Specificity for Nicotinamide Adenine Dinucleotide and Nicotinamide Adenine Dinucleotide Phosphate of Nitrate Reductase from the Salt-tolerant Alga Dunaliella parva

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ABSTRACT
Nitrate reductase of the salt-tolerant alga Dunaliella parva could utilize NADPH as well as NADH as an electron donor. The two pyridine nucleotide-dependent activities could not be separated by either ion exchange chromatography on DEAE-cellulose or gel filtration on Sepharose 4B. The NADPH-dependent activity was not inhibited by phosphatase inhibitors. NADPH was not hydrolyzed to NADH and inorganic phosphate in the course of nitrate reduction. Reduction of nitrate in vitro could be coupled to a NADPH-regenerating system of glycerol and NADP-dependent glycerol dehydrogenase. It is concluded that the nitrate reductase of D. parva will function with NADPH as well as NADH. This is a unique characteristic not common to most algae.

Nitrate reductase of most algae and higher plants is specific for, or has a preferential requirement for, NADH as electron donor (1, 6, 10). The ability of the enzyme from several higher plants to use NADPH as well as NADH (2, 4) was recently shown (13) to be an artifact caused by the presence of the extract of a phosphatase-like activity which converted NADPH to NADH and Pi. In an earlier communication (8), it was shown that the nitrate reductase of the salt-tolerant alga Dunaliella parva could utilize NADPH as well as NADH as an electron donor, and that the NADPH-dependent activity was insensitive to phosphatase inhibitors. Since the ability to utilize both pyridine nucleotides for nitrate reduction represented an uncommon characteristic among algae, a more detailed study was undertaken to determine the true electron donor specificity of the enzyme.

MATERIALS AND METHODS
Cells of D. parva were grown as previously described (7) on a synthetic medium (12) containing 2 mM NaCl. Nitrate reductase was extracted from cells at midlogarithmic phase as previously described (8). The crude extract was either dialyzed overnight against 0.1 M K-phosphate buffer (pH 7.5), containing 1 mM L-cysteine and then used as the enzyme source, or was further fractionated with ammonium sulfate. The protein fraction, which precipitated at 50% saturation of ammonium sulfate, was used as enzyme source after dialysis against 0.1 M K-phosphate buffer (pH 7.5), containing 1 mM L-cysteine.

Ion exchange chromatography on DEAE-cellulose was carried out according to Wells and Hageman (13). The column (1.5 × 40 cm) was equilibrated with 50 mM K-phosphate (pH 7.5), containing 1 mM L-cysteine and eluted with a linear gradient of 0 to 0.25 M K2SO4 in the equilibrating buffer. Sepharose 4B in a column (1.5 × 40 cm) was equilibrated and eluted with the same buffer as that used for the equilibration of the DEAE-cellulose. Nitrate reductase activity was assayed as previously described (14). Activity was determined by measuring either the amount of nitrite formed or the nitrate-dependent oxidation of the reduced pyridine nucleotides. The incubation mixture for the determination of Pi released from NADPH was free of added Pi and contained in 1 ml: 60 μmol tris-HCl (pH 7.5), 10 μmol KNO3; 6 μmol NADPH or NADH, and 2 to 5 mg of enzyme protein with specific activity of 20 to 40 nmol NO3− reduced/min·mg protein. Incubation period was 30 min. Inorganic phosphate was determined by the method of Fiske and SubbaRow (5). The incubation mixture for the coupling of nitrate reduction to glycerol oxidation contained in 1 ml: 50 μmol tris-HCl (pH 9), 10 μmol KNO3, 50 nmol NADH or NADPH, 3.3 mmol glycerol, and 2 to 3 mg crude extract with specific activity of 8 to 12 nmol NO3− reduced/min·mg protein. Protein was determined by the method of Lowry et al. (11).

The experiments were repeated at least three times with similar results.

RESULTS AND DISCUSSION
Nitrate reductase from D. parva can utilize NADH or NADPH as electron donors for nitrate reduction (8) with apparent Km values of 10 μM and 20 μM, respectively (Fig. 1). These Km values may point to a similar affinity of the enzyme for the two pyridine nucleotides. However, the ability to utilize NADPH as electron donor could be an artifact caused by the presence of a phosphatase in the crude extract which converted NADPH to NADH and Pi, as was recently shown for several higher plants (13). The following experiments were designed to determine whether this was the case for the nitrate reductase of D. parva as well.

As seen from Figure 2, neither ion exchange chromatography on DEAE-cellulose nor gel filtration on Sepharose 4B could eliminate or at least inhibit the NADPH-dependent activity. This result did not rule out the possibility that the phosphatase-like activity was tightly associated with the nitrate reductase. The effect of phosphatase inhibitors on the NADPH-dependent activity of a partially purified preparation was tested. There was no preferential inhibition of the NADPH-dependent activity by either inorganic phosphate or fluoride as compared with the NADH-dependent activity. Such an inhibition could be expected if a phosphatase were associated with the ability to use NADPH (13). Furthermore, NADPH was not hydrolyzed to NADH and Pi in the course of nitrate reduction. After a 30-min incubation period in the presence of NADPH, 4050 nmol NO3− and 480

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FIG. 1. Lineweaver-Burk plot of the rate of nitrate-dependent oxidation of NADH (•) and NADPH (○), as a function of their concentration in the assay mixture. Oxidation was assayed as the decrease of absorbancy at 340 nm.

FIG. 2. Gel filtration on Sepharose 4B (A) and ion exchange chromatography on DEAE-cellulose (B) of nitrate reductase. NADH (▲) and NADPH (△) nitrate reductase activities are expressed as nmol NO₂⁻ formed/ml·10 min. Broken line indicates the concentration of K₂SO₄. Fraction volume was 1.1 ml.
most algae is specific only for NADH (10). It remains to be seen whether this ability is related to the adaptation of this alga to extreme environmental conditions.

**LITERATURE CITED**