METABOLIC INTERCONVERSION OF FERREDOXIN-NITRATE REDUCTASE AND NADP REDUCTASE OF *Nostoc muscorum*

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SUMMARY. Ammonia is a physiological uncoupler of photophosphorylation which drastically inhibits the photosynthetic reduction of nitrate by *Nostoc muscorum* particles by promoting the conversion of ferredoxin-nitrate reductase into its reduced inactive form. Ammonia promotes also, after an initial stimulation, the total inhibition of the photosynthetic reduction of $NADP^+$. Like its nitrate counterpart, ferredoxin-NADP reductase is reversibly inactivated by reduction and reactivated by oxidation.

Ferredoxin-nitrate reductase and NADP reductase from blue-green algae are partner enzymes which catalyze, respectively, the ferredoxin-dependent photosynthetic reduction of nitrate and $NADP^+$ (1-3). For this reason, nitrate, like $NADP^+$, can be regarded as a genuine Hill reagent, whose reduction is coupled with the oxidation of water to molecular oxygen. The end product of nitrate photoreduction by the nitrate-reducing system is ammonia, a physiological uncoupler of photophosphorylation that promotes an increase in the cellular levels of the reduced form of nicotinamide nucleotides and of the uncharged forms of adenine nucleotides (3-5).

According to present evidence (6,7), the regulated step in the process of nitrate assimilation by the photosynthetic nitrate-reducing system is the reduction of nitrate to nitrite, catalyzed by the first enzyme of the pathway. Previous results

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from our laboratory (3,8) have shown that, in fact, ammonia regulates the activity of nitrate reductase from blue-green algae -as well as from other photosynthetic cells- by indirectly promoting the interconversion of the enzyme between an active oxidized form and an inactive reduced form. Vennesland's group, however, has challenged our thesis and come to the conclusion that cyanide controls physiologically the regulation of nitrate reductase (9).

The present paper reports the comparative effect of ammonia on the photosynthetic reduction by *Nostoc muscorum* particles of the two natural Hill-reagents nitrate and $NADP^+$ and of the artificial Hill-reagent ferricyanide. It also shows that *Nostoc* ferredoxin-NADP reductase, like ferredoxin-nitrate reductase, can be reversibly inactivated by reduction and reactivated by oxidation.

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.* (10) but with 20 mM KNO_3 as nitrogen source. The particulate systems B and C used in the experiments reported here were prepared as previously described (2). The crude extracts were prepared as follows: Algal cells were harvested by centrifugation and washed with 50 mM Tris-HCl buffer, pH 8.0. The washed cells were resuspended in the same buffer and disrupted in the cold with a Sonifier Branson 12 (20 Kc/70 W) for 40 sec. The broken material was then centrifuged at 5,000 x *g* for 10 min, and the supernatant used as crude extract.

Ferredoxin-NADP reductase activity was determined spectrophotometrically at 550 nm as described by Shin (11) by the assay method for the reverse reaction, involving the reduction of cytochrome *c*, as the terminal acceptor, in the presence of ferredoxin and NADPH. The reaction mixture contained in a total volume of 2 ml: 100 µmol of Tris-HCl buffer, pH 8.0; 1.5 mg of cytochrome *c*; 0.2 mg of *N. muscorum* ferredoxin; 0.5 µmol of NADPH, and enzyme preparation. The reaction was carried out at room temperature.

N. muscorum ferredoxin was prepared as described by Mitsui

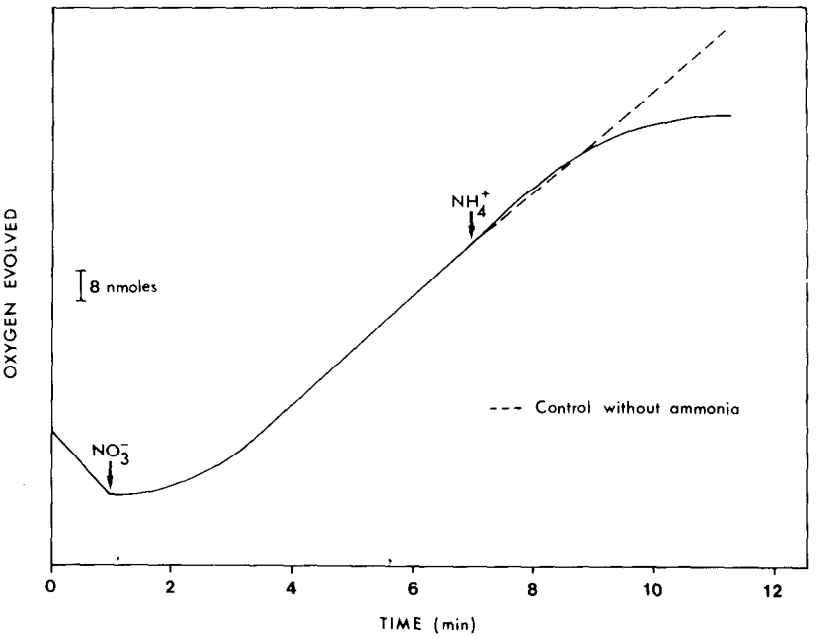

Fig. 1. *Inhibition by ammonia of photosynthetic nitrate reduction by N. muscorum particles.* The reaction mixtures included in a final volume of 3 ml: 150 µmol of Tricine-KOH buffer, pH 7.7; 30 µmol of $MgCl_2$; 0.3 mg of *N. muscorum* ferredoxin, and *N. muscorum* particles C containing 50 µg of chlorophyll. Where indicated, 120 µmol of KNO_3 and 30 µmol of NH_4Cl were added. The reaction was carried out at 30°C in the chamber of an oxygen electrode. Light intensity, 20,000 lux.

and Arnon (12). Chlorophyll was determined as described by Arnon (13). Protein was estimated using the Folin-phenol reagent as described by Lowry *et al.* (14). Oxygen evolution was measured with a Clark-type electrode.

RESULTS AND DISCUSSION. Figure 1 corroborates our previous results (3) and shows that, after an initial slight stimulation, the Hill reaction with nitrate as terminal electron acceptor by *N. muscorum* particles is rapidly and completely inhibited by ammonia. It can also be seen that, under the same experimental conditions, the photosynthetic reduction of nitrate proceeds linearly with time, for at least 10 min, in the absence of ammonia.

When nitrate is replaced by $NADP^+$ as Hill reagent, ammonia

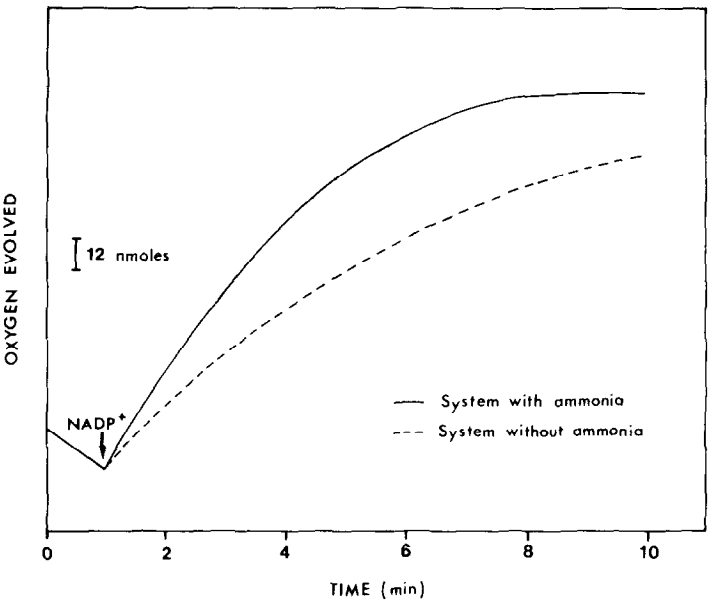

Fig. 2. *Inhibition of photosynthetic NADP$^+$ reduction by N. muscorum particles in the absence and in the presence of ammonia.* Experimental conditions were as described in the caption of Fig. 1, except that 4 µmol of NADP$^+$ were used instead of nitrate and that ammonia was present in the corresponding reaction mixture before the addition of NADP$^+$.

promotes also, after an initial significant stimulation, a total inhibition of NADP$^+$ photoreduction. However, by contrast with nitrate, the rate of photosynthetic NADP$^+$ reduction decreases also steeply in the absence of ammonia (Figure 2).

For a comparative analysis, ferricyanide was also assayed as Hill reagent in the absence and in the presence of ammonia. The experiment showed that ammonia does only induce a certain stimulation but no inhibition of ferricyanide photoreduction (results not shown).

The effect of ammonia on the photosynthetic reduction of NADP$^+$ might, in principle, be interpreted, like in the case of

Table I. INACTIVATION BY REDUCTION WITH LIGHT AND NAD(P)H OF FERREDOXIN-NADP REDUCTASE OF *N. muscorum*

Inactivating system	Relative activity (%)
Control (dark)	100
Control (light)	80
NADPH (dark)	58
NADPH (light)	40
NADH (dark)	78

The preincubation mixtures included in a final volume of 1 ml: 50 µmol of Tricine-KOH buffer, pH 8.0; 10 µmol of $MgCl_2$; 0.1 mg of *N. muscorum* ferredoxin; *N. muscorum* particles B containing 20 µg of chlorophyll, and, where indicated, 0.5 µmol of NADPH or NADH. Preincubation time, 20 min at 25°C. Light intensity, 20,000 lux. Ferredoxin-NADP reductase activity was determined in 0.1 ml aliquots of the preincubation mixtures as indicated in Materials and Methods. One hundred per cent activity corresponded to 6.6 nmol of cytochrome *c* reduced per min.

nitrate (3), as a consequence of its uncoupling action on non-cyclic photophosphorylation, which leads to an increased rate of generation of reducing power.

To test this hypothesis a preparation of *N. muscorum* particles was preincubated in the dark and in the light in the presence and in the absence of NAD(P)H as reductant, and ferredoxin-NADP reductase activity was determined afterwards in aliquots of the preincubation mixtures. Table I shows that light by itself and NADPH, rather than NADH, cause a marked inactivation of ferredoxin-nitrate reductase. The inactivated

Table II. REACTIVATION BY OXIDATION WITH $NADP^+$ OR FERRICYANIDE OF NADPH-INACTIVATED FERREDOXIN-NADP REDUCTASE OF *N.muscorum*

Addition	Relative specific activity (%)
None	42
$NADP^+$	92
Ferricyanide	110

N. muscorum crude extract containing 2.0 mg of protein was preincubated in a final volume of 5 ml with 500 µmol of Tris-HCl buffer, pH 8.0, and 2.5 µmol of NADPH. After 20 min at room temperature, $NADP^+$ (1 µmol) or potassium ferricyanide (0.5 µmol) were added to 0.5 ml aliquots of the preincubation mixture. After 10 min at room temperature ($NADP^+$ system) or 3 min at 0-4°C (ferricyanide system), the aliquots were passed through Sephadex G-25 columns, previously equilibrated with 50 mM Tris-HCl buffer, pH 8.0. Ferredoxin-NADP reductase activity was then determined as described in Materials and Methods. Activities are expressed as percentages of the activity of a control preincubated in the absence of NADPH and filtrated through Sephadex G-25. One hundred per cent activity corresponded to 420 nmol of cytochrome *c* reduced per min per mg of protein.

enzyme keeps its inactivation degree after passage through a Sephadex G-25 column and is apparently in its reduced form for it can be reactivated by oxidation with $NADP^+$ or ferricyanide (Table II).

Ferredoxin-NADP reductase of Shin, Tagawa and Arnon is the same flavoprotein enzyme as the NADPH diaphorase of Avron and Jagendorf, the transhydrogenase of Keister, San Pietro and Stolzenbach, the NADPH-cytochrome *f* reductase of Forti and the NADPH-plastocyanin reductase of Katoh, and can, therefore, use

Table III. PROTECTION OF *N. muscorum* FERREDOXIN-NADP REDUCTASE AGAINST INACTIVATION BY NADPH

Addition	Relative specific activity (%)
None	100
NADPH	46
NADPH, $NADP^+$	107
NADPH, NAD^+	54
NADPH, FAD	130
NADPH, Fd	83

The preincubation mixtures included in a final volume of 1 ml: 100 µmol of Tris-HCl buffer, pH 8.0; *N. muscorum* crude extract containing 0.53 mg of protein and where indicated, 0.5 µmol of NADPH, 2 µmol of $NADP^+$ or NAD^+, 20 nmol of FAD or 0.15 mg of *N. muscorum* ferredoxin (Fd). After 20 min at room temperature, 0.5 ml aliquots were taken and passed through Sephadex G-25 columns, equilibrated beforehand with 50 mM Tris-HCl buffer, pH 8.0. Ferredoxin-NADP reductase was then determined as described in Materials and Methods. One hundred per cent activity corresponded to 260 nmol of cytochrome *c* reduced per min per mg of protein.

NAD(P)H and reduced ferredoxin as reductants, and $NAD(P)^+$, oxidized ferredoxin, FAD and a variety of dyes as oxidants (15). Table III shows that inactivation by NADPH of ferredoxin-NADP reductase can in fact be protected by the oxidized forms of several of its physiological coenzymes or cofactors, specially $NADP^+$ and FAD. Forti has previously reported (16) that the diaphorase but not the cytochrome *f* reductase activity of the spinach enzyme is inhibited by preincubation with NADPH.

Inhibition of photosynthetic $NADP^+$ reduction may not be

limited in its mechanism to the inactivation by reduction of the enzyme ferredoxin-NADP reductase. In fact, Arnon and Chain (17) have recently shown that preincubation of chloroplasts with NADPH and ferredoxin greatly stimulates cyclic photophosphorylation by diminishing the electron flow from water, as tested by measuring its effect on the photoreduction of C_{550}, a primary indicator of photosystem II activity.

Our present results provide strong support for our proposed mechanism for the metabolic interconversion of photosynthetic nitrate reductase as well as NADP reductase. Ammonia induces the transformation of the active forms of the enzymes into their inactive ones by indirectly promoting, through uncoupling of photophosphorylation, the reversible reduction of the enzyme proteins. This interpretation differs fundamentally from the complicated model proposed recently by Solomonson (18) in order to try to explain the regulation of nitrate reductase by cyanide.

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