Photoreduction of NADP⁺ Sensitized by Synthetic Pigment Systems

(acridine/proflavine/ferredoxin-NADP reductase/ethylene diamine tetraacetic acid)

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ABSTRACT Two synthetic pigment systems capable of enzymatically photoreducing NADP⁺ are described. One system contains proflavine; the other, acridine. The complete system consists of ethylene diamine tetraacetic acid (EDTA), proflavine (or acridine), ferredoxin-NADP reductase (EC 1.6.99.4), and NADP⁺. The two pigments initiate the photoreduction of NADP⁺ in different portions of the electromagnetic spectrum. Proflavine photosensitizes in the visible portion; acridine, in the ultraviolet. Neither proflavine nor acridine is structurally related to chlorophyll. The acridine system has the attractive property that the enzyme, ferredoxin-NADP reductase, is the only component of the system that absorbs appreciably in the visible region of the spectrum.

PHOTOREDUCTION OF NADP⁺

The conversion of electromagnetic energy into chemically stored energy is the most important function of photosynthesis. One compound in which the free energy appears is the reduced form of nicotinamide adenine dinucleotide phosphate (NADPH). As shown schematically in Fig. 1, the electromagnetic energy (ℏw) is transformed into chemical energy by conversion of the "low-energy" form, NADP⁺, to the "high-energy" form, NADPH. The transformation involves the transport of an H⁺ from an electron donor via an electron transport system to NADP⁺. The "green box" must have three functions—it must capture the light, it must donate two electrons, and it must transport these two electrons, with a proton, to NADP⁺. In plants, NADP⁺ is photoreduced by photosystems I and II. These photosystems include light receptors and a complex electron transport chain consisting in part of an iron-sulfur protein, ferredoxin, and a flavoprotein, ferredoxin-NADP reductase (Fd-NADP reductase, EC 1.6.99.4). While the main aspects of photosynthesis are well known (1), some features of the electron transport chain are not yet fully understood; for example, there is no general agreement on the identity of the primary electron acceptors of photosystems I and II. Studies on model systems may help elucidate such features. Indeed, several investigations indicate that NADP⁺ can be photoreduced in vitro (see Table 1). Krasnovsky and Brin (2, 3) have demonstrated that chlorophylls dissolved in pyridine catalyze a photoreduction of NADP⁺ with ascorbate as the electron donor. Photoreduction of NADP⁺ in aqueous solution was shown by Vernon, San Pietro, and Limbach (4) with the enzyme Fd-NADP reductase transferring electrons to NADP⁺ from ascorbate; chlorophyll a, a water-soluble derivative of chlorophyll a, severed as the photosensitizing pigment. The earlier work on photoreduction of pyridine nucleotides used porphyrins and porphyrin derivatives, all of which are structurally related to chlorophyll (5).

In the present paper, we will describe two new pigment systems that can replace the chlorophyll or chlorophyll a listed in the first two reactions in Table 1. One system contains proflavine; the other, acridine. We will show that proflavine and acridine will serve as photosensitizing pigments for the photoreduction of NADP⁺. Proflavine photosensitizes in the visible region; acridine, in the ultraviolet. The range of wavelengths in the solar spectrum reaching the earth extends from about 310 to 2300 nm. An energy conversion system that uses ultraviolet radiation would, from a theoretical point of view, have potential for storing larger amounts of energy per quantum with the same number of steps than a system that converts visible radiation. This fact stems from Planck's law, E = ℏw, where ℏ is Planck's constant divided by 2π, and w is the angular frequency of the radiation. Radiation of wavelengths shorter than about 300 nm would be harmful to living organisms, which depend on proteins and nucleic acid for their continued existence.

PROFLAVINE PIGMENT SYSTEM

During our studies on hydroxylation mechanisms (6), we discovered that the methylene hydroxylase reaction of Pseudomonas putida (7) could be initiated photochemically with proflavine as a photosensitizing pigment. In the P. putida system, hydroxylation of camphor involves an electron transport chain consisting of cytochrome P₄₅₀, an iron-sulfur protein (putidaredoxin), and a flavoprotein (NADH dehydrogenase), and the reaction is initiated by NADH. NADH and NADH dehydrogenase can be replaced with an artificial electron donor consisting of ethylene diamine tetraacetic acid (EDTA),

<table>
<thead>
<tr>
<th>Solvent</th>
<th>Electron donor</th>
<th>Photosensitizer</th>
<th>Enzyme</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pyridine</td>
<td>Ascorbic acid</td>
<td>Chlorophyll</td>
<td>Fd-NADP reductase</td>
<td>2</td>
</tr>
<tr>
<td>Water</td>
<td>Ascorbate</td>
<td>Chlorophyll a</td>
<td>Fd-NADP reductase</td>
<td>4</td>
</tr>
<tr>
<td>Water</td>
<td>EDTA</td>
<td>Proflavine</td>
<td>Fd-NADP reductase</td>
<td>This work</td>
</tr>
<tr>
<td>Water</td>
<td>EDTA</td>
<td>Acridine</td>
<td>Fd-NADP reductase</td>
<td>This work</td>
</tr>
</tbody>
</table>

Abbreviation: Fd-NADP reductase, ferredoxin-NADP reductase.
proflavine, and methyl viologen. In this system a flash of light rapidly reduces methyl viologen, which then reduces the putidaredoxin; the iron-sulfur protein in turn reduces the cytochrome P450cam. It is clear that the photoreducing system consisting of EDTA and proflavine has sufficient potential to run the electron transport chain of the P450 system in either direction, i.e., oxidation or photoreduction of NAD+. A reaction similar to the photoreduction of NAD+ occurs in photosynthesis where NADP+ is converted to NADPH. It is this reaction that we chose to study with the hope of obtaining additional information concerning the role of flavoproteins in mediating the transport of electrons from two-electron donors (NADH) to one-electron acceptors (the iron-sulfur redox protein, putidaredoxin).

NADP+ can be photoreduced in aqueous solutions with EDTA serving as the electron donor and proflavine as the photosensitizing pigment. The flavoprotein, Fd-NADP reductase, is a necessary component. Presumably, this reduction is a Krasnovsky-type reaction in which the activated proflavine couples with the enzyme, Fd-NADP reductase, to transfer electrons from EDTA to NADP+. The fate of the oxidized EDTA molecule is not precisely known. The important fact, however, is that it no longer participates in the reaction. Proflavine (3,6-diaminoacridine) is a highly colored acridine dye. Its absorption spectrum consists principally of a broad, single peak with its maximum centered at $\lambda = 445$ nm (see Fig. 2, spectrum a). At this wavelength its extinction coefficient has a value of 35 mm$^{-1}$cm$^{-1}$. The structural formula of proflavine is indicated in Fig. 2, near spectrum a. The acridine dyes are relatively inert compounds and are noted for their resistance to reduction in the dark (8). This inertness is similar to that of chlorophyll, where the porphyrin-like structure contributes to the rigidity and stability of the molecule. In fact, porphyrins are among the most inert and stable molecules in organic chemistry. However, both chlorophyll and acridine dyes can become highly reactive when illuminated with light. Acridine dyes, under certain conditions, undergo photoreduction (9).

EDTA does not have sufficient reducing power to reduce proflavine in the dark. However, proflavine can be photo-reduced by EDTA. Presumably, the proflavine molecule in its excited state has the ability to abstract an electron from EDTA. If there are no other solute species in the aqueous solution, the proflavine molecule undergoes an irreversible bleaching. If, however, there are molecules that can accept electrons from proflavine, these will be reduced and most of the proflavine will return to its original oxidized form. In the present study the acceptor molecule is the FAD flavoprotein, Fd-NADP reductase. The experiment proceeded as follows: an 8-ml sample consisting of 500 mM EDTA, 50 $\mu$M proflavine, 0.15 $\mu$M Fd-NADP reductase, and 300 $\mu$M NADP+ was prepared in a solution of 50 mM Tris⋅HCl buffer adjusted to a pH of 7.4. The 8-ml sample was divided into two equal parts and placed into two matched, anaerobic cuvettes of 1-cm path length. A difference spectrum of the two identical samples was taken to establish the base line. One cuvette was then removed and exposed to visible radiation, while the control cuvette was maintained in darkness. The irradiated cuvette was exposed to successive periods of illumination. At the end of each period the molecular absorption of the irradiated mixture was recorded as a difference spectrum against the control cuvette on a Carey 14 spectrophotometer. NADPH is characterized by an absorption peak in the near ultraviolet at $\lambda = 339$ nm. At this wavelength the extinction coefficient is 6.22 mm$^{-1}$cm$^{-1}$. The absorption of NADP+ at $\lambda = 339$ nm is negligible compared to that of NADPH. In addition, the presence of NADPH can be determined chemically with the aid of 2,6-dichlorophenolindophenol. Fd-NADP reductase catalyzes the oxidation of NADPH with 2,6-dichlorophenolindophenol as electron acceptor. Upon addition of 2,6-dichlorophenolindophenol to the reaction cuvette, there is a decay in the peak at $\lambda = 339$ nm, corresponding to the oxidation of NADPH. The formation of NADPH as a function of time of irradiation is illustrated in Fig. 3. The reaction mixture of Fig. 3 is summarized in Table 2. The data of Fig. 3 show that there is a monotonic increase in the yield of product as a function of illumination. If any one of the components (EDTA, Fd-NADP reductase, or NADP+) is omitted from the system, NADP+ is not photoreduced.

The yield of reduced pyridine nucleotide as a function of proflavine concentration is shown in Fig. 4. The role of proflavine in this reaction is that of "photosensitizer." Presumably, this means that it traps some of the light passing through the solution in such a way that NADP+ may be photoreduced, resulting in an increase of free-energy of $32.7$ kcal/mol (2.3 eV/molecule), if O$_2$ is assumed to be the electron acceptor upon oxidation of NADPH (10). Fig. 4 shows an increase of photoreduced pyridine nucleotide with proflavine concentration for a given period of illumination. The yield of product appears to reach an asymptotic value. This saturation is reasonable if the reaction is driven by the absorption of light. One would expect increased yields of product with increasing

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**Fig. 1.** Schematic transformation of electromagnetic energy into chemical free energy. The photoreduction of NADP+ implies a corresponding oxidation, which is symbolized by the change in [A].

**Fig. 2.** Structure and molecular absorption spectra of (a) proflavine and (b) acridine.
light absorption. The amount of product should reach a limiting value as the absorption of light approaches 100%.

There is an additional interesting aspect of the data presented in Fig. 4: the amount of photoreduced NADPH is not zero when the concentration of proflavine is zero. The reason for this residual amount is that the FAD flavoprotein, Fd-NADP reductase, is itself a yellow pigment, having the characteristic yellow color of flavin-adenine dinucleotide. Fd-NADP reductase is a light-sensitive compound. Moreover, it is known that when flavins are illuminated with light in the presence of EDTA they are reduced to at least the semiquinone state (11).

ACRIDINE PIGMENT SYSTEM

The acridine pigment system is an ultraviolet analog of the proflavine system that was driven by visible light. The absorption spectrum of acridine in aqueous solution is shown in Fig. 5. Formulation of photoreduced NADPH as a function of steady-state ultraviolet illumination. The source is a mercury lamp with an intensity of 9 mW/cm² in the region 300–400 nm. The composition of the reaction mixture is indicated in Table 2.

in Fig. 2, spectrum b; it has an extinction coefficient of 17 mM⁻¹ cm⁻¹ at λ = 353 nm. The structural formula is also indicated in Fig. 2, near spectrum b. Acridine is not very soluble in water. However, it is possible to prepare a saturated solution of about 150 μM. From Fig. 2, spectrum b it is clear that acridine has negligible absorption in the visible region of the electromagnetic spectrum, but that it absorbs strongly in the near ultraviolet. The formation of photoreduced NADPH as a function of steady-state illumination is shown in Fig. 5; the composition of the reaction mixture is indicated in Table 2. A filter was introduced to cut off ultraviolet radiation below 300 nm. A simple, visual demonstration of the EDTA-acridine photosystem may be obtained by coupling it to the oxidation-reduction indicator, methyl viologen. Methyl viologen is colorless in the oxidized state and has a deep blue color in the reduced state. The reduced compound is quite stable in the absence of molecular oxygen or other electron acceptors. An aqueous solution of EDTA, acridine, and oxidized methyl viologen is colorless. If it is irradiated with ultraviolet light under anaerobic conditions, the initially colorless solution turns a deep blue, indicating the formation of the reduced methyl viologen radical cation. This reduction is analogous to the photoreduction of methyl viologen in an aqueous solution with EDTA and proflavine (12), where the photochemical activation is performed by visible light.

CONCLUSION

In this paper we have described two simple pigment systems that have certain properties similar to chlorophyll and its de-

![Fig. 3](image-url)  
Fig. 3. (a) Quantitative spectra of photoreduced NADPH. The composition of the reaction mixture is indicated in Table 2. (b) Formation of photoreduced NADPH as a function of steady-state ultraviolet illumination. The source is a mercury lamp with an intensity of 9 mW/cm² in the region 300–400 nm. The composition of the reaction mixture is indicated in Table 2.

![Fig. 4](image-url)  
Fig. 4. Yield of photoreduced NADPH as a function of proflavine concentration for a fixed time of irradiation (30 min.). The percentages refer to the fraction of light at λ = 445 nm that is absorbed in a 1-cm path length. [EDTA] = 500 μM; [Fd-NADP reductase] = 0.15 μM; [NADP⁺] = 300 μM.

![Fig. 5](image-url)  
Fig. 5. Formation of photoreduced NADPH as a function of steady-state ultraviolet illumination. The source is a mercury lamp with an intensity of 9 mW/cm² in the region 300–400 nm.

### Table 2. Composition of NADP⁺ photoreduction systems

<table>
<thead>
<tr>
<th>Compound</th>
<th>Proflavine system* (μM)</th>
<th>Acridine system† (μM)</th>
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</thead>
<tbody>
<tr>
<td>EDTA</td>
<td>500</td>
<td>500</td>
</tr>
<tr>
<td>NADP⁺</td>
<td>300</td>
<td>300</td>
</tr>
<tr>
<td>Fd-NADP⁺ reductase</td>
<td>0.15</td>
<td>0.15</td>
</tr>
<tr>
<td>Proflavine</td>
<td>50</td>
<td>—</td>
</tr>
<tr>
<td>Acridine</td>
<td>—</td>
<td>10</td>
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* See Fig. 3.  
† See Fig. 5.
rivatives. Perhaps more important from the standpoint of flavoprotein catalysis is the acridine system, which is a one-enzyme system that converts substrate to product and has the two important characteristics: (a) it can be initiated photo-chemically and (b) the only component of the system that absorbs appreciably in the visible is the enzyme itself. These features are attractive because they allow a spectral study of the enzyme as it goes through its catalytic cycle without interfering absorption from other components in solution.

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