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REVIEW

A MECHANISM FOR NITRATE TRANSPORT AND REDUCTION

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Key Word Index—Nitrate transport; nitrate reductase; nitrate reduction; nitrate-activated ATPase; membrane transport.

Abstract—It is proposed that a tetrahedron-shaped, transmembrane nitrate reductase tetramer functions as a carrier for nitrate transport. Reduction and transport are thereby brought about by the same enzyme complex. An ATPase is visualized to be closely associated with the nitrate reductase tetramer. The tetramer is apparently oriented such that one monomer is exposed to the outside of the plasmalemma while the other three are exposed to the cytoplasmic side. This orientation yields a reaction mechanism where the transport and reduction of one nitrate ion is accompanied by the transport of two additional nitrate ions (i.e. a 3:1 transport–reduction ratio). The proportion of transported nitrate that is reduced is apparently modulated by thiol reversible ADP inhibition of reduction. This inhibition, however, is probably the result of adenylate binding at sites on the proposed nitrate-activated ATPase to which nitrate reductase is tightly coupled. An analagous system consisting of a nitrate reductase dimer that spans a unit membrane plus an ATPase is proposed to be responsible for nitrate transport and reduction in some algae and chloroplasts.

INTRODUCTION

Active transport mechanisms can be divided into two major classes: carrier mediated transport and vesicle mediated transport (pinocytosis). At least three different mechanisms of energy coupling are involved in carrier mediated transport: (a) group translocation, (b) primary active transport and (c) coupled flow transport (see ref. [1] for review). A well-characterized example of group translocation is the phosphoenolpyruvate-dependent phosphotransferase system, where transport and chemical alteration appear to be coupled obligatorily. In this paper we summarize evidence from the literature to suggest that active transport of nitrate and nitrate reduction by nitrate reductase (EC 1.6.6.1, 1.9.6.1 and 1.6.6.2) may also be a group translocation type of active transport.

It is proposed that a membrane bound tetramer (native form) of nitrate reductase spans a unit membrane and acts as carrier for nitrate transport (Fig. 1). (Transport is meant to be movement of nitrate across the plasmalemma of microbial or plant cells). We envision the tetramer being oriented so that one monomer is exposed to the outside of the plasmalemma while the other three are exposed to the cytoplasmic side. Associated with each of the inner three monomers is an ATPase which has an adenylate binding site in very close proximity to the NAD(P)H binding site on the respective nitrate reductase monomer (Fig. 2). Binding of ADP at the adenylate binding site blocks NAD(P)H binding and thus reduction. It is further proposed that relative concentrations of ADP and thiol modulate the proportions of transported nitrate that is reduced since the ADP inhibition of reduction is thiol reversible. An analogous system consisting of nitrate reductase dimer associated with a single ATPase, spans a unit membrane also and is suggested to be responsible for nitrate transport and reduction in some algae and chloroplasts (transport in

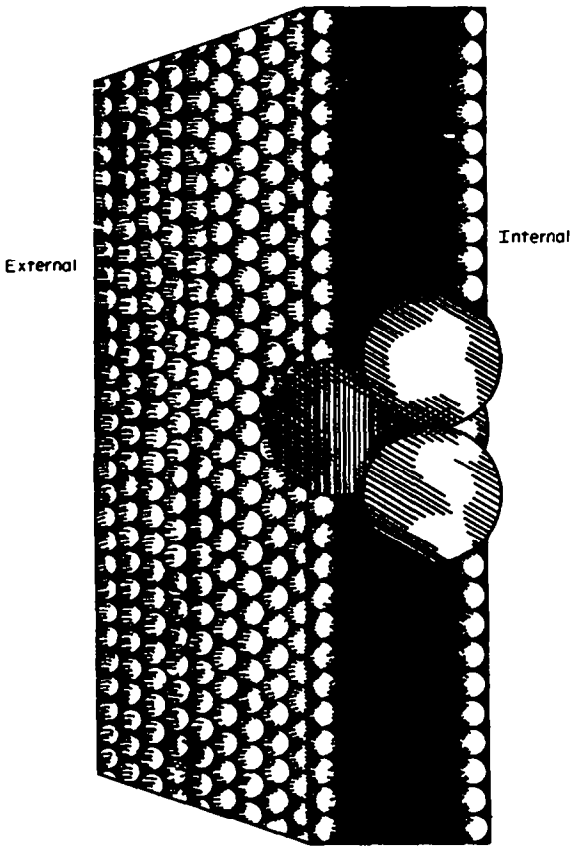

Fig. 1. Orientation of tetrahedron shaped nitrate reductase tetramer which spans a unit membrane. Three monomers are exposed to the cytoplasmic side of the plasmalemma while one extends out the other side.

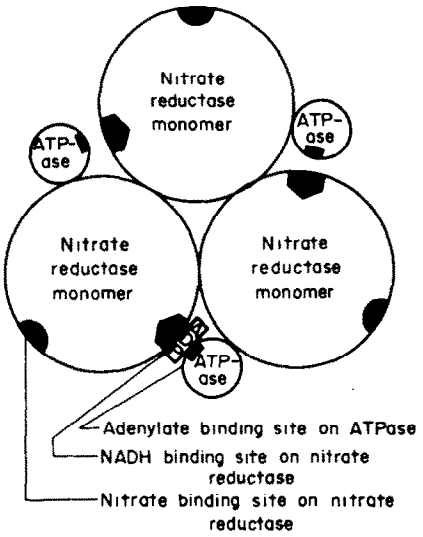

Fig. 2. Inner face (cytoplasmic side) of nitrate reductase tetramer–ATPase enzyme complex. The adenylate binding site on each of the three ATPase molecules is in close proximity to the NADH binding site on nitrate reductase so that ADP binding at the former blocks NADH binding and thus reduction.

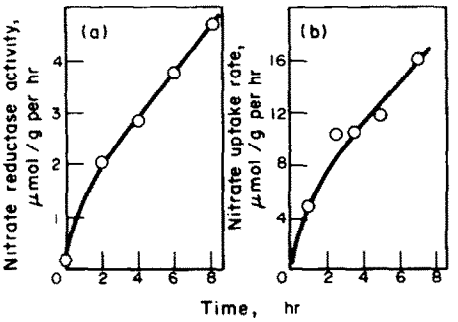

Fig. 3. A. Induction of nitrate reductase activity in decapitated corn seedlings harvested at 0, 2, 4, 6, 8 hr so that *in vitro* nitrate reductase activity could be measured at the same time nitrate uptake, translocation and accumulation were measured. This assay was essentially that described by Oaks *et al.*, [17] and Scholl *et al.*, [18]. Three % casein was added to the extraction solution. After homogenization the extract was filtered through 8 μm cellulosic filters instead of centrifugation and the filtrate used for enzyme source and tissue nitrate determination. Precision is such that three replicates are contained within the symbols. B. Induction of nitrate uptake activity corresponding to reductase activity in A. Procedures were very similar to those described by Jackson *et al.*, [6].

this case is defined as movement across the chloroplastic membrane).

Two necessary consequences of this proposal are: (a) nitrate reduction occurs only during the process of nitrate transport, and (b) intracellular nitrate is not readily reduced except during transport into chloroplasts. This concept contrasts with the view that the native form of nitrate reductase is a cytoplasmic monomer, and with the view that nitrate transport and nitrate reduction are separate events. The concept provides a rationale for the frequently-observed close relationship between these two activities.

INDUCTION OF NITRATE TRANSPORT AND NITRATE REDUCTASE

Nitrate reductase activity and nitrate transport activity appear to be obligatorily coupled [2, 3]. The rate of induction for both activities is increased by glucose or sucrose [2, 4, 5]. Inhibition of RNA and protein synthesis restricts the induction of nitrate transport [2, 6–8] and nitrate reductase [9, 10]. Both activities can be induced by nitrite [7, 11, 12] and coordinate regulation has been suggested [13]. Moreover, the simultaneous induction of nitrate transport, nitrate reductase and nitrite reductase has been reported [14, 15]. No one has shown induction of either nitrate reductase activity or transport activity without the other. Nitrate reductase and nitrite reductase are also significantly correlated ($r = 0.92$) in chlorophyll-deficient mutants of barley [16]. All these observations support the concept of a nitrate operon.

Figure 3 shows the induction of nitrate reductase activity (*in vitro*) and nitrate transport activity in roots of decapitated corn seedlings. The shape of the nitrate reductase and nitrate transport curves is similar to that found by Aslam and Oaks [4] for nitrate reductase of corn roots and that of Gealt and Axelrod [19] for nitrate reductase of the eucaryote *Aspergillus nidulans*. This shape is typical of non-gratuitous induction [20]. When the nitrate reductase activities in Fig. 3A are plotted against the corresponding transport activities in Fig. 3B a linear relationship which extrapolates to zero is obtained (Fig. 4). This is indicative of coordinate regulation [21] and implies that these two activities arise from common or neighbouring genes. In species examined to date, nitrate reductase activity is the product of a minimum of six genes [22–26; cf. 27]. Exactly which genes nitrate reductase and nitrate transport have in common remains to be elucidated. A possible mechanism of synthesis is shown in Fig. 5.

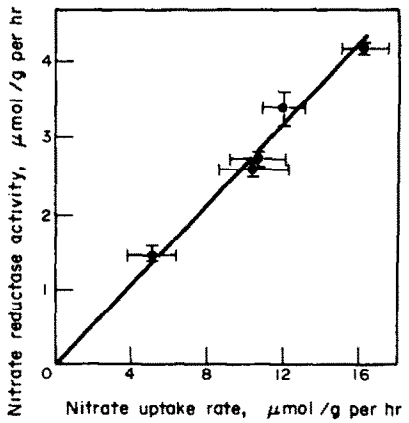

Fig. 4. Coordinate regulation of nitrate reductase activity and nitrate uptake activity. Ranges for nitrate reductase activity (vertical lines) are actual range of three replications while ranges for nitrate uptake activity (horizontal lines) are calculated standard deviation of uptake rate during time period preceding that where the estimate of rate is made.

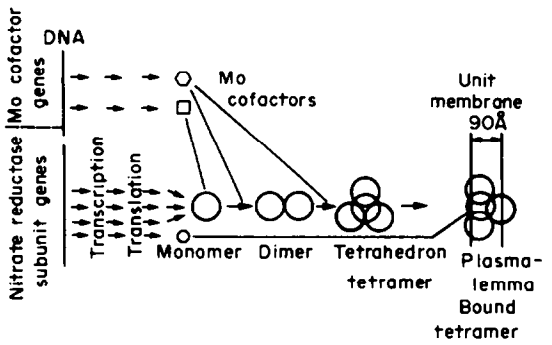

Fig. 5. Scheme for the synthesis of nitrate reductase species and function of multiple genes. The dimer is native to the chloroplast outer membrane and algae plasmalemma. The tetrahedron tetramer is native to the plasmalemma of bacteria and higher plant cells [28–32].

NITRATE REDUCTASE DIMER AND TETRAMER

A membrane bound nitrate reductase tetramer that spans a unit membrane (Fig. 1) and thus functions as both nitrate carrier and nitrate reductase appears to be a mechanism which accommodates many observations in the literature.

There is considerable evidence suggesting the existence of nitrate reductase tetramer and dimer as native forms [28, 30, 32–35]. Van't Riet and Planta [32] isolated two different homogenous nitrate reductase tetramers from bacterial membranes, which they designated nitrate reductase I and nitrate reductase II. They assumed both forms to be disimilatory (respiratory). The momomer MW of nitrate reductase I was 2.76×10^5 (9.8 S) while that of nitrate reductase II (1.74×10^5, 8.5 S) was very close to the monomeric MW of assimilatory nitrate reductase [30, 36].

The monomer of the assimilatory type has a MW of 1.52×10^5 in *Chlorella* [30] and 1.60×10^5 in maize [36]. Using the equation of Schachman [37] to calculate MW of nitrate reductase II from the sedimentation constant (S_{20w}) of 8.5 S, a value of 1.6×10^5 is obtained if the intrinsic viscosity is assumed to be 0.037 dl/g. On the other hand, the monomer of nitrate reductase I (dissimilatory) has two additional, identical subunits of MW 5.2×10^4 which contain iron [38]. It seems likely therefore that these subunits are involved in the electron flow from cytochrome *b*, (dissimilatory nitrate reduction) while the assimilatory nitrate reductase lacks this activity [39] simply because these subunits are lacking or nonfunctional. The species of monomer which has only one of these 5.2×10^4 MW subunits has a MW of 2.26×10^5 [32], and might be considered an intermediate between the assimilatory and dissimilatory forms. This corresponds well with the observed MW (2.3×10^5) of an assimilatory nitrate reductase monomer isolated from spinach [33] and barley [40]. In *E. coli*, Murray and Sanwal [41] found the purified assimilatory and dissimilatory forms to be serologically identical, but the species of nitrate reductase they were working with is not apparent.

Therefore it appears there is little difference between the assimilatory and dissimilatory nitrate reductase, but since the assimilatory form is well established as having a monomer MW of about 1.6×10^5 we will use this value for further calculations. The dimer would theoretically have a MW of 3.2×10^5 and the tetramer 6.4×10^5. Van't Riet and Planta [32] observed nitrate reductase II, which we presume to be the assimilatory enzyme, as having three sedimentation constants in phosphate or imidazole buffer: 13.9 S, 21.7 S, and 26.1 S. Calculating MW from the sedimentation constants as before yields: 3.3×10^5 (dimer, 6.5×10^5 (tetramer) and 8.6×10^5. The 8.6×10^5 species could be the tetramer of the assimilatory enzyme with its associated ATPase(s) as will be discussed later. What is important at present is the size and shape of the tetramer. Since the MW of the tetramer of the dissimilatory enzyme seems constant regardless of whether MW is determined by gel chromatography or summation of the subunit MWs after SDS treatment, gel electrophoresis, and subsequent gel chromatography [32], the tetramer must be fairly spherical cf. [42]. If it is assumed that the partial specific volume of nitrate reductase is 0.725 cm^3 g [30], then a hydrodynamic radius of the tetramer can be calculated [43] using the MW and sedimentation coefficient of Van't Riet and Planta [32]. The 6.4×10^5 MW (21.7 S) tetramer thus yields a radius of 7 nm. Therefore, a tetrahedral shaped tetramer capable of extending through both sides of a unit membrane to act as a nitrate carrier appears to be a plausible model. In fact, such a tetramer is capable of having part of 3 of its monomers extend from one side of a 9 nm thick membrane while the other extends out the opposite side (Fig. 1). In addition to the observations of particulate nitrate reductase in plasmalemma of bacteria [32, 42, 44], there have been a number of reports indicating the presence of particulate nitrate reductase in higher plants [45–48].

In the decapitated corn seedling experiment shown in Figs. 3 and 4, total nitrate in all tissue and xylem exudate as well as the amounts transported into the tissue were measured. Calculation of the total nitrate reduced during the experiment (Reduction = Uptake − [Translocation + Accumulation]) yielded values of 37% of the quantity transported. This is greater than indicated by integration of the *in vitro* nitrate reductase activities (Fig. 3) over time and implies that the *in vitro* assay, although very substantial for root tissue, underestimated the rate of reduction actually taking place. This experiment was conducted with complete nutrient solutions, and the uptake rates obtained after 8 hr of induction (16 μmol/g/hr based on fr. weight) were higher than those commonly reported for root systems [6, 8, 49–53]. The system was obviously extremely active. There are other reports indicating ratios of transport to reduction close to 3:1. After an induction period of about 5 hr, Chantarotwong *et al.* [53] observed rates of reduction in young barley seedlings to be 35% of the transport rate. Volk [54] has observed reduction to be 37% of transport using ^{15}N procedures during a 10 hr induction period with intact light grown maize seedlings. With nitrogen-deficient wheat seedlings, nitrate reduction was 35% of transport after 9 hr exposure to 0.5 mM Ca $(^{15}NO_3)_2$ [55]. In addition, a number of our unpublished experiments with decapitated maize seedlings yielded values around 40%.

As will be discussed later, transport across more than one plasmalemma (during translocation to other plant parts) is likely after several hr in these experiments. Hence, observation of a 3:1 transport–reduction ratio (33% reduced) is not expected. Also, if the soluble monomer, dimer or tetramer contribute to nitrate reductase activity, either during induction or decay, then a 3:1 ratio again

would not be expected. In view of the experimental complexities, however, a transport:reduction ratio of 3:1 during movement across a given cell membrane, in accord with the tetramer mechanism proposed here, appears reasonable.

KINETICS

The half-lives of nitrate transport and nitrate reductase activities should be similar if both activities are attributable to the membrane bound tetramer. However, if the soluble monomer, dimer, or tetramer also contributes to nitrate reductase activity then it might be expected to show a longer half-life than that of the transport activity. Goldsmith *et al.* [2] measured decay of both activities simultaneously and found half-lives of 0.6 and 2.2 hr for transport and reductase activities, respectively. Other investigators have found half-lives of 2.7 [7] and 5 [56] hr for transport activity while estimates of the half-life of reductase activity vary from 2 [17] to 4.3 hr [9, 57]. All these measurements have been made with non-synchronous cell cultures or developing tissue so that some variation in the half-life is expected. In addition, the presence of a constitutive protease which rapidly degrades nitrate reductase and which increases in activity as the tissue matures has been shown [58, 59]. The action of this protease is presumably responsible for the removal of nitrate reductase from membranes [42, 60–62]. Therefore, variable half-lives for both activities may be due to the use of non-synchronous cultures and/or the activity of this protease; so little can be concluded from these observations other than both activities have short half-lives.

Roustan *et al.*, [63] found the K_m for nitrate of a purified corn leaf nitrate reductase to be 2.0–3.5 $\times 10^{-4}$ M (depending on electron donor). Rao and Rains [51] and Chantarotwong *et al.*, [53] measured transport by solution depletion. This straight-forward procedure resulted in K_m's of 1.1 $\times 10^{-4}$ and *ca* 2.5 $\times 10^{-4}$ M in barley roots. Other investigators, using the same technique, have obtained similar results [7, 64, 65]. Brown *et al.*, [64] obtained a K_m of 2.6 $\times 10^{-4}$ for nitrate transport in marine bacteria and pointed out that this was of the same order as the K_m of nitrate reductase in these organisms (2.9 $\times 10^{-4}$ M) [66]. Neyra and Hageman [13], estimating transport by accumulation in corn root tissue, resolved multiple K_m's for nitrate transport in this same range. Heimer and Filner [14] measured transport as accumulation also, but in the absence of reductase activity by inhibiting it with 100 mM tungstate, and resolved a K_m of 4 $\times 10^{-4}$ M for nitrate transport. Tungstate, however, has an adverse effect on nitrate transport in root tissue [52, 54].

Earlier observations by Lycklama [67] and van den Honert and Hooymans [68] yielded smaller K_m values (*ca* 3 $\times 10^{-5}$ M) for nitrate transport with wheat and corn. Possible reasons for these lower values are discussed later.

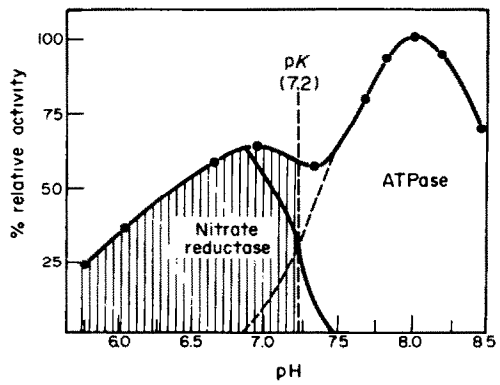

Fig. 6. The pH dependence of nitrate, chloride-activated ATPase [69, 70] and resolution into that of nitrate reductase and the ATPase.

INVOLVEMENT OF AN ATPase

If the proposed membrane bound dual-function nitrate carrier (nitrate reductase tetramer) is coupled to an ATPase then the resulting enzyme complex might exhibit properties of both enzymes. Falkowski [69, 70] measured the pH dependence of an ATPase which required both nitrate and chloride for maximal activity in crude preparations from two species of marine phytoplankton. Two relative maxima were observed which he assumed indicated the presence of two enzymes, the major peak (pH 8.0) representing the pH optimum of the nitrate, chloride-activated ATPase while the secondary peak (pH 6.9) was unexplained. If we assume this ATPase has a reaction mechanism like that of the sodium, potassium-activated ATPase, having an L-glutamyl-γ-phosphate residue at its active site [71] then the pH curve should have a pK near 7.2, the pK of phosphate. If we draw the left hand side of the primary peak of Falkowski's pH curve with this pK (dashed line, Fig. 6), and then resolve the right hand side of the secondary peak from the difference between the original and just drawn curve, the resulting curve (Fig. 6) is not unlike a pH curve for NADH:nitrate reductase from diatoms. Amy and Garret [72] purified nitrate reductase from a marine diatom with a pH optimum near 7.0. Thus the secondary peak observed by Falkowski [69, 70] is probably due to a nitrate reductase operating near its optimum pH so that the ATPase activity of this enzyme complex is relatively more efficient at pH 6.9 than at 6.5 or 7.3.

The assimilatory and dissimilatory forms of nitrate reductase apparently share the attribute of coupling to a nitrate-activated ATPase. In *Micrococcus* and *Pseudomonas* it is well established that dissimilatory reduction of nitrate to nitrite is coupled to oxidative phosphorylation [73–76]. With regard to ATPase activity, dissimilatory nitrate reduction appears to be the reverse of assimilatory reduction. Nitrite formed on the inside of the plasmalemma in the dissimilatory system presumably is extruded out of the cell, down its electrochemical potential gradient, accompanied by the synthesis of ATP. The nature of the electron donor does appear to be crucial since each pair of electrons donated by NADH yields two ATP molecules while donation by lactate or glycerol-1-phosphate yields one [77]. The well studied, ATP driven sodium, potassium transport system is partially or fully reversible [78] with resultant ATP synthesis [79].

Evidence from both assimilatory and dissimilatory nitrate reductase suggests that membrane-bound nitrate reductase is coupled to an ATPase and that it functions as a reductase as well as a carrier. The ATPase might be

expected to exert some form of control over nitrate reductase activity *per se*. Adenosine-5'-diphosphate (ADP), a product of ATPase activity, is a negative effector of nitrate reductase [80, 81]. Not only is this inhibition cooperative (with negative and positive phases) but 2',3'-cyclic AMP, 5'-AMP and ATP also induce cooperative kinetics with respect to NADH [82, 83].

We propose that the ADP inhibition of reduction is the modulator of the transport:reduction ratio. Eagle-sham and Hewitt [81] have shown that the ADP inhibition of nitrate reductase is reversible by thiol and NADH. Therefore, we might expect the transport:reduction ratio to be lower than 3:1 in a tissue where the endogenous thiol content is relatively high. This appears to be the case in corn scutellum tissue in which the thiol concentration is *ca* four-fold that of root tissue [84]. In an experiment with excised scutellum the transport:reduction ratio between 8 and 12 hr of exposure was 1.3:1 and 1.1:1 in 0.5 and 15 mM $K^{15}NO_3$, respectively [85]. Therefore it appears that physiological concentrations of thiols may be capable of modulating nitrate reduction by reversal of ADP inhibition [83].

The ADP inhibition of unpurified nitrate reductase [80–83] may actually be the result of ADP binding to the ATPase to which nitrate reductase is tightly coupled. Not only do ATPases possess separate regulatory and catalytic binding sites for nucleotides [86] but inactivation by ATP analogs is also thiol reversible [87]. The catalytic sites of ATPases contain two to six sulfhydryl groups each [88] whereas the NAD(P)H binding site of nitrate reductase has been shown to contain sulfhydryl groups only in crude extracts [9] and partially purified preparations (40-fold) [25]. The ATPase, or a subunit thereof, may have been physically coupled to nitrate reductase in these studies. In fact, the only observations of the adenylate inhibition of nitrate reductase [80–83] have been with unpurified or partially purified preparations. It seems likely therefore that the thiol reversible inhibition of nitrate reductase by various adenylates may be caused by binding at a catalytic or regulatory site on the ATPase.

What is important, however, is that this ADP inhibition prevents NAD(P)H binding to nitrate reductase apparently because the catalytic or regulatory binding site for ADP on the ATPase is presumably in very close proximity to the NAD(P)H binding site of nitrate reductase (Fig. 2). For this to be the case, there must be an ATPase molecule associated with each of three nitrate reductase monomers on the cytoplasmic side of the plasmalemma.

MECHANISM OF THE NITRATE REDUCTASE–ATPase COMPLEX

The tetramer is proposed to be oriented so that three of the monomers are exposed to the cytoplasmic side (Fig. 1) of the plasmalemma while one (external monomer) remains exposed to the ambient solution side. Nitrate transport appears to exhibit cooperatively [13, 51] supporting the assumption that a multimer is involved [89]. We visualize the monomer on the ambient solution side of the plasmalemma as binding nitrate ions and transferring them to each of the three monomers on the cytoplasmic side. It appears that at least two molybdenum atoms are located on the surface of each monomer, one (type A) functioning as a site for nitrate reduction and the other in monomer binding [28, 90]. The molybdenum atom (type B) involved in binding of the monomers may actually be 'shared' by two monomers. If both of these atoms function as nitrate binding sites, then the mechanism for nitrate transport may be visualized as a sequence in which the binding of nitrate to type A molybdenum of the external monomer is followed by transfer, via a conformational change (manifest as cooperative kinetics), to type B molybdenum which previously acted as monomer binder.

It seems reasonable to suggest that the transfer of nitrate from the one external monomer to the inner three would be rate limiting and would not readily occur unless the type B molybdenum sites on the inner three monomers have a higher affinity for nitrate than the type A molybdenum site on the external monomer. Hence, the K_m for transfer from the external solution to the interior of the tetramer would be dependent upon the affinity of nitrate binding to the type B molybdenum atoms. Two quite distinct K_m values for nitrate transport have been reported (see Kinetics). The low affinity transport K_m (*ca* 2×10^{-4} M) may be attributed to the type A molybdenum atoms serving as sites for reduction because the values are similar to the K_m for nitrate of purified nitrate reductase monomer [63]. The high affinity transport K_m (*ca* 3×10^{-5} M) would therefore reflect that of the type B molybdenum atoms involved in monomer binding. High affinity transport K_m values have been observed when relatively high molybdenum concentrations were present in the ambient medium [67, 68].

Hydrolysis of ATP, which has been suggested as an energy source for nitrate transport [51] may be responsible for the conformational change required to transfer nitrate from the external type A molybdenum site to the internal type B molybdenum sites. This concept is also consistent with the supposition that in the *lac* active transport system energy coupling is involved in one of the initial steps of transport [91]. A second conformational change could then transfer this nitrate ion, now located in the interior of the membrane, to the cytoplasmic side. Energy may also be involved in this movement.

This process is essentially repeated three times, transporting three nitrate ions across the membrane with the hydrolysis of three molecules of ATP (Fig. 7 shows a modified Cleland diagram [92] emphasizing the ordered nature of the proposed mechanism). The ADP formed during the first and second cycle (steps 1–7, Fig. 7) must stay bound on its respective ATPase (blocking reduction by preventing NAD(P)H binding). During the second cycle, binding of ADP is made easier by a conformational change induced by presence of ADP on the ATPase of NR1. In contrast, presence of ADP on the ATPases of both NR1 and NR2 makes ADP binding on the ATPase of NR3 extremely difficult (steps 10 and 13, Fig. 7); NAD(P)H, therefore, can bind to its site on NR3 and reduction occurs. The subsequent discharge of nitrite and $NAD(P)^+$ from NR3 could cause a further conformational change allowing the two nitrate ions on NR1 and NR2 to be released to the cytoplasm, followed by the release of the two ADP molecules from the ATPases of NR1 and NR2. The tetramer is then in a conformation suitable for repeating the entire reaction (Fig. 7). This proposed sequence is in accord with the ADP-induced cooperative kinetics with respect to NAD(P)H,

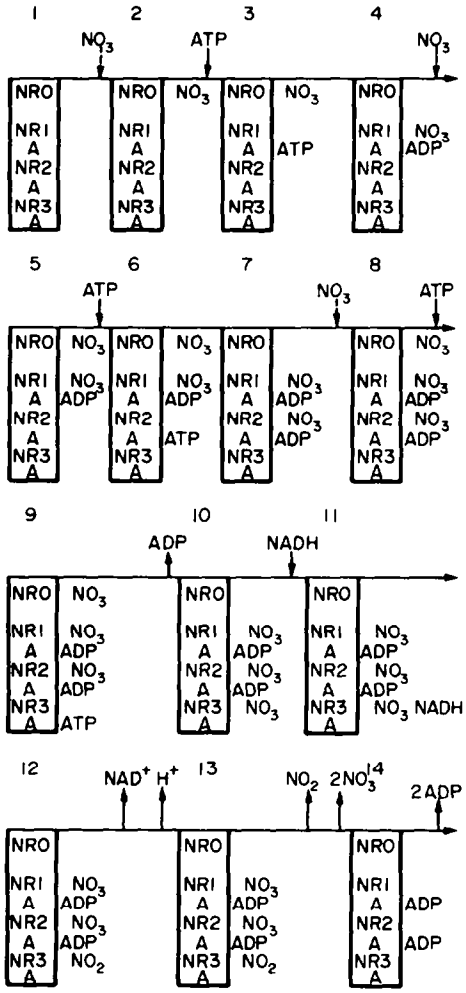

Fig. 7. Modified Cleland diagram [92], of the reaction mechanism of a membrane-bound nitrate reductase tetramer yielding a nitrate transport to reduction ratio of 3:1. Unbound substrates (NO_3^-, ATP, NADH) and products (NO_2^-, NO_3^-, ADP, NAD^+) are shown above the line while bound substrates and products and enzyme forms are located below the line. Dotted arrows represent nitrate originating from the outside of the plasmalemma while solid arrows represent substrates and products originating from or being released to, the cytoplasm. Hence, the only nitrate available for reduction is that which has been transported. NRO refers to the nitrate reductase monomer facing the outside of the plasmalemma while NR1, NR2 and NR3 refers to those monomers on the cytoplasmic side of the plasmalemma. The ATPase is of course envisioned as being on the cytoplasmic side also, one molecule associated with each nitrate reductase monomer on this side. These are represented by the A below NR1, NR2 and NR3.

exhibiting positive and negative phases, as observed by Eaglesham and Hewitt [82, 83]. This is therefore a group translocation type of transport; the only nitrate available for reduction is that which is being transported and, of a total of three transported, one is reduced.

Nitrate reductase (*in vitro*) is activated by phosphate [93]. In the reports by Eaglesham and Hewitt [81, 82] cooperative kinetics were obtained (Hill plot values $\geqslant 4$) indicative of a multimeric enzyme. This suggests a similarity between the nitrate transport–reduction system proposed here and the sodium–potassium transport system. In the latter the phosphorylated enzyme is the form to which potassium binds causing the discharge of sodium [94–96]. The enzyme is normally phosphorylated by ATP hydrolysis but this can also be accomplished with inorganic phosphate in the presence of ouabain [97, 98]. It seems plausible to suggest that a phosphoenzyme is involved in the nitrate transport–reduction system.

ALTERNATIVE ENERGY COUPLING TO PROTON TRANSLOCATION

There is evidence to suggest that active transport of nitrate can also be driven by a transmembrane pH gradient under certain conditions. As might be expected for anion transport, the protons presumably are transported inward through the membrane, the reverse of that observed with cation transport. When this type of energy coupling is functional, exogenous protons act as substrate so nitrate uptake is pH dependent. We visualize this process as utilizing the nitrate reductase tetramer as carrier; only the substrate for the ATPase differs from that described in the previous section. It sometimes has been observed that nitrate transport increases substantially as the ambient proton concentration increases from pH 8 to 4 [52, 68, 99]. The change in rate was not accompanied by a change in K_m and was observed over a wide concentration range [68], implying the system was not basically altered.

The inward translocation of protons by an ATPase coupled to ATP synthesis has been proposed [100]. Perhaps under some conditions the nitrate-activated ATPase is able to transport protons in this direction and thereby drive nitrate transport. Presumably, under normal physiological conditions proton transport in this direction would be exergonic when the cytoplasm is alkaline relative to the outside of the cell. Energy from this reaction might then be used in a more direct way (without ATP hydrolysis) by the nitrate-activated ATPase to bring about conformational changes in the nitrate reductase tetramer (carrier), thereby allowing transport. Apparently dissimilatory nitrate reductase, which was shown earlier (see Involvement of an ATPase) to be coupled to an ATPase, can also generate a transmembrane pH gradient [101, 102]. Haddock and Kendall-Tobias [102] proposed that ATP synthesis is driven by the reversal of the ATPase activity as described above, namely the outward extrusion of protons. An alternative possibility, however, is that ATP synthesis is coupled to the outward extrusion of nitrite (down its electrochemical potential gradient).

This mechanism of nitrate transport coupled to inward transport of protons is somewhat analogous to the mechanisms for anion transport proposed by Hodges [103] and Smith [104]. However, they suggested anion transport results from the exchange of anions for cellular hydroxide or bicarbonate ions. Experimentally it is nearly impossible to distinguish between the disappearance of a proton and the appearance of a hydroxide ion. The important feature is the pH gradient which can possibly serve as an alternative to ATP hydrolysis as a driving force for nitrate transport, utilizing the tetramer as carrier. The extent to which ATP hydrolysis serves as the driving force when there is a substantial pH gradient is not evident at this time. It is also unclear what proportion of the transported nitrate is reduced when the proton translocation energy coupling is functional.

NITRATE REDUCTASE DIMER AS CARRIER

Some organisms, such as certain algae, do not accumulate nitrate [105]. Nitrate or nitrite accumulation in chloroplasts would be detrimental (they are both strong oxidants) so a mechanism like the one proposed above where a transport to reduction ratio of 3:1 occurs would probably not exist in this organelle. Nitrate reductase has been shown to be associated with chloroplasts in wheat and spinach [47] and specifically with the chloroplast envelope membrane in *Eleusine coracana* [48]. In both algae [30, 106–108], and spinach leaves [109], nitrate reductase appears to exist as a dimer. A membrane bound dimer of molecular size such that it could span a unit membrane and therefore act as carrier and reductase at a 1:1 ratio (all the nitrate transported is reduced) would explain these observations. Solomonson, *et al.*, [30] estimated a Stokes radius of 8.9 nm for the nitrate reductase dimer (monomer MW = 1.52×10^5) they isolated from *Chlorella vulgaris*; the plasmalemma of algae is approximately 8 nm thick [110] while the chloroplast envelope membrane has an observed thickness of 6 nm [110]. The 5.0×10^5 MW nitrate reductase [109] isolated from spinach leaves is presumably a dimer [35] with associated ATPase or a dimer of the 2.3×10^5 MW monomer found in spinach [33]. Therefore, a nitrate reductase dimer could be responsible for active transport of nitrate into organelles or cells where no nitrate accumulation is observed.

Nitrate reduction in photosynthetic tissue is highly light dependent [110] and illumination increases the thiol concentration in chloroplasts [111]. These effects apparently are not due to enzyme synthesis or turnover because of the relatively short time in which they occur. It has been proposed [83] that the high chloroplastic thiol concentration is responsible for the lack of adenylate inhibition of nitrate reductase in the light. These observations are consistent with the hypothesis that nitrate transport and reduction occurs in a 1:1 ratio via a chloroplastic membrane nitrate reductase dimer–ATPase complex, and that the entire process is severely restricted in darkness by ADP inhibition owing to a low chloroplastic thiol concentration.

A possible mechanism is that the nitrate ion is bound to a type A molybdenum atom of the monomer on the outside of the membrane and transferred to a type B molybdenum atom functioning as binder of the monomers. A second transfer (or conformational change) brings the nitrate ion to the interior side of the membrane where it can be reduced to nitrate. In chloroplasts this nitrite may then be transferred directly to a nitrite reductase molecule which has been shown to occur in the chloroplast [48, 113–115]. It, therefore, is plausible that a membrane bound nitrate reductase dimer acts as a nitrate carrier and reductase in algae and chloroplasts resulting in a coupling of these two activities at a ratio of 1:1.

WHOLE PLANT DATA

As pointed out previously, overall transport to reduction ratios of about 3:1 (33% reduced) have been reported in several relatively short experiments with intact plants. According to the mechanism proposed here this ratio is only to be expected when the membrane bound tetramer is the dominant form (i.e. a fully induced system where the soluble forms are relatively few), when the majority of transport has taken place across only a single membrane, and when the ADP and thiol concentrations remain fairly constant. In an intact plant, the incoming nitrate must first pass the plasmalemma of root cells. Some of that which remains unreduced is deposited in the xylem by processes which may or may not involve transport across an additional membrane. Upon arrival in the leaf lamina, an additional plasmalemma transport process must occur, and the nitrate remaining unreduced upon deposition in the leaf cells now is available for the proposed chloroplast dimer 1:1 transport–reduction step. Hence, when time has passed for significant transport through more than one membrane, the reduced nitrate would be more than 33% of the total transported. A number of experiments with intact wheat seedlings yielded values ranging from 66 to 87% reduction of the nitrate absorbed during 24 hr [49, 116, 117]. Maize (4 inbreds and 6 hybrids) grown for 10.5 weeks in the greenhouse with nitrate as the sole source of nitrogen differed in the amount of nitrate absorbed but in each case reduced about 80% of the nitrate absorbed [118]. Most of the nitrate not reduced was accumulated in the stems. If the 3:1 transport to reduction ratio was strictly obeyed with each transport step, then 80% of the total amount absorbed would be reduced after passing through four membranes (Table 1). However, there are other possibilities to account for values of about 80% reduction. The 3:1 ratio may be decreased depending upon the relative ADP and thiol concentrations such that a proportion greater than 33% is reduced during transport across a given membrane. For example, transport across two membranes with a transport to reduction ratio of 3:2 would result in 89% reduction. In addition, it is likely that a sizeable amount of reduction occurs in chloroplasts because nitrite reductase, as well as nitrate reductase, are located there (see ref. [35]) and because nitrate reduction in photosynthetic tissue is highly dependent upon illumination [111]. For a significant proportion to be reduced in chloroplasts, no more than two 3:1 transport steps prior to chloroplast transport must be envisaged (cf Table 1).

Finally, it should also be pointed out that species apparently vary greatly with respect to the transport to reduction ratio in their root systems, as indicated by the percentage composition of nitrogen forms in bleeding xylem sap from a collection of herbaceous plants [119]. It would be interesting to determine if these differences could be explained by differences in the number of mem-

Table 1. Relationship between amount of nitrate reduced and unreduced after respective passes through a membrane assuming a 3.1 ratio of transport to reduction (one third of that which is transported is reduced at each passage through a membrane)

Number of membranes passed through	Percentage remaining unreduced	Percent reduced
0	100	0
1	67	33
2	44	22
3	30	15
4	20	10
Total		80

brane passes or by differences in the transport : reduction ratio at each membrane.

CONCLUSION

Various observations in the literature have led us to propose that a tetrahedron-shaped, transmembrane nitrate reductase tetramer functions as a carrier for nitrate; transport and reduction are thereby brought about by the same enzyme complex. Molecular size measurements calculated from sedimentation and MW data permit an orientation such that one monomer is exposed to the outside of the plasmalemma while the other three are exposed to the cytoplasmic side. Physiological studies showing that transport and reduction of one nitrate ion was accompanied by the transport of two additional nitrate ions (i.e. a 3 : 1 transport : reduction ratio) support the proposed orientation. Coupling of phosphorylation to nitrate reduction by dissimilatory nitrate reductase, an observation of a nitrate-activated ATPase, and various kinetic studies are used to develop the concept that an ATPase is associated with each of the three inner nitrate reductase monomers. Each ATPase is visualized as having an adenylate binding site in very close proximity to the NAD(P)H binding site on the respective nitrate reductase monomer; ADP binding at this site blocks NAD(P)H binding and thus reduction. This ADP binding is thiol reversible so that the relative concentrations of ADP and thiol modulate the proportion of transported nitrate that is reduced. Predictions of this mechanism are (a) only transported nitrate acts as substrate for reduction, and (b) intracellular nitrate is not readily reduced.

An analogous system consisting of a nitrate reductase dimer that spans a unit membrane plus an ATPase is proposed to be responsible for nitrate transport and reduction in some algae and chloroplasts. This system theoretically reduces all the nitrate that is transported so that no nitrate accumulation occurs.

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