PHOTOOXIDATION OF CYTOCHROMES BY A FLAVOPROTEIN FROM EUGLENA

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We report here a photooxidation of Euglena cytochrome 552 and mammalian cytochrome c by a flavoprotein from Euglena. The oxidized cytochromes are reduced in the dark by an enzyme utilizing TPNH. The Euglena extracts were prepared from light-grown cells (Lyman, et al., 1961), resuspended in 1.0M. phosphate buffer pH 7.0, subjected to freezing overnight followed by thawing at room temperature and centrifugation at 1,000 g. for 10 min. to remove whole cells. The supernatant was centrifuged at 86,000 g. for ½ hr. and was then dialyzed overnight at 4°C. against 0.01M. phosphate buffer, pH 7.0. Absorbancy changes were followed in a Cary model 14 recording spectrophotometer at 552 mu. or 550 mu. for cytochrome 552 and mammalian cytochrome c respectively. For excitation the reaction mixture in a 3 ml. quartz cuvette was exposed to a CH-3 high pressure mercury arc filtered by a combination of a pyrex filter and a Corning #5030 broad-band blue filter, at a distance of 20 cm.

Fig. 1 shows a typical experiment, with a complete reaction mixture lacking TPNH. A brief exposure to light caused an oxidation of the cytochrome 552. Re-reduction of the cytochrome in the dark occurred only when TPNH was added. If the light reaction is cycled more than the few times shown in the figure, the TPNH is exhausted and more must be added. DPNH shows less than one-tenth the activity of TPNH.

In these early experiments the extracts contained enough cytochrome 552 to carry out the reaction. We have since separated the cytochrome 552 from the other enzymatic components of the extract by ammonium sulfate fractionation followed by DEAE cellulose column separation, the details to be described in a later publication.

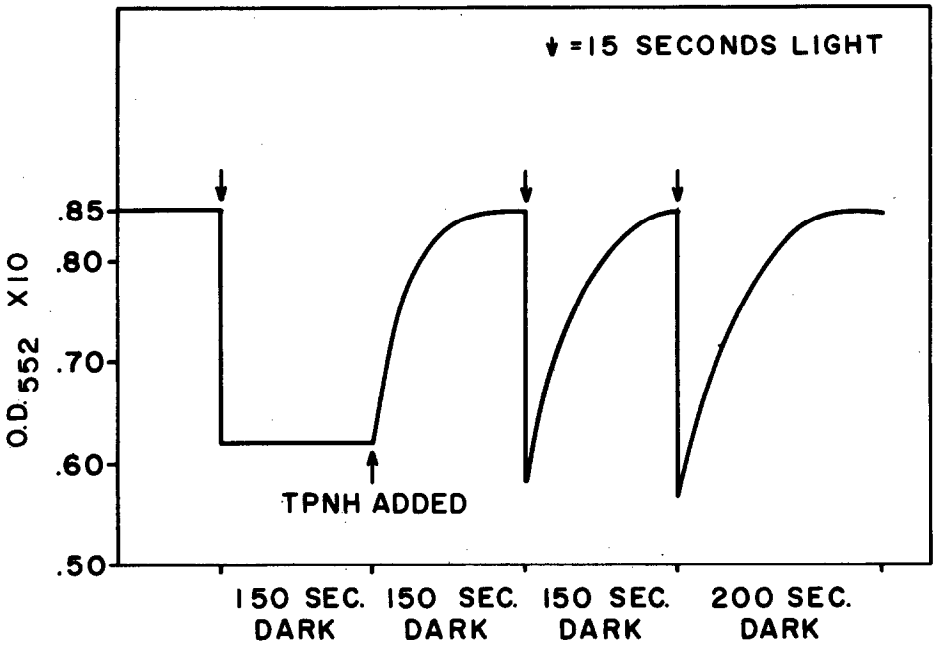

Fig. 1 The complete system contained 2.5 ml. dialyzed extract (containing endogenous reduced cytochrome 552), and 0.03 uM FAD. 0.1 uM TPNH was added later as indicated.

TABLE 1

Photooxidation of Cytochrome 552

Reaction mixture	% Photooxidation	% Dark Reduction
1. ±FMN, ±TPNH, ±Boiled extract	0	0
2. FMN, nonboiled extract	50	0
3. FMN, nonboiled extract, TPNH	50	50
4. FMN, nonboiled extract, anaerobic	0	-

Fractionated extracts lacked cytochrome 552. Cytochrome 552, 0.02 uM added throughout; FMN, 0.03 uM; TPNH, 0.1 uM where indicated.

The experiments summarized in table 1 establish the minimum requirements for the light reaction. These are: 1) Enzyme extract (boiled extract is

completely inactive), 2) Reduced Cytochrome 552 or reduced mammalian cytochrome c, 3) FMN or FAD (riboflavin is about one-third as active), and 4) Oxygen. The reduction of cytochrome in the dark requires the addition of TPNH.

To establish the identity of the photocatalyst, the complete reaction mixture lacking TPNH was irradiated with equal numbers of quanta in the following regions of the spectrum isolated by broad-band Wratten filters: 400-500 mu.; 500-600 mu.; and 600-700 mu. Blue light (400-500 mu.) alone was effective. These results are in complete agreement with our observation that chlorophyll and carotenoids are completely absent from the extract and with the interpretation that the reduced cytochrome is not the photocatalyst. A more detailed action spectrum showed peaks of effectiveness in the regions of 448 mu. and 367 mu. indicating that the photocatalyst is an oxidized flavin. Since boiled extract was inactive and since either FMN or FAD must be added, we conclude that a flavoprotein is the most likely chromophore.

An indication that the extract contains different enzymes or sites for the photooxidation of the cytochrome and for its dark reduction comes from the following experiment. The extract plus TPNH reduced oxidized cytochrome c in the dark in the absence of added flavin. Subsequent light excitation failed to photooxidize the reduced cytochrome. This indicates that added FMN or FAD is required specifically for the photooxidation and is not required for the dark reduction.

Our findings are summarized in the hypothetical reaction scheme shown in figure 2.

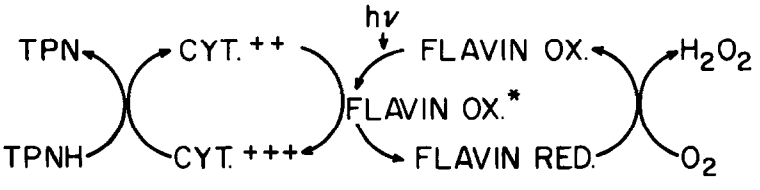

Fig. 2 Hypothetical Reaction Scheme

The potentials for Euglena cytochrome 552 and for mammalian cytochrome c are +0.36 v. (Nishimura, 1959) and +0.26 v. respectively (Lardy, 1949) so that their reduction

by TPNH at -0.32 v. is thermodynamically feasible. There remains the question of the photooxidation of the cytochrome. Since the potentials for many flavoproteins are approximately 0.00 v., these, in the ground state, could not oxidize the cytochromes in question. An einstein of blue light, however, is equivalent to approximately 60 k.cals. or approximately 2.7 electron volts. The excited state of the flavoprotein, therefore, could have a sufficiently positive potential to oxidize the cytochrome with much to spare. We have already mentioned above that the action spectrum implicates oxidized flavoprotein as the photocatalyst. Since oxygen is required we have tentatively placed it terminally in the formation of hydrogen peroxide during the reoxidation of the reduced flavin by analogy to other systems. There is reason to believe that the photooxidation described here is of physiological significance. Mehler (1951a, 1951b) and other workers have described a reaction in which O_2 is consumed by illuminated chloroplasts after exhaustion of added Hill reagent. This O_2 consumption is stimulated by the addition of FMN or FAD, among other compounds, and H_2O_2 is formed during this process. It is possible that we have demonstrated the Mehler reaction in the absence of other chloroplast materials not essential to the reaction.

A similar photooxidation of Porphyra tenera cytochrome 553 has been reported in cell-free extracts (Katoh, 1959). The photooxidase activity was found only in the particulate fractions of the cell-free extracts. Katoh states that chlorophyll and/or carotenoids remaining in the plastids are involved in carrying out the photooxidation.

It will be necessary to perform action spectra for the Mehler reaction and the photooxidation described by Katoh before stating that our reaction is the same as theirs.

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