

# Red-Light Effects Sensitized by Methylene Blue on Nitrate Reductase from Spinach (*Spinacia oleracea* L.) Leaves

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Nitrate reductase from spinach (*Spinacia oleracea* L.) leaves, which had been inactivated *in vitro* by incubation with NADH and cyanide, was fully reactivated in minutes when irradiated in anaerobic conditions with red light in the presence of methylene blue. Both the rate and the extent of reactivation increased with light intensity (6 to 100 W · m<sup>-2</sup>) and dye concentration (1 to 10 μM). On the contrary, photoreactivation was completely abolished when NADH or ethylenediaminetetra-acetic acid were present during irradiation. We propose that methylene blue, when photo excited, exhibits a redox potential positive enough to reoxidise the CN<sup>-</sup>-reduced molybdenum complex settled in the inactive enzyme, thus causing its reactivation.

On the other hand, prolonged irradiation of nitrate reductase, under air and in the presence of methylene blue, promoted an oxygen-dependent irreversible inactivation of the two partial activities of the enzyme. This inactivation was markedly enhanced in 77% deuterated water and greatly prevented by azide, which indicates that singlet oxygen is the species primarily involved in the photooxidative inactivation of the enzyme.

## 1. Introduction

Nitrate reductase (NADH-nitrate oxidoreductase, EC 1.6.6.1) from spinach leaves catalyses the reduction of nitrate to nitrite with NADH as physiological electron donor. Besides this overall reaction, the enzyme complex exhibits two partial activities which can be independently assayed: a) a NADH-dehydrogenase activity, which reduces cytochrome *c*, ferricyanide or dichlorophenolindophenol; and b) the so-called terminal NR activity, which catalyzes nitrate reduction with reduced flavins or viologens. The functionality of both partial activities is required for the reduction of nitrate with NADH. The enzyme molecule contains FAD, cytochrome *b*-557 and molybdenum as redox prosthetic groups. Whereas the flavin and, presumably, the heme components seem to be associated with the

dehydrogenase function, molybdenum is clearly involved in the catalytic reduction of nitrate [1–3].

NR from algae can exist in two metabolically interconvertible forms, either active or inactive, in response to changes in nutritional and environmental conditions [2, 4]. Except for the spinach enzyme, the interconversion mechanism of NR has been scarcely studied in higher plants [1, 2]. Spinach NR is inactivated *in vitro* by CN<sup>-</sup> or C<sub>2</sub>H<sub>2</sub> when previously reduced with a low potential reductant such as NADH or S<sub>2</sub>O<sub>4</sub><sup>2-</sup> [5, 6]. The inactivation affects the terminal NR but not to its dehydrogenase activity [5–7]. The CN–NR can be reactivated by ferricyanide [5, 7], peroxidase systems [8] or trivalent manganese ions generated by illuminated chloroplasts [7]. Apparently, these treatments reoxidise the CN<sup>-</sup>-reduced molybdenum complex, thus causing the release of the bound CN<sup>-</sup> and the concomitant restoration of the enzyme activity [3, 5, 6]. Inactivation by NADH *plus* cyanide, and corresponding reactivation by ferricyanide, have been also demonstrated on NR from rice [9] wheat [10], barley [11] and maize [12].

It has been established that *in vitro* inactivated NR from *Chlorella fusca* [13], *Chlamydomonas reinhardtii* [14], *Neurospora crassa* [15], spinach [5, 13, 16, 17], wheat [10] and maize [12], is likewise readily reactivated by exposure of the inactive enzyme to

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*Abbreviations:* NADH, nicotinamide adenine dinucleotide, reduced form; NR, nitrate reductase; FAD, flavin adenine dinucleotide; FMN, flavin mononucleotide; <sup>1</sup>O<sub>2</sub>, singlet oxygen; CN–NR, cyanide-inactivated nitrate reductase; MB, methylene blue; MV<sup>+</sup>, methyl viologen, reduced form; Tris, tris(hydroxymethyl)-aminomethane; EDTA, ethylene diaminetetra-acetic acid.

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light in the presence of flavins. Further, light *plus* FAD also promoted efficiently the reactivation of an inactive form of NR isolated from *Chlorella* [5, 13], *Chlamydomonas* [14] and wheat leaf [10]. On the other hand, it has been reported [18], that prolonged irradiation of spinach NR with blue light in the presence of FMN, provokes an irreversible damage of the enzyme due more likely to the harmful effect of photogenerated  $^1\text{O}_2$ .

We report herein that photoreactivation of spinach CN-NR can also be sensitized by MB. Moreover, in the presence of oxygen, the dye photosensitizes an irreversible inactivation of the two partial activities of NR complex where  $^1\text{O}_2$  seems to be involved.

## 2. Materials and Methods

### 2.1. Plant material and enzyme assays

NR from leaves of field-grown spinach (*Spinacia oleracea* L.) was partially purified as previously described [16]. The enzymatic assays for  $\text{MV}^+$ -nitrate reductase and NADH-dehydrogenase activities were reported elsewhere [6]. One unit of  $\text{MV}^+$ -nitrate reductase or NADH-dehydrogenase is the amount of enzyme which catalyzes the formation of 1  $\mu\text{mol}$   $\text{NO}_2^-$  or 1  $\mu\text{mol}$  reduced cytochrome *c* per min, respectively.

### 2.2. Inactivation of NR

To prepare CN-NR, active enzyme was incubated for 10 min at 4 °C in 0.2 M Tris-HCl buffer (pH 7.5), 0.6 mM NADH and 0.2 mM KCN. In order to remove the excess of NADH and  $\text{CN}^-$ , the preparation was filtered through a Sephadex G-25 column equilibrated with 0.1 M K-phosphate buffer (pH 7.5). The eluted inactive NR was used for experiments on photoreactivation.

### 2.3. Conditions of irradiation

Unless otherwise indicated, NR, in 0.5 ml of 0.1 M K-phosphate buffer (pH 7.5), was irradiated in open-air glass tubes (5 cm high  $\times$  1 cm diameter) placed in small transparent methacrylate baths. Temperature was kept at 4 °C by continuous circulation around the tubes of iced water. Actinic red-light was provided by an ordinary slide projector (24 V, 250 W lamp) fitted with a Balzer interference filter DT 606 transmitting from 606 nm into the

infrared. Red-light irradiance at sample position was 100  $\text{W} \cdot \text{m}^{-2}$  except in the experiment of Fig. 2 where different light irradiances were used, as indicated. Irradiances were measured with a Yellow-Spring 65A radiometer.

To establish anaerobic conditions, glucose (30 mM), glucose oxidase (1.5 units) and catalase (1400 units) were added to the corresponding mixtures. Immediately after, the tubes were fitted with rubber stoppers (Becton-Dickinson Vacutainer) and subjected to three 1 min-cycles of evacuation and flushing with  $\text{N}_2$ . Aliquots for determination of enzymatic activities were taken out in this case with the aid of hypodermic needles.

### 2.4. Analytical methods

Protein was determined according to the method of Lowry *et al.* [19], using bovine serum albumin as standard. Nitrite was measured by the diazo-coupling colorimetric assay of Snell and Snell [20].

Spectrophotometric enzyme assays were performed in a Beckman DK-2A spectrophotometer, and colorimetric measurements in a Bausch & Lomb Spectronic 100 spectrophotometer.

## 3. Results

### 3.1. The two light-effects mediated by MB on spinach NR

Results of Fig. 1 show that red-light irradiation, in the presence of MB, of CN-NR brought about two different effects on the terminal activity, depending on whether or not oxygen was present during irradiation. When irradiation was carried out under anaerobic conditions (Fig. 1A), a rapid reactivation of  $\text{MV}^+$ -nitrate reductase took place. Highest rates were attained at MB concentrations of 5 to 10  $\mu\text{M}$ , whereas at 1  $\mu\text{M}$  the rate decreased to about half. In the absence of sensitizer, photoreactivation could hardly proceed. Under air (Fig. 1B), the MB-photosensitized reactivation was greatly impeded and, moreover, upon extended irradiation, the reactivated enzyme was progressively inactivated, especially with 10  $\mu\text{M}$  MB.

In order to investigate separately both processes, photoreactivation was performed in anaerobic systems, whereas photoinactivation experiments were conducted under air and using fully active NR.

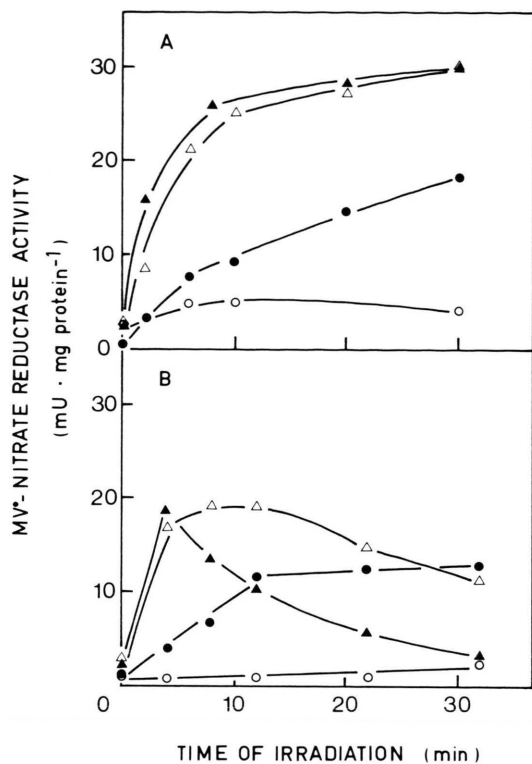


Fig. 1. Antagonistic effects of red-light irradiation on spinach CN-NR in the presence of MB. Samples of CN-NR were irradiated with red light under anaerobic (A) or aerobic (B) conditions, either alone (○) or in the presence of 1 μM (●), 5 μM (Δ) and 10 μM MB (▲). At the indicated times, 0.05 ml aliquots were taken out from the different mixtures to measure MV<sup>+</sup>-nitrate reductase activity. The specific activity of the enzyme preparation before inactivation with CN<sup>-</sup> was 32 mU · mg protein<sup>-1</sup>.

### 3.2. Reactivation of spinach NR photosensitized by MB

Fig. 2 shows the time courses of photoreactivation of CN-NR when the enzyme, in the presence of 5 μM MB, was irradiated with red light of 6, 25 and 100 W · m<sup>-2</sup>. The rate of MB-photosensitized reactivation increased with irradiance. Even at an irradiance as low as 6 W · m<sup>-2</sup>, a remarkable reactivation rate was observed. In the dark, however, the enzyme activity did not show any significant increase, at least within the period of time tested.

It has been reported [21, 22] that photoexcited MB is easily reduced by NADH or EDTA. Interestingly, the MB-mediated photoreactivation of spinach CN-NR was completely abolished when

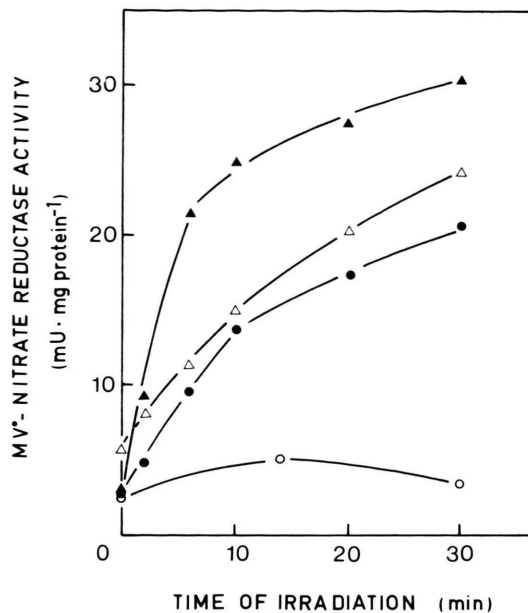


Fig. 2. Effect of light intensity on the MB-sensitized photoreactivation of spinach CN-NR. Samples containing CN-NR and 5 μM MB were made anaerobic and then either kept in the dark (○) or illuminated with red light at irradiances of 6 W · m<sup>-2</sup> (●), 25 W · m<sup>-2</sup> (Δ) or 100 W · m<sup>-2</sup> (▲). Other conditions as in Fig. 1.

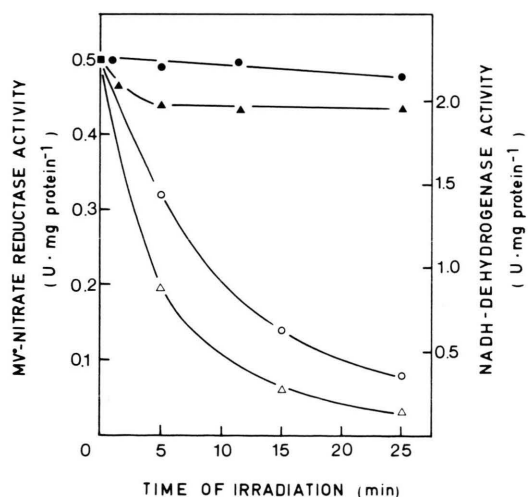


Fig. 3. Oxygen-dependent MB-sensitized photoinactivation of the partial activities of spinach NR. Samples containing active NR and 0.1 mM MB were irradiated with red light, either under air (○, Δ) or under anaerobic conditions (●, ▲). At the indicated times, 0.05 ml aliquots were taken out from the different mixtures to measure MV<sup>+</sup>-nitrate reductase (○, ●) and NADH-dehydrogenase (Δ, ▲) activities.

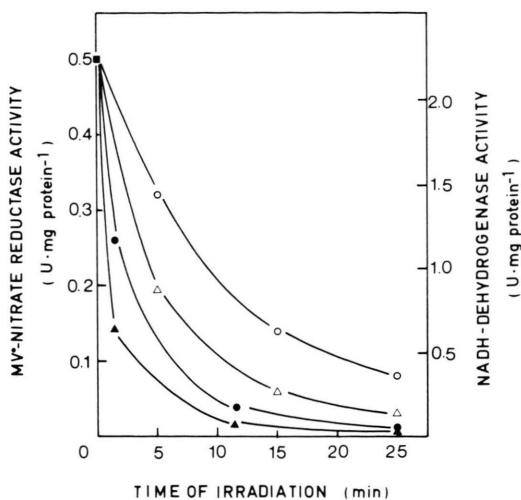


Fig. 4. Enhancement by deuterium oxide of the MB-sensitized photoinactivation of the partial activities of spinach NR. Samples containing active NR and 0.1 mM MB, in a medium without (○, △) and with (●, ▲) 77% D<sub>2</sub>O, were irradiated under air with red light. Other experimental conditions as in Fig. 3. MV<sup>-</sup>-nitrate reductase (○, ●); NADH-dehydrogenase (△, ▲).

Table I. Protection by azide of the partial activities of spinach NR against MB-sensitized photoinactivation.

Conditions	MV <sup>-</sup> -nitrate reductase NADH-dehydrogenase	
	U · mg protein <sup>-1</sup>	
Dark	0.49	2.25
Light	0.08	0.13
Light plus azide	0.25	0.90

Samples containing active NR and 0.1 mM MB were either kept in the dark or irradiated under air for 25 min with red light both in the absence and presence of 2 mM NaN<sub>3</sub>. Activities were then assayed on aliquots from the mixtures.

either EDTA or NADH at 5 mM concentration were included in the irradiation mixture (data not shown).

### 3.3. Photodynamic inactivation of the partial activities of spinach NR

Fig. 3 shows that irradiation with red light of active NR, under air and in the presence of 0.1 mM MB, provoked a marked inactivation of the two partial activities of the enzyme. In the absence of oxygen, the activities were practically not altered. The absolute requirement of oxygen seems to indicate that, in contrast to the photoreactivation

process, photoinactivation is not promoted by excited MB *per se* but rather by some reactive chemical species produced by interaction of the excited photosensitizer with molecular oxygen.

Many photosensitized biological reactions involving molecular oxygen (photodynamic reactions) take place by the action of the highly reactive <sup>1</sup>O<sub>2</sub> generated by direct energy transfer from the excited triplet state of the sensitizer to the ground triplet state of molecular oxygen [23]. The specific quenching effect of N<sub>3</sub><sup>-</sup> on <sup>1</sup>O<sub>2</sub> and the longer lifetime of <sup>1</sup>O<sub>2</sub> in D<sub>2</sub>O than in H<sub>2</sub>O are sensitive parameters successfully used to demonstrate the participation of <sup>1</sup>O<sub>2</sub> in photodynamic reactions [18, 23]. Table I shows that MB-photosensitized inactivation of the two partial activities of spinach NR was greatly prevented in the presence of 2 mM NaN<sub>3</sub>. On the other hand, photoinactivation rates substantially increased in a deuterated medium (77% D<sub>2</sub>O) as compared to regular water (Fig. 4). These results indicate that <sup>1</sup>O<sub>2</sub> is the major reactive oxygen species involved in the MB-sensitized photoinactivation of spinach NR.

## 4. Discussion

As demonstrated in this paper, spinach CN-NR is rapidly reactivated by red light in the presence of MB. In contrast to the flavin-mediated photoreactivation of *N. crassa* NR [15], photoreactivation of the spinach enzyme sensitized by either flavins [16] or MB (this paper) did not require oxygen. Moreover, reactivation was faster and more complete under anaerobic conditions. The experimental evidence presented above indicates that inhibition of photoreactivation by oxygen is due to the interaction of molecular oxygen with the excited dye, which yields two different effects. Firstly, molecular oxygen readily quenches the lowest excited triplet state of MB [24], thus diminishing the actual concentration of excited MB molecules, which appears to be the chemical species primarily involved in the photoreactivation process. This interpretation is further sustained by the fact that either EDTA or NADH, which efficiently reduce photoexcited MB [21, 22], completely impeded the MB-sensitized reactivation. Secondly, some reactive species, more likely <sup>1</sup>O<sub>2</sub>, generated from the interaction of molecular oxygen with excited MB, brings about a subsequent inactivation of the enzyme. Indeed, the experiments

carried out with active NR using  $D_2O$  and  $N_3^-$ , suggest that  $^1O_2$  is the major reactive species involved in the enzyme inactivation which takes place under air during extended light exposure. Incubation of photoinactivated NR with ferricyanide or dithioerythritol did not restore the original enzymatic activities (data not shown), indicating that, as in the FMN-mediated photoinactivation [18], NR might be irreversibly damaged.

By means of redox titrations with ferricyanide it has been demonstrated [25] that the reactivation process of CN-NR from *Ankistrodesmus braunii* shows a "midpoint potential" of +0.23 V at pH 7.5. On the other hand, from thermodynamic and kinetic aspects of photoinduced electron transfer reactions [21, 26], it has been proposed [21] that the redox potential of MB shifts upon irradiation from -0.23 V, in the ground state, to +1.6 V, in the excited triplet state. The redox potential of the photoexcited dye is therefore positive enough to reoxidise readily the  $CN^-$ -reduced molybdenum complex and, hence, reactivate the enzyme. Similar light-induced changes in the redox potential of flavins [27] might be responsible for the reported flavin-mediated photoreactivation [5, 13, 15, 16]. In fact, assuming that the photochemical active form of flavins is also the triplet state [28] and that the energy of the triplet state above the ground-state electronic level is 2.07 eV [29], it can be easily calculated [26] that the redox midpoint potential of flavins might shift from -0.24 V, in the dark, to about +1.83 V, in the light, namely their oxidizing power would be substantially increased upon photoexcitation.

As a comprehensive conclusion from results reported in this and previous related papers [5, 13, 16-18], in Fig. 5 are summarized the mechanisms proposed by ourselves for the sensitized light-effects on NR activity from spinach leaves: a) photoreactivation of the CN-NR, and b) irreversible photodynamic inactivation of its two partial activities. Both photoreactions can be triggered either by blue light *plus* flavins or red light *plus* MB. In the ground singlet state ( $S_0$ ), the sensitizer, either flavin or MB, by absorption of light becomes photoexcited to, eventually, its triplet state ( $^3S$ ). In this state, the sensitizer exhibits a redox potential positive enough to reoxidise the  $CN^-$ -reduced molybdenum complex settled in the inactive nitrate reductase (CN-NR), thus bringing about the restoration

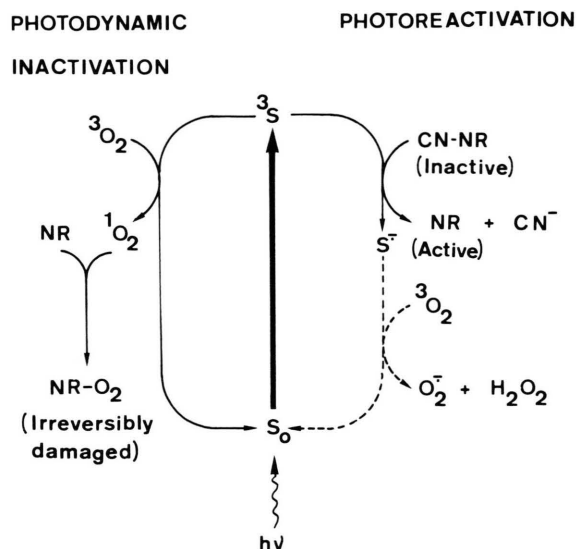


Fig. 5. Proposed mechanisms for the light effects and the roles of oxygen on the activity of spinach NR sensitized by either flavins or methylene blue.  $S_0$ : sensitizer in the ground singlet state;  $^3S$ : sensitizer in the excited triplet state;  $S^-$ : semiquinone form of the sensitizer;  $^3O_2$ : molecular oxygen (ground triplet state);  $O_2^-$ : superoxide radical; NR-O<sub>2</sub>: oxygenated nitrate reductase.

of the enzyme activity. The initial rate of the photoreactivation process increases under anaerobic conditions since  $^3S$  can be unenergized by ground triplet dioxygen ( $^3O_2$ ) through energy transfer (see left half of the figure). Notwithstanding, sustained photoreactivation might show a definite requirement of  $^3O_2$ , capable by itself of regenerating  $S_0$  from the semiquinone form of the sensitizer ( $S^-$ ), if the initial concentration of  $S_0$  were too low, which does not appear to be the case under the ordinary experimental conditions. On the other hand, under prolonged irradiation in air, the highly reactive  $^1O_2$ , generated by energy transfer from  $^3S$  to  $^3O_2$ , promotes oxidation or oxygenation of some aminoacids of the NR molecule (NR-O<sub>2</sub>) [27], thus causing the irreversible loss of its various activities.

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