Catalysis of the Phytochrome Dark Reaction by Reducing Agents*

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ABSTRACT: The promotion of $P_{FR} \rightarrow P_R$ reversion in darkness by reducing agents such as sodium dithionite, NADH, $FADH_2$, and reduced ferredoxin is reported. Reduced ferredoxin is a particularly potent promoter, causing an 800-fold increase in the reaction rate. In the presence of reduced ferredoxin the $P_{FR} \rightarrow P_R$ dark reaction is so fast that only partial photoconversion of P_R into P_{FR} by red light could be achieved. NADH and reduced ferredoxin appear to be true catalysts of the dark reaction since no net oxidation of reducing agent was seen during the $P_{FR} \rightarrow P_R$ transformation.

Phytochrome is believed to be the photoreceptor which mediates many of the red–far-red responses in plant growth and development (Borthwick *et al.*, 1952a,b; Hendricks *et al.*, 1956; Butler *et al.*, 1959; Hendricks and Borthwick, 1959; Siegelman and Butler, 1965; Hillman, 1967). Under red (~660 nm) irradiation phytochrome exists predominantly in a form, P_{FR}, which has an absorption maximum at 724 nm; under far-red (~725 nm) light in a second form, P_R, which absorbs at 664 nm (Mumford and Jenner, 1966). There is also evidence that P_{FR} spontaneously reverts to P_R in darkness.

$$P_R \underset{\text{far-red light or darkness}}{\overset{\text{red light}}{\rightleftharpoons}} P_{FR}$$

In previous *in vitro* studies (Mumford, 1966; Anderson *et al.*, 1969) we found that this dark reaction is first order in phytochrome and independent of pH from 6.9 to 8.6. However, as the pH is lowered below 6, the rate of the $P_{FR} \rightarrow P_R$ reversion is accelerated and there is a linear relationship between pH and the logarithm of the observed reaction rate. The reversion at these relatively low pH's apparently involves a protonated form of phytochrome, which we call FRH. This paper describes further work we have done which shows that the phytochrome dark reaction is catalyzed by a number of reducing agents including sodium dithionite, $FADH_2$, and reduced ferredoxin.

Experimental Section

Phytochrome Preparation. The phytochrome used in this work was extracted from *Avena sativa* L. cv Garry (oat) seedlings and purified by a published procedure (Mumford and Jenner, 1966). It had a specific activity of 200–300 units/g of protein.

Catalysis Studies. Dark reaction measurements (Table I) were made essentially as has been described in detail previously (Mumford, 1966). Unless otherwise specified in the text, the reactions were run at 10° in 0.025 M phosphate buffer (pH 7.8) in 10-cm path-length cells. The phytochrome concentrations ranged from 0.5×10^{-6} to 1×10^{-6} M.

In the work with ferredoxin all solutions were prepared and the spectrophotometer cells loaded in a glove box which was evacuated and filled with nitrogen five times before transfers were made. Boiled water was used for preparation of the solutions. Phytochrome and ferredoxin were present in these experiments at 1×10^{-6} M and approximately 10 moles of dithionite was added for each mole of ferredoxin. In spite of the precautions to remove all oxygen, oxidizing species were still present which rapidly consumed dithionite and made this amount of dithionite necessary for complete ferredoxin reduction. The ferredoxin was obtained from *Clostridium pasteurianum* (Lovenberg *et al.*, 1963) and in the oxidized state had an A_{390}/A_{280} of 0.65.

Catalysis with Tritiated NADH. Nicotinamide–adenine dinucleotide labeled with tritium in the 4 position of the pyridine ring (specific activity 29 mCi/mmole) was obtained from Amersham-Searle Co., Des Plaines, Ill. This was reduced to NADH by the published procedure (Beisenherz *et al.*, 1953) as follows.

The nicotinamide-4-T(N)–adenine dinucleotide sample, 0.7 mg (1.1 μmoles or 0.03 mCi), was dissolved in 10 ml of water containing 10 mg of sodium bicarbonate; the solution was treated with 5 mg of sodium dithionite and heated for 90 sec in a boiling-water bath. The solution was then cooled in an ice bath, 0.3 ml of a 1% sodium carbonate solution added, and excess sodium dithionite oxidized by ebullition with air.

Phytochrome, 0.14 μmole, and 0.077 μmole (0.7 ml of the above solution) of tritiated NADH were diluted with buffer to a total volume of 5 ml. The solution was sealed, irradiated with red light (Mumford, 1966) for 6 min, and allowed to stand in darkness at 10° for 17 hr. Phytochrome was then recovered from the incubation mixture by addition of 5 ml of 0.1 M sodium pyrophosphate solution saturated with ammonium sulfate followed by collection of the precipitate by centrifugation at 30,000*g* for 10 min. The pellet obtained was washed with 5 ml of 0.1 M sodium pyrophosphate solution saturated with ammonium sulfate, then redissolved in 5 ml of buffer and reprecipitated as before with ammonium sulfate. The final pellet was dissolved in 5 ml of buffer. Samples (0.1 ml) of the various fractions were counted in Bray's (1960) solution. The counts found for each solution are recorded in Table II.

Results and Discussion

The effect of several reducing agents on the rate of the $P_{FR} \rightarrow P_R$ reversion in darkness is shown in Table I. NADH by itself was not a particularly effective promoter, causing only a 3–4-fold increase in rate. However, if the electron-

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TABLE I: Effect of Reducing Agents on Rate of Phytochrome Dark Reaction.

Reducing Agent	Dark Period, T (sec)	% FR at Time, T	k_{obsd} (sec^{-1})
None	71,700	87.3	1.9×10^{-6}
5×10^{-3} M NADH	58,680	69.4	6.2×10^{-6}
	75,720	48.8	9.5×10^{-6}
1×10^{-3} M FAD	82,560	82.3	2.3×10^{-6}
5×10^{-3} M NADH	2,760	71.4	122.0×10^{-6}
$+ 1 \times 10^{-3}$ M FAD	4,260	63.2	107.0×10^{-6}
2.5×10^{-3} M sodium dithionite	900	64.2	491.0×10^{-6}
	1,800	39.5	513.0×10^{-6}
5×10^{-3} M sodium dithionite	900	49.2	786.0×10^{-6}
	1,920	21.0	812.0×10^{-6}
1×10^{-6} M ferredoxin	64,740	86.2	2.3×10^{-6}
1×10^{-6} M reduced ferredoxin	600	41.4	1470.0×10^{-6}

TABLE II: Distribution of Counts in Tritiated NADH Phytochrome Reaction.

Fraction Counted	Vol (ml)	cpm of 0.1-ml Sample	cpm Total
Reaction mixture	5	16,183	809,115
Supernatant from first $(NH_4)_2SO_4$ precipitate	10	7,205	720,500
Ammonium sulfate wash	5	502	25,100
Supernatant from second $(NH_4)_2SO_4$ precipitate	10	137	13,700
Final phytochrome solution	5	136	6,800

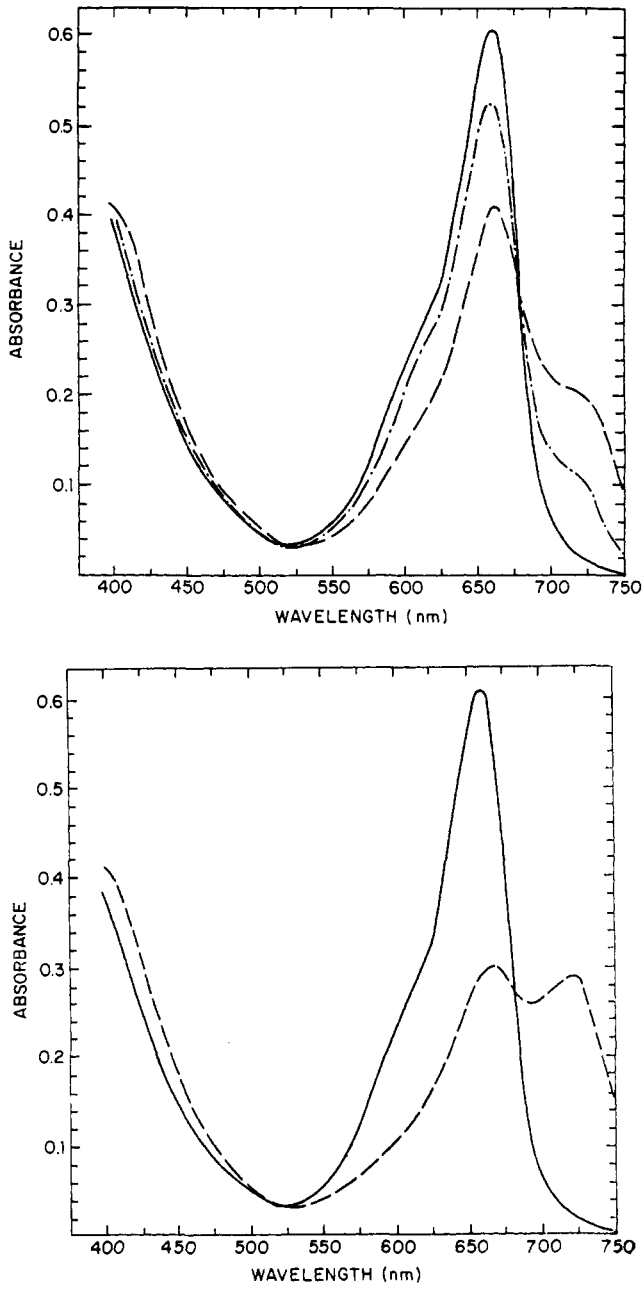


FIGURE 1: (A, upper) Effect of reduced ferredoxin on phytochrome photoreversibility and dark reaction. Spectra were taken with solutions 10^{-6} M in both phytochrome and reduced ferredoxin in a 10-cm path-length cell. (---) Spectrum after a saturating dose of red light ("P_{FR}"). (●–●) Spectrum of solution after "P_{FR}" stood 10 min in the dark at 10°. (——) Spectrum after a saturating dose of far-red light (P_R). (B, lower) Spectra of same solution 18 hr later after oxidation of reduced ferredoxin had taken place. (– – –) Spectrum after a saturating dose of red light (P_{FR}). (—) Spectrum after a saturating dose of far-red light (P_R).

carrier FAD was added along with the NADH, the dark reaction was stimulated 50-fold. The enhancement of NADH activity by FAD is not surprising in view of the fact that catalysis of electron transfer from reduced pyridine nucleotides by flavins is well known in many systems. Sodium dithionite produced up to a 400-fold increase in the reaction rate, but by far the best catalyst studied was reduced ferredoxin. Here nearly an 800-fold increase in dark reaction rate was obtained. In the presence of reduced ferredoxin the conversion to P_R was so rapid that only partial photoconversion of P_R to P_{FR} by red light could be achieved. This is clearly shown by comparing the spectra of P_R and "P_{FR}" in Figure 1A,B. In the presence of reduced ferredoxin (Figure 1A), the A_{663nm} of P_R minus that of P_{FR} was 0.196 while in subsequent spectra taken after the ferredoxin autooxidized (Figure 1B), it was 0.318.

The promotion of the phytochrome dark reaction by these reducing agents does appear to be a true catalysis in that several lines of evidence indicate no net oxidation of the reducing agent during the $P_{FR} \rightarrow P_R$ transformation. Thus if oxidation of reduced ferredoxin takes place during the reversion, one should observe no change in the absorbance at 425 nm. This is so because, coincidentally, the molar change in absorbancy (oxidized minus reduced) for ferredoxin at 425 nm, 8.2×10^3 (Tagawa and Arnon, 1962, but assuming mol wt 5500), is the same but in the opposite direction as the molar change in absorbancy for phytochrome ($P_{FR} - P_R$) at this wavelength. The observed change in absorbancy at 425 nm for the dark reaction in Figure 1A, 0.02, agrees closely

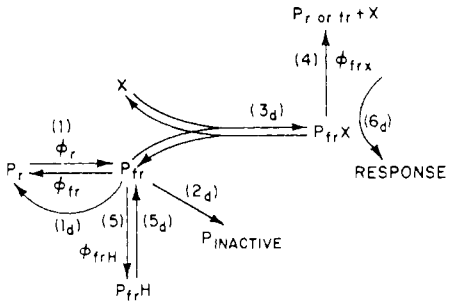


FIGURE 2: Scheme proposed by Borthwick *et al.* (1969) to explain high-energy responses in plants.

with the calculated value of 0.021 and argues against a net oxidation of ferredoxin.

Similarly, in studies on promotion of the dark reaction by NADH, spectral measurements gave no indication of oxidation of the NADH. For example, when a solution 10^{-5} M in both P_{FR} and NADH was incubated for 17 hr in the dark at 9°, 49% reversion to P_R occurred. However, the absorbance at 330 nm (the isosbestic point for P_R and P_{FR} and near the NADH maximum at 340 nm) remained constant. If oxidation of the NADH had taken place a drop in absorbance at this wavelength would be expected. To confirm that the NADH had not been oxidized during the reversion, the phytochrome was precipitated by ammonium sulfate, and the supernatant containing NADH (and any NAD) was treated with the reducing agent, form amidinesulfinic acid (Shashoua, 1964). No increase in the 340-nm maximum indicative of NAD reduction was found.

Experiments in which phytochrome was incubated with tritiated NADH also failed to produce evidence that a net reduction was taking place. Thus when a 2:1 molar ratio of phytochrome and tritiated NADH was incubated for 17 hr in the dark, less than 1% of the tritium present in the NADH appeared in the phytochrome fraction (Table II).

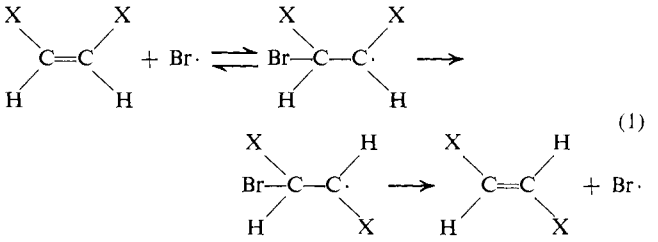
It might be expected, too, that if P_R is generated from P_{FR} by reduction, the reverse reaction, $P_R \rightarrow P_{FR}$, would be promoted by oxidizing agents. However, incubation of a 1×10^{-6} M solution of P_R with a 10-fold excess of potassium ferricyanide did not lead to P_{FR} formation. A 100-fold excess of potassium ferricyanide, although partially destroying photoreversibility, also did not yield a spectrophotometrically detectable concentration of P_{FR}.

Although net oxidation–reduction does not appear to occur, it does seem likely that a reduced species is formed as a transitory intermediate. For example, the addition of an electron to the conjugated system of the tetrapyrrole chromophore would give an intermediate radical ion which might well isomerize with ease. This process would be analogous

$$P_{FR} + e^- \rightleftharpoons \cdot P_{FR}^- \xrightarrow{\text{isomerization}} [\cdot P_R^-] \longrightarrow P_R + e^-$$

to the halogen-catalyzed cis–trans isomerization of olefins (Walling, 1957). In some respects such a mechanism would also resemble the enzymatic isomerization of UDP-galactose to UDP-glucose which requires NAD as a cofactor (Maxwell, 1961).

The physiological significance of these findings is unclear. It has been reported previously that redox chemicals can substitute for radiation in phytochrome-mediated photomorphogenesis (Klein and Edsall, 1966). However, in their



(1)

work, which used the bean leaf disk as an assay, reducing agents interacted synergistically with red light. The opposite, that is synergism of reducing agents with far-red light, would have been expected on the basis of the present *in vitro* investigation.

Obviously the rate at which P_{FR} reverts to P_R in darkness *in vivo* will be highly dependent on the reducing potential of the surrounding medium. After periods of high light intensity, for example, when the reducing capacity of the cell is high, it would be anticipated that the rate of the P_{FR} reversion would be considerably higher than after an extended period of darkness. It is also apparent that use of difference spectra to estimate phytochrome concentration may be unreliable in some situations *in vivo*. As is shown in Figure 1A,B the same phytochrome concentration will yield appreciably different $\Delta(\Delta A)$ values depending upon the reducing potential present.

Although the proposed phytochrome radical ion apparently does not undergo further reduction in the *in vitro* $FADH_2$ and reduced ferredoxin systems studied here, it is possible that such reductions do occur *in vivo*. Support for this possibility comes from the experiments with sodium dithionite. The rapid sodium dithionite catalyzed dark reactions are accompanied by slow losses in photoreversibility which are paralleled by the appearance of a new absorption maximum in the 580-nm region. It seems likely that this absorbance is that of the reduced chromophore. In D_2O the new 580-nm band grows in at a rate less than one-tenth the rate in H_2O. This large isotope effect suggests that in the sodium dithionite catalyzed process, the phytochrome radical ion (or other intermediate) can complete its reduction by abstraction of a hydrogen atom in a slow, rate-determining step.

The findings reported here may also bear on the high-energy response in plants (Mohr, 1962), and the scheme recently advanced (Borthwick *et al.*, 1969) to explain the high-energy phenomenon (Figure 2). On the basis of work done in etiolated tissue, where there is little chloroplast development, it has been assumed that extended red irradiation will lead *in vivo* to relatively high P_{FR}:P ratios. However, the present results point up the possibility that in tissue containing efficient chloroplasts (green tissue) extended red irradiation may actually lead to a lowered P_{FR}:P ratio. This would be so in the event that the strong reducing potential generated by the high-energy irradiation promoted the dark reaction, 1d in Figure 2, to a greater extent than ϕ_r photoconverts P_R back to P_{FR} in reaction 1, Figure 2. One can visualize situations *in vivo*, then, where doses of red light adequate to drive P_R to P_{FR}, but insufficient to produce electron flow in the chloroplast system, might yield concentrations of P_{FR} sufficient to induce a physiological response; for the reasons just mentioned, though, such concentrations of P_{FR} would be unattainable at higher irradiances. One can also speculate that the $P_{FR}X$ formed in dark reaction 3d, Figure 2, may be the postulated phytochrome radical ion.

On the basis of the action spectrum for the stem lengthening accompanying flowering of *Beta vulgaris*, it has been pre-

dicted that, when compared to P_{FR}, $P_{FR}X$ should have very low absorbancy in the 600–680-nm region but a high absorbancy at 720 nm (Borthwick *et al.*, 1969). However, *in vitro* spectral measurements that might help establish the equivalence of $P_{FR}X$ and the phytochrome radical ion have not been made as yet.

Another reaction that may tend to lower P_{FR}:P ratios in the light is reaction 5 in Figure 2 which leads to the protonated form of P_{FR}, designated $P_{FR}H$. Several physiological functions of $P_{FR}H$ have been alluded to previously (Anderson *et al.*, 1969; Borthwick *et al.*, 1969), but $P_{FR}H$ may also help explain the phytochrome paradoxes (Hillman, 1967). Thus $P_{FR}H$ has properties which would enable it to serve as a nonphotosensitive storage form of P_{FR} from which spectrophotometrically detectable and physiologically active P_{FR} could be generated in the dark.

Although ferredoxin is best known for its role in electron transport, recent work suggests that it may also serve a regulatory function. Thus it has been reported that reduced ferredoxin can activate fructose 1,6-diphosphatase, thereby providing a light-dependent mechanism for control of carbohydrate formation during photosynthesis (Arnon, 1969). Control of the $P_{FR} \leftrightarrow P_R$ equilibrium may be a second example of regulation of enzymatic activity by this nonheme iron protein.

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