Do the higher oxidation states of the photosynthetic O_2-evolving system contain bound H_2O?

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A modified mass spectrometer was used to determine whether the higher oxidation states of the photosynthetic O_2-evolving system contain substrate water that is not freely exchangeable with the external medium. Our data indicated that the higher oxidation states contain no appreciable bound, non-exchangeable H_2O. This suggests that H_2O oxidation takes place via a rapid, concerted, all-or-none mechanism rather than by a mechanism involving stable, partially oxidized, H_2O-derived intermediates. These findings set definite constraints on possible mechanisms of O_2 evolution.

Photosynthesis *Photosystem II* *Oxygen evolution* *Mass spectrometry* ^{18}O

1. INTRODUCTION

During the process of photosynthetic oxygen evolution (i.e., H_2O oxidation) the O_2 system cycles through 5 oxidation states (termed S_0–S_4) in the light. The most stable states are S_0 and S_1 which thus become predominant in the dark [1]. Oxygen is released during the $S_4 \rightarrow S_0$ transition [1] together with 2 of the 4 protons derived from the 2 H_2O molecules that are oxidized. A third proton is released during the $S_0 \rightarrow S_1$ transition and a fourth during the formation of S_3 [2–4].

Evidence suggests that the higher oxidation states of the O_2-evolving complex may contain bound (partially oxidized?) water [5]. Earlier, we showed that when chloroplasts were flashed in the presence of $H_2{}^{18}O$ (added in total darkness), the evolved O_2 entirely reflected the isotopic composition of the added H_2O rather than the H_2O in which the S_1 state was generated. These results indicated that the S_1 state did not contain tightly bound H_2O or an intermediate H_2O oxidation product [6].

In this communication, we describe experiments in which we used mass spectrometry to determine whether one or both of the higher oxidation states S_2 and S_3 contain water that is not freely exchangeable with the external medium. To this end, chloroplasts in $H_2{}^{18}O$ were pre-illuminated with 1 or 2 flashes (to achieve predominantly the S_2 or S_3 state, respectively). The chloroplasts were then rapidly washed in $H_2{}^{16}O$ (i.e., unlabelled H_2O) and the isotopic composition of the O_2 flash yields determined. The lifetime of the S_2 and S_3 states was sufficiently long under our condtions (>1 min, see also [7]) that deactivation during sample manipulation was not a major problem. Transport of H_2O across the chloroplast membrane is fast enough [8] ($\sim 1000\ s^{-1}$) to obviate problems due to H_2O access to the O_2-evolving site.

2. MATERIALS AND METHODS

The mass spectrometric apparatus and measuring technique used was a modification of a system described earlier [9]. The heart of the system is a 1-mm-thick silicone rubber membrane that admits gases dissolved in the liquid phase to the mass spectrometer vacuum. The experiments described in

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the following section require that we be capable of rapidly changing the milieu of the chloroplasts. To this end, we constructed a reaction vessel and ancillary equipment in which the chloroplast preparation was affixed to a membrane filter (0.45 μm pore size) captured in a portable plate. The chloroplast milieu was changed by injecting appropriate reaction mixtures onto the membrane system through a port while it was mounted on the mass spectrometer inlet. The sample was illuminated by using an FX101B xenon flash tube (EG&G, Salem, MA) enclosed in a tubular shield. The temperature of the inlet system and the enclosed membrane-supported sample was controlled by a surrounding water jacket and a thermostatted circulating bath (Neslab RTE-4). For each experiment the membrane with its deposited sample was mounted in retaining rings, the assembly affixed to the inlet, appropriate H_2O (^{18}O labelled or unlabelled) additions made, and the light shield put in place. The chloroplast sample was allowed to deactivate for 3 min at room temperature. The cooling system was then engaged, lowering the sample temperature to 11°C over a 6 min period. After 1 or 2 preflashes (3-s spacing), 10 ml unlabelled H_2O buffer was sprayed onto the back of the sample membrane via the injection port. After about 1 min, during which the signals were allowed to restabilize, the isotopic composition of each flash yield (3-s spacing) was determined.

Isotopic measurements were made by stepping (140 ms per step) the mass spectrometer to the maxima of *m/e* values 32, 34 and 36 (i.e. $^{16}O_2$, $^{18,16}O_2$ and $^{18}O_2$) during the train of actinic flashes, using a control system, built in-house. The envelopes of the 3 simultaneous flash-yield sequences were then reconstructed by hand.

Chloroplasts were prepared as described in [10]

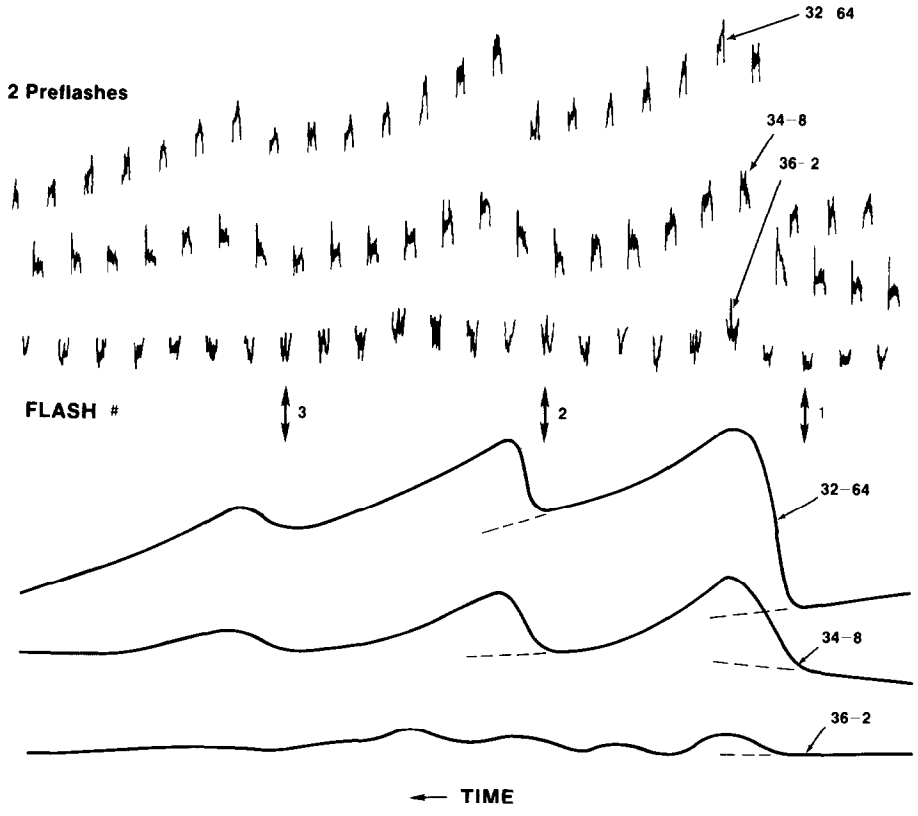

Fig.1. Flash-yield data obtained with chloroplasts subjected to 2 preflashes in the presence of $H_2{}^{18}O$ 46 s before analysis in unlabelled buffer. Top: recorder output, with traces between *m/e* values deleted. Bottom: reconstructed data derived by connecting the appropriate recorder output peaks. Each *m/e* value is shown at its own relative attenuation.

and suspended in buffer containing 0.4 M sucrose/ 20 mM Mes-NaOH (pH 6.2)/15 mM NaCl/5 mM $MgCl_2$. $H_2{}^{18}O$ (98.4 atom%) was obtained from Amersham (England).

3. RESULTS AND DISCUSSION

Fig.1 shows the results obtained when chloroplasts were subjected to 2 actinic flashes in the presence of $H_2{}^{18}O$, washed in the dark (to remove the $H_2{}^{18}O$) and then illuminated by a series of actinic flashes in the presence of unlabelled H_2O. The top panel of fig.1 is a tracing of the recorder output as the mass spectrometer was stepped through the *m/e* values of interest (i.e. 32, 34 and 36 corresponding to $^{16}O_2$, $^{16,18}O_2$ and $^{18}O_2$). The bottom panel is a reconstruction of the time courses of the 3 isotopic O_2 species. The most striking feature of these data is that there was no significant $^{18}O_2$ evolved, despite the fact that the O_2 system advanced to the S_3 (and S_2) states in the presence of 98 atom% $H_2{}^{18}O$.

Fig.2. shows the results of a similar experiment in which the chloroplasts were subjected to only one preflash with $H_2{}^{18}O$. Again, we note that there was no significant $^{18}O_2$ evolved, even though the S_2 state was formed in the presence of $H_2{}^{18}O$.

Fig.3 shows a control experiment: the chloroplasts were neither washed to remove the $H_2{}^{18}O$ nor preflashed. Note that in this case over one-half of the oxygen evolved on the third and fourth flashes was $^{18}O_2$ ($m/e=36$), and most of the remainder was singly labelled ($^{16,18}O_2$, $m/e=34$). These results demonstrate that our experimental system can detect labelled O_2 species when they are produced and provide a means to ascertain the amounts of labelled O_2 species one could expect to see in the experiments of figs 1 and 2.

The isotopic distribution of the evolved O_2 will be related to the isotopic composition of the H_2O according to:

$$32:34:36 = \alpha^2:2\alpha(1-\alpha):(1-\alpha)^2$$

where α is the atom fraction of ^{16}O ($=H_2{}^{16}O$). If we denote the measured *m/e* ratio (34/36) by *R*, then [11]

$$\alpha = R/R+2.$$

Using the data of fig.3, we can compute *R* to be

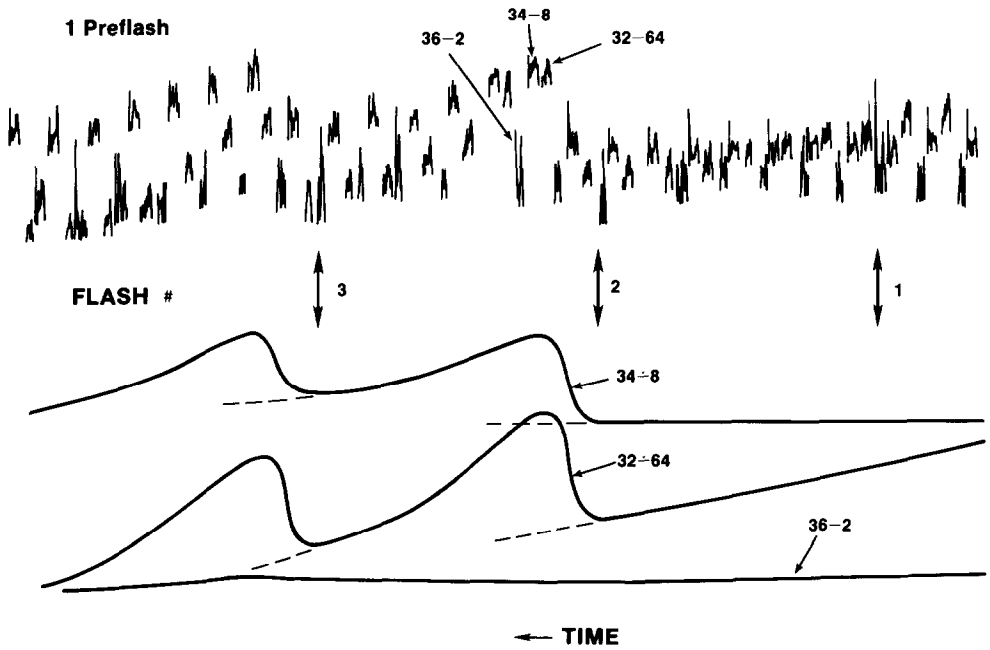

Fig.2. Flash-yield data obtained with chloroplasts subjected to one preflash in $H_2{}^{18}O$ 68 s before analysis in unlabelled buffer (see fig.1 legend).

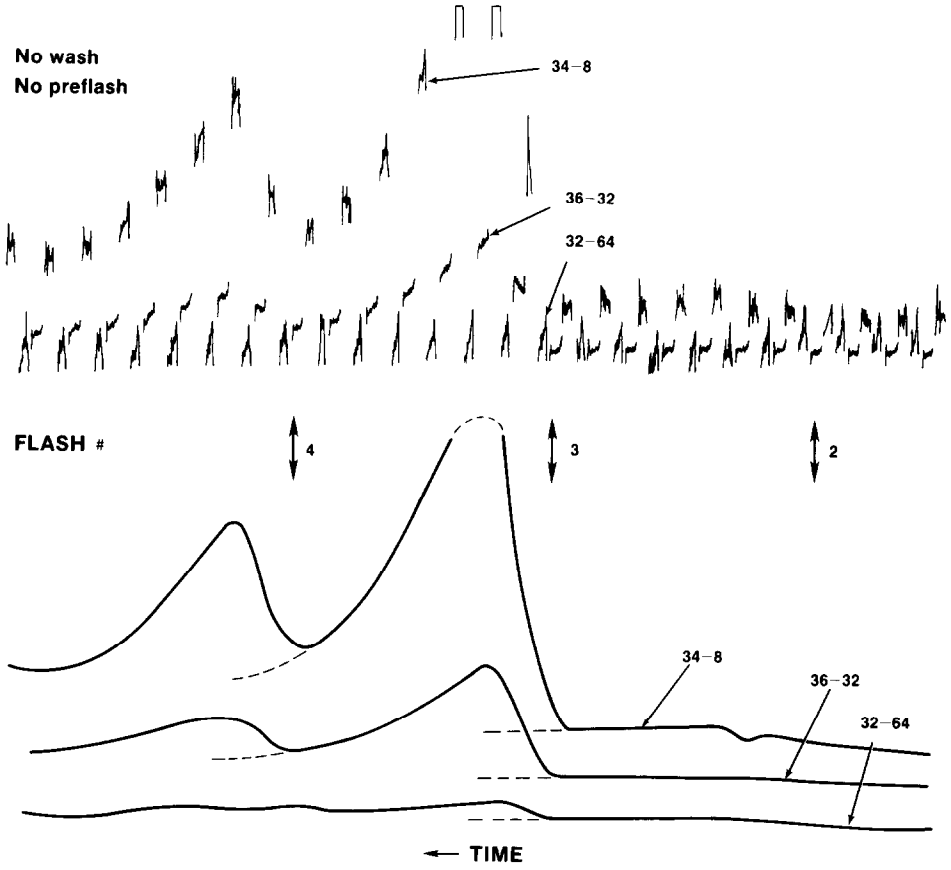

Fig.3. Flash-yield data obtained when chloroplasts were analyzed in the presence of $H_2{}^{18}O$ with no prior preflash or wash treatment.

0.70 and 0.86 for the third and fourth flash yields, respectively. Thus, α, the fraction of oxygen atoms that are ^{16}O, was ~0.26–0.30, and 70–74% of the water consumed to generate the O_2 was $H_2{}^{18}O$. We ascribe this less-than-quantitative isotopic yield to dilution by the wet (with $H_2{}^{16}O$) membrane and chloroplasts. The apparent isotopic difference between yields 3 and 4 is within the noise of our measurements and thus is probably not significant.

The above computations will set an upper limit on the amount of $^{18}O_2$ and $^{16,18}O_2$ that one could expect to observe if the S_2 and S_3 states do indeed contain bound non-exchangeable water. It is instructive to compare these values with the observed values summarized in table 1. If we assume that the same isotopic dilution will occur in the preflash experiments as we observed in the experiment of fig.3, we predict that the mole fraction of $^{18}O_2$ evolved should be ~0.49–0.55. As shown in table 1, we observed that the $^{18}O_2$ evolved was always $\leqslant$0.005 of the total O_2. Thus less than 1% of the H_2O was bound upon the generation of the S_2 and S_3 state and remained bound in the subsequent dark.

Table 1

Summary of flash-yield amplitudes computed from the data of figs 1 and 2

		Observed abundance		
	Flash no.	$^{16}O_2$	$^{16,18}O_2$	$^{18}O_2$
2 preflash	1	0.94	0.05	<0.005
	2	0.92	0.08	~0
1 preflash	2	0.92	0.08	~0

Although a spontaneous exchange of oxygen atoms between a putative bound intermediate and the added $H_2{}^{16}O$ cannot be ruled out a priori in these experiments, such exchange does seem unlikely. H_2O does not appreciably equilibrate labelled oxygen at room temperature with H_2O_2, permanganate, most metal oxides or anions of strong acids [12], compounds that could in some way or another be considered as model intermediates. Thus, lacking evidence to the contrary, we conclude that our results reflect a lack of bound H_2O intermediates rather than the presence of exchangeable intermediates.

4. CONCLUSION

Our data indicate that the S_2 and S_3 states do not contain bound, non-exchangeable H_2O in intermediate oxidation states. These findings, coupled with similar results reported earlier for the S_1 state [6], suggest that O_2 evolution, i.e. H_2O oxidation, takes place via a rapid concerted reaction during the $S4 \rightarrow S_0 + O_2$ transition and does not involve stable partially oxidized H_2O-derived intermediates. These findings are in accord with the report of Dekker et al. [13], who observed the successive oxidation of 3 Mn(III) to Mn(IV), which presumably reflected the stepwise oxidation of the O_2 system. Both of these experimental approaches lend substance to the idea that substrate H_2O is only involved in the $S_4 \rightarrow S_0$ transition; i.e. $H_2O + S_4 \rightarrow S_0 + O_2 + 4H^+$.

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