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MOLECULAR OXYGEN AS ELECTRON ACCEPTOR IN THE NADH-NITRATE REDUCTASE SYSTEM

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This paper describes first experimental evidence that dissolved molecular oxygen acts as an electron acceptor in the NADH-nitrate reductase system. The molecular mechanism and possible physiological implications on the induction mechanism of nitrate reductase by nitrate ion are discussed. © 1990 Academic Press, Inc.

Nitrate reductase (EC 1.6.6.1-3) catalyzes the reduction of nitrate to nitrite by reduced pyridine nucleotides:

$$NAD(P)H + NO_3^- + H^+ \rightleftharpoons NAD(P)^+ + NO_2^- + H_2O \qquad (R1)$$

In all higher plants reaction R1 is considered to be the rate-determining step in the transformation of nitrate to organically-bound nitrogen, a process where nitrate reductase appears to play an important regulatory role [1-4]. In addition to process R1 a variety of other, probably mostly unphysiological activities have been found for nitrate reductases [5, 6]. Several schematic models with the subunits FAD, a Cyt b_{557} heme group, and a molybdenum containing cofactor have been proposed [7, 8] to account for these activities (Fig. 1). It is now generally accepted that electrons from NAD(P)H are transferred from FAD via Cyt b_{557} heme to the Mo-cofactor, where nitrate is reduced (Fig. 1) [6-9].

During a stoichiometric study of process R1 catalyzed by corn leaf NADH:nitrate reductase we found that dissolved molecular oxygen accepts electrons from the NADH-nitrate reductase complex. This is the first explicit experimental finding that nitrate reductase catalyzes the reduction of molecular oxygen by NADH.

MATERIALS AND METHODS

Corn leaf nitrate reductase was purified to electrophoretic homogeneity as described earlier [10, 11]. Specific activities of the purified enzyme were about 80-90 units/mg, which are comparable to the specific activities obtained by Howard and Solomonson [12] for the *Chlorella vulgaris* nitrate reductase. Kinetic experiments

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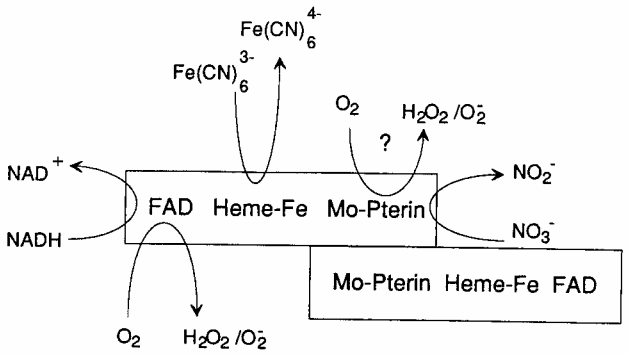


Figure 1. A schematic model of a higher plant nitrate reductase dimer [11]. It should be noted, however, that suggested nitrate reductase models [6-9, 11] differ in the stoichiometric composition of the cofactors and in the number of monomers which form the nitrate reductase oligomer. Proposed sites for electron acceptance by molecular oxygen are indicated.

were performed at 30°C (± 0.1°) in 0.1 M Tris-HCl buffer (pH 7.5, 1 mM EDTA). Reaction R1 was followed spectrophotometrically under air or pure oxygen atmospheres by recording the amount of reacting NADH at 340 nm. To exclude oxygen from the solution the reaction was also run under an inert argon atmosphere. Argon is preferred because it is heavier than air. The reaction was run in a special cuvette where argon or oxygen passed a water-containing washing bottle to avoid water take-up by the dry gas. Before start of the reaction the gas was bubbled vigorously through the reactant mixture for about 5 minutes. The reaction was started by adding nitrate reductase to the reactants. The reaction was then recorded spectrophotometrically while a steady stream of gas was blown on the reaction mixture from above.

β-NADH was purchased from Sigma (Grade III, 98%). All other chemicals were of analytical quality. All commerically available chemicals were used without further purification. The water used was first distilled and finally led through a deionizer column.

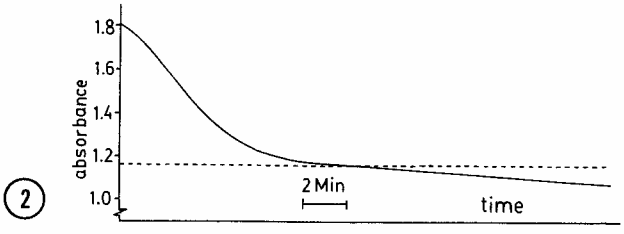
1 unit of nitrate reductase is defined as the production rate of 1 µmol nitrite per minute.

Results and Discussion

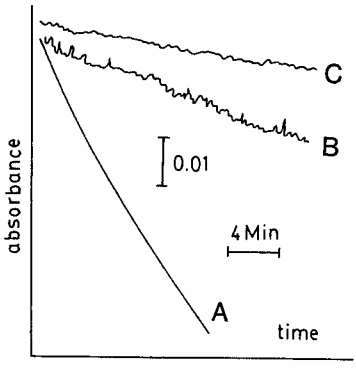
During a stoichiometric and kinetic study of reaction R1 we found that NADH was still consumed even when all nitrate has apparently been converted to nitrite (Fig. 2). A variation of the amount of dissolved oxygen showed that the rate of this "background reaction" was dependent upon the dissolved oxygen concentration, and that NADH was consumed even in absence of initial nitrate ion as long as nitrate reductase and dissolved oxygen were present. No measureable consumption of NADH could be observed for the uncatalyzed process R1.

Fig. 3 shows consumptions of NADH in the NADH-nitrate reductase-air, NADH-nitrate reductase-oxygen, and NADH-nitrate reductase-argon systems.

In any liquid-phase reaction where dissolved atmospheric oxygen is one of the reactants, the reaction rate in a pure oxygen atmosphere will increase by a factor of 4.76 compared with the rate in the air atmosphere (with 21 vol% oxygen) because,



(2)



(3)

Figure 2. Detected deviation from expected stoichiometry R1 in the NADH-nitrate reductase-NO_3^- system. The figure shows the consumption of NADH recorded at 340 nm. Initial concentrations: $[NADH]_0$ = 300 μM, $[NO_3^-]_0$ = 100 μM. Amount of active nitrate reductase, 0.1 units. Dashed line shows the 200 mM NADH concentration mark at which the system should have established equilibrium. Instead, NADH consumption continues in presence of air or oxygen.

Figure 3. NADH consumption in the NADH-nitrate reductase system with (A) oxygen atmosphere, (B) air atmosphere, and (C) argon atmosphere. Same NADH concentration and same amount of nitrate reductase as in Fig. 2. Determined NADH consumption rates: (A) 7.4×10^{-5} s^{-1}, (B) 1.7×10^{-5} s^{-1}, (C) 5×10^{-6} s^{-1}.

due to Henry's law [13], the dissolved oxygen concentration under a pure oxygen atmosphere will be higher by the factor of 100/21. Our experimentally found ratio of 4.4 between air and oxygen rates (Fig. 3A, 3B) provides clear evidence that dissolved molecular oxygen is a reacting species in this system and that dissolved oxygen accepts electrons from the NADH-nitrate reductase complex. The slight decrease in absorbance under an argon atmosphere (Fig. 3 C) may be due to a reaction between nitrite ion and NADH which can be also catalyzed by nitrate reductase [14].

A possible molecular mechanism may first involve the reduction of nitrate reductase-FAD by NADH to $FADH_2$ with a final electron transfer to oxygen to generate H_2O_2 and recycle the FAD [15]. This process can be represented by equation R2 and is also shown schematically in Fig. 1 [15]:

$$FAD + NADH \rightleftharpoons (FAD\cdot\cdot NADH) \xrightarrow{H^+} FADH_2 + NAD^+ \xrightarrow{O_2} FAD + H_2O_2 \qquad (R2)$$

The physiological reactions between $FADH_2$ and molecular oxygen can be divided into three classes, dependent whether oxygen acts as a 1-electron, 2-electron, or 4-electron acceptor [16].

In case of a 1-electron transfer, the superoxide anion O_2^- and a semiquinone is generated, while in case of a 2- or 4-electron transfer H_2O_2 or H_2O is formed, respectively.

The attempt to detect H_2O_2 in presence of excess NADH using the method of Ovenston and Rees [17] was not successful, probably due to interference with NADH.

However, Maldonado et al. [18] concluded that H_2O_2 has to be produced in a coupled NADH-nitrate reductase-peroxidase system in some way or the other in order to explain reactivation of a in vitro NADH-cyanide-inactivated nitrate reductase. It may therefore still be possible that some of the necessary H_2O_2 in the Maldonado et al. system has been produced by the NADH-nitrate reductase complex with molecular oxygen as an electron acceptor. The additional observation that the oxidative reactivation of nitrate reductase was enhanced by superoxide dismutase added before peroxidase but in the absence of glucose oxidase [18] supports this view and indicates that superoxide ion appears to be another intermediate in the NADH-nitrate reductase-O_2 system.

It has been reported that molecular oxygen is able to reactivate in vitro NADH-inactivated nitrate reductase with or without CN^- ion present [19]. Although the oxygen reactivation of a NADH-CN inhibited nitrate reductase is a considerably slower process than for example the ferricyanide reactivation [20, 21], it may involve the direct oxidation of molybdenum at the Mo-CN complex site creating an unstable Mo-CN complex with the subsequent release of cyanide ion [20]. This view, however, implies that molecular oxygen could possibly accept electrons at the molybdenum cofactor site if a direct oxidation of the Mo(V) in the cofactor to Mo(VI) by O_2 occurs. An interesting task appears to design experiments which can decide whether a two-site reduction of oxygen is taking place in the NADH-nitrate reductase system.

As can be seen from Fig. 2, the nitrate reductase-catalyzed removal of NADH by oxygen is a much slower process than the corresponding nitrate reduction by NADH. Therefore, only at low nitrate levels the competition between nitrate ion and dissolved oxygen for the reduced form of the enzyme will allow a sufficiently high rate in the reduction of oxygen. If O_2^- or H_2O_2 are intermediates or products in the reduction of oxygen by NADH-nitrate reductase, this may have physiological implications, since O_2^- and H_2O_2 are of considerably toxicity to living cells [22]. The mechanisms for O_2^- and H_2O_2 detoxification in the cytosol where nitrate reductase is believed to be located [23], is not well characterized [24, 25]. Inadequate mechanisms for detoxification of O_2^- and H_2O_2 in the cytosol may be a reason why nitrate reductase is only present when sufficiently high nitrate ion levels are available.

Further, Ninnemann and coworkers [28, 29] have shown that the cytochrome b 557 of nitrate reductase is very autoxidizeable and, in fact, formation of singlet oxygen is found in the presence of nitrate reductase. Singlet oxygen is detrimental to nitrate reductase, but this is a much slower process and cannot be seen on the time scale in our experiments.

Although there are a variety of other substances like ammonium [5, 30], ethylene [31], cytokinins [32, 33] and other growth regulators [32-34] which have influence on the nitrate reductase activity, it is the simultaneous presence of nitrate ion and light which has the greatest effects on the induction of nitrate reductase in green plants. More work is needed to understand the molecular mechanism of O_2 reduction in the NADH-nitrate reductase system and to see whether the avoidance of toxic

oxygen species may be part of the explanation why nitrate induction of nitrate reductase has evolved as one of the major control mechanisms.

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REFERENCES

[1] Hewitt, E. J. and Cutting, C. V. eds (1979) Nitrogen Assimilation of Plants, Academic Press, London.

[2] Ullrich, W. R., Aparicio, P. J., Syrett, P. J., and Castillo, F. eds (1987) Inorganic Nitrogen Metabolism, Springer-Verlag, Berlin.

[3] Rajasekhar, V. K. and Oelmüller, R. (1987) Physiol. Plant. 71, 517-521.

[4] Solomonson, L. P. and Barber, M. J. (1990) Ann. Rev. Plant Physiol. Plant Mol. Biol. 41, 225-253.

[5] Knobloch, O. and Tischner, R. (1989) Plant Physiol. 89, 786-791.

[6] Campbell, W. H. and Smarelli, J. (1986) in: Biochemical Basis of Plant Breeding (Neyra, C. ed.) pp. 1-45, CRC, Boca Raton.

[7] Hewitt, E. J. and Notton, B. A. (1980) in: Molybdenum and Molybdenum-Containing Enzymes (Coughlan, M. ed.) pp. 273-325, Pergamon Press, Oxford.

[8] Solomonson, L. P. and Barber, M. J. (1987) in: Inorganic Nitrogen Metabolism (Ullrich, W. R., Aparicio, P. J., Syrett, P. J., and Castillo, F. eds) pp. 71-75, Springer-Verlag, Berlin.

[9] De la Rosa, M., Roncel, M., and Navarro, J. A. (1989) Bioelectrochem. Bioenerg. 22, 355-364.

[10] Ruoff, P., Lillo, C., and Campbell, W. H. (1989) Biochem. Biophys. Res. Commun. 161, 496-501.

[11] Hyde, G. E., Wilberding. J. A., Meyer, A., Campbell, E. R., and Campbell, W. H. (1989) Plant Mol. Biol. 13, 233-246.

[12] Howard, W. D., and Solomonson, L. P. (1981) J. Biol. Chem. 256, 12725-12730.

[13] Castellan, G. W. (1983) Physical Chemistry, 3rd Ed, Addison-Wesley, Reading, page 311.

[14] Dean, J. V. and Harper, J. E. (1988) Plant Physiol. (1988) 88, 389-395.

[15] Klotz, I. M. (1987) in: Enzyme Mechanisms (Page, M. I. and Williams, A. eds) pp. 14-34, The Royal Society of Chemistry, London.

[16] Walpole, C. S. J. and Wrigglesworth, R. (1987) in: Enzyme Mechanisms (Page, M. I. and Williams, A. eds) pp. 506-533, The Royal Society of Chemistry, London.

[17] Ovenston, T. C. and Rees, W. T. (1950) Analyst 75, 204-208.

[18] Maldonado, J. M., Notton, B. A., and Hewitt, E. J. (1982) Planta 156, 289-294.

[19] Jetschmann, K., Solomonson, L. P., and Vennesland, B. (1972) Biochem. Biophys. Acta 275, 276-278.

[20] Solomonson, L. P. (1979) in: Nitrogen Assimilation of Plants (Hewitt, E. J. and Cutting, C. V. eds) pp. 199-205, Academic Press, New York.

[21] Echevarria, C., Maurino, S. G., and Maldonado, J. M. (1984) Phytochemistry 23, 2155-2158.

[22] Fridovich, I. (1986) Advances in Enzymology, 58, 61-97.

[23] Dalling, M. J., Tolbert, N. E., and Hageman, R. H. (1972) Biochem. Biophys. Acta 283, 505-512.

[24] Elstner, E. F. (1987) in: Biochemistry of Metabolism (Davies, D. D. ed) The Biochemistry of Plants, vol. 11, pp. 253-315, Academic Press, San Diego.

[25] Salin, M. L. (1987) Physiol. Plant. 72, 681-689.

[26] Ninnemann, H. (1982) Photochem. Photobiol. 35, 391-398.

[27] Fritz, B. J. and Ninnemann, H. (1985) Photochem. Photobiol. 41, 39-45.

[28] Lips, S. H. and Roth-Bejerano (1969) Science 166, 109-110.

[29] Kende, H., Hahn, H., and Kays, S. E. (1971) Plant. Physiol. 48, 702-706.

[30] Dilworth, M. F. and Kende, H. (1974) Plant. Physiol. 54, 821-825.

[31] Knypl, J. S. (1974) in: Mechanisms of Regulation of Plant Growth (Bieleski, R. L., Ferguson, A. R., Cresswell, M. M., eds.) The Royal Society of New Zealand, Wellington, pp. 71-76.

[32] Rao, L. V. M., Datta, N., Mahadevan, M., Guha-Mukherjee, S., and Sopory, S. K. (1984) Phytochemistry 23, 1875-1879.