INACTIVATION BY AMMONIA OF THE PHOTOSYNTHETIC REDUCTION OF NITRATE IN *Nostoc muscorum* PARTICLES

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SUMMARY. Ammonia, as well as other uncouplers of photophosphorylation, strongly inhibit the photosynthetic reduction of nitrate by particles of the blue-green alga *Nostoc muscorum*. The enzyme responsible for nitrate reduction, nitrate reductase, can be reversibly inactivated by reduction in a ferredoxin-dependent reaction. Nitrate protects against this inactivation, and molecular oxygen restores the original activity.

The reduction of nitrate to ammonia with water carried out by blue-green algae is one of the most simple and relevant examples of photosynthesis (1-3). Since both nitrate photoreduction through the ferredoxin-dependent nitrate-reducing system and $NADP^+$ photoreduction through ferredoxin-$NADP^+$ reductase are similar reactions, nitrate can be regarded, like $NADP^+$, as a genuine natural Hill reagent. Moreover, ammonia, the end product of photosynthetic non-cyclic electron flow with nitrate as Hill reagent, is a physiological uncoupler of photophosphorylation, and thus promotes an increase in the cellular levels of the reduced form of nicotinamide nucleotides and of the uncharged forms of adenine nucleotides (4,5). According to present evidence from our laboratory, these fluctuations in reducing power and energy charge seem to be critical for the metabolic interconversion

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Abbreviations: CMU: 3-(4-chlorophenyl)-1,1-dimethylurea; MVH: reduced methyl viologen; NO_3Rase: nitrate reductase.

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of nitrate reductase, the first enzyme of the assimilatory pathway leading to ammonia formation, which can exist in an active oxidized form and in an inactive reduced form (6,7).

The present paper reports the inhibition exerted by ammonia and other uncouplers on the photoreduction of nitrate by *Nostoc muscorum* particles. The paper deals also with the mechanism of the reversible inactivation of nitrate reductase from this blue-green alga.

MATERIALS AND METHODS

N. muscorum cells (strain 7119 from the Department of Cell Physiology, Berkeley, USA, a gift of Dr. D.I. Arnon) were cultivated autotrophically in the light (5,000 lux) at 25°C on 5% CO_2 in air, using the culture medium of Arnon *et al.* (8) but with KNO_3 (20 mM) as nitrogen source. The particulate systems A and B used in the experiments reported here were prepared as previously described (2) and lacked nitrite reductase activity. The standard assay for nitrate reductase using reduced methyl viologen as electron donor was carried out at 30°C for 7 min in a reaction mixture containing in a final volume of 1 ml: 100 µmol of glycine-KOH buffer, pH 10.5; 40 µmol of KNO_3; 1.6 µmol of methyl viologen; 8 µmol of $Na_2S_2O_4$ neutralized with 15 µmol of $NaHCO_3$, and a convenient amount of active particles. The reaction was stopped by destroying dithionite with vigorous shaking in a Cyclomixer and then the nitrite formed was determined colorimetrically according to Snell and Snell (9). In reactivation experiments with ferricyanide (FeCy), the incubation mixtures were treated with 0.3 mM potassium ferricyanide for 2 min at 0°C prior to determination of activities. Special experimental conditions are described in the captions of the corresponding Tables and Figure. *N. muscorum* ferredoxin was prepared as indicated by Mitsui and Arnon (10). Chlorophyll was determined as described by Arnon (11). Protein was estimated with the Folin-phenol reagent (12).

RESULTS AND DISCUSSION

In 1970 we found that addition of ammonia to *Chlorella* cells growing in the light on nitrate led to a rapid inactivation of nitrate reductase and that subsequent removal of ammonia led

to its reactivation (13). *Clamydomonas reinhardi*, another green alga, showed the same behaviour (14). It was an unexpected finding that the *in vivo* inactivated enzyme appeared to be in its reduced state and could be reactivated by oxidation with ferricyanide (14,15). Although these and other results (16,17) were explained on the basis of uncoupling by ammonia, the validity of this interpretation had to await more critical experiments to be performed at the subcellular level, particularly since Vennesland's group challenged our thesis and came to the conclusion that the inactivation process was associated with the binding of cyanide to the enzyme protein and the reactivation process with its release (18,19).

Since chlorophyll-containing particles prepared from blue-green algae under the specified conditions contain firmly bound ferredoxin-dependent nitrate-reductase, they represent an excellent material for testing at the subcellular level the light-effect of ammonia on nitrate reductase inactivation. As Table I shows, ammonia drastically inhibits the photosynthetic reduction of nitrate by *N. muscorum* particles. Since ammonia (results not shown) uncouples photophosphorylation and does not itself affect nitrate reductase activity assayed in the dark with either dithionite-methyl viologen or NADPH-ferredoxin NADP reductase as electron donor, its effect on nitrate photoreduction can be best explained as an indirect result of its uncoupling action. For a comparison, Table I shows also that, under the same conditions, the Hill reaction with $NADP^+$ as electron acceptor is not at all inhibited, but slightly stimulated, in the presence of ammonia.

The interpretation that the ammonia effect on nitrate photore-

TABLE I

EFFECT OF AMMONIA ON NITRATE AND $NADP^+$ PHOTOREDUCTION BY PARTICLES OF *Nostoc muscorum*

Reaction system	Nitrite formed (nmoles)	NADPH formed (nmoles)
Nitrate system		
Complete	177	-
plus ammonia	17	-
$NADP^+$ system		
Complete	-	118
plus ammonia	-	137

The complete system contained in a final volume of 1 ml, 50 µmol of Tricine-KOH buffer, pH 7.7; 40 µmol of KNO_3 or 1 µmol of $NADP^+$; 10 µmol of $MgCl_2$; 2 µmol of ADP; 2 µmol of potassium phosphate; 0.1 mg of *N. muscorum* ferredoxin; *N. muscorum* particles B containing 26 µg of chlorophyll, and, where indicated, 10 µmol of NH_4Cl. The reaction was carried out in air at 30°C for 20 min. Light intensity, 20,000 lux.

duction is not directly on nitrate reductase but rather indirectly through non-cyclic electron flow, which supplies reducing power to the enzyme, is further reinforced by the experiments presented in Tables II and III. We observed 1) that two other well-known uncouplers of photophosphorylation, methylamine and arsenate, behave like ammonia in inhibiting nitrate photoreduction (Table II), and 2) that preincubation of the particles in the light leads to a strong inactivation of their bound nitrate reduct

TABLE II

EFFECT OF AMMONIA AND OTHER UNCOUPLERS ON NITRATE PHOTOREDUCTION BY PARTICLES OF *Nostoc muscorum*

System	NO_2^- formed (nmoles)
Complete	124
plus ammonia	19
plus arsenate	45
plus methylamine	27

The complete system was as described in the legend of Table I. Where indicated, 10 µmol of NH_4Cl, 40 µmol of sodium arsenate or 10 µmol of methylamine were added. In the arsenate experiment, potassium phosphate was omitted from the reaction mixture.

ase (Table III). Furthermore the inactivation process depends on photosynthetically generated reducing power, since it does not occur when an inhibitor of the non-cyclic electron flow is added or when ferredoxin, which mediates electron transfer between illuminated particles and the enzyme, is omitted. Nitrate, the enzyme substrate, provides a high degree of protection against inactivation (5-7,19).

As previously shown (5-7) for nitrate reductase from eukaryotic cells, which contains an aditional NAD(P)H-diaphorase activity and depends on NAD(P)H both as electron donor and inactivating re-

TABLE III

CHARACTERIZATION OF NITRATE REDUCTASE INACTIVATION BY ILLUMINATION IN *Nostoc muscorum* PARTICLES

System	Relative Activity (%)
Complete, dark	100
Complete, light	34
plus CMU	95
plus KNO_3	94
minus Fd	88

The preincubation mixtures included in a final volume of 1 ml: 50 µmol of Tricine-KOH buffer, pH 7.7; *N. muscorum* particles B containing 10 µg of chlorophyll; 10 µmol of $MgCl_2$ and, where indicated, 0.1 mg of *N. muscorum* ferredoxin, 100 nmol of CMU and 40 µmol of KNO_3. Preincubation time, 30 min at 30°C. Light intensity, 20,000 lux. Nitrate reductase activity was determined as indicated in Materials and Methods. If necessary, activity was corrected by subtracting the nitrite formed during preincubation. One hundred percent activity corresponded to 48 nmol of nitrite formed.

ductant, inactivation by reduction of the *Nostoc* enzyme can also be reversed by reoxidation. Figure 1 shows that when an active preparation of *N. muscorum* pigment-containing particles is illuminated its nitrate reductase becomes inactivated, but recovers its initial activity on exposure to oxygen in the dark.

N. muscorum ferredoxin-nitrate reductase can also be inactivated

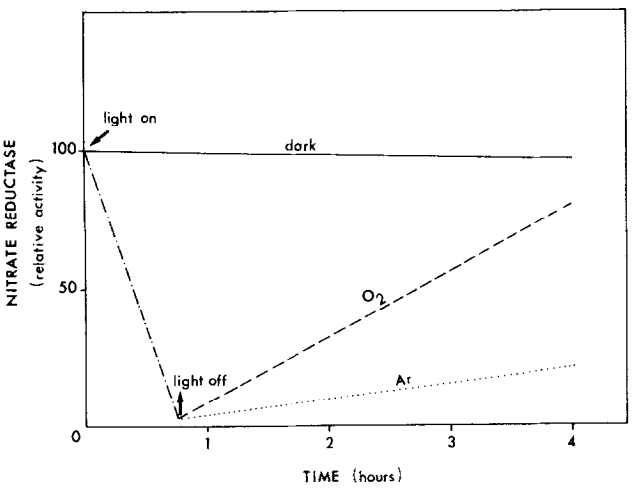

Figure 1. *Inactivation by light and reactivation by oxygen of Nostoc muscorum nitrate reductase.* The preincubation mixture, including in a final volume of 2 ml: 100 µmol of Tricine-KOH buffer, pH 7.7; 20 µmol of $MgCl_2$; 0.2 mg of *N. muscorum* ferredoxin and *N. muscorum* particles B containing 0.1 mg of chlorophyll, was kept at 30°C for 45 min in the light (20,000 lux). The treated particles were then maintained in the dark either under Ar or under O_2 up to 4 h. MVH-NO_3Rase activities were determined in 0.2 ml aliquots as described in Materials and Methods and are expressed as percent of the initial activity.

in the dark when preincubated with ferredoxin reduced by the NADPH-NADP reductase system (Table IV). Here again nitrate protects against inactivation, and the reduced inactive enzyme can be immediately reactivated by treatment with ferricyanide.

Finally, it is worth mentioning that *Nostoc* nitrate reductase can also be chemically and reversibly inactivated in the absence of ferredoxin by direct reduction with dithionite, and that cyanate, an artificial analogue that binds to the enzyme protein at the same site as nitrate (5-7), protects against this inactivation (results not shown).

TABLE IV

FERREDOXIN-DEPENDENT REVERSIBLE INACTIVATION OF *Nostoc muscorum* NITRATE REDUCTASE BY NADPH AND ITS PROTECTION BY NITRATE

Addition	Relative Activity (%)	
	-FeCy	+FeCy
None	100	100
NADPH	78	95
NADPH, Fd	18	79
NADPH, Fd, KNO_3	85	95

The preincubation mixtures included in a final volume of 0.5 ml: 25 µmol of Tris-HCl buffer, pH 8.5; *N. muscorum* particles A containing 4 mg of protein, and, where indicated, 0.1 mg of *N. muscorum* ferredoxin, 4.8 µmol of NADPH, and 20 µmol of KNO_3. Preincubation time, 2 h at 30°C. Nitrate reductase activity with and without FeCy was determined as indicated in Materials and Methods. If necessary activity was corrected by subtracting the nitrite formed during preincubation. One hundred percent activity corresponded to 55 nmol of nitrite formed.

Our present results provide strong support for our proposed mechanism for the metabolic interconversion of nitrate reductase: ammonia induces the transformation of the active form into the inactive one by indirectly promoting through uncoupling the reduction of the enzyme protein, and nitrate preserves the active form in its functional oxidized state by binding to it.

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REFERENCES

1. Manzano, C., Candau, P., Gómez-Moreno, C., Relimpio, A.M. and Losada, M. (1976) Mol. Cell. Biochem. 10, 161-169.
2. Ortega, T., Castillo, F. and Cárdenas, J. (1976) Biochem. Biophys. Res. Commun. 71, 885-891.
3. Candau, P., Manzano, C. and Losada, M. (1976) Nature 262, 715-717.
4. Chaparro, A., Maldonado, J.M., Diez, J., Relimpio, A.M. and Losada, M. (1976) Plant Sci. Letters 6, 335-342.
5. Losada, M. (1976) J. Molec. Catal. 1, 245- 264
6. Losada, M. (1974) Metabolic Interconversion of Enzymes 1973 (Fisher, E.H., Krebs, E.G., Neurath, H. and Stadtman, E.R., eds.), pp. 257-270,Springer,Berlin.
7. Losada, M. (1976) Reflections on Biochemistry (Kornberg,A., Horecker, B.L., Cornudella, L. and Oró, J., eds.), pp. 73-84 Pergamon Press, Oxford.
8. Arnon, D.I., McSwain, B.D., Tsujimoto, H.Y. and Wada, K. (1974) Biochim. Biophys. Acta 357, 231-245.
9. Snell, E.D. and Snell, C.T. (1949) Colorimetric Methods of Analysis, p. 804, D. Van Nostrand Company, Academic Press, New York.
10. Mitsui, A. and Arnon, D.I. (1971) Physiol. Plant. 25, 135-140.
11. Arnon, D.I. (1949) Plant Physiol. 24, 1-15.
12. Lowry, O.H., Rosebrough, M.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265-275.
13. Losada, M., Paneque, A., Aparicio, P.J., Vega, J.M., Cárdenas, J. and Herrera, J. (1970) Biochem. Biophys. Res Commun. 38, 1009-1015.
14. Herrera, J., Paneque, A., Maldonado, J.M., Barea, J.L. and Losada, M. (1972) Biochem. Biophys. Res. Commun. 48, 996-1003.
15. Losada, M.,Herrera, J., Maldonado, J.M. and Paneque, A. (1973) Plant Sci. Lett. 1, 31-37.
16. Moreno, C.G., Aparicio, P.J., Palacián, E. and Losada, M. (1972) FEBS Lett. 26, 11-14.
17. Maldonado, J.M., Herrera, J., Paneque, A. and Losada, M. (1973) Biochem. Biophys. Res. Commun. 51, 27-33.
18. Lorimer, G.H., Gewit, H., Völker, W., Solomonson, L.P. and Vennesland, B. (1974) J. Biol. Chem. 249, 6074-6079.
19. Pistorius, E.K., Gewitz, H., Voss, H. and Vennesland, B. (1976) Planta 128, 73-80.