Photoreduction of Pyridine Nucleotides by Ascorbate: Catalysis by Chlorophylls and Porphyrins in Aqueous Media *

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1. In the presence of ascorbate and partially purified photosynthetic pyridine nucleotide reductase (PPNR) ***, a photoreduction of pyridine nucleotides was catalyzed by chlorophyll *a*, chlorophyll *b*, hematoporphyrin IX, protoporphyrin IX, coproporphyrin III, and to a lesser extent by pheophytin *a* and pheophytin *b*. Iron porphyrins and heme proteins were inactive. Phycocyanin was moderately active.

2. In general, TPN was photoreduced at a faster rate than DPN. With purified chlorophylls, however, the reverse was true.

3. Addition of 2,6-dichlorophenolindophenol or quinacrine resulted in an inhibition of the photoreductions.

4. In the absence of PPNR, hematoporphyrin catalyzed a slow photoreduction of DPN. Under the same conditions with TPN, a photoreduction of the porphyrin was observed.

The previous paper in this series describes the photoreduction of methyl red and tetrazolium blue by ascorbate in aqueous solution, which reactions are catalyzed by chlorophylls, pheophytins and porphyrins ¹. These reactions are also catalyzed by spinach chloroplasts and by chromatophores of *Rhodospirillum rubrum* ², showing that similar reactions can be catalyzed by the chlorophylls in the free form or when contained in the organized structure found in chloroplasts and chromatophores. The reactions described have significance in terms of the mechanism of the primary photochemical act of photosynthesis, but the reagents involved are not those which take part in the natural photosynthetic process. Of more interest would be the demonstration of a photoreduction of pyridine nucleotides by the isolated chlorophylls in an

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*** The abbreviations used are: DPIF, 2,6-dichlorophenolindophenol; DPN, diphosphopyridine nucleotide; TPN, triphosphopyridine nucleotide; PPNR, photosynthetic pyridine nucleotide reductase; Tris, tris(hydroxymethyl)aminomethane, and FMN, flavin mononucleotide.

aqueous medium. Krasnovsky^{3,4} has shown that chlorophylls dissolved in pyridine catalyze a photoreduction of pyridine nucleotides by ascorbate. The data presented in this paper show that the photoreduction of pyridine nucleotides proceeds in the presence of PPNR in an aqueous medium, and a few of the characteristics of this reaction are presented.

MATERIALS AND METHODS

The experimental methods employed, the preparative methods for chlorophylls and pheophytins, the methods used for estimation of photocatalyst concentration and a description of most of the materials used have been previously described¹. DPN and TPN were purchased from Sigma Chemical Co. of St. Louis. PPNR was prepared from spinach leaves according to the directions of San Pietro and Lang⁵, and the preparations used correspond to their "extract of acetone precipitate". The enzyme preparation was lyophilized and stored at -20°C in a drying chamber in the dark. The alcohol dehydrogenase was a twice recrystallized preparation from horse liver, and was a gift from Dr. C. L. Woronick. Illumination was with a 500 W tungsten reflector flood lamp, which gave a light intensity of approximately 4500 foot candles at the reaction site.

RESULTS

In view of the requirement for PPNR for pyridine nucleotide photoreduction with fresh chloroplasts in a regular Hill reaction⁵ and by aged chloroplasts utilizing ascorbate as the electron donor⁶, it would be expected that the photoreduction of pyridine nucleotides in an aqueous medium by photocatalysts outside the biological photosynthetic unit would also require the presence of

Fig. 1. Photoreduction of pyridine nucleotides by non-purified chlorophyll preparation. The chlorophyll preparation was an acetone extract of spinach which had been transferred into petroleum ether and adsorbed on sucrose. The experimental conditions were the same as given for Table 1. At the first arrow, 0.05 ml of water was added to both the illuminated and non-illuminated sides of the reaction vessel, and at the second arrow 50 μmoles of acetaldehyde and 0.05 μmole of alcohol dehydrogenase were added in sequence to both the illuminated and non-illuminated sides. The resultant sharp decrease in the optical density shows the presence of DPNH in the illuminated system.

this enzyme. Fig. 1 shows the results obtained when a non-purified chlorophyll preparation (added to the system adsorbed on sucrose) was illuminated in the presence of ascorbate, pyridine nucleotide and PPNR. A photoreduction of both DPN and TPN was observed, with TPN giving the faster reaction. This type of nucleotide preference would be expected in view of the characteristics of the PPNR preparation used ⁵. To show that the absorbancy change at 340 μ was a measure of the reduced pyridine nucleotide formed, acetaldehyde and alcohol dehydrogenase were added after illumination of the reaction system containing DPN. The immediate drop in absorbancy can be attributed to the DPNH present in the illuminated system. Similar experiments were not performed with the TPN system, but it is logical to assume that here also the absorbancy changes were an accurate measurement of the formation of reduced pyridine nucleotide.

The extensive work of Krasnovsky and coworkers ⁷ shows the ability of protoporphyrin and hematoporphyrin to catalyze the same photoreactions as the chlorophylls and pheophytins. Both protoporphyrin and hematoporphyrin are also active in the photoreduction of methyl red and tetrazolium blue previously reported ¹. Therefore, it is not surprising that a photoreduction of the pyridine nucleotides would also be catalyzed by these porphyrins in the presence of PPNR. Fig. 2 shows the activity of hematoporphyrin in such a reaction. Compared to the chlorophylls, higher concentrations of hematoporphyrin could be utilized, thus allowing a faster rate of pyridine nucleotide photoreduction. The addition of acetaldehyde and alcohol dehydrogenase again showed the presence of DPNH in the system geared for its production in the light. The same nucleotide preference was shown as in the case when chlorophylls were used, emphasizing that this is a function of the PPNR preparation used.

Fig. 2. Photoreduction of pyridine nucleotides catalyzed by hematoporphyrin. The experimental conditions were the same as listed in Table 1. At the first arrow 0.05 ml of water was added to both the illuminated and non-illuminated sides of the reaction vessel, and at the second arrow 50 μ moles of acetaldehyde and 0.05 μ mole of alcohol dehydrogenase were added in sequence to both sides of the reaction vessel, showing the presence of DPNH.

The reaction system employed for the experiments represented in Figs. 1 and 2 contained glutathione, since as shown below, glutathione stimulated the reaction. In both experiments, there was a slow rate of DPNH formation in the dark. The shorter illumination period utilized with hematoporphyrin minimized this dark reaction, but it was observed to proceed at about the same rate in both cases. This reduction in the dark was related to the PPNR preparation used, since in separate experiments removal of this compound prevented the reduction of DPN in the dark. Thus, it is apparently caused by a contaminating dehydrogenase in the PPNR preparation.

The relationship between the rate of pyridine nucleotide photoreduction and the concentration of PPNR is shown in Fig. 3. With both photocatalysts, and with both pyridine nucleotides, maxima were observed. The inhibition caused by excess enzyme was more pronounced and commenced at a lower enzyme concentration when TPN was used. One explanation for this behavior is to be found in the fact that the PPNR preparations used were not extensively purified, and probably contained glutathione reductase. Since glutathione was added to the system, a coupling of the reduced pyridine nucleotide and the glutathione system would be expected at higher enzyme concentrations. This would explain the greater decrease in activity with TPN, since glutathione reductase from plants is specific for TPN⁸. The saturation of the hematoporphyrin system at lower PPNR concentrations reflects a better interaction between the hematoporphyrin and the enzyme than occurs in the case of chlorophyll. This is logical, since the hematoporphyrin is in solution, while the chlorophyll is present as a very fine colloidal suspension. That saturation occurs in these experiments indicates that under these conditions the donation of electrons to the PPNR complex from the pigment molecules becomes the limiting factor.

Fig. 3. The effect of PPNR concentration upon the photoreduction of pyridine nucleotides catalyzed by a non-purified chlorophyll preparation and by hematoporphyrin. The experimental conditions were the same as given in Table 1 except the chlorophyll concentration was $7.6 \mu\text{M}$. The experiments performed with chlorophyll were for 30 min and the experiments with hematoporphyrin were 20 min in the case of DPN and 10 min in the case of TPN.

In the reactions catalyzed by chlorophylls, no photoreduction was observed when PPNR was absent. In the case of hematoporphyrin however, it was seen that with DPN there was a significant reaction in the absence of added enzyme. In the case of TPN with hematoporphyrin, negative absorbancy changes were observed in the absence of the enzyme. In the latter case, these reflect a photoreduction of the porphyrin itself by ascorbate. The previous paper¹ reports such a reaction and although the maximum absorbancy change upon hematoporphyrin photoreduction occurred at 375 m μ , there was nevertheless considerable change at 340 m μ , where pyridine nucleotide reduction was followed. In the experiments with TPN and no PPNR, a visual bleaching of the hematoporphyrin was observed.

The effect of concentration of photocatalyst upon the photoreduction reactions is shown in Fig. 4. The reactions saturated with respect to the photocatalyst, indicating that under these conditions some other component becomes rate limiting, and the most logical substance to suspect in this regard would be the enzyme. One would expect a further increase in the rate of reaction by increasing the concentration of both enzyme and photocatalyst. When such experiments were performed, a decrease in activity was noted. This suggests the presence of an inhibitor in the PPNR preparation. It is apparent that clarification of this problem depends upon the use of pure PPNR in reactions of this sort.

The requirements for the photoreduction of pyridine nucleotides by hematoporphyrin and the chlorophylls are shown in Table 1. Also included in this table are results obtained with pure chlorophyll *a*. One significant result of these experiments is the fact that DPIP inhibits the reactions. This was

Fig. 4. The effect of photocatalyst concentration upon the photoreduction of pyridine nucleotides. The experimental conditions were the same as given for Table 1 and the exposure times were the same as set forth in Fig. 3. A non-purified chlorophyll preparation was used in these experiments, with a final concentration of 7.5 μ M.

Table 1. Requirements for pyridine nucleotide photoreduction by chlorophyll and hematoporphyrin. The reaction mixture contained 5.5 mM ascorbate, 1.8 mM glutathione, 18 mM Tris buffer pH 7.8 and either 22 μ M hematoporphyrin (with 2 mg PPNR), 4.2 μ M chlorophyll *a* (with 5 mg PPNR) or 8.2 μ M crude chlorophyll (with 5 mg PPNR). The volume was 5.5 ml and anaerobic conditions were employed. The crude chlorophyll preparation was from an acetone extract of spinach and consequently contained both chlorophylls and other acetone-soluble substances, including carotenoids. For the experiments involving the crude chlorophyll, 4 mg of sodium deoxycholate were also added, since otherwise the chlorophyll showed a tendency to precipitate from solution. When present, DPIP was 91 μ M. β -carotene was 15 μ M and pyridine was added to give a 1 % pyridine solution. Illumination was performed with a 500 W tungsten reflector flood lamp. The chlorophylls were added to the system dissolved on sucrose and consequently these solutions were also approximately 0.5 M in sucrose.

Conditions	Absorbancy change, 340 m μ , light minus dark				
	Hematoporphyrin 20 min		Crude Chphyll 30 min		Chphyll <i>a</i> 40 min
	DPN	TPN	DPN	TPN	DPN
Complete system	0.495	0.542	0.217	0.302	0.176
— ascorbate	0.283	0.301	0.063	0.123	0.054
— glutathione	0.328	0.410	0.169	0.207	0.113
— ascorbate and glutathione	0.024	0.015	0.011	0.016	0.007
Boiled PPNR	0.098	—0.158	0.003	0.002	0.003
— PPNR	0.118	—0.163	0.003	0.001	0.001
— pyridine nucleotide	—0.115	—0.110	0.004	0.003	0.003
— photocatalyst	0.001	0.002	0.001	0.002	0.001
+ DPIP	0.238	0.375	—0.068	—0.021	—0.135
+ pyridine	—	—	—	—	0.018
+ β -carotene	—	—	—	—	0.156

unexpected, since this compound serves to stimulate the photoreduction of tetrazolium blue and methyl red by these same photocatalysts, and is almost an absolute requirement for the photoreduction of pyridine nucleotides by ascorbate using chloroplasts which are aged or inhibited with 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU)⁶. The inhibition was particularly severe when the chlorophyll mixture or chlorophyll *a* were used. In both of these cases an absorbancy decrease was observed in the illuminated systems. This cannot be explained at present, but it could reflect a photoreduction of the chlorophyll molecule itself. The mode of inhibition by DPIP must await further experimentation, but it is expected that its ability to function either as an oxidant⁹ or a reductant¹ to the chlorophyll system is the basis for this behavior. In this regard, it should be pointed out that DPIP is a potent inhibitor of DPN photoreduction by chromatophores of *R. rubrum*, and its action there is attributed to its ability to act as a shunt between the photoreducing and photooxidizing sites¹⁰.

Removal of either ascorbate or glutathione from the reaction systems caused a decrease in activity, and removal of both compounds almost stopped the reactions. It should be noted that the small residual activity in the latter case is probably due to the fact that in preparation of the enzyme, sufficient glutathione was added just prior to lyophylyzation to make the solution mM

in this compound, and consequently some glutathione would be added with the PPNR preparation. The glutathione can function both as an electron donor¹ or as a protective agent for the PPNR, which is inactivated by illumination in the presence of chlorophyll¹¹.

No photoreduction of pyridine nucleotides was observed in the absence of photocatalyst. Addition of β -carotene caused a slight reduction in the activity of the chlorophyll *a* system, and addition of pyridine resulted in a marked drop in activity. Removal of the PPNR allowed no reaction to proceed in the case of the chlorophylls, but had opposite effects on the hematoporphyrin catalyzed reactions. Some photoreduction of DPN was observed with no PPNR present, while in the case of TPN an absorbancy decrease was noted subsequent to illumination. This was due to photoreduction of the hematoporphyrin itself by ascorbate as discussed above. A photoreduction of hematoporphyrin was also observed in the case where there was no pyridine nucleotide added.

In Table 2 a comparison is made of various porphyrins and porphyrin derivatives concerning their ability to catalyze the photoreduction of pyridine nucleotides. In general, the same pattern was obtained as was previously observed for the photoreduction of tetrazolium blue and methyl red¹. In the present case, however, hematoporphyrin was more active than either protoporphyrin or coproporphyrin. For the chlorophylls, the best reaction was observed with the non-purified mixture of chlorophylls. This preparation also contained other acetone-soluble components, and it may be that these stimulate the reaction. When comparing this chlorophyll preparation with the purified

Table 2. A comparison of several porphyrins and porphyrin derivatives in pyridine nucleotide photoreduction. The experimental conditions were the same as those described in Table 1, with different photocatalysts added in the amounts indicated. The observed photoreduction rates have been adjusted to give a rate for 60 minutes with 10 μ M photocatalyst.

Photocatalyst	Absorbancy change, 340 m μ for 60 min for 10 μ M photocatalyst, light — dark	
	TPN	DPN
Crude chlorophyll	0.734	0.530
Chlorophyll <i>a</i>	—0.007	0.487
Chlorophyll <i>b</i>	0.135	0.428
Pheophytin <i>a</i>	0.004	0.030
Pheophytin <i>b</i>	0.038	0.056
Hematoporphyrin IX	0.740	0.676
Protoporphyrin IX	—	0.408
Coproporphyrin III	—	0.484
Hematochrome	—	0.007
Hemin	—	0.004
Cytochrome <i>c</i>	—	—0.008
Catalase	—	—0.002
Peroxidase	—	—0.003
FMN	—	—0.006
Bilirubin	—	0
Phycocyanin	—	1.08

Table 3. Effect of various compounds upon DPN photoreduction catalyzed by hematoporphyrin. The experimental conditions were the same as given for Table 1, with the additions as listed below. Different PPNR preparations were used during the course of these experiments, and accordingly the activities are expressed as a percent activity related to a control.

Addition	Final concentration mM	Activity %
None	—	100
Ethylenediamine tetraacetic acid	0.18	65
KCN	1.8	97
Sucrose	530	102
Quinacrine	0.17	28
Mn ⁺⁺	0.18	76
Mg ⁺⁺	0.18	92
Cu ⁺⁺	0.18	69
Fe ⁺⁺⁺	0.18	9
None, aerobic	—	61

chlorophylls, one significant difference is observed. With chlorophyll *a* no photoreduction of TPN was observed, and with chlorophyll *b* the rate of TPN photoreduction was less than that of DPN. This is opposite to the nucleotide preference ordinarily found⁵. The reason for this odd behavior toward TPN is not known, but it has a corollary in the fact that with hematoporphyrin and no added PPNR, a slow photoreduction of DPN was noticed, but none was observed with TPN (Table 1). Thus it would appear that in opposition to the preference of the PPNR preparation for TPN photoreduction, there is a greater potential for DPN photoreduction in the absence of the enzyme.

The results given in Table 2 again show the iron porphyrins, the heme-proteins and FMN to be inactive in the reaction. Bilirubin was also inactive, but phycocyanin was found to be moderately effective. The phycocyanin was prepared from *Cyanidium caldarium*, was chromatographically purified, and was made available by Dr. L. Bogorad. The high value reported for phycocyanin must be considered in light of the fact that the values are reported for 10 μ M photocatalyst. Since the phycocyanin molecule has 8 or 16 light absorbing groups per molecule¹², the rate per absorbing group would be smaller by that factor. The phycocyanin concentration was determined from absorbancy measurements using the constant given by Lemberg and Legge¹².

The effect of various compounds upon the photoreduction of DPN by hematoporphyrin is shown in Table 3. Sucrose was included since the experiments involving the chlorophylls contained that quantity of sucrose in the reaction system. It was found to have no inhibitory effect upon the reaction catalyzed by hematoporphyrin. Quinacrine, which markedly stimulates the photoreduction of methyl red and tetrazolium blue by *R. rubrum* chromatophores² was inhibitory in the present case. Anaerobic conditions were best, as shown by the decreased activity in the presence of air. The presence of some selected metal ions inhibited the reaction, with iron being the most effective. Cyanide ion was without effect, while ethylenediamine tetraacetic acid was mildly inhibitory.

DISCUSSION

The data presented above show that chlorophylls and porphyrins can catalyze a photoreduction of both DPN and TPN when ascorbate is present as an electron donor and PPNR is present to facilitate electron transfer to the pyridine nucleotides. The rates of the photoreductions observed to date are relatively low when compared to the rates obtained with the intact chloroplast¹³. The data of Table 2 show that with non-purified chlorophyll, 12 moles of TPN were reduced per hour for each mole of chlorophyll. The rate observed for hematoporphyrin, the most active porphyrin, was only slightly higher. The results are significant, however, since they show this important reaction can proceed in aqueous solution in the absence of the complex organization of the chloroplast or chromatophore. The ability of the chlorophylls to function in this simplified system strongly supports the concept advanced by Krasnovsky⁷ that in the chloroplast also the primary photochemical act involves the photoreduction of chlorophyll by means of some physiological electron donor (ultimately water) and a subsequent transfer of the electrons to either pyridine nucleotide or the enzymatic components of the cyclic electron transfer system. In comparing the photoreduction of pyridine nucleotides by chlorophylls and chloroplasts, two differences are noted. With the purified chlorophylls an inverse nucleotide preference is shown, with TPN being photoreduced at the lower rate (and not at all with chlorophyll *a*). Also, addition of DPIP to the chlorophyll system caused complete inhibition of the reactions, whereas it stimulates the ascorbate coupled photoreduction of pyridine nucleotides by chloroplasts⁶. As mentioned above, this is most likely due to the ability of DPIP to act as either an oxidant or a reductant with the photoactivated chlorophyll depending upon experimental conditions, and thus it has the potential of short-circuiting the electron flow and preventing any net photoreduction.

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