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NITRATE REDUCTASE FROM Azotobacter chroococcum. INACTIVATION BY OXIDIZING AGENTS AND REACTIVATION WITH DITHIOERYTHRITOL

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SUMMARY. Treatment of a partially purified nitrate reductase preparation from the aerobic bacterium Azotobacter chroococcum with a variety of oxidizing agents, such as glutathione, ferricyanide and illuminated flavins, results in inactivation of the enzyme. Independently of the mode of inactivation, incubation in the presence of dithioerythritol causes almost full recovery of nitrate reductase activity. Our data suggest that Azotobacter nitrate reductase might be regulated through an interconversion process between an oxidized inactive form and a reduced active one.

The assimilatory nitrate reductase from green cells, blue-green algae, and fungi can exist in vitro in two interconvertible forms: an oxidized active form and a reduced inactive one. The interconversion process appears to be of physiological significance and, in fact, nitrate reductase from different sources can be isolated, according with the selected experimental conditions, either in the active or the inactive form (1).

Buchanan's group has recently described a new regulatory system of chloroplast whereby enzymes are activated in the light by reduction and are inactivated in the dark by oxidation. This group has demonstrated that the light-dependent activation of several enzymes of the reductive pentose phosphate cycle is regulated via the ferredoxin/thioredoxin system. On the other hand, oxidants native to chloroplasts, such as oxidized glutathione or dehydroascorbate, have been implicated in the dark inactivation of those chloroplast enzymes (2,3).

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Thus, it turns up that the metabolic regulation of CO_2 and NO_3^- assimilation in green cells is governed by the redox state of the cell. However, the regulatory enzymes of both processes are affected in just the opposite way by the changes in the intracellular redox state (4).

We report now that the assimilatory nitrate reductase from the aerobic bacterium Azotobacter chroococcum is very sensitive towards different oxidants. Ferricyanide, glutathione and illuminated flavins inactivate the enzyme. Incubation of the inactive enzyme with dithioerythritol restores the initial activity.

MATERIALS AND METHODS. Azotobacter chroococcum (strain ATCC-4412 from the Salamanca University collection) was used throughout this study. Stock cultures of bacteria were kept as 3 ml suspensions in 60% (v/v) glycerol at -20°C. Preparation of a preculture from this stock culture and its inoculation into the liquid medium was as previously described (5). 12-14 h old cells of a nitrate-containing liquid culture coming from a stock culture in glycerol were always used for the preparation of cell-free extracts and the soluble nitrate reductase. The experimental conditions for the preparation of the partially purified nitrate reductase, as well as its routine assay, were carried out as previously described by Guerrero et al. (6). All the incubations took place in 0.1 M potassium phosphate buffer, pH 7.0. Appropiate controls demonstrated that the concentration of the reagents carried over into the assay mixtures did not have any interfering effect on the assay of activities.

RESULTS AND DISCUSSION. We observed that the levels of nitrate reductase activity in crude extracts of A. chroococcum significantly increased after incubation, at room temperature, with dithioerythritol (cf. 7,8). By contrast, treatment of crude extracts with ferricyanide -a compound which brings about the in vitro reactivation of the inactive form of nitrate reductase from plants and algae (1)- originated the inactivation of the Azotobacter nitrate reductase. These unexpected results prompted us to study the effect of several oxidizing agents on nitrate reductase from A.chroococcum.

Table I shows the effect of a variety of oxidants on the partially purified nitrate reductase. As can be seen, glutathione, ferricyanide,

Table I. Inactivation of nitrate reductase from A. chroococcum by oxidants and its reversal with dithioerythritol

Reagent	Concentration (mM)	Percentage of activity	+ Dithio-erythritol
None		100	110
Glutathione	15.0	40	90
Ferricyanide	0.3	8	90
2,6-Dichlorophenol-indophenol	0.5	8	65
NAD^+	0.5	100	100
$NADP^+$	0.5	100	100

Nitrate reductase (2 mg/ml) was incubated at 30°C in the presence of the compounds indicated in the table during 10 min. After incubation, nitrate reductase activity was determined on aliquots of the inactivation mixture. The inactivation mixture was made 5 mM in dithioerythritol, incubated for 60 min and then assayed for nitrate reductase activity.
The activities are expressed as percentages of the activity of the control without additions. Activity of the control was 420 nmoles nitrite formed/min per mg protein.

and 2,6-dichlorophenol-indophenol effected a strong inactivation of nitrate reductase activity. NAD^+ and $NADP^+$ were without effect. Once inactivation had taken place, nitrate reductase activity could not be restored by dialysis or gel filtration through Sephadex G-25. However, as shown in the table, incubation in the presence of dithioerythritol restored almost full activity. Reduced glutathione or cysteine were ineffective under the same experimental conditions.

Hepatic sulfite oxidase, another molybdenum-containing enzyme, is also inactivated by ferricyanide (9). Nevertheless, the inactivation of sulfite oxidase by ferricyanide was irreversible and evidence was provided to implicate the molybdenum center of the enzyme as the

site of inactivation. In the case of nitrate reductase just discussed, ferricyanide - and the other oxidants- might affect the sulfhydryl groups(s) of the active enzyne, an interpretation that is further strengthened by the experimental evidence presented below. As a matter of fact, ferricyanide has been used routinely as a reagent for sulfhydryl groups of proteins (10).

Due to the high electron affinity of the excited triplet state of flavin nucleotides (11), we investigated the effect of these irradiated pigments on nitrate reductase from A. chroococcum. It had been previously shown (12) that the inactive form of nitrate reductase from spinach leaves and from the green alga Chlorella fusca could be reactivated by blue-light, and that this reactivation process was enhanced in the presence of catalytic amounts of either FMN or FAD. As we shall now demonstrate the illumination of Azotobacter nitrate reductase in the presence of flavin nucleotides causes the loss of its catalytic properties.

Table II shows that nitrate reductase is inactivated by illuminated FMN and FAD. In the absence of flavin nucleotides no inactivation occurred. When any substance which can donate electrons to the excited flavin, such as EDTA (11), was included in the illuminated mixture, the enzyme was protected against inactivation. The significance of this experiment is that nitrate reductase itself might act as a reductant for the excited flavin nucleotide.

As in the above case of inactivation by chemical oxidants, the inactive enzyme formed by illumination in the presence of flavin nucleotide could be reactivated by dithioerythritol. As can be seen in Table II, after treatment with 5 mM dithioerythritol all of the nitrate reductase activity was recovered. Under the same experimental conditions, reduced glutathione or cysteine were unable to reverse the photoinactivation of nitrate reductase.

Table II. Photoinactivation of nitrate reductase from A. chroococcum by flavin nucleotides and its reversal with dithioerythritol

Addition	Percentage of activity	
		+ Dithio-erythritol
None	100	125
FMN	10	110
FAD	15	100
FMN, plus EDTA	105	125
FMN, in the dark	100	125

Nitrate reductase (2 mg/ml) was incubated at 30°C under an atmosphere of argon in Warburg manometer flask during 8 min. Illumination of 25,000 lux, at the botton of the vessels, was provided from below by a bank of cold beam 100 W lamps. Where indicated, 0.2 mM of either FMN or FAD and 1 mM EDTA was added. At the end of the illumination time, nitrate reductase was determined on aliquots of the incubation mixture. The incubation mixture was then made 5 mM in dithioerythritol, incubated under air for 60 minutes, without illumination, and finally assayed for nitrate reductase activity. The activities are expressed as percentage of the activity of the control at the time of incubation zero. Activity of the control was 440 nmoles nitrite formed/min per mg protein.

By using blue and red plexiglas filters it could be ascertained that the photoinactivation of nitrate reductase was mediated by blue but not red light. As it is known, several flavoproteins were also found to be inactivated by blue light (13).

Our results strongly suggest that the site of action of illuminated flavin nucleotide might be SH-groups which are essential for the structure and catalysis of nitrate reductase (6). A similar proposal has been formulated for the inhibition of some enzymes by excited flavins (14).

It is worthy of mention that the soluble nitrate reductase from A. chroococcum has the characteristic features of Pichinoty's type B

nitrate reductase, i.e., the rate of nitrate reduction diminishes as a function of time during its assay with dithionite -reduced methyl viologen as the reductant (15,5,6). This decay in rate of nitrate reduction could be hindered by the presence of cyanate in the reaction mixture. Furthermore, cyanate was also able to reactivate the reaction to its maximal rate if added once inactivation had occurred. The mechanism of action of cyanate, which must be present in the reaction mixture in order to exercise its peculiar effect, remains to be clarified (6).

The results of the present paper have shown that the bacterial nitrate reductase is very sensitive towards natural and artificial oxidants. These compounds might act upon SH- groups, and the opening and closing of disulfide bonds would explain the interconversion process between a reduced active form and an oxidized inactive one. Since it has been shown that thioredoxin is the natural electron donor implicated in the reduction of disulfides bonds and that it can be reduced by dithiols (2,16), it is tempting to assume the involvement of this electron transferring cofactor in the metabolic regulation of Azotobacter nitrate reductase.

In order to verify this possibility, the crude enzyme preparation used in this work is now being fractionated. Preliminary experiments have shown that nitrate reductase can be inactivated by oxidants after gel filtration on Sephadex G-100. However, the dithioerythritol activation of this inactive enzyme depended on some protein factor(s) that emerged from the Sephadex column after the nitrate reductase peak. Work is in progress to identify this protein factor(s).

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