

Mechanism of reactivation of cyanide-inactivated nitrate reductase by flavins in light

N. Jawali and P.V. Sane*

Biology and Agriculture Division, Bhabha Atomic Research Centre, Bombay 400 085, India

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1. INTRODUCTION

Nitrate reductase (EC 1.6.6.1) from higher plants *in vitro* performs the reduction of NO_3^- to NO_2^- using NADH as the reductant. It can also reduce NO_3^- to NO_2^- using an artificial electron donor such as reduced flavin mononucleotide. The latter activity, usually designated as $\text{FMNH}_2\text{-NR}$, involves the molybdenum-containing moiety of the nitrate reductase (NR) and is easily inactivated by cyanide, when molybdenum is in a reduced state [1]. The cyanide (CN^-) is a very potent ($K_i = 0.2 \mu\text{M}$) but reversible inhibitor with a dissociation constant of 10^{-10} M for the molybdenum-cyanide complex [2, 3]. Cyanide has been implicated in the physiological regulation of NR in *Chlorella* [4]. Ferricyanide rapidly and almost completely reactivates the CN^- -inhibited enzyme [5]. The CN^- -inactivated enzyme can also be reactivated by flavins; e.g., flavin mononucleotide (FMN) or flavin adenine dinucleotide (FAD) in light [6,7]. More recently, reactivation by Mn^{3+} pyrophosphate has also been reported [8].

The mechanism of reactivation by ferricyanide and Mn^{3+} pyrophosphate appears to involve chemical oxidation of molybdenum; i.e., Mo^{IV} to Mo^{VI} . In [8] it was proposed that this oxidation results in the formation of an unstable cyanide Mo^{VI} coordinated state from a very stable cyanide Mo^{IV} coordinated state causing dissociation of cyanide. The mechanism of reactivation of CN^- -inactivated NR by flavins in light has not yet been elucidated. Considering the fact that flavins

in light can generate superoxide anions ($\text{O}_2^{\cdot-}$) and singlet oxygen, we felt that one of these two species of oxygen may mediate the oxidation of Mo^{IV} of the CN^- -inactivated enzyme, leading to the reactivation of the enzyme. The data presented here provide evidence for the involvement of $\text{O}_2^{\cdot-}$ in the reactivation of CN^- -inhibited NR by flavins in light.

2. MATERIALS AND METHODS

Nitrate reductase was isolated and partially purified from spinach leaves as in [9]. The enzyme preparation had an activity of about 30 nmol NO_2^- produced $\text{mg protein}^{-1} \cdot \text{min}^{-1}$ using NADH as the reductant. The $\text{FMNH}_2\text{-NR}$ activity was measured in a total volume of 1 ml containing 100 mM phosphate buffer (pH 7.6), 10 mM potassium nitrate, 0.2 mM FMN and 0.1 ml sodium dithionite (from 8 mg NaHCO_3 + 8 mg sodium dithionite in 1 ml water). After 5 min (or as necessary), the reaction was terminated by vigorous shaking and NO_2^- was estimated as in [10].

The CN^- -inactivated enzyme was prepared by treating NR with 0.4 mM CN^- in the presence of dithionite for 5 min. The enzyme was passed through a column of Sephadex G-25 equilibrated with 0.1 M phosphate buffer (pH 7.6) to remove excess cyanide and dithionite. The enzyme eluted from the Sephadex G-25 column was used as a cyanide-inactivated enzyme. Such an enzyme showed <10% activity in terms of NO_2^- produced using NADH or FMNH_2 as the reductant, as compared to the enzyme not inactivated by CN^- . Ferricyanide fully reactivated the CN^- -inactivated enzyme.

* To whom correspondence should be addressed

The reactivation of the enzyme was carried out in a volume of 0.2 ml in the presence of 0.2 mM FAD and light obtained from a 500 W tungsten lamp of a projector. A 10-cm thick water filter was used to prevent heating of the sample by the lamp.

3. RESULTS AND DISCUSSION

The CN^- -inactivated enzyme could be fully reactivated by ferricyanide, either in light or in the dark, in the presence or absence of oxygen (table 1). In all the reactions FAD (0.2 mM) was present. In the absence of ferricyanide, FAD in the dark, under aerobic conditions, could not reactivate the enzyme. However, in light under aerobic conditions, the reactivation was well over 90%. Under strict anaerobic conditions, FAD could not reactivate the enzyme in light although ferricyanide could fully restore the enzyme activity. This indicates the necessity of the presence of oxygen for

Table 1

Reactivation of CN^- -inactivated FMNH₂-NR by FAD in the presence and absence of oxygen, light and ferricyanide

Treatment	FMNH ₂ -NR (% activity)	
	- Ferricyanide	+ Ferricyanide
Dark, aerobic	9	100
Light, aerobic	93	100
Light, anaerobic	10	100
Light anaerobic made aerobic	100	100

The CN^- -inactivated enzyme along with 0.2 mM FAD in a volume of 0.5 ml was kept in a serum-capped vial. The solution was made anaerobic by blowing, for 1 h, nitrogen gas passed through a pyrogallol trap over it. The vials were incubated for 10 min in light or dark, as necessary. After incubation, aliquots of enzyme were further incubated for 2 min in the presence or absence of 1 mM potassium ferricyanide. The FMNH₂-NR activity of the aliquots was assayed as given in section 2. For aerobic treatment, the air was blown over the sample instead of oxygen-free nitrogen gas. In treatment 4 the irradiated sample under anaerobic conditions was further irradiated for 10 min after making the sample aerobic by blowing air over it. The 100% rate corresponds to 40 nmol NO_2^- produced.mg protein⁻¹.min⁻¹.

reactivation by FAD in light. However, presence of oxygen is not essential for ferricyanide activation. The incubation of the enzyme with FAD in light first in the absence of oxygen followed by its incubation in light in the presence of oxygen, leads to full reactivation. Thus the enzyme was not irreversibly inactivated by incubation in light under anaerobic conditions. The 10% activity observed in the dark in the presence of FAD is the residual activity of the CN^- -inactivated enzyme. The results presented in table 1 bring out the absolute requirement of oxygen during photoreactivation of CN^- , inactivated enzyme by FAD. As expected, chemical oxidation of Mo^{IV} of the enzyme by ferricyanide does not need the presence of oxygen. The data also show that reactivation of CN^- -inactivated FMNH₂-NR by FAD in light is not due to direct interaction of the photoexcited FAD molecules with the enzyme.

The requirement of the presence of oxygen during reactivation by FAD in light suggests that a species of oxygen produced by photoexcited FAD must be mediating the reactivation. Photoexcited flavins have been shown [11] to convert oxygen to superoxide anion (O_2^-). This superoxide anion can give rise to singlet oxygen. Under the experimental conditions used for the reactivation by FAD, we expect both superoxide anion and singlet oxygen to be present, and either or both could mediate reactivation of the enzyme by oxidation of the stable cyanide Mo^{IV} coordinated state to the unstable cyanide Mo^{VI} coordinated state.

In order to distinguish which of the two species of oxygen, viz. superoxide anion or singlet oxygen, was responsible for reactivation purposes, scavenger of superoxide anion, viz. ascorbate [12], and of singlet oxygen, viz. tryptophan and histidine [13], were used during photoreactivation of the enzyme by FAD. Data presented in table 2 show that the scavenger of superoxide anion, namely, ascorbate at 5 mM, inhibited the reactivation of the enzymes by FAD by about 95%. However, scavengers of singlet oxygen, viz. tryptophan and histidine, even at 60 mM did not inhibit the reactivation of the enzyme at all. These observations strongly suggest that the photoreactivation by FAD is mediated by O_2^- , and not by singlet oxygen. The observations in [14] that ammonium persulphate which liberates O_2^- reactivates CN^- -inactivated NR under aerobic condi-

Table 2

Effect of scavengers of superoxide anions and singlet oxygen on the photoreactivation of cyanide-inactivated enzyme by FAD

Addition	FMNH ₂ -NR % activity
None (control)	100
Ascorbate, 5 mM	4
Tryptophan, 60 mM	100
Histidine, 60 mM	100

The CN⁻-inactivated enzyme along with 0.2 mM FAD and different compounds at required concentrations were exposed to light for 10 min in the presence of 0.1 M potassium phosphate buffer, pH 7.6. The enzyme was then assayed for FMNH₂-NR activity as given in section 2. The control rates of activity were 40 nmol NO₂⁻ produced .mg protein⁻¹ .min⁻¹. None of the scavengers used by themselves inactivate the active FMNH₂-NR under identical conditions.

tions also supports the conclusion that O₂⁻ can oxidize the Mo^{IV} cyanide coordinate state and relieve inhibition.

The involvement of O₂⁻ during photoreactivation of CN⁻-inactivated NR by FAD is further supported by the data presented in fig. 1. We argued that if O₂⁻ was the species involved, the addition of superoxide dismutase (which specifically uses superoxide anions) to the reactivation mixture would delay reactivation. Data presented in fig. 1 show a time-dependent significant decrease in the extent of photoreactivation. Complete inhibition of reactivation in the presence of superoxide dismutase is not seen because the number of superoxide anions produced by FAD in light are in great excess, and the superoxide dismutase molecule may not be able to reach the site of molybdenum on the NR enzyme where superoxide anion is created by FAD in light.

The above results demonstrate that the reactivation of CN⁻-inactivated NR by FAD in light is mediated by O₂⁻. The superoxide anion may be oxidizing the stable Mo^{IV} cyanide coordinate state to the unstable Mo^{VI} cyanide coordinate state resulting in the liberation of CN⁻ and reactivation of the enzyme. It is tempting to suggest a physiological significance to the reactivation of the

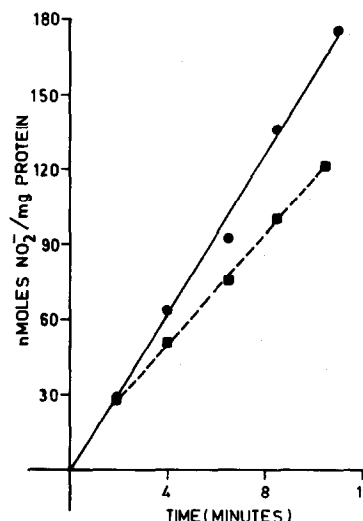


Fig. 1. Effect of superoxide dismutase on photoreactivation of CN⁻-inactivated enzyme by FAD. The inactivated enzyme along with 0.08 mM FAD and superoxide dismutase (75 units) in potassium phosphate buffer, in 0.1 ml aliquots, was exposed to white light. At different time intervals FMNH₂-NR activity was assayed as given in section 2 by adding 0.9 ml assay mixture. The rates on the Y-axis are for a 5-min period: (●—●) in the absence of superoxide dismutase; (■—■) in the presence of superoxide dismutase.

cyanide-inactivated enzyme by O₂⁻, as O₂⁻ is created in light by the light reactions of chloroplast. However, one has to consider the fact that NR is not located in the chloroplast and that the life time of O₂⁻ is short. It is unlikely that O₂⁻ produced in the chloroplast can interact with the NR located outside the chloroplast.

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