

Viewpoint:**Isotopic fractionation by plant nitrate reductase, twenty years later**Guillaume Tcherkez^{A,B,C} and Graham D. Farquhar^B^ALaboratoire d'Ecophysiologie Végétale, Bâtiment 362, Université Paris XI, 91405 Orsay, France.^BEnvironmental Biology Group, Research School of Biological Sciences, Australian National University, GPO Box 475, Canberra, ACT 2601, Australia.^CCorresponding 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 $^{14}\text{N}/^{15}\text{N}$ and also the $^{16}\text{O}/^{18}\text{O}$ 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 (Solomonson and Barber 1990). Moreover, 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. So 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 ($^{14}\text{N}/^{15}\text{N}$) 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 *v.* 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 $^{14}\text{N}/^{15}\text{N}$ 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

Abbreviations used: KIE, kinetic isotope effect; NR, nitrate reductase; SO, sulfite oxidase.

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 Mo^{IV} is oxidised to Mo^{VI} .

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 (Mo^{V}), 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 NO_2 , which is subsequently reduced to NO_2^- (Taylor *et al.* 1979). In addition, during the reduction of nitrate by dihydroxo-molybdenum-triazacyclononane monohydrate (Mo^{III}), it was suggested (Weighardt *et al.* 1985) that the replacement of H_2O by NO_3^- 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 diphenylmercaptoethyl pyridine complexes of molybdenum (Mo^{IV} , 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 ^{18}O -labelling and phenyl pyridylmethanolate as a complex of molybdenum (Mo^{IV}) (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-ONO_2^- bond formation and (ii) the O-N cleavage with oxidation of Mo^{IV} to Mo^{VI} (Fig. 1A, 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 SO_3^{2-}

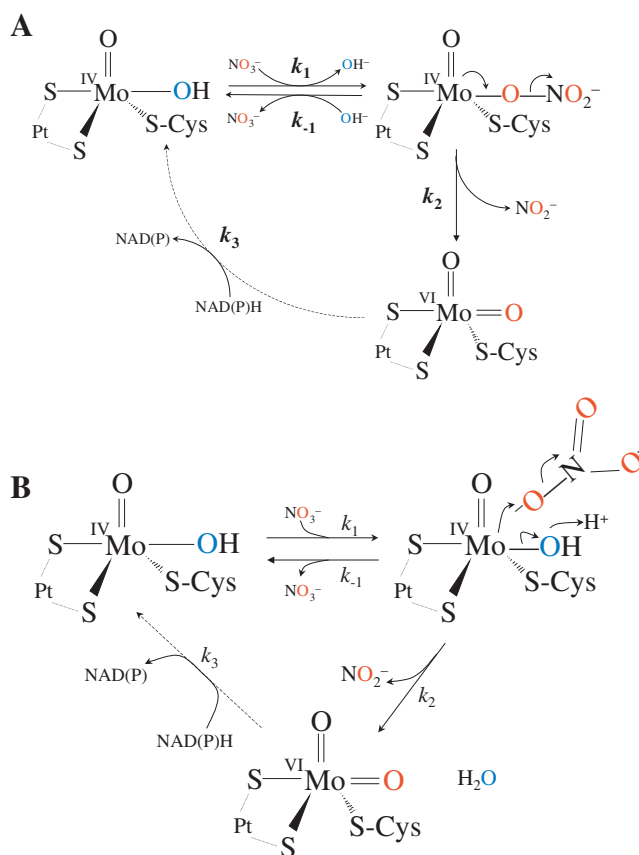


Fig. 1. Plausible mechanism of nitrate reduction catalysed by nitrate reductase, involving a bridging oxygen (A) or involving a direct O transfer (B). The molybdopterin cofactor is not completely drawn, the pterin part being symbolised by Pt. Cys stands for the residue of Cysteine 191 (*Arabidopsis*). For simplicity, reduction of molybdenum is summarised as a single step by k_3 (whether or not reduction involves several steps does not have any consequence for N and O isotopes). Panel (A) is stylised from Fischer *et al.* (2005); panel (B) describes the mechanism proposed in the present paper (see the text for further details). S stands for sulfur.

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 (V/K) of the 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 k_{cat} 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 ($\sim 500 \text{ s}^{-1}$), 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 kinetics 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 are described elsewhere (Berti 1999) and are not detailed here. Briefly, for a reaction that is made up of several elemental steps, the observed KIE ($k_{\text{light}}/k_{\text{heavy}}$, that is, for nitrogen, $^{14}k/^{15}k$, which is denoted below as α_{obs}) is a function of both (i), the intrinsic isotope effect α_i , that is, the KIE of the isotope-sensitive step, and (ii), kinetic limitations due to the other steps, represented by the commitment to catalysis (or partition factor) c . Assuming that the reaction has a Michaelis–Menten mechanism (composed of reversible substrate binding followed by an irreversible isotope-sensitive step), the relationship is as follows (O'Leary 1980):

$$\alpha_{\text{obs}} = \frac{\alpha_i + c}{1 + c}. \quad (1)$$

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 c 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 $^{14}\text{N}/^{15}\text{N}$ and $^{16}\text{O}/^{18}\text{O}$ isotope effects on k_2 (these isotope effects are $^{14}k_2/^{15}k_2$ and $^{16}k_2/^{18}k_2$, respectively, and, for brevity, will be denoted as α_2 in Eqn 2), because a N–O bond is broken. The first step (rate constants k_1 and k_{-1}) may also be subjected to a secondary ($^{14}\text{N}/^{15}\text{N}$ and $^{16}\text{O}/^{18}\text{O}$) 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:

$$c = \frac{k_2}{k_{-1}}.$$

We note that if steps k_1 and k_{-1} are also associated with an isotope effect, the overall observed isotope effect is given by:

$$\alpha_{\text{obs}} = \frac{\alpha_2 \times \alpha_1 / \alpha_{-1} + \alpha_1 \times c}{1 + c}, \quad (2)$$

where α_n is the (intrinsic) isotope effect associated with step k_n and so is given by $\alpha_n = ^{14}k_n/^{15}k_n$ and $^{16}k_n/^{18}k_n$ 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 (\neq), the isotope effect, defined as the rate constant ratio $^{14}k/^{15}k$, is given by (Bigeleisen 1949):

$$\frac{^{14}k}{^{15}k} = \left(\frac{^{14}M^{\neq} \cdot ^{15}M^R}{^{15}M^{\neq} \cdot ^{14}M^R} \right)^{3/2} \times \left(\frac{^{14}\mu^{\neq} \cdot ^{15}\mu^R}{^{15}\mu^{\neq} \cdot ^{14}\mu^R} \right)^{1/2} \times \prod_i^{3N-6} \frac{\sinh(^{14}u_i^R/2)}{\sinh(^{15}u_i^R/2)} \cdot \prod_i^{3N^{\neq}-7} \frac{\sinh(^{15}u_i^{\neq}/2)}{\sinh(^{14}u_i^{\neq}/2)} \times \frac{^{14}\kappa}{^{15}\kappa}, \quad (3)$$

where M are the molecular masses, μ are the moments of inertia components and κ the transmission coefficient (fraction of molecules that passes the energy barrier in the forward direction). The superscripts R and \neq refer to the reactants and the transition state, respectively. u_i is given by $u_i = h\nu_i/k_B T$ where ν_i is the frequency in the vibrational mode i , h is Planck's constant, k_B is Boltzmann's constant, and T the absolute temperature. \sinh is the hyperbolic sine and is defined as: $\sinh(x) = 0.5 \times (e^x - e^{-x})$. 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 $^{14}\text{N}/^{15}\text{N}$ or $^{16}\text{O}/^{18}\text{O}$ so that the isotope effect on κ 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 Wolfsberg 1958; Melander 1960):

$$\frac{{}^{14}k}{{}^{15}k} = \prod_{i=1}^{N^{\ddagger}} \left(\frac{{}^{14}m_i^{\ddagger}}{{}^{15}m_i^{\ddagger}} \right)^{3/2} \cdot \prod_{i=1}^N \left(\frac{{}^{15}m_i^R}{{}^{14}m_i^R} \right)^{3/2} \times \frac{{}^{14}u_L}{{}^{15}u_L} \prod_{i=1}^{3N^{\ddagger}-7} \frac{{}^{14}u_i^{\ddagger}}{{}^{15}u_i^{\ddagger}} \prod_{i=1}^{3N-6} \frac{{}^{15}u_i^R}{{}^{14}u_i^R} \times \prod_{i=1}^{3N-6} \frac{\sinh({}^{14}u_i^R/2)}{\sinh({}^{15}u_i^R/2)} \cdot \prod_{i=1}^{3N^{\ddagger}-7} \frac{\sinh({}^{15}u_i^{\ddagger}/2)}{\sinh({}^{14}u_i^{\ddagger}/2)}, \quad (4)$$

where the m_i are the atomic masses and u_L 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 ($-\text{O}-\text{NO}_2^-$), with vibrational frequencies of NO_3^- and $\text{Metal}-\text{O}-\text{NO}_2^-$ 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 $^{14}\text{N}/^{15}\text{N}$ 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 $\delta^{15}\text{N}$ 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 $^{16}\text{O}/^{18}\text{O}$ isotope effect has been measured once in maize and is of the same order of magnitude as for ^{15}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 1. The $^{14}\text{N}/^{15}\text{N}$ and $^{16}\text{O}/^{18}\text{O}$ kinetic isotope effects (denoted as ${}^{14}k/{}^{15}k$ and ${}^{16}k/{}^{18}k$, respectively) associated with the reduction of nitrate

When available, the standard error given by the authors is reported

Reaction	Species	${}^{14}k/{}^{15}k$	${}^{16}k/{}^{18}k$	Reference
NO_3^- uptake by plants	<i>Tagetes erecta</i>	1.005 ^A	–	Kohl and Shearer (1980)
NO_3^- reduction by NR	<i>Zea mays</i>	1.029 ± 0.005 ^B	1.015 ± 0.008	Olleros-Izard (1983)
		1.024 ± 0.001 ^C		
	<i>Spinacia oleracea</i>	1.015	–	Ledgard <i>et al.</i> (1985)
	<i>Spinacia oleracea</i>	1.015	–	Werner and Schmidt (2002)
	<i>Brassica campestris</i>	1.019 ^D	–	Yoneyama <i>et al.</i> (2003)
Inorganic NO_3^- reduction by Fe^{2+} and Ag^+	<i>Thalassiosira weissflogii</i> (alga)	1.022 ^E	–	Needoba <i>et al.</i> (2004)
	–	1.075 ± 0.004	–	Brown and Drury (1967)

^AValue calculated using the difference in the nitrogen isotope composition between source NO_3^- and whole-plant material.

^BValues obtained *in vitro* on the purified enzyme at constant NADH concentration.

^CValues obtained *in vitro* on the purified enzyme at decreasing NADH concentration.

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

^EValue inferred by 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_{-1} , 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_{-1}) $^{14}\text{N}/^{15}\text{N}$ isotope effect (Urey 1947). One may calculate estimates of the intrinsic $^{14}\text{N}/^{15}\text{N}$ isotope effect associated with the nitrate reduction step *per se* (labelled k_2) assuming (i), that data published in the literature for related compounds such as KNO_3 , NaNO_3 , AgNO_3 , CuNO_3 or CoNO_3 somehow reflect the vibrational modes around the N atom and (ii), a N–O bond order value in the transition state. One may hypothesise (Hammond's postulate) that the transition state is reactant-like as it has been found that the activation energy of NO_2^- production is $\sim 30 \text{ kJ mol}^{-1}$ (Lillo *et al.* 1997) while the reduction of nitrate to nitrite has a large negative free energy of -143 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 Farquhar (2005) [that only takes into account the isotope ratio of $\sinh(u_L)$, where u_L 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_{-1}) may be subjected to a non-negligible isotope

Table 2. Orders of magnitude of the $^{16}\text{O}/^{18}\text{O}$ and $^{14}\text{N}/^{15}\text{N}$ kinetic isotope effects (denoted as $^{14}k/^{15}k$ and $^{16}k/^{18}k$, respectively) associated with several steps of nitrate reduction

Kinetic isotope effects were obtained with the values indicated in the footnotes below. We recall that the (Pauling) bond order relates to the structure and is equal to $e^{(r_s-r_a)/0.3}$ where r_s is the length of a single bond and r_a the actual length (in Å)

Reaction	$^{16}k/^{18}k$	$^{14}k/^{15}k$
Formation of $\text{Mo}\sim\text{O}\sim\text{NO}_2^-$ (k_1)	1.055–1.065 ^A	1.002–1.004
Cleavage of $\text{Mo}\sim\text{O}\sim\text{NO}_2^-$ (k_{-1})	~ 1.080 ^B	1.002–1.004
Cleavage of $\text{Mo}\sim\text{O}\sim\text{NO}_2^-$ (k_2)	1.050 ^C	1.037 ^C
Direct cleavage of $\text{O}\sim\text{NO}_2^-$	1.090 ^D	1.075 ^E

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

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

^{C,D}A O–N stretching frequency of 800 and 1350 cm^{-1} , respectively, and a decrease of the O–N bond order of 0.1 in the transition state.

^EValue measured by Brown and Drury (1967).

effect. However, giving an estimate for both bridging oxygen formation and breakdown ($^{16}k_1/^{18}k_1$ and $^{16}k_{-1}/^{18}k_{-1}$) 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 $^{16}k_1/^{18}k_1$ of approximately 1.060 and a maximum value of $^{16}k_{-1}/^{18}k_{-1}$ (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 $^{18}\text{O}/^{16}\text{O}$ 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 $^{16}\text{O}/^{18}\text{O}$ isotope effect on k_2 of approximately 1.050 (Table 2).

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 Eqns (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):

$$c = \frac{\alpha_2 \alpha_1 / \alpha_{-1} - \alpha_{\text{obs}}}{\alpha_{\text{obs}} - \alpha_1}$$

It gives $c \approx \frac{1.037-1.015}{0.015} \approx 1.5$ for $^{14}\text{N}/^{15}\text{N}$ isotope effects, while $c = \frac{1.050 \times 1.060 / 1.080 - 1.015}{1.015 - 1.060} \approx 0$ with $^{16}\text{O}/^{18}\text{O}$ 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 α_1 were larger.

Alternatively, calculations can be made assuming a one-step process for oxygen exchange (from $\text{Mo}\sim\text{OH} + \text{NO}_3^-$ to $\text{Mo}=\text{O} + \text{NO}_2^-$), the Michaelis equilibrium being without any covalent bond (Fig. 1B). In such a case, the intrinsic N isotope effect associated with N–O 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 $^{14}\text{N}/^{15}\text{N}$ isotope effect on k_2 using Bigeleisen's Eqns (4) and (5) in a simplified form (Brown and Drury 1967). The $^{16}\text{O}/^{18}\text{O}$ 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:

$$c = \frac{\alpha_2 - \alpha_{\text{obs}}}{\alpha_{\text{obs}} - 1},$$

then we find a partition factor c of $\frac{1.075-1.015}{0.015} = 4$ and $\frac{1.090-1.015}{0.015} = 5$ 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_{-1} 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_{\max} (higher rate constant k_2) and lower K_m values, unless steric constraints disfavour binding (particularly for iodine, quite a large atom). However, evidence is to the contrary: the V_{\max} value of borate only is slightly higher, and K_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 complexation event only (explaining the predominance of steric effects on K_m), followed by a ‘direct’ reaction of oxygen exchange between the substrate and the Mo atom (explaining the slight effect only of nucleophilicity, 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_3 (if $k_3=0$, 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* NR 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

K_A is the acidic dissociation constant at ambient temperature. E_1 is the energy of the X–O bond, X being the atom considered

Substrate	V_{\max} (s^{-1}) ^A	K_m ($\mu\text{mol L}^{-1}$) ^A	K_A ^B	E_1 of the X–O bond (kJ mol^{-1})
NO_3^-	150	13	4.6×10^{-4}	368
ClO_3^-	150	221	≥ 10	184
BrO_3^-	250	739	5	200
IO_3^-	100	10 751	1.7×10^{-1}	221

^AData derived from Barber and Notton (1990).

^BData 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 complexation 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 crystallised. 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 $^{14}\text{N}/^{15}\text{N}$ fractionations 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 ^{15}N – ^{18}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

- Amberger A, Schmidt HL (1987) Natürliche Isotopengehalte von Nitrat als Indikatoren für dessen Herkunft. *Geochimica et Cosmochimica Acta* **51**, 2699–2705. doi: 10.1016/0016-7037(87)90150-5
- Barber MJ, Notton BA (1990) Spinach nitrate reductase. *Plant Physiology* **93**, 537–540.
- Berti PJ (1999) Determining transition states from kinetic isotope effects. *Methods in Enzymology* **138**, 355–397.
- Bigeleisen J (1949) The relative reaction velocities of isotopic molecules. *Journal of Chemical Physics* **17**, 665.
- Bigeleisen J, Wolfsberg M (1958) Theoretical and experimental aspects of isotope effects in chemical kinetics. *Advances in Chemical Physics* **1**, 15–76.
- Brown LL, Drury JS (1967) Nitrogen isotope effects in the reduction of nitrate, nitrite and hydroxylamine to ammonia. I. In sodium hydroxide solution with Fe (II). *Journal of Chemical Physics* **46**, 2833–2837. doi: 10.1063/1.1841123
- Campbell WH (1999) Nitrate reductase structure, function and regulation: bridging the gap between biochemistry and physiology. *Annual Review of Plant Physiology and Plant Molecular Biology* **50**, 277–303. doi: 10.1146/annurev.arplant.50.1.277
- Cleland WW (2005) The use of isotope effects to determine enzyme mechanisms. *Archives of Biochemistry and Biophysics* **433**, 2–12. doi: 10.1016/j.abb.2004.08.027

- Craig JA, Holm RH (1989) Reduction of nitrate to nitrite by molybdenum-mediated atom transfer: a nitrate reductase analogue reaction system. *Journal of the American Chemical Society* **111**, 2111–2115. doi: 10.1021/ja00188a026
- Farquhar GD, Barbour MM, Henry BK (1998) Interpretation of oxygen isotope composition of leaf material. In 'Stable isotopes, integration of biological, ecological and geochemical processes'. (Ed. H Griffiths) pp. 27–62. (Bios Scientific Publishers: Oxford)
- Fischer K, Barbier GG, Hecht HJ, Mendel RR, Campbell WH, Schwarz G (2005) Structural basis of eukaryotic nitrate reduction: crystal structure of the nitrate reductase active site. *The Plant Cell* **17**, 1167–1179. doi: 10.1105/tpc.104.029694
- George GN, Mertens JA, Campbell WH (1999) Structural changes induced by catalytic turn-over at the molybdenum site of *Arabidopsis* nitrate reductase. *Journal of the American Chemical Society* **121**, 9730–9731. doi: 10.1021/ja9903101
- Hille R, Rétey J, Bartlewski-Hof U, Reichenbecher W, Shink B (1999) Mechanistic aspects of molybdenum-containing enzymes. *FEMS Microbiology Reviews* **22**, 489–501.
- Kohl DH, Shearer G (1980) Isotopic fractionation associated with symbiotic N₂ fixation and uptake of NO₃⁻ by plants. *Plant Physiology* **66**, 51–56.
- Ledgard SF, Woo KC, Bergersen FJ (1985) Isotopic fractionation during reduction of nitrate and nitrite by extracts of spinach leaves. *Australian Journal of Plant Physiology* **12**, 631–640.
- Lillo C, Kazazaic S, Ruoff P, Meyer C (1997) Characterization of nitrate reductase from light and dark-exposed leaves. *Plant Physiology* **114**, 1377–1383.
- Lin H, Zhao Y, Ellingson BA, Pu J, Truhlar DG (2005) Temperature dependence of carbon-13 kinetic isotope effects of importance to climate change. *Journal of the American Chemical Society* **127**, 2830–2831. doi: 10.1021/ja0434026
- Melander L (1960) 'Isotope effects on reaction rates.' (Ronald Press Company: New York)
- Meyer MP, DelMonte AJ, Singleton DA (1999) Reinvestigation of the isotope effects for the Claisen and aromatic Claisen rearrangements: the nature of the Claisen transition states. *Journal of the American Chemical Society* **121**, 10865–10874. doi: 10.1021/ja992372h
- Needoba JA, Sigman DM, Harrison PJ (2004) The mechanism of isotope fractionation during algal nitrate assimilation as illuminated by the ¹⁵N/¹⁴N of intracellular nitrate. *Journal of Phycology* **40**, 517–522.
- National Institute of Standards and Technology (2005) NIST Chemistry Webbook. <http://webbook.nist.gov/chemistry> [verified 23 March 2006]
- O'Leary MH (1980) Determination of heavy atom isotope effects on enzyme-catalyzed reactions. *Methods in Enzymology* **64**, 83–104.
- Olleros-Izard T (1983) Kinetische Isotopeneffekte der Arginase und Nitratreduktase Reaktion: ein Beitrag zur Aufklärung der entsprechenden Reaktionsmechanismen. PhD thesis, Technischen Universität München, Freising-Weihenstephan.
- Paneth P (1995) Theoretical calculations of heavy-atom isotope effects. *Computers & Chemistry* **19**, 231–240. doi: 10.1016/0097-8485(95)93589-S
- Robinson R (2001) δ¹⁵N as an integrator of the nitrogen cycle. *Trends in Ecology & Evolution* **16**, 153–162. doi: 10.1016/S0169-5347(00)02098-X
- Saunders L (1986) Kinetic isotope effects. In 'Investigation of rates and mechanisms of reactions, Part I, C'. (Ed. F Bernosconi) pp. 211–255. (Wiley and Sons: New York)
- Schmidt HL, Medina R (1991) Possibilities and scope of the double isotope effect method in the elucidation of mechanisms of enzyme catalyzed reactions. *Isotopenpraxis* **27**, 1–4.
- Schmidt S, Dennison WC, Moss GJ, Stewart GR (2004) Nitrogen ecophysiology of Heron Island, a sub-tropical coral cay of the Great Barrier Reef, Australia. *Functional Plant Biology* **31**, 517–528. doi: 10.1071/FP04024
- Schrader N, Fischer K, Theis K, Mendel RR, Schwarz G, Kisker C (2003) The crystal structure of plant sulfite oxidase provides insights into sulfite oxidation in plants and animals. *Structure* **11**, 1251–1263.
- Schultz BE, Gheller SF, Muetterties MC, Scott MJ, Holm RH (1993) Molybdenum-mediated oxygen atom transfer: an improved analog reaction system of the molybdenum oxotransferases. *Journal of the American Chemical Society* **115**, 2714–2722. doi: 10.1021/ja00060a021
- Shearer G, Kohl DH (1986) N₂ fixation in field settings: estimations based on natural ¹⁵N abundance. *Australian Journal of Plant Physiology* **13**, 699–756.
- Sims LB, Lewis DE (1984) Bond order methods for calculating isotope effects in organic reactions. In 'Isotope effects, recent development in theory and experiment'. (Eds E Buncl, CC Lee) pp. 161–259. (Elsevier: New York)
- Skipper L, Campbell WH, Mertens JH, Lowes DJ (2001) Pre-steady-state kinetic analysis of recombinant *Arabidopsis* NADH-nitrate reductase. *Journal of Biological Chemistry* **276**, 26995–27002. doi: 10.1074/jbc.M100356200
- Solomonson LP, Barber MJ (1990) Assimilatory nitrate reductase: functional properties and regulation. *Annual Review of Plant Physiology and Plant Molecular Biology* **41**, 225–253. doi: 10.1146/annurev.pp.41.060190.001301
- Taylor RD, Spence JT (1975) Reduction of nitrate by diammonium oxopentachloromolybdate (V) in dimethylformamide. *Inorganic Chemistry* **14**, 2815–2818. doi: 10.1021/ic50153a043
- Taylor RD, Todd PG, Chasteen ND, Spence JT (1979) Reduction of nitrate by monomeric molybdenum (V) complexes in dimethylformamide. *Inorganic Chemistry* **18**, 44–48. doi: 10.1021/ic50191a008
- Tcherkez G, Farquhar GD (2005) Carbon isotope effect predictions for enzymes involved in the primary carbon metabolism of plant leaves. *Functional Plant Biology* **32**, 277–291. doi: 10.1071/FP04211
- Urey HC (1947) The thermodynamic properties of isotopic substances. *Journal of the Chemical Society* **1**, 562–581.
- Van Hook A (1971) Kinetic isotope effects: introduction and discussion of the theory. In 'Isotope effects in chemical reactions'. (Eds CJ Collins, NS Bowman) pp. 20–35. (Van Nostrand Reinhold Co.: New York)
- Weast R (1971) 'Handbook of chemistry and physics.' (The Chemical Rubber Co.: Cleveland)
- Weighardt K, Woeste M, Roy PS, Chaudhuri P (1985) Kinetics and mechanism of nitrate reduction by dimeric aquamolybdenum(III) complex in aqueous solution: direct evidence for an oxo-group transfer. *Journal of the American Chemical Society* **107**, 8276–8277. doi: 10.1021/ja00312a085
- Werner RA, Schmidt HL (2002) The *in vivo* nitrogen isotope discrimination among organic plant compounds. *Phytochemistry* **61**, 465–484. doi: 10.1016/S0031-9422(02)00204-2
- Yakir D (2002) Global enzymes: sphere of influence. *Nature* **416**, 795. doi: 10.1038/416795a
- Yoneyama T, Ito O, Engelaar WMHG (2003) Uptake, metabolism and distribution of nitrogen in crop plants traced by enriched and natural ¹⁵N: progress over the last 30 years. *Phytochemistry Reviews* **2**, 121–132. doi: 10.1023/B:PHYT.0000004198.95836.ad

Manuscript received 24 November 2005, accepted 10 March 2006