Light and Dark Reduction of Nitrite in a Reconstituted Enzymic System*

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Kessler (1) has studied the reduction of nitrite to ammonia by the green alga Ankistrodesmus both in the light in an atmosphere of nitrogen and in the dark under hydrogen. No specific conclusion could be drawn as to the mechanism of the process, although it was assumed that reduced pyridine nucleotides might serve as the immediate hydrogen donors for the reduction of nitrite and that energy-rich phosphate bonds were specifically required for the occurrence of the reaction. The production of "assimilatory power" by noncyclic photophosphorylation (2) was, therefore, considered a prerequisite for nitrite reduction. Nicholas (3), working with cell-free enzyme preparations from Neurospora, found also that phosphorylations were involved in the reduction of nitrite.

Rousso and Nason (4) purified from extracts of soybean leaves a soluble "pyridine nucleotide-nitrite enzyme" which, in the presence of an unidentified, heat-stable, organic factor obtained from the same extracts, required nitrite in order to catalyze the oxidation of reduced di- or triphosphopyridine nucleotide. However, they could not demonstrate any disappearance of nitrite concomitant with the enzymic oxidation of pyridine nucleotide, and the fate of nitrite remained unknown.

More recently, Hageman, Creswell, and Hewitt (5) have shown that the reduction of nitrite to ammonia by marrow leaf enzymes was required in addition to grana for the photochemical reduction of nitrite. The production of "assimilatory power" by noncyclic photophosphorylation (2) was considered a prerequisite for nitrite reduction. Nicholas (3), working with cell-free enzyme preparations from Neurospora, found also that phosphorylations were involved in the reduction of nitrite.

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Huzisige and Satoh (8) isolated from spinach leaves a soluble enzyme preparation, "photosynthetic nitrite reductase," which was required in addition to grana for the photochemical reduction of nitrite. In previous work from our laboratory (9, 10), it has been shown, however, in spinach chloroplasts, that the reduction of nitrite itself is a dark reaction. The mechanism involved in the process was found to be similar to the one implicated in the photosynthetic reduction of TPNH (11). To take place, the reduction of nitrite required, in addition to light- or dark-reduced spinach ferredoxin, a thermostable factor (nitrite reductase) also present in the chloroplast extract.

The present report is concerned with the purification of the enzymes involved in the dark and light reduction of nitrite, and with the characterization of the process in a reconstituted enzymic system. Of particular interest in the present investigation is that energy-rich phosphate is not required for the reduction of nitrite, but when nitrite is reduced in the light, adenosine triphosphate is produced simultaneously in stoichiometric amounts.

**EXPERIMENTAL PROCEDURE**

**Preparation of Chloroplast Fragments and Ferredoxins**—Broken chloroplasts or once-washed broken chloroplasts were prepared from spinach according to Whatley, Allen, and Arnon (12). Spinach and C. pasteurianum ferredoxins were obtained as described by Tagawa and Arnon (11).

**Purification of Spinach Nitrite Reductase**—A crude homogenate of spinach leaves was prepared as described by San Pietro and Lang (13) except that, as indicated by Tagawa and Arnon (11), the pH was adjusted with Tris buffer to pH 7.3. Precipitation with acetone, extraction of the precipitate, and dialysis were also carried out according to San Pietro and Lang (13), but at pH 7.3.

The dialyzed extract was supplemented with sufficient NaCl to give a final concentration of 0.2 M, and passed through a DEAE-cellulose bed, 3 × 6 cm, equilibrated with 0.15 M Tris (pH 7.3)-O.2 M CI- in order to adsorb the ferredoxin. The nitrite reductase fraction which went through the column was precipitated with ammonium sulfate between 0 and 70% saturation. After standing for 20 minutes, the suspension was centrifuged for 10 minutes at 27,000 × g, and the supernatant solution was discarded. The sediment was suspended in Tris 0.05 M, pH 7.3, and dialyzed overnight against the same buffer in the cold. This purified spinach nitrite reductase preparation exhibited ferredoxin-TPN reductase activity (14) as assayed by the method of Avron and Jagendorf (15), but was free of ferredoxin.

**Purification of C. pasteurianum Hydrogenase**—C. pasteurianum strain W-5 was obtained from the American Type Culture Collection, Washington, D. C. The lyophilized culture was transferred to the potato medium of Jensen and Spence (16) and then to the medium of Carnahan and Castle (17), with ammonia as the nitrogen source. A crude extract of C. pasteurianum was prepared by autolysis of the dried cells according to the technique of Carnahan et al. (18), except that 0.05 M Tris buffer, pH 7.3, was used. In order to adsorb the ferredoxin, the crude
extract was passed through a DEAE-cellulose bed equilibrated with the same buffer. The hydrogenase-containing fraction which came through the column was treated with calcium phosphate gel (2 mg per mg of protein), allowed to stand for 10 minutes, and centrifuged for 5 minutes at 2000 x g. The supernatant liquid was discarded, and the sediment washed by suspending it in Tris, 0.7 M, pH 7.3. The suspension was allowed to stand for 10 minutes with occasional mixing and centrifuged as before, and the supernatant solution was discarded. The pellet was suspended in 0.1 M sodium pyrophosphate buffer, pH 7.0, in order to elute the hydrogenase, and the suspension was allowed to stand for 10 minutes with occasional stirring. After centrifugation at 2000 x g for 5 minutes, the sediment was discarded; the resulting supernatant solution contained the hydrogenase. All steps were performed at 0-4° in the presence of 3 mM cysteine. In the presence of ferredoxin the purified Clostridium hydrogenase did not catalyze either TPN+ or nitrite reduction with molecular hydrogen (6, 7, 19). The enzyme was also unable to reduce methylene blue; however, it liberated H2 in the presence of reduced methyl viologen.

**Chemicals—**ADP, TPN+, TPNH, DEAE-cellulose, and L-cysteine were purchased from Sigma Chemical Company. Methylene viologen was purchased from Mann Research Laboratories. Na2HPO4 was obtained from the J. E. N., Madrid, Spain. The calcium phosphate gel was prepared according to Keilin and Hartree (20), but Na2HPO4 was used instead of Na2HPO4 and NaOH was added to adjust the pH to 7.4.

**Analytical Methods—**Oxygen evolution or hydrogen uptake was estimated in Warburg manometer flasks. When the reactions were carried out in light, illumination was provided from below by a 100-watt fluorescent lamp and from above by a bank of 100-watt reflector flood lamps at distances of 3 and 14 cm, respectively, from the reaction vessels.

Nitrite was estimated by the method of Novak and Wilson (21). Ammonia was determined by nesslerization after diffusion and absorption of the gas in 0.01 N H2SO4 in Conway units (22). Radioactive adenocose triphosphate was measured by the technique of Arnon, Whatley, and Allen (23). Protein concentration was assayed by the method of Lowry et al. (24).

**RESULTS AND DISCUSSION**

The experiments to be described on the mechanism of nitrite reduction in a reconstituted enzymic system confirm previous investigations from this laboratory (9, 10) and reveal that ferredoxin is always involved in the process. Ferredoxin itself is not the enzyme which catalyzes the reduction of nitrite, but the electron carrier which functions in collaboration with the spinach nitrite reductase in the transfer of electrons to nitrite.

**Photochemical Reduction of Nitrite**—The reduction of nitrite in the light required spinach ferredoxin and spinach nitrite reductase in addition to illuminated grana (Table I). No stimulation of nitrite reduction by Mn++ ions was observed (4, 5).

<table>
<thead>
<tr>
<th>Reaction system</th>
<th>NO3- reduced</th>
<th>Oxygen evolved</th>
</tr>
</thead>
<tbody>
<tr>
<td>Complete</td>
<td>1.4</td>
<td>4.8</td>
</tr>
<tr>
<td>Spinach ferredoxin omitted</td>
<td>0.2</td>
<td>0.7</td>
</tr>
<tr>
<td>Nitrite reductase omitted</td>
<td>0.1</td>
<td>0.9</td>
</tr>
<tr>
<td>Nitrite omitted</td>
<td>0</td>
<td>0.7</td>
</tr>
<tr>
<td>Complete, dark</td>
<td>0.1</td>
<td>0</td>
</tr>
</tbody>
</table>

**FIG. 1.** Stoichiometry of oxygen evolution and ATP formation in noncyclic photophosphorylation with nitrite as the terminal electron acceptor. The experimental conditions were the same as in Table I, except that broken chloroplasts containing 0.2 mg of chlorophyll were used, and the following were added: MgCl2, 10 µmoles; Na2HPO4, 10 µmoles; and ADP, 10 µmoles. The gas phase was argon. The mixture was incubated at 20° for 15 minutes. Other experimental conditions are indicated under "Experimental Procedure."

The complete reaction mixture included, in a final volume of 3 ml, once washed broken chloroplasts containing 0.2 mg of chlorophyll, spinach ferredoxin, 1 µg; spinach nitrite reductase, 2 µg; Tris buffer, pH 8.0, 150 µmoles; and sodium nitrite, 6 µmoles. The gas phase was argon. The mixture was incubated at 20° for 15 minutes. Other experimental conditions are indicated under "Experimental Procedure."

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<td>0.7</td>
</tr>
<tr>
<td>Complete, dark</td>
<td>0.1</td>
<td>0</td>
</tr>
</tbody>
</table>
evolution is explained by the uncoupling effect of ammonia on ATP formation during the photochemical reduction of nitrite. Nitrite itself does not affect the photophosphorylation coupled to ferricyanide reduction, but its reduction product, ammonia, effectively uncouples photosynthetic phosphorylation from ferricyanide (25) and $\text{TPN}^+$ reduction (26).

The reduction of nitrite in the light is thus a new type of noncyclic, ferredoxin-dependent photophosphorylation reaction in chloroplasts, similar to but not identical with the one studied by Arnon et al. (2, 27) with $\text{TPN}^+$ as the terminal oxidant. At the electron donor end, the similarities are total, since both $\text{TPN}^+$ (27, 28) and $\text{NO}_2^-$ (9, 10) can use either water or ascorbate-dichlorophenol indophenol as the reductant. At the electron acceptor end the difference is most pronounced since, as shown in Table III, the noncyclic electron flow was not affected by 1 mM KCN in the $\text{TPN}^+$ system (cf. Avron and Jagendorf (15)), whereas it was completely inhibited in the $\text{NO}_2^-$ system (cf. Roussos and Nason (4) and Hageman, Creswell, and Hewitt (5)). Both systems are, therefore, identical up to the ferredoxin level. Reduced ferredoxin has been recently identified by Whatley, Tagawa, and Arnon (29) as the earliest chemically isolated reductant formed at the expense of radiant energy trapped during photosynthesis. Once ferredoxin has been reduced, the electrons can be transferred either to $\text{TPN}^+$ by $\text{TPN}$ reductase (14) or to nitrite by nitrite reductase.

Tagawa and Arnon (11) found that the proteins from green plants (13) and photosynthetic bacteria (27), previously known as photosynthetic pyridine nucleotide reductases, are ferredoxins which share chemical and functional similarities with the ferredoxins from nonphotosynthetic microorganisms (19), and are capable of transferring the electrons coming from hydrogen gas or from light-activated chlorophyll to the chloroplast $\text{TPN}^+$-TPN reductase system. It was interesting, therefore, to determine whether Clostridium ferredoxin is capable of replacing spinach ferredoxin in the photochemical reduction of nitrite. As shown in Fig. 2, Clostridium ferredoxin can substitute for spinach ferredoxin but is a far less effective electron carrier.

**Table III**

<table>
<thead>
<tr>
<th>Acceptor</th>
<th>Addition</th>
<th>Oxygen evolved (μmols)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\text{NO}_2^-$</td>
<td></td>
<td>3.1</td>
</tr>
<tr>
<td>$\text{NO}_2^-$</td>
<td>Cyanide</td>
<td>0</td>
</tr>
<tr>
<td>$\text{TPN}^+$</td>
<td></td>
<td>3.2</td>
</tr>
<tr>
<td>$\text{TPN}^+$</td>
<td>Cyanide</td>
<td>3.1</td>
</tr>
</tbody>
</table>

**Fig. 2.** Ferredoxins (Fd) as electron carriers in the light reduction of nitrite by chloroplasts. Experimental conditions were as in Table I, except that 1.5 mg of Clostridium ferredoxin were used where indicated.

**Fig. 3.** Ferredoxins (Fd) and methyl viologen (MV) as electron carriers in the dark reduction of nitrite with hydrogen gas as the electron donor. The reaction mixture contained, in a final volume of 3 ml, Clostridium hydrogenase, 1 mg; spinach nitrite reductase, 10 μg; Tris buffer, pH 8.0, 200 μmoles; cysteine, 10 μmoles; and sodium nitrite, 15 μmoles. Where indicated, 1 μg of spinach ferredoxin, 1.5 mg of Clostridium ferredoxin, or 1 μmole of methyl viologen was added. Gas phase, hydrogen. Temperature, 20°C.

The transfer of electrons (cf. Valentine et al. (7)). The specificity of Clostridium and spinach ferredoxin is, therefore, more rigid for the electron donor system than for the nitrite-spinach nitrite reductase system.
With the hydrogen-Clostridium hydrogenase system, it was possible to demonstrate that spinach nitrite and TPN+ reductases differed not only in their sensivities to KCN (Table III), but also in their inactivation by heating. As shown in Table IV, nitrite reductase activity disappeared after the enzyme was heated for 10 minutes at 60°, whereas TPN reductase was not affected by the same treatment.

Losada et al. (10) found that the dark reduction of nitrite with TPNH, catalyzed by the spinach chloroplast extract, was ferredoxin-dependent. They suggested that TPNH transferred its electrons to ferredoxin with the TPN reductase also present in the chloroplast extract in a reaction which would be the reverse of that studied by Tagawa and Arnon (11); i.e. spinach ferredoxin could mediate the transfer of electrons from the TPNH-TPN reductase system to the nitrite-nitrite reductase system. Recent reports from Arnon’s laboratory (14) have shown that spinach TPN-reductase in fact catalyzes the reversible reduction of TPN+ by ferredoxin. In C. pasteurianum, however, the reaction seems not to be reversible. According to Valentine ferredoxin does not mediate the evolution of H2 (19) or the reduction of nitrite (30) from TPNH. Since our spinach nitrite reductase preparation contained also TPN reductase, we have tested its ability to reduce nitrite from TPNH. As Table V shows, the enzymic oxidation of TPNH by nitrite required the presence of spinach ferredoxin.

**Table IV**

**Effect of heating on spinach nitrite reductase and TPN reductase**

The experimental conditions were the same as in Fig. 3, except that all the vessels included 1 mg of Clostridium ferredoxin, and 4 μmoles of NaNO₂ or TPN+ were added as indicated. The heat treatment of the spinach nitrite reductase preparation containing TPN reductase was carried out at 60° for 10 minutes.

<table>
<thead>
<tr>
<th>Acceptor</th>
<th>Treatment</th>
<th>H₂ uptake</th>
<th>μmoles</th>
</tr>
</thead>
<tbody>
<tr>
<td>NO₂⁻</td>
<td>None</td>
<td>2.6</td>
<td></td>
</tr>
<tr>
<td>NO₂⁻</td>
<td>Heat</td>
<td>0.3</td>
<td></td>
</tr>
<tr>
<td>TPN+</td>
<td>None</td>
<td>3.0</td>
<td></td>
</tr>
<tr>
<td>TPN+</td>
<td>Heat</td>
<td>3.0</td>
<td></td>
</tr>
<tr>
<td>TPN+</td>
<td>TPN+</td>
<td>0.0</td>
<td></td>
</tr>
</tbody>
</table>

**Table V**

**Ferredoxin as electron carrier in dark oxidation of TPNH with nitrite as electron acceptor**

The reaction mixture included, in a final volume of 3 ml, spinach ferredoxin, 0.5 mg; spinach nitrite reductase (containing TPN reductase) 2 mg; Tris buffer, pH 8.0, 150 μmoles; sodium nitrite, 6 μmoles; and TPNH, 4 μmoles. The reaction was run at 20°, under argon, for 20 minutes. The oxidation of TPNH was measured by the change in optical density at 540 μg. The endogenous oxidation in the absence of nitrite was subtracted from the observed rates.

<table>
<thead>
<tr>
<th>Reaction system</th>
<th>TPNH oxidized</th>
<th>μmoles</th>
</tr>
</thead>
<tbody>
<tr>
<td>Complete</td>
<td></td>
<td>1.0</td>
</tr>
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