Electron Transport to Assimilatory Nitrate Reductase in *Azotobacter vinelandii*

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Assimilatory nitrate reductase was particle-bound in extracts from *Azotobacter vinelandii*. Nitrate reduction by particle fractions was dependent on NADPH and a particle-bound electron carrier. When the enzyme was solubilized from the particles by treatment with detergents, the particle-bound electron carrier could be substituted by ferredoxin or flavodoxin. Flavodoxin reduced at the expense of photoreduced deazaflavin was much more efficient than ferredoxin in transferring electrons to nitrate reductase. The addition of both ferredoxin and flavodoxin to the assays with photoreduced deazaflavin gave additive effects. With the solubilized enzyme, NADPH only poorly supported nitrate reduction even after the addition of electron carriers. The experiments indicate that *A. vinelandii* utilizes an electron transport chain between NADPH and nitrate reductase with some properties similar to those described for the generation of reductants to nitrogenase.

Introduction

Compared to nitrogen fixation, rather little is known about nitrate assimilation of *Azotobacter*. Assimilatory nitrate reductase, catalyzing the reduction of nitrate to nitrite, was reported to be inducible and bound to large particles in extracts of *Azotobacter vinelandii* [1]. Nitrate reduction by such preparations was achieved with either NADH or NADPH, when the assays were supplemented with artificial dyes or with FMN or FAD [1]. Substantial nitrate reduction was also obtained by the artificial system NADPH, ferredoxin from spinach, NADPH: ferredoxin oxidoreductase from spinach and nitrate reductase from *A. vinelandii* [2]. Extracts from *A. vinelandii* partially replaced ferredoxin from spinach but were not characterized for electron carrier composition [2]. In contrast to *A. vinelandii, Azotobacter chroococcum* apparently forms a soluble nitrate reductase [3]. The enzyme does not possess the NAD(P)H diaphorase moiety, a characteristic feature of the soluble assimilatory nitrate reductase of plants [4]. When photoreduced by spinach chloroplasts, ferredoxin from *A. chroococcum* coupled to the enzyme from the bacterium [5]. Significant nitrate reduction was also obtained in a model system consisting of NADPH, NADPH: ferredoxin oxidoreductase from spinach, ferredoxin and nitrate reductase both from *A. chroococcum*. As was the case with *A. vinelandii* [2], spinach NADPH: ferredoxin oxidoreductase could not be substituted by an enzyme native to *A. chroococcum*, and flavodoxin was not tried. In later experiments [6], most of the assimilatory nitrate reductase activity was found to be incorporated into the cytoplasmic membrane, when *A. chroococcum* was repeatedly subcultured in liquid media with nitrate as the nitrogen source.

Thus neither the immediate electron carrier to nitrate reductase (ferredoxin, flavodoxin) nor the electron donor (NADH, NADPH or an unidentified compound) nor the enzyme catalyzing the reaction between electron donor and carrier is known for *Azotobacter* at present.

This communication describes the distribution of the assimilatory nitrate reductase among particulate and soluble fractions in *A. vinelandii*. Ferredoxin and flavodoxin reduced by several methods are found to stimulate nitrate reduction by a particulate fraction. Finally, experiments to elucidate the electron transport chain to assimilatory nitrate reductase are reported.

Abbreviations: BV, benzyl viologen; EDTA, ethylene dinitrilotetra acetic acid – sodium salt; MV, methyl viologen.

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Materials and Methods

Growth of the organism. Lyophilized stock material of *Azotobacter vinelandii* CCM 289 was a kind

gift of the Czechoslovak Collection of Microorganisms at Brno. *Azotobacter* was grown in 1 l flasks under vigorous aeration at 30 °C. The medium consisted of Burk's salts [7] supplemented with 2% sucrose and 8 mM KNO_3. Cells were diluted into fresh medium every day and grown in the presence of nitrate for at least 3 weeks. The organism was then grown in a 10 l fermenter (5% inoculum) for 24 h, centrifuged quickly and stored at −20 °C prior to use.

Preparation of nitrate reductase (see scheme 1). Cells were rethawed, washed once by resuspending in 50 mM K-phosphate buffer pH 7.0, centrifuged and resuspended in the same buffer (10 ml of buffer/g cells, wet weight). The paste was then passed through a French Press at 5000 psi (= approx. 35000 kPa) and centrifuged at $3000 \times g$ for 20 min. The supernatant was then centrifuged at $39000 \times g$ for 20 min. The resulting pellet ("large particle fraction") contained most of the nitrate reductase and was devoid of nitrite reductase activity. The supernatant was centrifuged further at $110000 \times g$ for 150 min to find out the distribution of the remaining activity among the soluble and the particulate fraction. The large particle fraction ($39000 \times g$ pellet) was suspended in a solution of 50 mM phosphate buffer supplemented with 1% Triton-X-100, incubated for 5 min at room temperature and centrifuged at $39000 \times g$ for 20 min. The supernatant was then absorbed on a DE-52 Whatman cellulose column (3 × 1 cm) preequilibrated with 50 mM phosphate buffer pH 7.0 at 4 °C. Nitrate reductase was eluted by washing the column with 0.3 M NaCl dissolved in 50 mM phosphate buffer pH 7.0 and dialysed for 2 h at 4 °C.

Determination of nitrate reductase activity. The standard assay was performed in test tubes under air containing in a final volume of 2 ml in µmol: K-phosphate buffer pH 7.0, 150; KNO_3, 10; BV, 1.5; 7.5 mg $Na_2S_2O_4$ dissolved in 0.3 ml of a 2.5% (w/v) solution of $NaHCO_3$. The reaction was performed at 30 °C for 15–30 min during which the solution kept dark blue due to reduced BV. The assay was terminated by oxidizing BV and $Na_2S_2O_4$ by vigorous shaking with air. The samples were centrifuged at $5000 \times g$ for 10 min, and the supernatant was assayed for nitrite content, using the diazo-coupling reagents [8].

Other experimental procedures. Spinach chloroplasts (P_1 s_1) were prepared as described by Whatley and Arnon [9]. When EDTA/deazaflavin was the photoreductant, the assays were performed under argon in Thunberg cuvettes equipped with two side arms. The reaction mixture contained in a final volume of 3 ml in µmol: EDTA, 7.5; deazaflavin, 0.3; tris-HCl buffer pH 7.0, 300; KNO_3, 10, added from one side arm; solubilized nitrate reductase, 0.3–0.5 mg. The reaction was performed at 30 °C for 15 min during which the Thunberg cuvette was illuminated with white light of an intensity of approximately 100000 lux at the cuvette in the water bath. One side arm of the cuvette contained 10% pyrogallol dissolved in 10% Na_2CO_3. Nitrate reductase activity was determined by the amount of nitrite formed [8]. NADPH was generated from glucose-6-phosphate dehydrogenase and NADH from galactose and galactose dehydrogenase (see [10], Table IV). Isolation procedures and extinction coefficients of ferredoxins from *Azotobacter* and spinach and flavodoxin from *Azotobacter* are given in [11]. Protein was determined by the Biuret method (for particulate fractions) or by a modification of the Lowry procedure (for the solubilized nitrate reductase).

Chemicals. Fine chemicals were purchased from Boehringer, Mannheim, benzyl- and methyl-viologen from Serva, Heidelberg, all other from Merck, Darmstadt. Deazaflavin was the K-salt of 7,8 bisnor-5-deazalumiflavin-3-propane sulfonic acid and was a kind gift of Prof. P. Hemmerich, Konstanz.

Results

Assimilatory nitrate reductase was particulate in extracts of *A. vinelandii* grown on nitrate as the nitrogen source for weeks (Table I). No activity was detectable in the $110000 \times g$ supernatant after centrifugation, and the major part of the enzyme was associated with large particles centrifuging at $39000 \times g$, in agreement with previous observations for *A. vinelandii* [1]. Since the large particle fraction was devoid of any nitrite reductase activity, nitrate reductase could conveniently be assayed by the appearance of nitrite from nitrate.

Nitrite formation by the large particle fraction was optimal between pH 6.5 and 8.5 and between 38 and 42 °C. Nitrate reduction was proportional to protein concentrations up to 20 mg in the vessels and to time for at least 1 h. In contrast, nitrite formation by the soluble nitrate reductase from *A.*

Table I. Distribution of the assimilatory nitrate reductase from *Azotobacter vinelandii* among fractions after differential centrifugation.

Fraction	Volume [ml]	Total protein [mg]	Specific activity nmol NO_3^-/h × mg protein reduced	Total activity µmol NO_3^-/h reduced	[%]
1. 3000 × *g* supernatant	24	350.4	62.6	21.9	100
2. 39000 × *g* supernatant	24	307.2	11.3	3.4	15.5
3. 39000 × *g* pellet	2	38.0	430.2	16.3	74.4
4. 110000 × *g* supernatant	24	199.2	0.0	0.0	0.0
5. 110000 × *g* pellet	2	90.0	36.0	3.2	14.6

chroococcum drastically diminished after 3 min incubation [3]. The particulate nitrate reductase from *A. vinelandii* did not require a special activating agent in contrast to the enzyme from *A. chroococcum* [12].

$Na_2S_2O_4$ was an efficient reductant for nitrate reductase, and the reaction was stimulated by viologen dyes (Table II). Among the natural electron donors, NADPH but virtually not NADH supported nitrite formation. NADPH-dependent nitrite formation by the particulate nitrate reductase was enhanced by viologen dyes, also by ferredoxin from spinach or *Azotobacter*, but only slightly by flavodoxin from *Azotobacter* (Table II).

Half maximal nitrite formation by the particulate nitrate reductase was obtained at approximately 0.75 mM benzyl viologen in the vessels (Fig. 1). Ferredoxin and flavodoxin could not be assayed at such high protein concentrations. Fig. 1 and Table II indicate that nitrite formation by the large particle fraction was not strictly dependent upon the addi-

Table II. Nitrate reduction by the particle-bound nitrate reductase.

Electron donor system	nmol NO_3^- reduced/h × mg protein
1. $Na_2S_2O_4$	220
2. $Na_2S_2O_4$ + MV	510
3. $Na_2S_2O_4$ + BV	604
4. NADH	9
5. NADH + MV	8
6. NADH + BV	7
7. NADH + ferredoxin spinach	0
8. NADH + ferredoxin Azotobacter	0
9. NADH + flavodoxin Azotobacter	0
10. NADPH	114
11. NADPH + MV	211
12. NADPH + BV	501
13. NADPH + ferredoxin spinach	193
14. NADPH + ferredoxin Azotobacter	168
15. NADPH + flavodoxin Azotobacter	132

The experiment was performed with the large particle fraction having 5–9 mg protein. Carrier concentrations were 25 µM for ferredoxin and flavodoxin and 0.75 mM for viologens. For other experimental details see Materials. The results represent average values of at least three determinations.

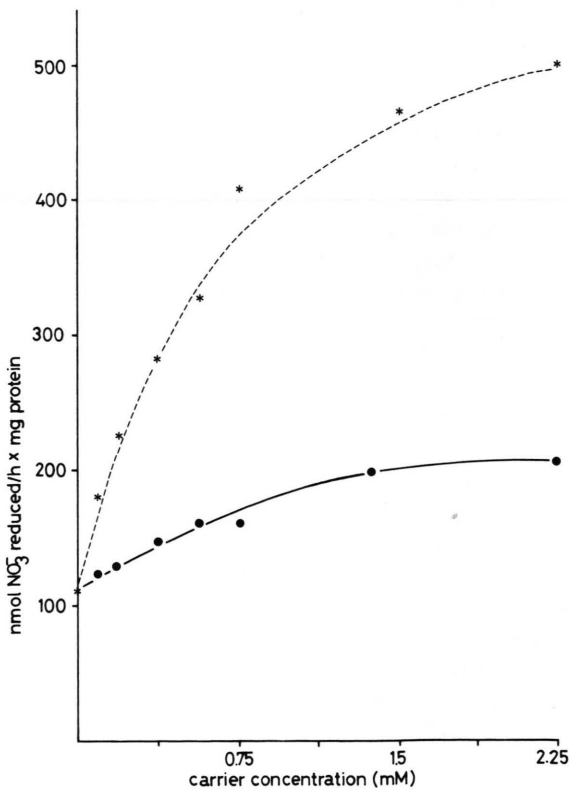
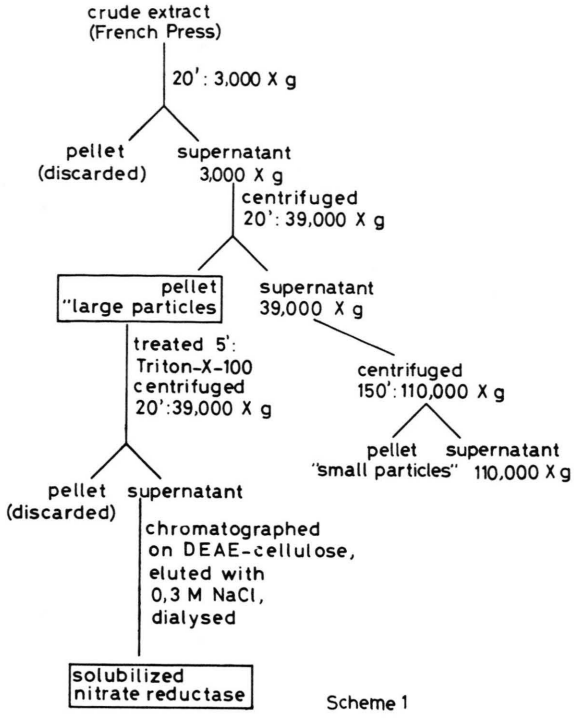


Fig. 1. NADPH-dependent nitrate reduction by the large particle fraction from *Azotobacter vinelandii*. *- - - * BV-dependent nitrate reduction; ●—● MV-dependent nitrate reduction. The reaction was performed for 20 min at 30 °C, and the mixture contained in a final volume of 2 ml in µmol: K-phosphate buffer pH 7.0, 150; KNO_3, 10; BV or MV, as indicated; glucose-6-phosphate, 50; $NADP^+$, 1; glucose-6-phosphate dehydrogenase, 0.01 mg; 7.5 mg $Na_2S_2O_4$ dissolved in 0.3 ml of a 2.5% (w/v) solution of $NaHCO_3$; large particles, 7.8 mg.



Scheme 1

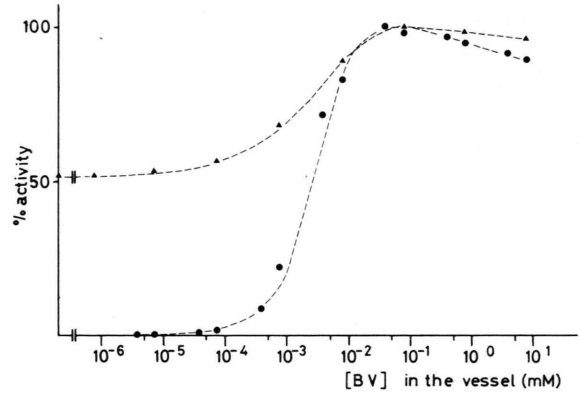


Fig. 2. $Na_2S_2O_4$ and BV-dependent nitrate reduction by particulate and solubilized nitrate reductase. ▲- - -▲ Particulate nitrate reductase, large particle fraction, untreated, 8.4 mg protein/vessel; 100% activity = 470 nmol NO_3^- reduced/h × mg protein. ● - - - ● solubilized nitrate reductase, obtained by treating the large particle fraction with Triton-X-100, 0.4 mg protein/vessel; 100% activity = 3560 nmol NO_3^- reduced/h × mg protein. The reaction was performed for 20 min at 30 °C and the mixture contained in a final volume of 2 ml in μmol: K-phosphate buffer pH 7.0, 150; KNO_3, 10; BV as indicated, and 7.5 mg $Na_2S_2O_4$ dissolved in 0.3 ml of a 2.5% (w/v) solution of $NaHCO_3$.

tion of an electron carrier (viologen dye, ferredoxin or flavodoxin). This could mean either that NADPH served as an immediate reductant to nitrate reductase (as in the case of the enzyme in plants) or that the particles contained a membrane-bound electron carrier.

To resolve these two possibilities, nitrate reductase from the large particle fraction was solubilized by treatment with detergents followed by chromatography on DE-cellulose (Scheme 1). Specific nitrate reductase activity was enhanced about tenfold by this treatment. Nitrite formation catalyzed by

Table III. Nitrate reduction by the solubilized nitrate reductase.

Electron donor system	nmol NO_3^- reduced/ h × mg protein
1. $Na_2S_2O_4$	0
2. $Na_2S_2O_4$ + BV	3480
3. $Na_2S_2O_4$ + MV	2800
4. $Na_2S_2O_4$ + ferredoxin spinach	0
5. spinach chloroplasts	2
6. spinach chloroplasts + ferredoxin spinach	85
7. spinach chloroplasts + ferredoxin Azotobacter	114
8. spinach chloroplasts + flavodoxin Azotobacter	63
9. Deazaflavin EDTA	0
10. Deazaflavin EDTA + ferredoxin Azotobacter	51
11. Deazaflavin EDTA + flavodoxin Azotobacter	1390
12. NADH + BV or MV	0
13. NADPH	51
14. NADPH + MV	165
15. NADPH + BV	369

Solubilized nitrate reductase was obtained by treating the large particle fraction with Triton-X-100 and DE-cellulose. Protein concentration of nitrate reductase was 0.3–0.5 mg in the assays.

the solubilized enzyme was now strictly dependent upon the addition of an electron carrier using $Na_2S_2O_4$ as the reductant (Fig. 2). In addition, activity with NADPH was now marginal (Table III). Obviously, nitrate reductase cannot directly accept electrons from $Na_2S_2O_4$ or NADPH, and treatment with detergents solubilizes membrane-bound carrier(s) mediating the electron transfer between electron donors and nitrate reductase.

Different reductants were tried for nitrite formation by the solubilized enzyme in an attempt to establish components that substituted for the membrane-bound carrier(s) (Table III). With illuminated spinach chloroplasts as reductant, ferredoxin from Azotobacter stimulated nitrite formation, whereas the activity with flavodoxin from *Azotobacter* was low. This is probably due to the fact that spinach chloroplasts reduce flavodoxin to the hydroquinone form (the electron donating species) only to a limited extent [11]. A more powerful reductant for flavoproteins and iron sulfur clusters is deazaflavin photoreduced at the expense of EDTA. The deazaflavin-EDTA system reduces flavodoxin from *Azotobacter* completely to the fully reduced form [13] and thus supports nitrite formation with activities comparable to those obtained with viologen dyes reduced by $Na_2S_2O_4$ (Table III).

When EDTA-deazaflavin was the photoreductant, nitrate reduction by the solubilized nitrate reductase was proportional to flavodoxin concentrations up to 0.1 mM (not documented). *Azotobacter* ferredoxin, on the other hand, when reduced by EDTA-deazaflavin, only poorly supported nitrite formation (Table III). Activities were also low with viologen dyes reduced by NAD(P)H in the case of the solubilized enzyme (Table III).

Table IV gives a selection of experiments where the activities of ferredoxin and of low concentrations of flavodoxin were compared in the nitrate reduction by the solubilized enzyme using EDTA-deazaflavin as photoreductant. Ferredoxin and flavodoxin stimulated additively, indicating that both electron carriers could substitute for the membrane-bound carrier independently of each other.

Table IV. Ferredoxin and flavodoxin photoreduced by EDTA-deazaflavin: Activities in the nitrate reduction catalyzed by the solubilized nitrate reductase.

	Electron carrier	nmol NO_3^- reduced/ h × mg protein
a)	50 nmol ferredoxin	52
	2 nmol flavodoxin	31
	50 nmol ferredoxin + 2 nmol flavodoxin	103
b)	100 nmol ferredoxin	78
	4 nmol flavodoxin	102
	100 nmol ferredoxin + 4 nmol flavodoxin	149
c)	200 nmol ferredoxin	149
	8 nmol flavodoxin	177
	200 nmol ferredoxin + 8 nmol flavodoxin	298

Ferredoxin and flavodoxin (both from *Azotobacter*) were photoreduced by the deazaflavin/EDTA system. Protein content of the solubilized nitrate reductase was 0.49 mg.

Discussion

Assimilatory nitrate reductase is usually a soluble protein, and the dissimilatory enzyme is bound to membranes [4]. *A. vinelandii* assimilatory nitrate reductase is an exception to this general rule, since it was found to be bound to the large particle fraction in the present investigation. The enzyme did not react directly with NADPH, but the transfer was mediated by ferredoxin, flavodoxin or artificial low-potential dyes. This indicates that the enzyme from *A. vinelandii* as well as the one from *A. chroococcum* [5] does not possess the FAD-dependent NAD(P)H diaphorase moiety. In addition to *A. vinelandii*, particulate and ferredoxin-dependent assimilatory nitrate reductases have unambiguously been demonstrated only in cyanobacteria [14] and in *Rhodopseudomonas capsulata* [15].

The present communication indicates that the electron transport chains to nitrate reductase and nitrogenase apparently have much in common. N_2-fixation also requires a membrane-bound electron carrier which can be substituted by flavodoxin or ferredoxin in experiments with solubilized nitrogenase [16]. As with nitrate reduction (present communication), flavodoxin photoreduced by EDTA-deazaflavin was much more effective than ferredoxin in supporting nitrogen fixation [16]. Such observation does not necessarily mean that flavodoxin is the natural electron carrier to both nitrate reductase and nitrogenase, sine a reductant capable of producing fully reduced flavodoxin as effectively as EDTA-deazaflavin has not been demonstrated in *Azotobacter*. There is no immediate explanation for

the observation that ferredoxin is a better electron donor than flavodoxin when reduced by chloroplasts but is weakly active when reduced by EDTA-deazaflavin both in nitrate reductase (Table III) and nitrogenase assays [11,·16].

Currently, two concepts have been published to explain the generation of reducing equivalents. According to Benemann *et al.* [17], ferredoxin and flavodoxin are part of a complex electron transport chain between NADPH and nitrogenase. Their NADPH-dependent C_2H_2-reduction was, however, low, even when the assays were supplemented with NADPH: ferredoxin oxidoreductase from spinach. An enzyme with comparable properties to the spinach protein could not be identified. According to Veeger and coworkers [16], the immediate electron carrier to nitrogenase is flavodoxin which is reduced by NADH and a membrane-bound NADH: flavodoxin oxidoreductase [18]. These authors concluded from studies with inhibitors that the generation of reduced flavodoxin is dependent on the energized state of the membrane. However, they have not been able to demonstrate a reduction of flavodoxin beyond the semiquinone state in the membrane and in dependence of the membrane potential. It appears from the results presented here that both concepts apply equally for the electron transport to nitrate reductase.

In the present communication, the NADPH-dependent nitrate reduction rate by the particulate fraction accounted for approximately 20% of the *in vivo* activity. Nitrite formation by the particles did not require the presence of ATP in the vessels and was, therefore, independent of the proton motive force. These experiments may, however, not reflect the situation in the intact cells. Nitrate reductase was easily solubilized form the particles, but resolution and reconstitution of the electron transport chain to nitrate reductase (as to nitrogenase) was difficult to achieve. The chain may be composed of ferredoxin, flavodoxin, reductase(s), menadione [6], cytochrome b [6] and other factors. On the whole, assimilatory nitrate reduction should provide a better chance to elucidate the electron transport chain than nitrogen fixation, since all nitrogenase reactions have the additional complication of their dependence on ATP.

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