

An estimation of the size of the water cluster present at the cleavage site of the water splitting enzyme

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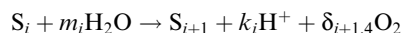
Abstract In time-dependent measurements of oxygen evolution in tobacco thylakoid membranes we varied the fraction of H_2^{18}O and the temperature and measured water splitting as $^{18}\text{O}_2$, $^{16}\text{O}^{18}\text{O}$, and $^{16}\text{O}_2$ by mass spectrometry. We show that the approach to the equilibrium of the system after H_2^{18}O addition can be very well understood in terms of the diffusion of water molecules. The equilibrium states of $^{16}\text{O}_2$, $^{16}\text{O}^{18}\text{O}$, and $^{18}\text{O}_2$ evolution differ from the theoretical binomial distributions, which are expected under the prerequisite of ideal mixing of the water molecules and that of the chemical equivalence of H_2^{18}O and H_2^{16}O for an infinite cluster. The presence of this deviation means that there is a typical size of water clusters having access to cleavage by the water splitting enzyme. We estimated that this cluster contains about 12 ± 2 water molecules. © 2001 Federation of European Biochemical Societies. Published by Elsevier Science B.V. All rights reserved.

Key words: Photosystem II; Water splitting enzyme; Water cluster; Oxygen evolution; Mass spectroscopy

1. Introduction

The mechanism of water splitting is one of the most interesting topics in photosynthesis research. Despite a variety of approaches it still remains a puzzle. The oxygen evolving complex (OEC) of photosystem II (PSII) catalyzes the light-driven reaction of extraction of four electrons from water molecules. In this process four protons and O_2 are released. Based on the observation of periodic oscillations of oxygen evolution in dark-adapted chloroplasts under short saturating flashes [1] a linear four-step model for the oxidizing cycle has been proposed by Kok et al. [2]. It is believed that a four-manganese cluster is involved in the accumulation of positive charges. According to the Kok et al. model, the state S_i is usually assigned to a certain arrangement and oxidation state of a Mn cluster, where the subscript describes the number of accumulated charges. Water oxidation is supposed to occur only after formation of the S_4 state. However, thermodynamic considerations favor a sequence of two-electron oxidation steps rather than a concerted four-electron abstraction [3] with formation of peroxide at a redox level below S_4 [4]. The organization of the OEC is not really established but the observation that Mn ions are released and recombined

pairwise suggests that there are two manganese dimers bound to the OEC [5]. It is not known at which reaction stage and how the oxygen–oxygen bond is formed. Besides this there is an even more fundamental problem, namely how water molecules enter into the catalytic site and how big this water entity is. There are two possible scenarios: one assumes that the cleavage site of water splitting enzyme remains ‘dry’ until a specific mechanism permits water access to the catalytic site [6]; the other hypothesis insists that water molecules are present in the vicinity of the splitting center independently of the redox state of the manganese complex [7]. The latter theory is in accordance with the Yachandra et al. model of oxygen evolution [8] and the finding of Evans et al. [9] that at least one of the two Mn dimers is exposed to an aqueous environment. The free access of water molecules to the water splitting enzyme can be summarized in the following formula of a four-step univalent oxidative pathway:



where S_i symbolizes the redox state of the water splitting enzyme, m_i is the net uptake of m water molecules coupled with one-electron abstraction from the redox oxidase in the redox state S_n , δ_{ij} is the Kronecker symbol ($ij=1$ for $i=j$, otherwise 0) [7].

There is experimental evidence that the *entrance* of the water cleaving hole would be of a size between the size of glycerol and ethylene glycol molecules, namely about 5 Å [10]. It is important to note that the size of a cluster containing five to six water molecules would have roughly such a diameter. Data from electron paramagnetic resonance measurements, in which a broadening of the signal of the S_2 state was induced by ^{17}O , gave good agreement with the used H_2^{17}O enrichment only when at least six water molecules were assumed in the vicinity of the Mn complex [11].

In this paper we present investigations on the mechanism of water splitting by means of mass spectrometry, a method which allows us to follow directly the process at the donor side of PSII. We have observed differences between the experimental data of the evolution of the various isotopic fractions of O_2 and the theoretically expected signals at masses 32, 34, and 36. The assumption of the existence of a finite number of water molecules in/at the cleavage site of the OEC explains these discrepancies.

2. Materials and methods

Thylakoid membranes were prepared from *Nicotiana tabacum* var.

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Abbreviations: PSII, photosystem II; OEC, oxygen evolving complex

John William's Broadleaf (JWB) according to Homann and Schmid [12] with minor modifications. Only freshly isolated thylakoids were used. Samples containing 100 μg chlorophyll were suspended in Tricine (0.06 M)–KCl (0.03 M) buffer, pH 7.5. Various amounts of labeled water were added to the sample keeping the final volume always at 3 ml (the thickness of the sample was 0.43 cm). Ferricyanide (1 mM) was used as an exogenous acceptor.

Mass spectrometric measurements were performed by means of a modified magnetic sector field spectrometer 'type Delta' from Finnigan MAT (Bremen, Germany). The details of the adapted set-up for our highly sensitive photosynthetic experiments have been described earlier [13,14]. 32 ($^{16}\text{O}_2$), 34 ($^{16}\text{O}^{18}\text{O}$), and 36 ($^{18}\text{O}_2$) signals were simultaneously detected in Faraday cups and recorded on a SE 130-03 BBC Metrawatt 3-channel recorder. The holder of the sample was cooled via connection to a thermostat, stabilizing the temperature to within 0.1°C . Every 10 min, after H_2^{18}O injection, 10 short saturating flashes were given. Flashes were provided by a stroboscope 1539A from General Radio (xenon flash lamp) with a flash duration of 5 μs at half intensity. Flashes were spaced 300 ms apart. H_2^{18}O was obtained from CEA–Oris, Bureau des Isotopes Stables, Gif-sur-Yvette, France.

3. Results and discussion

In the time-dependent measurements of oxygen evolution in tobacco thylakoid membranes we varied the fraction of H_2^{18}O (which we denote with α) and the temperature T . Fig. 1 shows an example of mass spectrometric signals at masses 32, 34, and 36 for a sample suspended in buffer containing 15.9% labeled water (H_2^{18}O). The measurements were done at 22°C .

Under the assumption of ideal mixing of the water molecules and of the chemical equivalence of the two types of water (H_2^{18}O and H_2^{16}O) from the point of view of the water

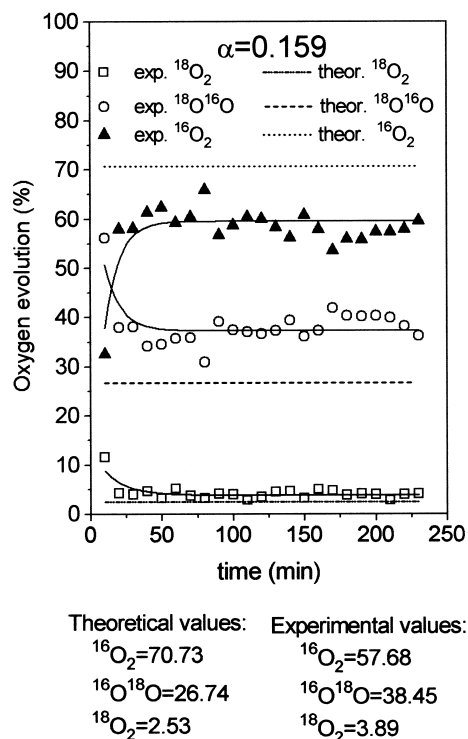


Fig. 1. Photosynthetic oxygen evolution in thylakoid membranes of *N. tabacum* as a consequence of 10 short saturating flashes given in intervals of 10 min over 240 min. The measurement was started 10 min after the addition of H_2^{18}O . The experiment shows the mass spectrometric signals at masses 32, 34, and 36 for a H_2^{18}O enrichment of 15.9%. Measurements were carried out at 22°C .

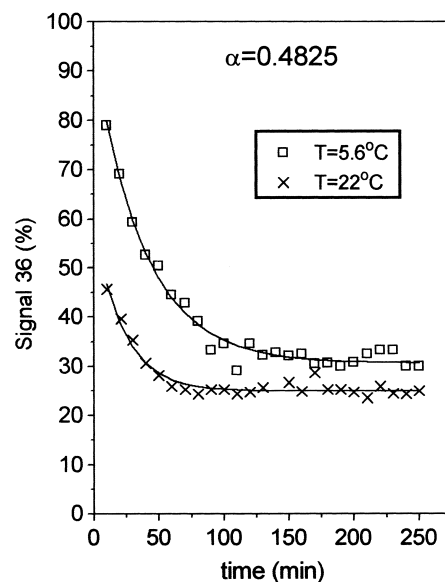


Fig. 2. Mass spectroscopic signals at mass 36 for the H_2^{18}O enrichment of 48.25%. Temperatures of the measurements are indicated. Experimental set-up as in Fig. 1. Points are experimental values; the solid curves represent the theoretical fits.

splitting reaction and of all mechanisms leading to it, one expects that the probabilities of yielding the molecules O_{36} , O_{34} and O_{32} are given by the binomial probability distribution $P^*_{36} = \alpha^2$, $P^*_{34} = 2(1-\alpha)$, and $P^*_{32} = (1-\alpha)^2$, independently of the temperature.

However, the experimental data of the probabilities P^{exp}_{36} , P^{exp}_{34} , and P^{exp}_{32} show deviations from the probabilities P^* . The oxygen evolution under 10 short saturating flashes was measured in time intervals of 10 min over 3–4 h. There are two apparent features of the mass spectroscopic signals at masses 32, 34, and 36. (a) Initially the curves deviate strongly from the values which are reached after a few measurements. The steady states are approached from above for P^{exp}_{36} and from below for P^{exp}_{32} . (b) In the course of time the curves relax to some equilibrium values P_{eq} around which they start to statistically fluctuate without showing any further drift. However, the values P_{eq} differ from the binomial probabilities P^* (Fig. 1).

The dynamics of the experiment (see (a)) are related to a particular experimental set-up, and can be explained by the experimental conditions. The deviation of P_{eq} from P^* (see (b)), however, can not. It carries the information about the PSII centers operating at a given α and T .

Before the experiment starts, the layer of thylakoids is on the bottom, covered with buffer containing only H_2^{16}O water. The experiment is initiated by a single act of adding H_2^{18}O water in the proportion given by α . In our set-up this is done by the injection of H_2^{18}O into the vessel containing only H_2^{16}O . By gravity and inertia H_2^{18}O goes down to the bottom. The first measurement of the oxygen yield is taken 10 min after the injection. At this moment, the mixture of water is not yet uniform in the whole vessel. Thus, in the lower part, where the thylakoid layer is, the fraction of H_2^{18}O is still larger than α . It is what the data points show. Then the uniformity of the mixture is restored by the diffusion of water molecules. This hypothesis can be tested by the analysis of the velocity of the approach of equilibrium by the experimental curves. In

Table 1
Cluster sizes and quantities of H_2^{18}O in the cluster

H_2^{18}O enrichment α	Temperature ($^{\circ}\text{C}$)	$\langle k \rangle$ molecules of H_2^{18}O in the cluster	Size $\langle N \rangle$ of the water cluster
0.319	5.6	5.33 ± 0.38	14.7 ± 1.4
	8.3	3.21 ± 0.38	7.2 ± 0.9
	10.5	3.19 ± 0.23	6.7 ± 0.7
	11.3	4.69 ± 0.61	13.4 ± 2.2
	11.8	5.06 ± 0.54	14.0 ± 1.5
	22	3.77 ± 0.27	10.0 ± 0.8
0.4825	5.6	9.87 ± 0.65	18.7 ± 3.0
	10.8	5.21 ± 0.43	9.7 ± 1.2
	22	6.10 ± 0.43	11.7 ± 1.3
0.106	5.6	3.58 ± 0.42	19.4 ± 2.5
	22	3.22 ± 0.40	12.9 ± 1.5
0.159	22	3.42 ± 0.28	13.8 ± 0.9

Calculation according to Eqs. 9 and 10; average size of the water cluster 11.0 ± 1.3 over the temperature range of 5.6°C to 22°C for $\alpha = 0.319$ and average size of the water cluster 13.4 ± 2.2 for $\alpha = 0.4825$.

the simplest case the dynamics can be described by an exponential decay with relaxation coefficients corresponding to the diffusion constant D . We have fitted the function $P^{\text{exp}}(t) = P_{\text{eq}} + A \exp(-Ct)$ with three free parameters, P_{eq} , A , and C , to the data points. The constant A is a prefactor of the exponential decay. In Fig. 2 we show the experimental data of $^{18}\text{O}_2$ (points) and the corresponding fits (solid curves) for $\alpha = 0.4825$ at $T = 22^{\circ}\text{C}$ and $T = 5.6^{\circ}\text{C}$. The estimated values of the decay coefficients are $C(22.5) = 0.0447 \pm 0.0045 \text{ min}^{-1}$ and $C(5.6) = 0.0261 \pm 0.0016 \text{ min}^{-1}$, respectively. The ratio $C(22.5)/C(5.6) = 1.71 \pm 0.20$ is comparable to the ratio of diffusion constants, which we found in the literature [15] to be $D = (2.57 \pm 0.022) \times 10^{-5} \text{ cm}^2/\text{s}$ for 25°C and $D = (1.426 \pm 0.018) \times 10^{-5} \text{ cm}^2/\text{s}$ for 5°C , which gives $D(25)/D(5) = 1.80 \pm 0.04$. This agreement, despite the simplicity of the approach for the relaxation, supports the hypothesis of diffusional mixing. Taking into account the diffusion constants at 5°C and 10°C $((1.675 \pm 0.025) \times 10^{-5} \text{ cm}^2/\text{s})$, 15°C $((1.97 \pm 0.02) \times 10^{-5} \text{ cm}^2/\text{s})$ and 25°C we estimated the time in which equal distribution of H_2^{18}O molecules within the whole volume of the studied system is achieved. We obtained

as time scales 197, 167, 142, and 109 min for the temperatures 5, 10, 15, and 25°C , respectively. In reality, the redistribution in our experiments is much faster (see Figs. 1 and 2) as the injected labeled water has a large initial velocity because of the fast injection.

An immediate question arises: why do the equilibrium data points P_{eq} differ from the binomial values P^* ?

As discussed by Bader et al. [13], the probabilities P_{36} , P_{34} , and P_{32} are given by the binomial distribution α^2 , $2(1-\alpha)$ and $(1-\alpha)^2$, when single water molecules are picked up with probabilities α (H_2^{18}O) and $(1-\alpha)$ (H_2^{16}O) from the solution. Let us assume that two molecules entering a single water splitting center are taken up in two steps. In the first step, a (N, k) group of N molecules with k (^{18}O) and $N-k$ (^{16}O) is picked up from the solution, and in the second step, two molecules out of the total N are taken. Denote the probability distribution of the first step by $P(N, k)$. The probability of choosing two H_2^{18}O molecules is $k(k-1)/(N(N-1))$ which means that the first one is picked up with the probability k/N and the second with $(k-1)/(N-1)$. Similarly the probabilities of picking the mixed pair $\text{H}_2^{18}\text{O} \text{H}_2^{16}\text{O}$ and the pair of two H_2^{16}O are $2k(N-k)/(N(N-1))$ and $(N-k)(N-k-1)/(N(N-1))$, respectively. Using the expression for conditional probabilities we get for P_{36} , P_{34} , and P_{32} :

$$P_{36} = \sum_{\{k, N\}} \frac{k(k-1)}{N(N-1)} P(N, k) \quad (1)$$

$$P_{34} = \sum_{\{k, N\}} 2 \frac{k(N-k)}{N(N-1)} P(N, k) \quad (2)$$

$$P_{32} = \sum_{\{k, N\}} \frac{(N-k)(N-k-1)}{N(N-1)} P(N, k) \quad (3)$$

The expressions are general, as long as the probabilities $P(N, k)$ are not specified. In the following we discuss the natural cases:

(1) The water groups of size N are picked up from uniformly mixed water. In this case $P(N, k)$ is given by the binomial distribution:

$$P(k, N) = \binom{N}{k} \alpha^k (1-\alpha)^{N-k} \quad (4)$$

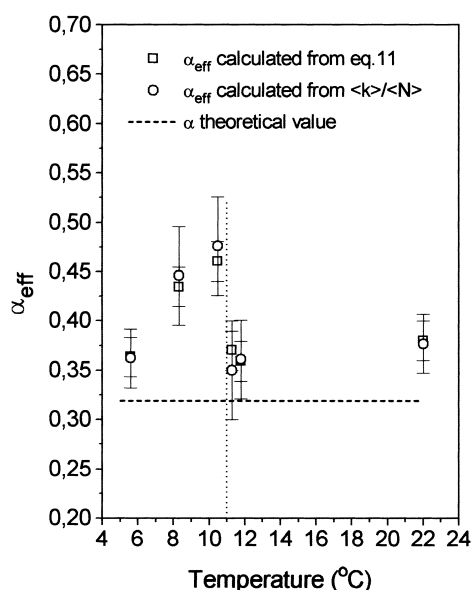


Fig. 3. Temperature dependence of the effective enrichment, seen by the studied system as calculated from experimental data. The sample contained 31.9% of labeled water.

Inserting this form into Eqs. 1–3 and summing over k (keeping N fixed) we get after some algebra:

$$P_{36} = \alpha^2, P_{34} = 2\alpha(1-\alpha), P_{32} = (1-\alpha)^2, \quad (5)$$

which agrees with the previous results. Remarkably, the resulting probabilities do not depend on the cluster size N , which means that with this simple assumption the information about the size of the water clusters in the bulk cannot be obtained.

(2) In the extreme case of isotopically uniform clusters the probabilities $P(N,k)$ vanish if k differs from N or 0. An immediate consequence is that P_{34} vanishes. Later, when molecules in clusters mix, P_{34} grows. We abandon this approach, since we do not see any evidence for any suppression of the P_{34} probabilities in the experiments.

(3) It is difficult to make any general assumption about $P(N,k)$ which would take into account any non-trivial distribution of the cluster size N and the distribution of k within clusters. So here we take a phenomenological approach and approximate Eq. 1 by:

$$P_{36} = \frac{\langle k \rangle (\langle k \rangle - 1)}{\langle N \rangle (\langle N \rangle - 1)} \quad (6)$$

$$P_{34} = 2 \frac{\langle k \rangle (\langle N \rangle - \langle k \rangle)}{\langle N \rangle (\langle N \rangle - 1)} \quad (7)$$

$$P_{32} = \frac{(\langle N \rangle - \langle k \rangle) (\langle N \rangle - \langle k \rangle - 1)}{\langle N \rangle (\langle N \rangle - 1)} \quad (8)$$

where $\langle N \rangle$ is the average cluster size entering the reaction pocket, and $\langle k \rangle$ is the average number of molecules $\langle k \rangle$ in the cluster. The ratio $\langle k \rangle / \langle N \rangle$ plays the role of the effective concentration α_{eff} seen by the reaction centers. It actually represents the biologically effective mole fraction. The approximation has certain limitations and it is a priori not clear how good it is. However, as we will see it gives stable results. Table 1 contains $\langle N \rangle$ values calculated from Eqs. 6–8, where P_{36} , P_{34} , and P_{32} are substituted by the experimentally obtained fractions of the evolved molecule species of $^{18}\text{O}_2$, $^{16}\text{O}^{18}\text{O}$, and $^{16}\text{O}_2$, respectively:

$$\langle N \rangle = \frac{2 + x + y}{1 - xy}, \text{ where } x = \frac{2 \cdot P_{36}^{\text{exp}}}{P_{34}^{\text{exp}}} \text{ and } y = \frac{2 \cdot P_{32}^{\text{exp}}}{P_{34}^{\text{exp}}} \quad (9)$$

The average number of H_2^{18}O water molecules is given by:

$$\langle k \rangle = \frac{1 + x}{1 - xy} \quad (10)$$

Without doubt, the average cluster size depends on the temperature (see Table 1). It is supported by the independent

estimation of α_{eff} from the equation:

$$\alpha_{\text{eff}} = \frac{2 \cdot P_{36}^{\text{exp}} + P_{34}^{\text{exp}}}{2} \quad (11)$$

The temperature dependence of α_{eff} for the H_2^{18}O enrichment of $\alpha = 0.319$ is shown in Fig. 3. It seems that the accessibility of substrate water to the oxygen evolving center depends on the temperature showing discontinuities at about 11°C . This transition temperature of the oxygen yield has been found using a complementary polarographic method of high resolution investigating the O_2 evolution under short saturating flashes [13,16,17].

Summarizing, the deviation of the steady states of mass spectroscopic signals from ideal mixing predictions can be explained by the finite size of water clusters entering the catalytic site of the water splitting enzyme. By a simple phenomenological approach, we estimated that there are about 12 ± 2 water molecules in the vicinity of the OEC.

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