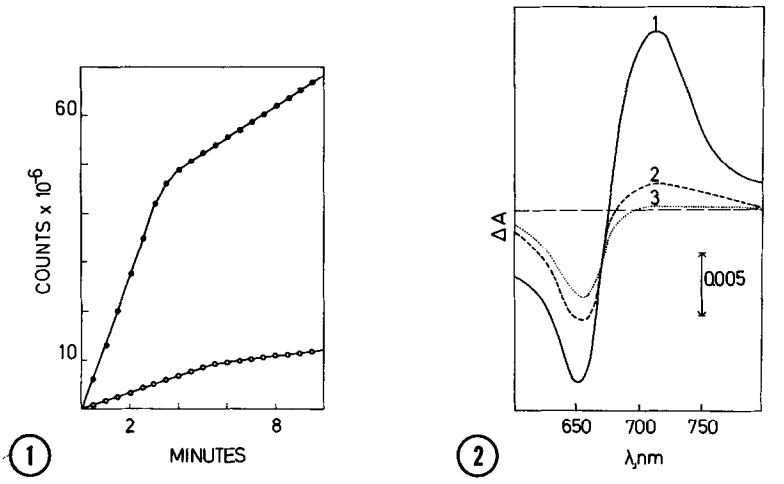
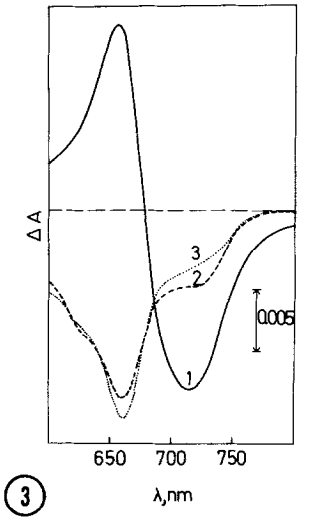


Figure 1 - Integrated emission from the enzymatic system: (•-•-•) alone; (o-o-o) in the presence of 6.4 x 10^{-7}M P_r. The transmittance at 435 nm of the P_r solution is 97%.

Figure 2 - Difference spectra of phytochrome, i.e. P_{fr} versus P_r, obtained by: (1) irradiation; (2) incubating the enzymatic system for 3 min. with 6.4 x 10^{-7}M P_r; eight minutes elapsed after the beginning of the incubation before the spectrum was taken. (3) Three minutes irradiation with far-red light of the sample in (2) while the corresponding blank was kept in the dark.

(Fig. 3), further confirming the reversible "photo"-transformation of phytochrome in the dark.

It should be noted that there is a substantial spectral shape difference between spectra 1 and 2 in Figure 2 and 3; i.e., in both enzymatically promoted conversions, the decrease in the absorbance at 667 nm is not quantitatively compensated by changes at 727 nm. This is partially, but not wholly, due to differences in the HRP absorption between the "resting" (in the blank) and the active state (Fig. 4); therefore, the occurrence of side processes other than energy transfer is confirmed (vide supra). Control experiments showed that they are not related to the reaction between ground state aldehyde and phytochrome (20). Independently of the chemical nature of the side processes, the observed decrease in the absorbance at 667 nm might be due to some



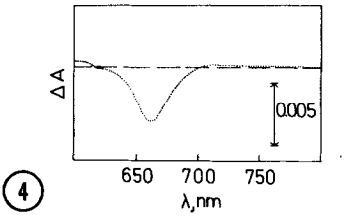


Figure 3 - Difference spectra of phytochrome, i.e. P_r versus P_{fr} obtained by: (1) irradiation; (2) incubating the enzymatic system for 10 min. with 9.4 x 10^{-7}M P_{fr}; twenty minutes elapsed after the beginning of the incubation before the spectrum was taken; (3) three minutes irradiation with red light of the sample in (2) while the blank was kept in the dark.

Figure 4 - Difference spectrum of the enzymatic reaction mixture in the presence of phytochrome obtained 15 min. after IBAL addition to the sample.

denaturation and precipitation of phytochrome. However, in the supernatant protein, whether in the P_r of P_{fr} form, the photoreversibility ratio ($\Delta A_{667}/\Delta A_{727}$) remains essentially unity, further confirming the photoreversible transformation of phytochrome in the dark by the enzymatic system.

Although it is premature to discuss details of the energy transfer process, it may nevertheless be inferred that a long range triplet-singlet process must be operative. The efficiency with which phytochrome quenches the chemiphosphorescence of triplet acetone (Fig.1) excludes a trivial energy transfer process and indicates that the rate constant of the energy transfer is on the order of $10^{12}M^{-1}s^{-1}$.

Triplet-singlet energy transfer, though spin forbidden, can indeed occur efficiently even at long distances provided that the donor

has a long lifetime (18) and the acceptor absorbs in the region of the donor phosphorescence (18,19). These conditions are satisfied in the present investigation since the emission spectrum of the long-lived acetone (λ_{max} = 435 nm; $\tau \sim 10^{-5}$ s) overlaps the absorption spectra of P_r and of P_{fr} in the blue region (λ_{max} = 380 and 400 nm respectively).

In conclusion, our results indicate that phytochrome may also suffer phototransformation in the dark. Although the "in vitro" dark reversion has been well studied, the inverse dark reversion has never been reported "in vitro". Although these reversions are far from understood (11,13,21), the possibility exists that they are dark photochemical processes catalyzed by peroxidatic enzymes, which may account for the apparent $P_r \rightarrow P_{fr}$ dark transformation "in vivo" (22,23).

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