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Quantification of bound bicarbonate in photosystem II[#]

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Abstract

In this study, we presented a new approach for quantification of bicarbonate (HCO_3^-) molecules bound to PSII. Our method, which is based on a combination of membrane-inlet mass spectrometry (MIMS) and ^{18}O-labelling, excludes the possibility of "non-accounted" HCO_3^- by avoiding (*1*) the employment of formate for removal of HCO_3^- from PSII, and (*2*) the extremely low concentrations of HCO_3^-/CO_2 during online MIMS measurements. By equilibration of PSII sample to ambient CO_2 concentration of dissolved CO_2/HCO_3^-, the method ensures that all physiological binding sites are saturated before analysis. With this approach, we determined that in spinach PSII membrane fragments 1.1 ± 0.1 HCO_3^- are bound per PSII reaction center, while none was bound to isolated PsbO protein. Our present results confirmed that PSII binds one HCO_3^- molecule as ligand to the non-heme iron of PSII, while unbound HCO_3^- optimizes the water-splitting reactions by acting as a mobile proton shuttle.

Additional key words: hydrogen carbonate; inorganic carbon; mass spectrometry; Mn-stabilizing protein; non-heme iron; oxygen-evolving complex.

Introduction

All photosynthetic organisms utilize atmospheric carbon dioxide (CO_2) to store solar energy within the energy-rich chemical bonds of carbohydrates. In oxygenic organisms, such as cyanobacteria, algae, and higher plants, the electrons required for this process are extracted from water. Water oxidation is catalyzed by the inorganic Mn_4CaO_5 cluster that forms the center of the oxygen-evolving complex (OEC) of PSII, a large multi-component pigment–protein enzyme (Vinyard *et al*. 2013, Shen 2015). PSII is responsible for the light-induced generation of a stable and directed charge separation followed by electron transfer from water to plastoquinone (PQ). This leads to the sequential oxidation of the Mn_4CaO_5 cluster, which is coupled to the stepwise liberation of protons until, in the last step, two water molecules are oxidized to dioxygen (O_2) and the system is set back to the lowest oxidation state of the electron-donor side of PSII. Meanwhile, PQ is reduced to plastoquinol (PQH_2) on the electron-acceptor side (Rappaport and Diner 2008) (Fig. 1). The PSII electron transfer has long been suggested to be regulated by bicarbonate ions (HCO_3^-, recommended term by IUPAC is hydrogen carbonate, but here we used its traditional term bicarbonate). Extensive data accumulated over the last few decades reveal that HCO_3^- ions affect the electron flow on both the acceptor and the donor side of PSII (*see* reviews by Blubaugh and Govindjee 1988, van Rensen *et al.* 1999, 2002, Stemler 2002, van Rensen and Klimov 2005, McConnell *et al*. 2012, Shevela *et al*. 2012).

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Abbreviations: Chl – chlorophyll, MIMS – membrane-inlet mass spectrometry, OEC – oxygen-evolving complex, PQ – plastoquinone; PQH_2 – plastoquinol; RC – reaction center, NHI – non-heme iron.
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[#]This paper is dedicated to the memory of Prof. Vyacheslav V. Klimov (12 January 1945 – 9 May 2017), our dear colleague and co-author, a world leader in the field of photosynthesis research for almost five decades. Among many scientific achievements, Vyacheslav Klimov (known to many as "Slava") was one of the discoverers of pheophytin as the primary electron acceptor in PSII, and rediscoverer of "bicarbonate effect" on the electron donor side of PSII.
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The site and mode of HCO_3^- interactions/binding with the water-splitting donor side of PSII were, however, very controversial for a long time. The effects of HCO_3^- on the donor side reactions are discussed since the early 1970s (Stemler and Govindjee 1973, Stemler *et al.* 1974, Stemler 2002), and many different roles for bicarbonate were suggested. For example, HCO_3^- was shown to be a transient ligand to Mn ions during the photoassembly process of the Mn_4CaO_5 cluster in the OEC-depleted PSII centers (Baranov *et al*. 2004, Dasgupta *et al*. 2007, Kozlov *et al.* 2010). On the other hand, no bound HCO_3^- is seen in the structure of intact (assembled) Mn_4CaO_5 cluster, as shown by infra-red spectroscopy (Aoyama *et al*. 2008), mass spectrometry (Shevela *et al*. 2008, Ulas *et al*. 2008), and by high-resolution crystallographic studies (Guskov *et al.* 2010, Umena *et al.* 2011). Nevertheless, a large body of experimental data suggest stabilizing and protective effects of HCO_3^- on the activity of the OEC (Klimov *et al.* 1995, 1997, 2003, Klimov and Baranov 2001). Several recent reports demonstrate that easily exchangeable HCO_3^- ions improve water oxidation by acting as specific acceptors of protons during water oxidation (Villarejo *et al*. 2002, Ananyev *et al.* 2005, Shutova *et al.* 2008, Shevela *et al.* 2013, Koroidov *et al.* 2014). This role may, at least in part, explain the protective/stabilizing effect of bicarbonate on PSII observed in the earlier studies. Additionally, it was suggested that HCO_3^- may stabilize the OEC *via* binding to the extrinsic proteins, and specifically to the manganese stabilizing PsbO protein (Fig. 1) (Pobeguts *et al.* 2007, 2010).

The binding site of bicarbonate on the acceptor site and several functional aspects of it are well-established (for the latest reviews, *see* McConnell *et al.* 2012, Müh *et al.* 2012, Shevela *et al.* 2012). Wydrzynski and Govindjee (1975) provided the first experimental evidence for a PSII acceptor side effect of bicarbonate. Numerous subsequent experiments by Govindjee and his co-workers (and, later, by other groups) confirmed this discovery, and it was shown that one bound HCO_3^- molecule accelerates the electron transfer between the quinones Q_A and Q_B of PSII by facilitating the protonation of reduced Q_B (Q_B^{2-}) [for historical perspective, *see* Shevela *et al*. (2012) and references therein]. Recently, Brinkert *et al.* (2016) suggested that reduced Q_A (Q_A^-) can accelerate the release of bicarbonate from the acceptor side, and that this down-regulates PSII, together with the donor side effects (Koroidov *et al.* 2014), thereby protecting PSII against photodamage. Recent x-ray crystallographic and cryo-EM studies have firmly established that HCO_3^- binds as a bidentate ligand to the non-heme iron (Fe^{2+}; NHI) between Q_A and Q_B in cyanobacteria (Fig. 1), algae, and higher plants (Guskov *et al.* 2010, Umena *et al.* 2011, Ago *et al*. 2016, Wei *et al*. 2016). First evidences for a bound HCO_3^- in PSII came from numerous functional and spectroscopic studies (Blubaugh and Govindjee 1988, van Rensen *et al.* 1999, 2002). Among them, a study using a mass spectrometer and an infra-red gas analyzer detected the slow release of 0.5–1.3 HCO_3^- (as CO_2) from plant (maize, pea, spinach) PSII after addition of formate (≥ 100 mM) (Govindjee *et al.* 1991, 1997), indicating at least one bound HCO_3^- per PSII RC. However, it was suggested that about 50% of all bound HCO_3^- might escape the detection

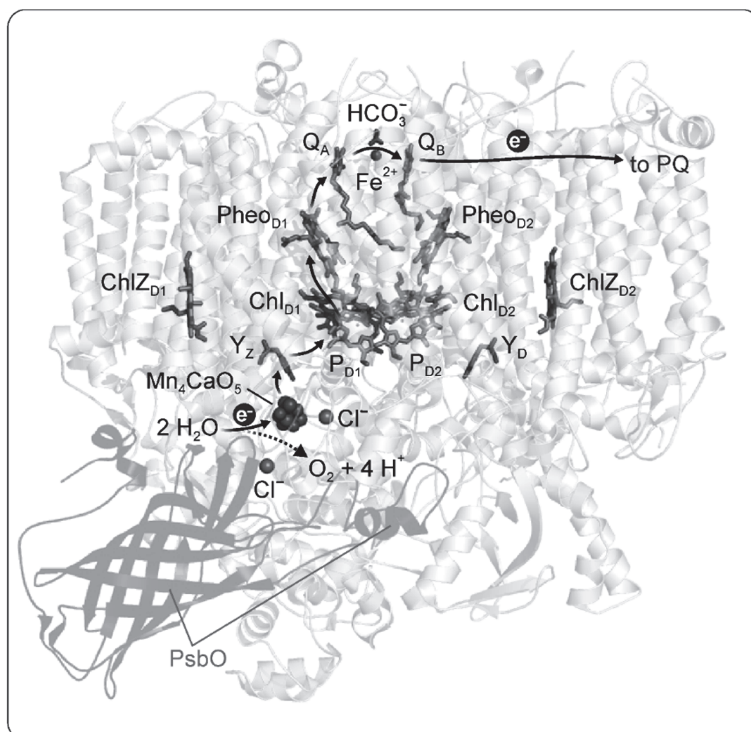

Fig. 1. PSII structure and its redox-active cofactors. The arrows within PSII indicate the direction of electron transfer from H_2O to plastoquinone molecule (PQ). The electron transfer chain within PSII comprises the following redox-active cofactors: inorganic Mn_4CaO_5 cluster, tyrosine Z (Y_Z), the ensemble of Chls *a* (P_{D1} and P_{D2}) and two accessory Chls (Chl_{D1} and Chl_{D2}), known as P680, the primary pheophytin ($Pheo_{D1}$) acceptor, the primary (Q_A) and the secondary (Q_B) quinone acceptors. Single HCO_3^- is known to bind to the NHI (Fe^{2+}) between Q_A and Q_B. PsbO protein is highlighted by dark grey. The long phytyl tails of the Chls and Pheos, and the isoprenyl chains of the quinones have been cut for clarity. The PSII structure is based on the PSII crystal structure at a resolution of 1.9 Å (PDB entry 3ARC; Umena *et al*. 2011).

by this method, since not all bicarbonate may be released by the formate injection (Govindjee *et al.* 1997). Subsequently, employing membrane-inlet mass spectrometry (MIMS) combined with ^{18}O-isotope labelling, we observed the release of ~0.3 HCO_3^- per PSII RC in spinach BBY samples (Shevela *et al.* 2008). All detected HCO_3^-/CO_2 was concluded to originate solely from the acceptor side, and not from the PSII donor side. The substoichiometric amount of less than one HCO_3^- per PSII obtained in that study was explained by the loss of some PSII-bound HCO_3^- into the bulk medium during the degassation of the sample in the MIMS chamber before formate addition. Therefore, one cannot exclude that the HCO_3^-, which escaped the detection in these previous reports, may originate not only from its binding site at the NHI, but also from another weaker binding site in PSII.

In the present study, we presented an improved MIMS-based method for quantification of the entire amount of inorganic carbon (HCO_3^-/CO_2) bound to PSII in spinach PSII membranes. This new method excludes the possibility of losing HCO_3^- from PSII or isolated PsbO protein before the MIMS detection. The release of HCO_3^- and its quantitative and rapid conversion into CO_2 is facilitated by injecting the sample into a pH 2.7 solution, avoiding possible uncertainties if PSII can release bicarbonate from all its binding sites.

Materials and methods

Sample preparations: PSII membranes ('BBY' preparations) were isolated from fresh leaves of a laboratory-grown spinach (*Spinacia oleracea*) as described earlier (Ford and Evans 1983). After isolation, the PSII membranes were resuspended and homogenized in a final storage/assay medium (0.33 M sucrose, 35 mM NaCl, 20 mM MES-NaOH, pH 6.5; thereafter termed as SNM medium) containing ~15% (v/v) glycerol. Control rate of photosynthetic O_2 evolution of the isolated samples was ~400 µmol(O_2) mg(Chl)$^{-1}$ h^{-1} (as measured by a Clark-type electrode at 25°C under the light intensity of ~2,000 µmol(photon) m^{-2} s^{-1} (red LED) in the presence of 0.1 mM 2,5-dichloro-*p*-benzoquinone and 1 mM $K_3[Fe(CN)_6]$). The isolated samples were stored at −80°C until used. Shortly before the measurements, PSII membranes were thawed in the dark on ice. Thereafter, 100-µl aliquot of these thawed samples was transferred into an open vessel, and, for ^{18}O-labelling, further addition of 3 µl of $H_2^{18}O$ (97%; *Larodan Fine Chemicals AB*, Solna, Sweden) was made. The resulting sample suspension [Chl_{final} = 3.5 mg(Chl) ml^{-1}] was then incubated in the dark while continuously stirring it in an open vessel at 20°C for 10 min. During this incubation, the PSII membranes were equilibrated with air and the ^{18}O-label was distributed between water and dissolved CO_2.

PsbO protein was isolated from PSII membrane fragments according to the procedure described previously by Pobeguts *et al.* (2010) and suspended in medium containing 40 mM MES-NaOH, pH 6.5 (PsbO medium) at concentrations of 17 µM or 35 µM. The PsbO protein samples were equilibrated with air and enriched with ^{18}O-label following the procedure described above for PSII membranes. As controls, identical manipulations were performed with assay media (SNM and PsbO media) without the biological material.

MIMS measurements of the CO_2 content in the sample preparations were performed with an isotope ratio mass spectrometer (*Thermo Finnigan DELTA^{Plus}XP*, Bremen, Germany) connected to an in-house membrane-inlet cell similar to that described earlier (Messinger *et al.* 1995, Shevela and Messinger 2013). The sample volume inside the MIMS cell (150 µl) was separated from a high vacuum (~3 × 10^{-6} kPa) of the mass spectrometer by a gas-permeable silicon membrane (25 µm thick; type *MEM-213*, *MemPro*, Troy, USA) that was hold on a porous Teflon support (Ø 10 mm; *Small Parts Inc.,* Miami Lakes, USA). A cryogenic trap installed between the mass spectrometer and the MIMS cell was filled with dry ice and ethanol (~200 K). The MIMS cell was thermostated to 20°C and constantly stirred at high speed with a magnetic stir bar. Before the assays, a phosphate buffer (1 M NaH_2PO_4-HCl, pH 2.7, not labelled with ^{18}O) was loaded into the MIMS cell and degassed for ~12 min until only slightly sloping baseline was reached. Thereafter, a 50-µl aliquot of the air-equilibrated sample (PSII membranes, PsbO protein, assay medium) was collected with a gas-tight Hamilton syringe and injected into the MIMS cell. The amplitude of the CO_2 signal was simultaneously monitored as non-labelled ($C^{16}O_2$; m/z = 44), single-labelled ($C^{16}O^{18}O$; m/z = 46), and double-labelled ($C^{18}O_2$; m/z = 48) CO_2-isotopologues for 10 min. Additionally, ^{40}Ar (m/z = 40) was collected and served as a probe for observing possible injection artifacts. Data with injection artifacts were excluded from data analysis. The CO_2 signals were calibrated by the subsequent injections of defined volumes of air-equilibrated water [15.3 µM(CO_2) at 20°C and an atmospheric pressure of ~101.3 kPa] plastoquinol that was slightly acidified with 5 mM MES (pH 4.3) in order to avoid the accumulation of inorganic carbon in form of HCO_3^-. The CO_2 solubility value in such medium was taken from Diamond and Akinfiev (2003). The amount of PSII RC was determined from the Chl in the PSII membranes, assuming 250 Chl/RC (Berthold *el al*. 1981, Enami *et al.* 1989). The analysis of the MIMS data was performed by using *Origin* software.

Results

Quantification of HCO_3^- bound to PSII membrane fragments: Fig. 2 shows kinetic traces of CO_2 evolution upon the addition of the air-equilibrated/^{18}O-labelled PSII membrane fragments (black curves 1) into the MIMS cell filled with degassed phosphate buffer (pH 2.7). The low pH of the phosphate buffer assured a rapid and complete conversion of HCO_3^- into CO_2, and, thus, facilitated the release of bound HCO_3^- ions from the PSII samples, as long as there is an equilibrium with the buffered medium, as indicated previously in our online MIMS experiments (Shevela *et al.* 2008). Exposure of the PSII samples to a low pH (3.0) is known to cause a reversible extraction of Ca^{2+} and of all extrinsic proteins from PSII (Shen and Katoh 1991). Three CO_2-isotopologues were recorded simultaneously at *m/z* = 44 ($C^{16}O_2$; Fig. 2*A*), *m/z* = 46 ($C^{16}O^{18}O$; Fig. 2*B*), and *m/z* = 48 ($C^{18}O_2$; Fig. 2*C*). The areas covered by the signal curves are directly proportional to the total amounts of CO_2-isotopologues in the samples. However, the total amount of CO_2 evolved from the PSII membranes (black curves 1) includes both CO_2 originating from PSII and CO_2 dissolved in the medium. To determine the dissolved fraction of CO_2 in the PSII preparations, we measured the amount of CO_2 in the same volume of air-equilibrated sample medium (SNM medium) (gray curves 2 in Fig. 2). A comparison of the *m/z* = 44 signals obtained (Fig. 2*A*) clearly revealed a lower content of non-labelled CO_2 in the "blank" medium as compared to the PSII membrane suspension. An inspection of the *m/z* = 46 and 48 signals (Fig. 2*B*,*C*) showed that the difference in levels of the ^{18}O-labelled CO_2 isotopologues in the "blank" medium and the PSII sample was significantly higher than that observed for the non-labelled CO_2. This apparent discrepancy can be readily explained by different aqueous volumes in the analyzed sample aliquots: in the PSII sample suspension a significant volume was occupied by membrane fraction [*ca*. 24% at 3.5 mg(Chl) ml^{-1}according to the estimation presented below; this value was also confirmed by the measurement of the volume of supernatant of precipitated PSII membrane fragments]. Accordingly, the water fraction in the PSII samples was smaller than in the "blank" medium. Therefore, after an equal addition of $H_2^{18}O$ (*see* Materials and methods), its mole fraction was higher in the PSII preparations, which resulted in the elevated amounts of the ^{18}O-labelled CO_2 isotopologues observed in Fig. 2*B*,*C*, with peak amplitudes described by equation 1:

$$^{44}x : {}^{46}x : {}^{48}x = (1 - {}^{18}x)^2 : 2\,{}^{18}x\,(1 - {}^{18}x) : {}^{18}x^2 \quad (1)$$

where ^{44}x, ^{46}x, and ^{48}x are the mole fractions of the corresponding CO_2-isotopologues in the injected sample, and, the total CO_2 pool ($^{44}x + {}^{46}x + {}^{48}x$) is given as 1. Using this equation we calculated the ^{18}x values both in the blank sample buffer ($^{18}x_{buf}$) and in the PSII sample suspension ($^{18}x_{susp}$) (Table 1), which was then converted into the volume ratio of PSII to water of 0.236 : 0.764 stated above. Accordingly, the amount of CO_2 bound to PSII (n_{PSII}) was determined using the following equation:

$$n_{PSII} = n_{susp} - n_{buf} \times {}^{18}x_{buf} / {}^{18}x_{susp} \quad (2)$$

where n_{susp} is the total amount of CO_2 in the sample suspension and n_{buf} is CO_2 dissolved in the medium. Thus, the isotope labelling was crucial for a precise determination of the volume of water in the PSII sample and thus for correct background subtraction.

For simplicity, the total amount of the CO_2 evolved after an injection was calculated from the area under the curve of the *m/z* = 44 signal corrected by the molar fraction of non-labelled CO_2 isotopologue at the given enrichment. Using the number of 250 Chls per PSII complex we obtained a value of 1.1 ± 0.1 HCO_3^-/CO_2 bound per PSII RC (with the significance level p=0.05).

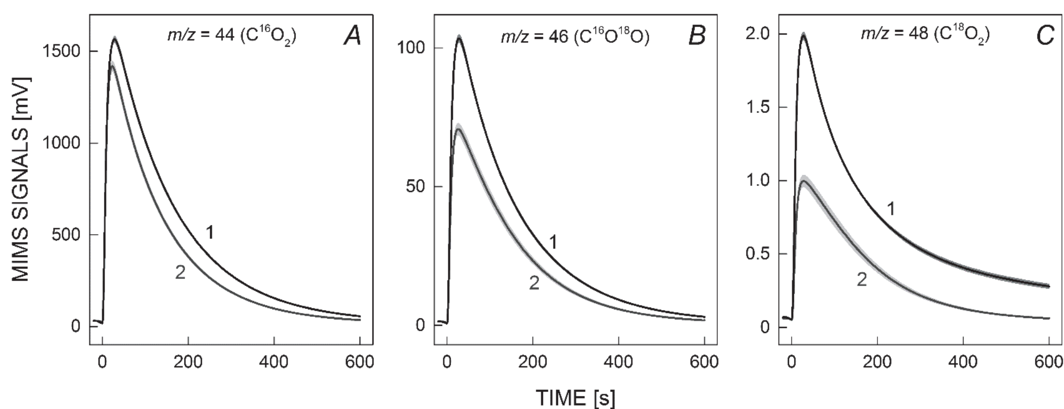

Fig. 2. Off-line MIMS measurements of HCO_3^-/CO_2 content dark-adapted PSII membrane fragments (*black curves 1*) and blank medium solution (*gray curves 2*) at 20°C and pH 6.5. Before the injection of 50 µL sample aliquot into the MIMS cells filled in with the degassed phosphate buffer (pH 2.7), the samples were air-equilibrated and enriched with $H_2^{18}O$ as described in Materials and methods. The [Chl] of the injected PSII membranes was 3.5 mg ml^{-1}. The CO_2 isotopologues were simultaneously measured at *m/z* = 44 ($C^{16}O_2$; panel *A*), *m/z* = 46 ($C^{16}O^{18}O$; panel *B*), and *m/z* = 48 ($C^{18}O_2$; panel *C*). Shaded areas indicate SE of the mean signals ($n \geq 3$) indicated by solid curves.

Table 1. Estimation of ^{18}x in aqueous fraction of blank medium (SNM buffer) and in PSII membrane fragments suspension. Each value represents the mean of 3-4 replicates ±SE.

m/z	Blank medium Maximum of the MIMS signal [mV]	x of CO_2 isotopologue	$^{18}x_{buf}$	PSII membranes suspension Maximum of the MIMS signal [mV]	x of CO_2 isotopologue	$^{18}x_{susp}$
44	1,412 ± 15	0.9514 ± 0.0003		1,535 ± 20	0.9366 ± 0.0002	
46	71.2 ± 1.1	0.0480 ± 0.0003	0.0246 ± 0.0002	102.1 ± 1.5	0.06227 ± 0.00015	0.0322 ± 0.0001
48	0.97 ± 0.02	0.00065 ± 0.00001		1.92 ± 0.03	0.00117 ± 0.00001	

Table 2. Estimation of ^{18}x in aqueous fraction of blank medium (PsbO medium) and in PsbO protein solution. Each value represents the mean of 3-4 replicates ±SE.

m/z	Blank medium Maximum of the MIMS signal [mV]	x of CO_2 isotopologue	$^{18}x_{buf}$	PsbO protein suspension Maximum of the MIMS signal [mV]	x of CO_2 isotopologue	$^{18}x_{susp}$
44	1,321 ± 144	0.9640 ± 0.0011		1,083 ± 129	0.9632 ± 0.0009	
46	48 ± 4	0.0356 ± 0.0011	0.0182 ± 0.0006	41 ± 4	0.0364 ± 0.0009	0.0186 ± 0.0005
48	0.53 ± 0.04	0.00039 ± 0.00003		0.47 ± 0.04	0.00042 ± 0.00002	

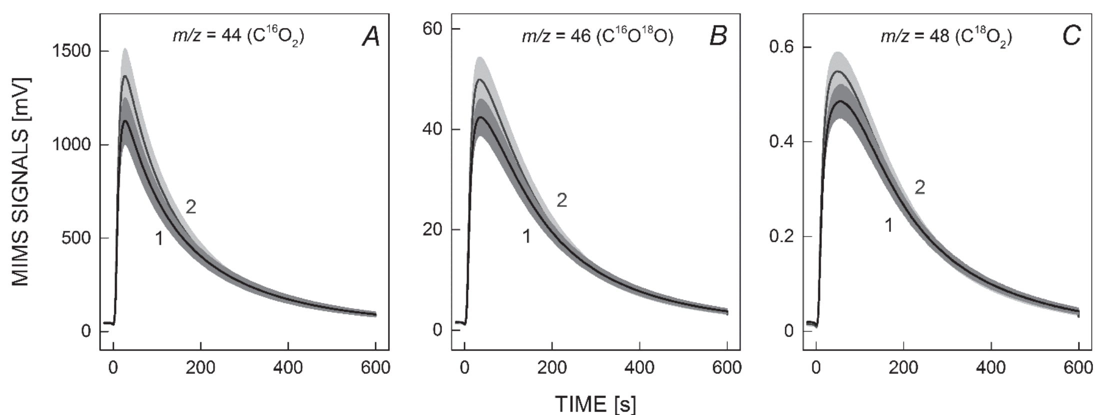

Fig. 3. Off-line MIMS measurements of HCO_3^-/CO_2 content PsbO sample suspension (*black curves 1*) and blank medium solution (*grey curves 2*) at 20°C and pH 6.5. Other conditions: see Fig. 2, but [PsbO] in the injected sample aliquot was 17 µM or 35 µM. Shaded areas indicate SE of the mean signals ($n \geq 3$) indicated by solid curves.

Probing of HCO_3^-/CO_2-binding to PsbO protein: In earlier studies, some of us suggested that the PsbO protein binds HCO_3^- close to the OEC of PSII (Pobeguts *et al.* 2007, 2010). Employing the quantitative approach described above, we probed the possible binding of HCO_3^-/CO_2 to the PsbO protein isolated from PSII (Table 2, Fig. 3). As seen in Fig. 3, the CO_2 concentration detected in air-equilibrated PsbO suspensions (black curves 1) was surprisingly a bit lower than that in the medium (gray curves 2). Unfortunately, we are presently unable to provide a conclusive explanation for this phenomenon. Nevertheless, our data do not support a specific HCO_3^- binding to isolated PsbO protein.

Discussion

The results obtained in this quantitative MIMS study show that even PSII samples, which are fully equilibrated to ambient CO_2/HCO_3^- concentrations (at pH 6.5), bind only one HCO_3^- per PSII. This HCO_3^- molecule is likely the one detected in crystal structures of PSII as ligand of the NHI (Guskov *et al.* 2010, Umena *et al.* 2011, Ago *et al.* 2016, Wei *et al.* 2016).

Our data advance previous reports that 0.3–1.3 HCO_3^- are bound per PSII RC (Govindjee *et al.* 1991, 1997, Shevela *et al.* 2008). Unlike these earlier reports, our approach does not rely on the competitive replacement of HCO_3^- by formate (HCO_2^-), and thus avoids the

uncertainty of a HCO_3^- binding site within PSII that is not competitive with formate. Instead, our method relies on the presence of the equilibrium of bound HCO_3^- with that in the solution. Our data thus support our previous suggestion that bicarbonate bound to the NHI on the acceptor side of PSII is, even in the dark, released into the surrounding medium if that has a very low bicarbonate content. This finding extends the recent suggestions by us (Koroidov *et al.* 2014) and by Brinkert *et al.* (2016) of a feedback regulation of PSII by CO_2 (*via* HCO_3^-). Such a feedback control would help to prevent over-reduction of the PQ pool and tune the recombination reactions within PSII, thereby reducing the risk of photodamage to PSII and other components of the light reactions in case of low CO_2 concentrations inside the leaf and thus slow CO_2-fixation.

Our present data do not exclude the existence of a non-exchangeable HCO_3^- within PSII. However, this is unlikely, since crystal structures of PSII would likely have revealed such a binding site already. This is in line with numerous previous studies, which excluded that HCO_3^- is a tightly bound ligand of the Mn_4CaO_5 cluster (Aoyama *et al*. 2008, Shevela *et al*. 2008, Ulas *et al*. 2008, Guskov *et al*. 2010, Umena *et al*. 2011). Similarly, we demonstrated in this study that the PsbO protein, at least in isolated and thus structurally slightly modified form, cannot bind HCO_3^- tightly.

Presently available data thus show that bicarbonate binds, with high affinity, only to the NHI on the acceptor side of PSII and that the NHI-bound HCO_3^- remains exchangeable even in dark-adapted PSII. At this binding site, it promotes the electron transfer from Q_A to Q_B by making the protonation of $Q_B^{-/2-}$ more efficient (McConnell *et al.* 2012, Shevela *et al.* 2012). Additionally, mobile HCO_3^- promotes water oxidation, by acting as a proton acceptor (Ananyev *et al.* 2005, Shutova *et al.* 2008, Koroidov *et al.* 2014). Low dissolved HCO_3^-/CO_2 concentrations lead to a removal of bicarbonate form PSII. This makes PSII less efficient and promotes safe dissipation of access energy.

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