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NADH SUBSTRATE INHIBITION AND ENHANCED THERMAL STABILITY OF HIGHER PLANT NITRATE REDUCTASE IMMOBILIZED VIA A MONOCLONAL ANTIBODY

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The molecular basis of light-induced circadian rhythms of higher plant NADH:nitrate reductase (EC 1.6.6.1) activity is presently not understood. We have investigated whether the regulatory properties of NADH:nitrate reductase would allow oscillatory or related dynamic behavior. We report here the first example of NADH substrate inhibition of higher plant nitrate reductase in solution and for an immobilized enzyme using a novel immobilization technique with a monoclonal antibody. According to current theories on chemical oscillatory reactions, substrate inhibition will allow bistable and oscillatory behavior when the substrate-enzyme reaction is carried out in an open system. We also found a significant enhanced thermal stability of the immobilized enzyme.

It has recently been shown that the NADH:nitrate reductase activity in leaves of barley, wheat, oats and corn can reach its maximum within 1 or 2 hours and subsequently exhibit circadian rhythm when plants are transferred from darkness to continuous light (1-4). Although the time scale of the switch-on/switch-off behavior in nitrate reductase activity is considerably shorter than the 24 h period length of the rhythm, it was possible to simulate the oscillations almost quantitatively (5). The light-induced switch-on behavior in nitrate reductase was related to a positive feedback regulation, while the switch-off behavior in nitrate reductase activity when plants are transferred from light to continuous darkness was treated as a negative feedback loop (5). However, this approach does not allow for identification of the molecular processes which cause this feedback behavior. While such feedback control can be realized at various metabolic levels, we investigated whether regulatory properties of the enzyme itself would allow oscillatory or related dynamic

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behavior. In this paper we report the first example of NADH substrate inhibition for a higher plant nitrate reductase. In general (6), substrate inhibition will allow bistability (7) and oscillatory behavior (8) when the enzyme-substrate reaction proceeds in an open system. To obtain an open system for later study, we have immobilized the nitrate reductase via a monoclonal antibody bound to a Sepharose matrix. The immobilized enzyme shows the same substrate inhibition as the soluble enzyme, but in addition has increased thermal stability as compared to the soluble nitrate reductase.

MATERIALS AND METHODS

The procedure to purify corn nitrate reductase using immunoaffinity chromatography with a monoclonal antibody was similar to a method developed earlier (9). Corn seeds (*Zea mays* L. W64A x W182E, Wisconsin Seed Foundation, Madison, WI) were allowed to swell in deionized water with slight air bubbling for 24 h, planted in Vermiculite, and grown in a growth chamber at 30°C with a 16 h light and a 8 h dark period. Deionized water was given to the plants until the end of the third light period when Hoagland's solution (10) containing 35 mM NH_4NO_3 and 1 mM $(NH_4)_2SO_4$ was given at about 1 L/ 250 seedlings. At the end of the fourth light period, an identical nutrient treatment was given to the seedlings and the leaves were harvested 4-5 h into the fifth light period when nitrate reductase activity had reached its maximum. Crude extracts were prepared by blending the leaves in an extraction buffer made of 0.1 M K-phosphate, pH 7.5, 1 mM EDTA, and 10 mM cysteine hydrochloride with a 2:1 ratio of extraction buffer to the weight of the tissue, together with 0.1 g polyvinylpolypyrrolidone per g tissue. After filtration through 4 layers of cheesecloth and 1 layer of Miracloth, the extract was centrifugated at 15,000 g for 15-20 min. Fat floating on the surface was removed by suction, and the extract recentrifuged. The crude extract was then mixed with monoclonal antibody CM15(11) (raised against purified squash nitrate reductase and covalently bound to Sepharose 4B) and gently stirred for 1-1.5 h (9). Then the Sepharose beads were filtered, and washed several times with water and 0.1 M Tris-HCl buffer (pH 7.5, 1 mM EDTA). A column was packed with the antibody-enzyme loaded Sepharose beads, and nitrate reductase was eluted using 1 M NaCl in 0.1 M Tris, pH 7.5. Nitrate reductase activity was assayed with NADH, and nitrite was determined colorimetrically (11). The same assay conditions were used for the immobilized enzyme. Initial rates of the homogeneous reaction R1 were determined in 0.1 M Tris-HCl (pH 7.5, 30°C) by the amount of consumed NADH (measured by optical density at 340 nm), while initial rates of the heterogeneous reaction R1 were determined by the amount of nitrite produced (11). One unit of nitrate reductase (NR) activity is defined as 1 µmol nitrite produced in 1 minute.

$$NADH + NO_3^- + H^+ \xrightarrow{NR} NAD^+ + NO_2^- + H_2O \qquad (R1)$$

RESULTS AND DISCUSSION

Figure 1A shows inital rates of the homogeneous reaction R1 as a function of initial NADH concentration. Substrate inhibition for initial NADH concentrations higher than 100 µM is clearly observed and more pronounced for

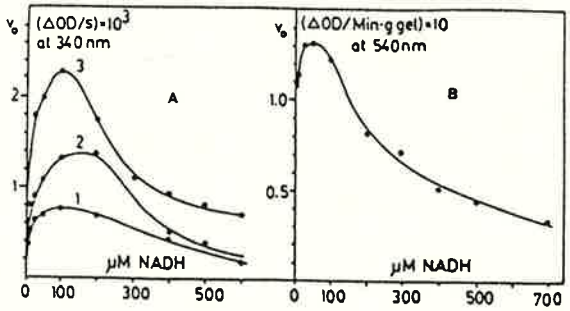

Figure 1. A. Initial velocity, v_o (measured as optical density changes (ΔOD) at the absoption maximum of NADH), as a function of initial NADH concentrations. Initial nitrate concentrations: 1, 50 μM; 2, 150 μM; 3, 300 μM. Fifty μg of purified nitrate reductase were used in each experiment. B. Initial velocity, v_o, as a function of initial NADH concentration where nitrate reductase was immobilized on Sepharose-antibody beads and suspended in the reaction mixture. The initial velocity was measured as the optical density change per minute at 540 nm using the nitrite assay (11). Sepharose particles with immobilized enzyme were rapidly removed by filtration prior to the colorimetric nitrite determination. Initial nitrate concentration was 300 μM.

increasing initial nitrate concentrations. These findings lead to the possibility that earlier observed (1-2) oscillatory behavior in nitrate reductase activity could be related to the NADH substrate inhibition of the enzyme. A number of experimental and theoretical studies have shown that under nonequilibrium conditions substrate inhibition can lead to bistability and oscillatory behavior (7,8,12,13). Most of these studies obtain a "far from equilibrium situation" by the use of a Continuous Stirred Tank Reactor where the enzyme is homogeneously distributed in a certain reaction volume while substrates are pumped into the volume and products and unreacted substrates are pumped out. While such a situation is very different from a biological system, many chemical oscillators and related systems show an astonishing analogy to biological dynamic behavior, for example, excitability (14), facilitation-like behavior (15), stimulus-time relationships (16), and wave propagation on excitable tissues (17).

To do subsequent studies of the nitrate reductase system in an open system, we have immobilized the enzyme on a solid support. We found that not all antibody-bound nitrate reductase was eluted by 1 M NaCl, but that a substantial amount of enzyme remained bound to the antibodies (approximately 50%) and was catalytically active even after NaCl concentrations as high as 2 M were applied. In fact, the use of monoclonal antibodies has quite recently been recognized as a novel tool for immobilization of enzymes (18). We found the same NADH substrate inhibition for our immobilized nitrate reductase (Figure

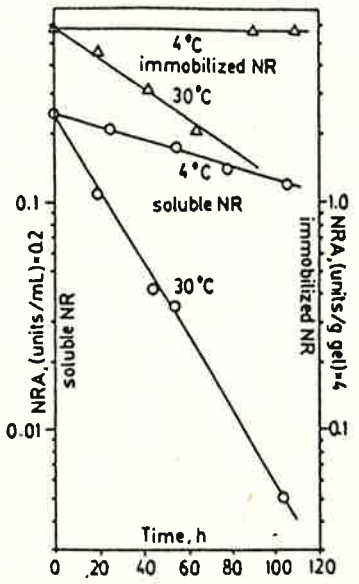

Figure 2. Thermal stability of soluble and immobilized nitrate reductase (NR) preparations in 0.1 M Tris, pH 7.5. Calculated half-life times for soluble NR: 4°C, 106 h; 30°C, 19 h; immobilized NR: 4°C, no signifcant decrease in activity observed during a six day period; 30°C, 46 h.

18). However, the immobilized enzyme had greater thermal stability than the enzyme in solution (Figure 2). This enhanced thermal stability of antibody-bound enzyme might be of general interest, because many nitrate reductase extraction procedures, like the blue-Sepharose method (19), often result in quite unstable enzyme preparations.

While we have observed NADH substrate inhibition of nitrate reductase for a higher plant system, NADH substrate inhibition in algae (20) and NADPH substrate inhibition in barley (21) have been found earlier. It appears that NAD(P)H inhibition of nitrate reductase activity is a more general phenomenon and might not only be important in relation to possible oscillatory behavior in light-induced enzyme activity, but also might be a necessary regulatory property to balance reduced pyridine nucleotide utilization in plant cells. In this respect it is interesting to note that NAD(H) and NADP(H) concentrations in the different plant cell compartments are of the order of 0.1-1 mM (22), which falls within the concentration range where this substrate inhibition is observed.

Further experimental and computational work to obtain possible oscillatory and bistable behavior of reaction R1 under nonequilibrium conditions together with an understanding of the molecular basis of the observed substrate inhibition is being carried out.

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