Isotopic fractionation by plant nitrate reductase, twenty years later

Guillaume Tcherkez\textsuperscript{A, B, C} and Graham D. Farquhar\textsuperscript{B}

\textsuperscript{A}Laboratoire d’Ecophysiologie Végétale, Bâtiment 362, Université Paris XI, 91405 Orsay, France.
\textsuperscript{B}Environmental Biology Group, Research School of Biological Sciences, Australian National University, GPO Box 475, Canberra, ACT 2601, Australia.
\textsuperscript{C}Corresponding author. Email: guillaume.tcherkez@ese.u-psud.fr

Abstract. Plant nitrate reductase, the enzyme that reduces nitrate (NO$_3^-$) to nitrite (NO$_2^-$), is known to fractionate N isotopes, depleting nitrite in $^{15}$N compared with substrate nitrate. Nearly 20 years ago, the nitrogen isotope effect associated with this reaction was found to be around 1.015. However, the relationships between the isotope effect and the mechanism of the reaction have not yet been examined in the light of recent advances regarding the catalytic cycle and enzyme structure. We thus give here the mathematical bases of the $^{14}$N/$^{15}$N and also the $^{16}$O/$^{18}$O isotope effects as a function of reaction rates. Enzymatic nitrate reduction involves steps other than NO$_3^-$ reduction itself, in which the oxidation number of N changes from $^+$V (nitrate) to $^+$III (nitrite). Using some approximations, we give numerical estimates of the intrinsic N and O isotope effects and this leads us to challenge the assumptions of nitrate reduction itself as being a rate-limiting step within the nitrate reductase reaction, and of the formation of a bridging oxygen as a reaction intermediate.

Introduction

Many plant physiological processes, such as growth, depend upon N supply. Apart from symbiotic N$_2$-fixing species, the major pathway by which nitrogen is converted to an organic form in plants involves the key enzyme nitrate reductase (NR), which reduces nitrate (NO$_3^-$) to nitrite (NO$_2^-$). It is thought that this reaction, together with subsequent NO$_2^-$ reduction, uses as much as 25% of the energy from photosynthesis and enzyme structure. NR appears to be the rate-limiting and regulated step of nitrate assimilation and, consequently, it is likely that NO$_3^-$ reduction is a limiting factor for growth and development. NR is an enzyme of fundamental importance, and immense effort is currently being made to understand the structure and the mechanism of NR, and to develop methods for its improvement.

Stable nitrogen isotopes ($^{14}$N/$^{15}$N) are useful for understanding biological processes, from enzymatic mechanisms to global signals (Yakir 2002). For example, nitrate assimilation by plants is strongly related to $^{15}$N natural abundance in plant communities (Robinson 2001). Making correlations between the $^{15}$N natural abundance of various N-containing metabolites helps to understand metabolic fluxes and N allocation in plants (Yoneyama et al. 2003). At a larger scale, the nitrogen isotope composition of organic matter is an important tool for estimating the contribution of N$_2$-fixation vs nitrate uptake and reduction in soil and vegetation (Shearer and Kohl 1986), for assessing the use of N fertilisers in crops (Amberger and Schmidt 1987) or for investigating the absorption of guano-derived N by plants in coral cay ecosystems (Schmidt et al. 2004). In this context, the reduction of nitrate by NR, which is the first step of N assimilation, is an important component of N use by plants. Plants inherit the depletion in $^{15}$N induced by NR (Werner and Schmidt 2002), unless subsequent $^{15}$N-enriching processes occur or the observed isotopic fractionation becomes negligible because of N-limited conditions (in such a case, the fractionation indeed disappears because all the NO$_3^-$ molecules are assimilated). In particular, we note that the final isotope abundance in plant organs is the result of several steps, namely, nitrate absorption and reduction, nitrite reduction, and N metabolism and losses, and may not reflect the intrinsic isotope fractionation by NR.

It is now nearly twenty years since the $^{14}$N/$^{15}$N isotope effect associated with nitrate reduction by plant NR was reported in the Australian Journal of Plant Physiology (Ledgard et al. 1985) with a fractionation of 15% against $^{15}$N. Isotope effects ($k_{\text{light}}/k_{\text{heavy}}$) are closely related to the pathways of the reactions considered (limiting steps, transition state structure) and so are a useful tool for elucidating chemical mechanisms (Cleland 2005). This may be helpful in the case of NR as its mechanism is still somewhat uncertain (see below), and is why we give here an isotopic
viewpoint on the NR-catalysed reaction. Values of isotope effects and correlations with other kinetic properties are reviewed and an updated version of the kinetics of NR is proposed.

Nitrate reductase and its assumed mechanism

Nitrate reductase is a complex, multimeric enzyme that contains an internal electron transfer system reduced by NAD(P)H (there are two forms in nature: one NADH-specific, one non-specific). The structure and the biochemical mechanism of the enzyme are largely independent of whether the plant source tissue is leaf or root. NR contains the molybdopterin cofactor, which contains the Mo atom responsible for nitrate reduction: nitrate is reduced to nitrite (that is, N oxidation number goes from +V to +III) while molybdenum MoIV is oxidised to MoVI.

In order to elucidate the biochemical mechanism of NR and to determine limiting steps, some studies have focused on reactions that occur between molybdenum and nitrate, using non-biological complexes of molybdenum. With oxopentachloromolybdate (MoV), the reduction of nitrate proceeds in two steps, namely, complexation (complex formation) and oxygen transfer (from nitrate to Mo), and classical kinetic investigations suggested that O transfer is considerably more rapid than complexation (Taylor and Spence 1975). The oxygen transfer, in this non-biological case, is thought to involve NO2−, which is subsequently reduced to NO2− (Taylor et al. 1979). In addition, during the reduction of nitrate by dihydroxo-molybdenum-triazacyclononane monohydrate (MoIII), it was suggested (Weighardt et al. 1985) that the replacement of H2O by NO3 in the coordination sphere of Mo (complexation) might be limiting.

Uncertainties remain as to whether the reaction proceeds via a bridging oxygen (that is, the formation of a Mo–O–N bond) or not. It appeared that the reaction with dihydroxo-molybdenum-triazacyclononane monohydrate did not involve a bridging oxygen (Weighardt et al. 1985), but an oxygen transfer involving a bridging oxygen was observed during nitrate reduction by diphenylmercaptethyl pyridine complexes of molybdenum (MoIV), which is the natural reduced Mo state in the active site of NR (Craig and Holm 1989). It was further demonstrated that the oxygen atom of nitrate is transferred to Mo, using 18O-labelling and phenyl pyridylmethanolate as a complex of molybdenum (MoIV) (Schultz et al. 1993).

Thus from the present overview of non-biological complexes a plausible two-step mechanism emerges with (i) nitrate binding, possibly involving a Mo–ON02− bond formation and (ii) the O–N cleavage with oxidation of MoIV to MoVI (Fig. 1), adapted from Fischer et al. 2005). This plausible mechanism is consistent with what has been found in the similar enzyme sulfite oxidase (SO), which involves an oxygen transfer and a bridging oxygen between SO32− and Mo (for a recent review, see Hille et al. 1999). Analysis of the crystal structure of NR further showed that the nitrate-binding domain is similar to the sulfite-binding domain of SO. However, there are differences in some residues of the active site, the most striking being the substitution of SO’s Arg (374) by Met (427) and of Tyr (241) by Asn (272) (Fischer et al. 2005). The functional consequence of the first substitution is unsurprising, as the Arg residue is responsible for a salt-bridge with an oxyanion group of sulfite in SO, while the binding of nitrate does not involve such a bridge. Rather, one oxygen atom of nitrate should point towards the molybdenum centre (Schrader et al. 2003). The functional impact of the Tyr-to-Asn substitution is, by contrast, still to be established, but one may hypothesise that it stabilises nitrate binding.

X-ray absorption fine structure studies also showed that unlike SO, NR of *Arabidopsis* undergoes (minor)
changes in the Mo–S coordination (bonds) during catalytic turnover (George et al. 1999). Thus, although some data indicate that NR’s mechanism may be similar to that of SO, the extent to which the mechanisms are identical, and the details of NR’s intrinsic mechanism, are not established.

There are also still uncertainties about which step limits the reaction. NAD(P)H and nitrate bind to the enzyme at two separated active sites (Solomonson and Barber 1990). The catalytic ratio of this active site for pyridine nucleotide electron donation is much greater than that of the active site for nitrate reduction (Skipper et al. 2001). In other words, NR (with a catalytic rate constant of 150–200 s⁻¹) does not appear to be limited by its rate of reduction. In addition, recombinant cytochrome reductase fragments of NR have very high FAD reduction rates (~500 s⁻¹), suggesting that NR catalytic activity is limited either by internal electron transfer to molybdenum, by the rate of nitrate reduction, or by the rate of nitrite release. Recent in vitro work on NR suggests that the rate of nitrate reduction varies from high rates in pre-steady-state conditions (nearly fully-reduced enzyme) to lower values at the steady state, similar to the steady electron transfer rate (Skipper et al. 2001). However, apart from this study, data on the mechanism and the limiting step of the enzyme are scarce. In addition, it is likely that the enzyme is sensitive to molybdenum concentration in solution, a factor that may drastically change kinetics of the reaction (Skipper et al. 2001).

Classical theory of isotope effects applied to nitrate reductase

The general relationship between the kinetic isotope effect (KIE) and the mechanism of a reaction or the nature of transition states is described elsewhere (Berti 1999) and are not detailed here. Briefly, for a reaction that is made up of several elemental steps, the observed KIE is equal to the product of the stepwise KIEs of each reaction step. Classical theory of isotope effects applied to nitrate reductase is given by Equation (1) for the reaction rate constants.

\[
\alpha_\text{obs} = \frac{\alpha_i + \varepsilon}{1 + \varepsilon}.
\]  

(1)

Equation (1) allows one to see the two main parameters that influence the observed KIE. The partition factor is generally expressed using the rates of the individual steps, so that the more limiting the isotope-sensitive step, the lower the ε value. The intrinsic isotope effect mainly depends upon the bond rearrangements during the isotope-sensitive step and the structure of the transition state.

The mechanism of the nitrate reductase reaction is modelled by a three-step process (Fischer et al. 2005) as described in Fig. 1A. There are primary 14N/15N and 16O/18O isotope effects on k2 (these isotope effects are denoted as α2 (14N) and k2 (14O), respectively, and, for brevity, will be denoted as α2 and k2 in Eqn 2). Because a N–O bond is broken.

The first step (rate constants k1 and k−1) may also be subjected to a secondary 14N/15N and 16O/18O isotope effect, that is, caused by changes of bonds that do not involve the atom considered; this is opposed to a primary isotope effect that is due to changes in bonds directly involving the atom of interest) because of the formation of the Mo–O bond. While being initially neglected here, it will be discussed later. In such a mechanism, the commitment to catalysis is:

\[
e = \frac{k_1}{k_{-1}}.
\]

We note that if steps k1 and k−1 are also associated with an isotope effect, the overall observed isotope effect is given by:

\[
\alpha_{\text{obs}} = \frac{\alpha_2 \times \alpha_i}{\alpha_{-1} \times \alpha_i \times (1 + \varepsilon)}.
\]  

(2)

where \(\alpha_i\) is the (intrinsic) isotope effect associated with step ki and so is given by \(\alpha_i = 14k_{i}/15k_{i}\) for nitrogen and oxygen isotopes, respectively.

The intrinsic isotope effect

For a chemical step that converts reactants R into products P and which proceeds through a transition state (φ), the isotope effect, defined as the rate constant ratio \(k_2/15k_2\), is given by (Bigeleisen 1949):

\[
\frac{k_2}{k_{15}} = \left(\frac{14\mu_2}{15\mu_2}\right)^{\frac{3}{2}} \left(\frac{14\mu_0}{15\mu_0}\right)^{\frac{1}{2}} \left(\frac{14\mu_F}{15\mu_F}\right)^{\frac{1}{2}} \left(\frac{\Pi \sinh(15\kappa_0)}{\Pi \sinh(14\kappa_0)}\right)^2.
\]  

(3)

where M are the molecular masses, μ are the moments of inertia components and κ is the transmission coefficient (fraction of molecules that pass the energy barrier in the forward direction). The superscripts R and φ refer to the reactants and the transition state, respectively. ν is given by νi = hνi/k0T where νi is the frequency in the vibrational mode i, k0 is Planck’s constant, and T the absolute temperature. sinh is the hyperbolic sine and is defined as: sinh(λ) = e^(λ) − e^(-λ). For oxygen isotopes, superscripts 14 and 15 are changed to 16 and 18, respectively.

The component κ includes effects of tunneling (that is, the possibility that a particle of small mass passes through...
the energy barrier as a consequence of the Heisenberg uncertainty principle as well as of recrossing of the energetic barrier. Generally, it is thought that the tunneling effect is negligible at ordinary temperatures with heavy atoms like $^{14}$N/$^{15}$N or $^{16}$O/$^{18}$O so that the isotope effect on $k$ is considered to be of minor importance (Van Hook 1971; Saunders 1986, but see Meyer et al. 1999 and Lin et al. 2005 for chlorine and carbon). Simplification of the component associated with moments of inertia in Eqn (3) with the Teller–Redlich rule gives (Bigeleisen 1949; Bigeleisen and Saunders 1958; Melander 1960):

$$\frac{14_k}{15_k} = \prod_{i=1}^{N} \frac{(14_{\mu_i})^{3/2}}{(15_{\mu_i})^{3/2}} \prod_{i=1}^{N} (\frac{14_{\mu_i}}{15_{\mu_i}})^{3/2}$$

$$\frac{14_{\mu_i}}{15_{\mu_i}} = \frac{1}{2} \sum_{\sigma=\pm 1} \sinh(15_{\mu_i} / 2) \sinh(14_{\mu_i} / 2)$$

where the $\mu_i$ are the atomic masses and $u_i$ the reaction coordinate frequency factor. Provided that all the frequencies are known in both reactant and transition state, the kinetic isotope effect can be calculated (for a recent review, see Paneth 1995). Kinetic isotope effects are almost insensitive to changes in structural parameters, unless structural changes also induce changes in bonding parameters such as force constants and frequencies.

So the important factors in determining the calculated isotope effect are vibrational frequencies. Provided one has an estimate of frequencies, an estimate of the kinetic isotope effect can be calculated. Simple calculations for the NR-catalysed reaction are made here using a simple model composed of four atoms (–O–NO$_2$), with vibrational frequencies of NO$_3^−$ and Metal–O–NO$_2^−$ taken from the literature (National Institute of Standards and Technology 2005), corrected for bond orders (Sims and Lewis 1984).

### Nitrate reductase’s observed and intrinsic isotope effects

The $^{14}$N/$^{15}$N isotope effect associated with nitrate reduction in different organisms is shown in Table 1. When the isotopic composition of whole-plant material and source nitrate are compared, the isotope effect is as low as 1.005, probably because of N limitation during growth and other steps subsequent to nitrate fixation (for example, amino-acid synthesis, N losses), that influence the $^{15}$N abundance of plant material. Except for the value of 1.029 found in maize and reported in the PhD thesis of Olleros-Izard (1983), the in vitro isotope effect of NR has been measured to be 1.015 in spinach. The value found in maize is nevertheless sensitive to NADH concentration and decreases from 1.029 to 1.024 when NADH is not constant and progressively consumed by the reaction with the maize enzyme. The isotope effect inferred from the isotope composition of total organic N in algae (fed with NO$_3^−$) is around 1.022 and close to the value inferred from the comparison of the natural $\delta^{15}$N value of different compounds in Brassica leaves (1.019). Although not explained yet, the variability from one organism to another might originate from different NR regulatory properties, which influence the rate of the reaction and the isotope effect. The isotope effect associated with the inorganic reduction of nitrate by silver and iron is much greater, approximately 1.075 at 25°C. This reaction is thought to proceed through a one-step transfer of the oxygen atom from nitrate to Ag (Ag$^+$ is previously reduced by Fe$^{2+}$, producing AgO and nitrite. The $^{16}$O/$^{18}$O isotope effect has been measured once in maize and is of the same order of magnitude as for $^{14}$N (1.015, Table 1). Both N and O isotope effects are low, indicating that the reduction of nitrate and the exchange of oxygen are not the

<table>
<thead>
<tr>
<th>Reference</th>
<th>$^{14}k/^{15}k$</th>
<th>$^{14}k/^{15}k$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Olleros-Izard (1983)</td>
<td>1.029 ± 0.005</td>
<td>1.015 ± 0.008</td>
</tr>
<tr>
<td>Ledgard et al. (1985)</td>
<td>1.024 ± 0.001</td>
<td>1.015</td>
</tr>
<tr>
<td>Werner and Schmidt (2002)</td>
<td>1.015</td>
<td>1.015</td>
</tr>
<tr>
<td>Yoneyama et al. (2003)</td>
<td>1.022</td>
<td>1.015</td>
</tr>
<tr>
<td>Brown and Deury (1967)</td>
<td>1.075 ± 0.004</td>
<td>1.045</td>
</tr>
</tbody>
</table>

$k$ values calculated using the difference in the nitrogen isotope composition between source NO$_3^−$ and whole-plant material.

Values obtained in vitro on the purified enzyme at constant NADH concentration.

$^{15}$N values inferred from the comparison of $^{15}$N natural abundance in supplied nitrate, leaf nitrate and amino acids.

Values inferred from the authors from the comparison of internal nitrate with supplied nitrate.
limiting steps of the catalytic cycle. The extent of the kinetic impact of these steps on the reaction rate may be numerically assessed by comparing the observed isotope effects with the intrinsic values.

Both N and O intrinsic isotope effects are unknown, but some calculations can be made (see the previous section). For the intrinsic isotope effects associated with $k_1$ and $k_2$, a secondary isotope fractionation (the fractionation is the deviation of the isotope effect from 1) of a few per mil at most is envisaged for both (Table 2), with a very small resulting equilibrium ($k_1 / k_2$) of approximately 1.000 and a maximum value of $k_1 / k_2 = 1.015$ (destruction of the bond) of 1.080 (Table 2).

One may hypothesise (Hammond’s postulate) that the transition state is reactant-like as it has been found that the activation energy of NO production is $30 \text{kJ mol}^{-1}$ (Lillo et al. 1997) while the reduction of nitrate to nitrite has a large negative free energy of $-143 \text{kJ mol}^{-1}$ (Campbell 1999). As a consequence, one may expect a low intrinsic isotope effect (Berti 1999) that we estimated to be around 1.037 (Table 2). This value is similar to what would be obtained with the simplified equation used by Tcherkez and Faquhar (2005) [that only takes into account the isotope ratio of $\sinh(u)$, where $u$ is the imaginary frequency corresponding to the motion of the O–N bond that is broken by the reaction] with a frequency value between 700 and 800 cm$^{-1}$, a realistic range for this bond.

The oxygen isotope effect of the overall reaction needs more caution as the equilibrium of bridging oxygen formation ($k_1 / k_2$) may be subjected to a non-negligible isotope effect. However, giving an estimate for both bridging oxygen formation and breakdown ($k_1 / k_2$) is rather difficult as there are no available clues to the transition state structures. The vibrational frequency associated with a Mo–O bond is approximately 670 cm$^{-1}$, giving a value for $k_1 / k_2$ of approximately 1.000 and a maximum value of $k_1 / k_2 = 1.015$ (destruction of the bond) of 1.080 (Table 2).

We note that these values give an equilibrium isotope fractionation of approximately $-20\%$ (i.e. an isotopic enrichment of the bonded O), a value that is not very different from the already known $^{18}O / ^{16}O$ thermodynamic isotope effect associated with oxygen exchange between a carbonyl-bonded O and water (Farquhar et al. 1998). For nitrate reduction, using a small bond order decrease in the transition state (that is, a small change of bond frequency between the reactant and the transition state), as we did for $^{15}N$ (see above), we obtain a $^{18}O / ^{16}O$ isotope effect on $k_2$ of approximately 1.050 (Table 2).

### Table 2. Orders of magnitude of the $^{16}O / ^{18}O$ and $^{14}N / ^{15}N$ isotope effects (denoted as $k_1 / k_2$) respectively associated with some steps of nitrate reduction

<table>
<thead>
<tr>
<th>Reaction</th>
<th>$^{16}O / ^{18}O$</th>
<th>$^{14}N / ^{15}N$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Formation of Mo–O–NO$_3^-$</td>
<td>1.055–1.065$^a$</td>
<td>1.080$^a$</td>
</tr>
<tr>
<td>Cleavage of Mo–O–NO$_3^-$</td>
<td>~1.000</td>
<td>1.002–1.004</td>
</tr>
<tr>
<td>Direct cleavage of O–NO$_3^-$</td>
<td>1.050$^b$</td>
<td>1.037$^b$</td>
</tr>
</tbody>
</table>

$^a$Mo–O stretching frequency of 670 cm$^{-1}$ and a bond order range of 0.1–0.3.

$^b$A decrease of the Mo–O bond order between 0.1 and 0.5 in the transition state.

$^{18}O$–O–N stretching frequency of 800 and 1330 cm$^{-1}$, respectively, and a decrease of the O–N bond order of 0.1 in the transition state.

$^c$Value measured by Brown and Drury (1967).

### Isotopes and nitrate reductase’s mechanism

The comparison of the observed and the intrinsic isotope effects allows one to calculate the commitment to catalysis, $c$, using Eqs (1) and (2) and thus, to check the consistency of the reaction mechanism when comparing $c$ values obtained with $^{15}N$ and $^{18}O$ (double isotope effect method, Schmidt and Medina 1991). The commitment $c$ can be calculated, re-arranging Eqn (2):

$$c = \frac{\alpha_{14} - \alpha_{15}}{\alpha_{iso} - \alpha_1}$$

It gives $c \approx 1.037–1.045$ for $^{15}N / ^{14}N$ isotope effects, while $c = \frac{\alpha_{18} - \alpha_{16}}{\alpha_{iso} - \alpha_1} \approx 0$ with $^{18}O / ^{16}O$ isotope effects. This discrepancy would be even larger (with large negative $c$ values) if the transition state structure for the formation of the bridging oxygen ($k_1$) were very advanced (more product-like), that is, if $\alpha_1$ were larger.

Alternatively, calculations can be made assuming a one-step process for oxygen exchange (from Mo–OH $\rightarrow$ NO$_3^-$ to Mo=O+NO$_3^-$), the Michaelis equilibrium being without any covalent bond (Fig. 1B). In such a case, the intrinsic N isotope effect associated with O–N cleavage of nitrate is certainly similar to that observed for the inorganic reduction (1.075, Table 1), a value that is consistent with calculations of the $^{18}O / ^{16}O$ isotope effect on $k_2$ using Biegelisen’s Eqs (4) and (5) in a simplified form (Brown and Drury 1967). The $^{18}O / ^{14}O$ isotope effect can be calculated in the same way, that is, assuming a frequency of approximately 1350 cm$^{-1}$, giving a value of 1.090. Equation (1) can be re-arranged to:

$$c = \frac{\alpha_{18} - \alpha_{14}}{\alpha_{iso} - \alpha_1}$$

then we find a partition factor $c$ of $1.075–1.015$ from N and O data, respectively.
Both calculations (with and without the bridging oxygen hypothesis) using N isotope data indicate that nitrate reduction per se is not a limiting step of the reaction, because the partition factor \( k_2/k_{-2} \) is not negligible compared to 1. It also emerges from the observed isotope data that the mechanism that does not involve a bridging oxygen (illustrated in Fig. 1B) is more likely, because a unique value of the commitment to catalysis satisfactorily explains both observed \(^{14}\text{N}/^{15}\text{N} \) and \(^{16}\text{O}/^{18}\text{O} \) isotope effects.

Further arguments against the formation of a bridging oxygen during the first reaction intermediate might be given by the use of alternative substrates, such as halogenic acids (e.g. HClO\(_3\), HBrO\(_3\)), that are also reduced by NR. Halogenic acids have lower O–X bond energies than that of O–N in nitrate and are better nucleophiles than nitrate (Table 3). So if that bridging oxygen were involved, one may predict higher \( V_{\text{max}} \) (higher rate constant \( k_1 \)) and lower \( K_{\text{m}} \) values, unless steric constraints disfavour binding (particularly for iodine, quite a large atom). However, evidence is to the contrary: the \( V_{\text{max}} \) value of borate only is slightly higher, and \( K_{\text{m}} \) values are strikingly high (Table 3; Barber and Notton 1990). This simply suggests that the first step may not involve a covalent bond (that is, a bridging oxygen), but rather, a complexion event only (explaining the predominance of steric effects on \( K_{\text{m}} \), followed by a ‘direct’ reaction of oxygen exchange between the substrate and the Mo atom (explaining the slight effect only of nucleophily, the best nucleophile IO\(_3^−\) having a higher bond energy compared with the other two halogens).

We recognise that additional factors may contribute to diminishing the \(^{14}\text{N}/^{15}\text{N} \) nitrogen isotope effect to 1.015, such as a strong limitation on \( k_1 \) (if \( k_1 = 0 \), the reaction eventually consumes all the intermediary molecules and step \( k_2 \) cannot fractionate). This view would accord with the observation that the \(^{14}\text{N}/^{15}\text{N} \) isotope effect is lower when NADH concentration decreases as the reaction proceeds. However, kinetic data of Skipper et al. (2001) on recombinant Arabidopsis nitrate reductase showed that the nitrate reduction rate and the internal electron transfer rate are of the same order of magnitude.

**Table 3. Biochemical properties of alternative substrates of nitrate reductase**

<table>
<thead>
<tr>
<th>Substrate</th>
<th>( V_{\text{max}} ) (s(^{-1}))</th>
<th>( K_{\text{m}} ) (μmol L(^{-1}))</th>
<th>( K_{\text{e}} )</th>
<th>( E_l ) of the X–O bond (kJ mol(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>( \text{NO}_3^- )</td>
<td>150</td>
<td>13</td>
<td>4.6 × 10(^{-4})</td>
<td>368</td>
</tr>
<tr>
<td>( \text{ClO}_3^- )</td>
<td>150</td>
<td>221</td>
<td>≥10</td>
<td>184</td>
</tr>
<tr>
<td>( \text{BrO}_3^- )</td>
<td>250</td>
<td>739</td>
<td>5</td>
<td>200</td>
</tr>
<tr>
<td>( \text{IO}_3^- )</td>
<td>100</td>
<td>10 751</td>
<td>1.7 × 10(^{-4})</td>
<td>221</td>
</tr>
</tbody>
</table>

\(^{a}\)Data derived from Barber and Notton (1990).

\(^{b}\)Data derived from Weast (1971).

Conclusions and future prospects

The present overview of isotopic aspects of the NR-catalysed reaction is consistent with nitrate reduction per se (labelled \( k_2 \) in Fig. 1B) as a non-limiting step of the catalytic cycle. Further, it suggests that the first reversible step (classical Michaelis–Menten nitrate binding, \( k_1/k_{-1} \)) is not accompanied by the formation of a covalent bond but rather, is a simple complication reaction (Fig. 1B). The oxygen exchange would then take place in a single step (possibly involving a ‘concerted’ mechanism) (Fig. 1B). Nevertheless, more experimental data are obviously needed to establish all the details of the mechanism, and much may be expected in the future if a nitrate-complexed NR is crystalised. This would in turn give a better framework for understanding the isotopic biology of the enzyme. More isotopic investigations are indeed needed for environmental studies. For example, the amount of nitrate found in nature is affected by assimilatory and (microbial) dissimilatory NR activity and so the knowledge of their associated \(^{14}\text{N}/^{15}\text{N} \) fractations is necessary to correctly interpret the nitrogen isotope composition of sampled nitrate. This topic is of particular interest for studies on water pollution that focus on the double \(^{14}\text{N}/^{18}\text{O} \) signal in nitrate from natural springs, lakes or rivers to detect water adulteration by fertilisers (Amberger and Schmidt 1987).

Acknowledgments

GT thanks the Australian Department of Education, Science and Training for its financial support through an Endeavour Post-doctoral Fellowship of the Australian Education International Department. GF acknowledges the support of the Australian Research Council.

References


G. Tcherkez and G. D. Farquhar


George GN, Wright J (1999) Mechanistic changes induced by catalytic turn-over at the molybdenum site of *Azotobacter* nitrite reductase. *Journal of the American Chemical Society* 121, 9730–9731. doi: 10.1021/ja990310r


Kohl DH, Sheraer G (1980) Isotopic fractionation associated with symbiotic N₂ fixation and uptake of NO₂⁻ by plants. *Phytochemistry* 19, 537–543


Peterson BL, Somerville CA (1990) The crystal structure of plant sulfite oxidase provides insights into sulfite oxidation in plants and animals. *Science* 247, 1251–1263


Manuscript received 24 November 2005, accepted 10 March 2006

http://www.publish.csiro.au/journals/fpb