

**Short communication:****On the  $^{16}\text{O}/^{18}\text{O}$  isotope effect associated with photosynthetic  $\text{O}_2$  production**Guillaume Tcherkez<sup>A,B,D</sup> and Graham D. Farquhar<sup>C</sup><sup>A</sup>Plateforme Métabolisme-Métabolome, IFR87, Université Paris-Sud XI, Orsay 91405, France.<sup>B</sup>Laboratoire d'écophysologie végétale, CNRS UMR 8079, Université Paris-Sud XI, Orsay 91405, France.<sup>C</sup>Environmental Biology Group, Research School of Biological Sciences, Australian National University, Canberra, ACT 2601, Australia.<sup>D</sup>Corresponding author. Email: guillaume.tcherkez@u-psud.fr

**Abstract.** While photosynthetically evolved  $\text{O}_2$  has been repeatedly shown to have nearly the same oxygen isotope composition as source water so that there is no corresponding  $^{16}\text{O}/^{18}\text{O}$  isotope effect, some recent  $^{18}\text{O}$ -enrichment studies suggest that a large isotope effect may occur, thus feeding a debate in the literature. Here, the classical theory of isotope effects was applied to show that a very small isotope effect is indeed expected during  $\text{O}_2$  production. Explanations of the conflicting results are briefly discussed.

**Introduction**

Understanding the structure and operation of the water-splitting system of photosystem II (PSII), the oxygen evolving complex (OEC), is one of the most enduring goals of plant biology. Major advances have been made in the last 10 years (e.g. Haumann *et al.* 2005) that open new perspectives on OEC structure and chemistry. OEC is also the most significant catalytic activity producing atmospheric  $\text{O}_2$ . Consequently, this has considerable importance for oxygen balance studies, such as those that use  $^{16}\text{O}/^{18}\text{O}$  oxygen isotopes. Atmospheric  $\text{O}_2$  has a greater oxygen isotope composition than (ocean) water by 23‰ (the so-called Dole effect). Several processes are known to contribute to this effect, such as  $\text{O}_2$  consumption by respiration and photorespiration. The latter metabolic pathway is indeed accompanied by a  $^{16}\text{O}/^{18}\text{O}$  isotope effect, the best known of which is the  $\text{O}_2$ -addition to ribulose-1,5-bisphosphate by Rubisco, which fractionates by  $\sim 21\text{‰}$  (for a recent study, see Helman *et al.* 2005). Nevertheless, it is critical to know whether the Dole effect is also related to an isotope fractionation associated with water photolysis by the OEC. Surprisingly, while several studies have experimentally shown that there is nearly no isotope effect during  $\text{O}_2$  generation from water by photosynthesis, some debate remains in the literature (McEvoy and Brudvig 2006). This paper gives an isotopic viewpoint of the reaction, in light of the advances made on the structure of the OEC. From a theoretical point of view, we show that the reaction is indeed weakly fractionating, in agreement with experimental studies. Plausible explanations of the conflicting results are proposed.

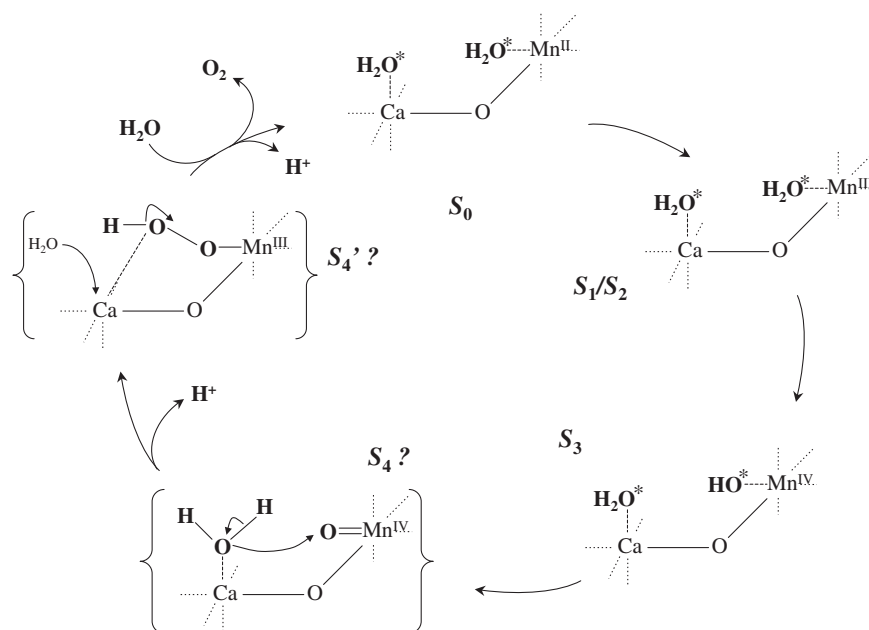
**The mechanism of water oxidation**

The mechanism by which the OEC generates  $\text{O}_2$  from water is still a matter of debate, and important reviews may be found

elsewhere (Hillier and Messinger 2005; McEvoy and Brudvig 2006). Figure 1 depicts a plausible mechanism with emphasis on substrate water molecules. The structure and reduction state of Mn atoms will not be discussed.

The reaction catalysed by the OEC proceeds through four recognised states (maybe five with  $S_4$ , also quoted as  $S_3\text{YZ}_{\text{ox}}$ ) which correspond to the flashes-induced oscillations of  $\text{O}_2$  evolution found by Joliot (see Joliot 1968 and the references therein). The starting dark-adapted step is  $S_1$ .  $\text{O}_2$  formation occurs during the  $S_3 \rightarrow S_0$  transition. In all the  $S_0$  to  $S_3$  states, there is water exchange with the bulk, as revealed by  $^{18}\text{O}$ -enrichment experiments (Hillier and Wydrzynski 2001, 2004). Water exchange seems bi-phasic, suggesting that there are two water binding sites: water exchange is indeed kinetically resolved for one site in the  $S_0$ ,  $S_1$ ,  $S_2$  and  $S_3$  intermediates while the other site is seen in the  $S_2$  and  $S_3$  intermediates. The exchange rates on both sites vary during the reaction but they are generally larger on one site ( $40\text{--}120\text{ s}^{-1}$ ) than on the other site ( $0.02\text{--}10\text{ s}^{-1}$ ). It is believed that no water exchange is possible in the  $S_4$  state (if one may define such a state) so that there is likely to be no water exchange when  $\text{O}_2$  production occurs. We note that the exchange rates are always slower than transitions between  $S$  states by at least one order of magnitude ( $750$  to  $34\,000\text{ s}^{-1}$ ) (Hillier and Messinger 2005).

In the mechanism of Fig. 1, one water molecule is deprotonated and gives an  $\text{O}=\text{Mn}$  oxo bond. The latter is attacked by the other bound water molecule to give a peroxo intermediate. This intermediate then decomposes to  $\text{O}_2$ . Water replacement follows, providing two new substrate water molecules. This simple scheme may be modified because some uncertainties remain, such as whether an oxo- or peroxo-intermediate exists, or whether one of the exchangeable water molecule binds the OEC within the Ca-coordination sphere or makes a Mn–Ca bridge. However, from



**Fig. 1.** Hypothetical mechanism of O<sub>2</sub> generation by the oxygen evolving complex. The Mn cluster is simplified in order to show substrate water molecules more clearly. Asterisks show the exchangeable oxygens with bulk water. Question marks stand for the uncertainty in S<sub>4</sub> intermediate(s). For clarity, the e<sup>-</sup> (and H<sup>+</sup> losses not involving substrate water molecules) are not indicated here. Scheme simplified from McEvoy and Brudvig (2006).

an isotopic point of view, this should not change the conclusions reached below.

### Order of magnitude of isotope effects involved in individual steps

In the framework of the mechanism discussed above, the overall isotope effect associated with O<sub>2</sub> production may be caused by water exchange between the bulk and the OEC, and the chemical rearrangements leading to O<sub>2</sub> formation (proton loss, (per)oxo and O=O bond formation). While the exchange of water molecules with the bulk may occur at several *S* states, the production of O<sub>2</sub> is rapid and happens during the S<sub>3</sub> → S<sub>0</sub> transition. There are many reasons to assume that the latter process is accompanied by a kinetic isotope effect (a thermodynamic isotope effect is *not* plausible as the reaction is certainly not at equilibrium). The kinetic isotope effect associated with O<sub>2</sub> addition to metals (forming a Me–O<sub>2</sub> intermediate) falls between 1.010 and 1.020 (Smirnov *et al.* 2006). If a similar chemical pathway for the reverse reaction were assumed (similar transition state), the same order of magnitude would be obtained. A value of around 1.020 would also be consistent with the kinetic isotope effect of 1.022 associated with proton abstraction leading to O<sub>2</sub><sup>-</sup> production from H<sub>2</sub>O<sub>2</sub> (when the reaction is reversible) by Cu–Zn-dependent superoxide dismutase (Smirnov and Roth 2006).

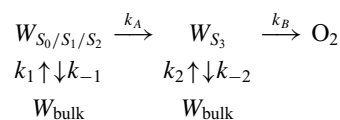
In addition, the equilibrium isotope effect associated with O<sub>2</sub> release from Mn-bound dioxygen is within the range 1.020–1.050 (calculated by Burda *et al.* 2003) so that the kinetic isotope effect in the forward direction may be less

than 1.040 (= 1.050/1.010). Indeed, such values would be consistent with the rapid calculations that can be made (Tcherkez 2006; Tcherkez and Farquhar 2006), based on the stretching frequency modes associated with the Mn–O<sub>2</sub> intermediate: with  $\nu_{O-O} \approx 800 \text{ cm}^{-1}$  and  $\nu_{O-Mn} \approx 500 \text{ cm}^{-1}$  for the Mn–O<sub>2</sub> peroxo-molecule and a bond order change of 0.25 (giving  $\nu_{O-O} \approx 1000 \text{ cm}^{-1}$  and  $\nu_{O-Mn} \approx 300 \text{ cm}^{-1}$  in the transition state, the isotope effect is within the range 1.020–1.030).

Water exchange, which occurs until the S<sub>3</sub> state of the cycle, is also presumably accompanied by an isotope effect, due to OEC–water complex formation. In other words, H<sub>2</sub>O entrance into the coordination sphere of catalytic Mn or Ca atoms likely fractionates between isotopes. However, such an isotope effect is very small: the <sup>16</sup>O/<sup>18</sup>O isotope effect associated with hydration equilibrium of cations such as CaCl<sub>2</sub> has been shown to be around 0.9995 at 25°C (Truesdell 1974). It is similarly around 0.9995 with dilute solutions of LiCl at 25°C (Bopp *et al.* 1977).

### The overall isotope effect during O<sub>2</sub> production from water

The question that remains is then the amplitude of the isotope effect during the overall water-splitting reaction. For this purpose, a reaction scheme is needed. As a summary of what was discussed above, the following, although simplified, may be used:



where  $W$  stands for water. In such a framework, two main kinetic cases have to be distinguished.

First, both exchange and transitions between states (that is,  $k_{-1}$  and  $k_A$ , and  $k_{-2}$  and  $k_B$ ) may occur simultaneously (these can compete). In such a case, the overall isotope effect depends on the ratio between the (isotopically sensitive) rate  $k_B$  and  $k_{-2}$ . Indeed, the rate of O<sub>2</sub> production, assuming a steady-state on bound water molecules and first-order kinetics is:

$$k = k_B \frac{\frac{k_A k_1}{k_{-1} + k_A} + k_2}{k_B + k_{-2}}.$$

Water exchanges are slower than transitions between  $S$  states (see above). In other words,  $k_1$ ,  $k_{-1}$ ,  $k_2$  and  $k_{-2}$  are small compared to  $k_A$  and  $k_B$ . The equation above can then be simplified to:  $k \approx k_1 + k_2$ . The kinetic isotope effect is then simply given by:  $\alpha \approx ({}^{16}k_1 + {}^{16}k_2)/({}^{18}k_1 + {}^{18}k_2)$ , which is the kinetic isotope effect associated with hydration. The hydration isotope effect is certainly small, in the per mil range or less (see above). On the other hand, we note that if the transition between  $S$  states ( $k_A$  and  $k_B$ ) were very slow (e.g. if the whole process were strongly light-limited), the overall isotope effect would tend to  $\alpha = ({}^{16}k_B/{}^{18}k_B) \times ({}^{16}K_2/{}^{18}K_2)$  where  $K_2$  is the equilibrium constant equal to  $k_2/k_{-2}$ . While the isotope effect would be relatively large (near  $1.025 \times 0.9995 = 1.0245$ ), its occurrence is very unlikely: as soon as photons arrive at PSII, the  $S_3$ -to- $S_0$  transition occurs with a high rate. In addition, water exchange and O<sub>2</sub> chemistry do not happen simultaneously (see below).

Second, both water exchange and the  $S_3$ -to- $S_0$  transition (O<sub>2</sub> production) cannot compete, simply because water cannot leave the reactional site as it is already involved in the O<sub>2</sub> production of the transition. In other words, this means that the  $S_3$ -to- $S_0$  transition uses the water molecule exchanged before, with no possibility of isotopic choice: such a system would be committed. This case is by far the most likely because: (i) the chemical pathway (Fig. 1) is not consistent with water exchange going on during O<sub>2</sub> production, and (ii) O<sub>2</sub> production is irreversible (McEvoy and Brudvig 2006; but see Clausen and Junge 2004), a fact inconsistent with continuous water exchange. From an isotopic point of view, the consequence is obvious – oxygen atoms in O<sub>2</sub> inherit the isotopic signature of the water exchange equilibrium so that the isotope effect is  ${}^{16}K_2/{}^{18}K_2$ . The latter is indeed very small, around 0.9995 as discussed above.

**Table 1.** The  $\delta^{18}\text{O}$  difference (in ‰) between photosynthetic evolved O<sub>2</sub> and source water, as found using isotope-ratio mass spectrometry measurements

| Species                          | $\Delta\delta^{18}\text{O}$ (‰) | Reference                  |
|----------------------------------|---------------------------------|----------------------------|
| <i>Anacystis nidulans</i>        | +0.38                           | Guy <i>et al.</i> 1993     |
| <i>Phaeodactylum tricorutum</i>  | +0.62                           |                            |
| <i>Spinacia oleracea</i>         | -0.06                           |                            |
| <i>Synechocystis</i> PCC 6803    | +0.46                           | Helman <i>et al.</i> 2005  |
| <i>Agmenellum quadruplicatum</i> | -0.46                           | Stevens <i>et al.</i> 1975 |
| <i>Chlorella pyrenoidosa</i>     | -0.80                           |                            |

Unsurprisingly then, one may expect a very small isotope effect during O<sub>2</sub> formation by the OEC, and there are several strong pieces of experimental evidence for this (Table 1). When algal suspensions are incubated in an O<sub>2</sub>-free and high-CO<sub>2</sub> medium (in such conditions, photorespiration is inhibited), O<sub>2</sub> evolved in the light has the same oxygen isotope composition ( $\delta^{18}\text{O}$ ) as source water (Stevens *et al.* 1975; Guy *et al.* 1993). The same is true with the *fv3* mutant of *Synechococcus* (deficient in O<sub>2</sub> photoreduction) (Helman *et al.* 2005) or with purified spinach (*Spinacea oleracea* L.) thylakoids (Guy *et al.* 1993). Taken as a whole, these results show that the  $\Delta\delta^{18}\text{O}$  value between O<sub>2</sub> and source water falls between +0.6‰ (slightly <sup>18</sup>O-enriched O<sub>2</sub>) and -0.8‰ (slightly <sup>18</sup>O-depleted O<sub>2</sub>) (Table 1). This difference may be insignificant if within the measurement noise. Thus, clearly, water oxidation by the OEC does not fractionate between oxygen isotopes.

A couple of studies (Burda *et al.* 2001, 2003) have, however, suggested that there is a very large isotope effect ( $\geq 1.100$ ) in the photosynthetic water splitting process. The experiments involved the use heavy water (H<sub>2</sub><sup>18</sup>O) enrichment in the assay cell. The authors then studied the time course of <sup>18</sup>O<sub>2</sub>, <sup>16</sup>O<sub>2</sub> and <sup>16</sup>O-<sup>18</sup>O evolution with mass spectrometry, over 250 min. While very close to the statistical distribution (if the water <sup>18</sup>O abundance is  $p$ , one expects a <sup>18</sup>O<sub>2</sub> : <sup>16</sup>O<sub>2</sub> : <sup>16</sup>O-<sup>18</sup>O distribution of  $p^2 : (1-p)^2 : 2p(1-p)$ ), there is a slight deviation (namely, more <sup>18</sup>O<sub>2</sub> than expected) that has been thought to be due to an isotope effect associated with O<sub>2</sub> production.

A kinetic isotope effect is, however, unlikely to be so large – intrinsic isotope fractionations involving heavy isotopes such as <sup>16</sup>O/<sup>18</sup>O are generally less than 100‰ (Buncel and Saunders 1992). Thus, other processes may be involved such as a lag phase for water exchange that would slow the replacement of the experimentally added H<sub>2</sub><sup>18</sup>O. Although plausible, this is not very likely on such a time scale because of the exchange rates found by Hillier and Wydrzynski (2001, 2004). In addition to mixing problems (the added heavy water is not mixed with light water, only diffusion occurs), it is possible that there is an isotope effect associated with O<sub>2</sub> transfer through the inlet membrane of the device used by the authors and described in Bader *et al.* (1987) (for the evidence of such a membrane fractionation, see Hillier *et al.* 2006). In addition, other reactions that consume O<sub>2</sub> and fractionate against <sup>18</sup>O may be involved, such as xanthophyll oxidation. The tobacco (*Nicotiana tabacum* L.) *Su/su* mutant line, which is known to have a larger amount of xanthophylls (Schindler *et al.* 1994), has indeed a larger deviation from the statistical O-isotope distribution in evolved O<sub>2</sub> (Burda *et al.* 2003). Whatever the technical reason for the results, a large isotope effect is doubtful. Additionally, a thermodynamic isotope effect, as suggested by these authors, is clearly unlikely because the catalytic cycle is certainly not at equilibrium between intermediates and O<sub>2</sub>.

Nevertheless, the water coordination sphere around the OEC is probably complex. While it remains possible that as many as 12 water molecules (as suggested by Burda *et al.* 2003) are more or less associated with the OEC Ca/Mn containing structure, assuming a large <sup>16</sup>O/<sup>18</sup>O isotope effect during water splitting is irrelevant. This is not necessarily a good piece of news: if an isotope effect were to occur, the variation of its value with assay conditions would have provided information on the

intrinsic mechanism of water oxidation, as isotopes have proved to be powerful tools for revealing enzyme kinetics (for a review, see Cleland 2005).

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