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High and unique carbonic anhydrase activity of Photosystem II from *Pisum sativum*: Measurements by a new and very sensitive fluorescence method

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ABSTRACT

Carbonic anhydrase (CA) activity, associated with Photosystem II (PSII), has been shown to enhance water oxidation. However, CA activity was thought to be a side effect or even a "contamination" of other CAs because of the relatively low rates of CA reactions in PSII measured previously. Here, by using 8-hydroxy-pyrene-1,3,6-trisulfonate (pyranine), a fluorescent dye, as a pH indicator, we show that PSII preparations (**BBYs) from *Pisum sativum* have a high CA activity (as measured by HCO_3^- dehydration), which is close to that of highly active CAs. This fluorescence method is new for BBYs giving at least ten times higher activity than the other methods used earlier, as well as being highly sensitive and, thus, more convenient to use for BBYs than any other approach. We show here that the pH range of 5.0–7.5 is optimum for the pyranine measuring system, in general, and this pH range is suitable not only for the CA in BBYs but also for other CAs. Further, the CA activity of BBYs has the following unique properties: (1) low sensitivity to some known, and otherwise, effective CA inhibitors; (2) an opposite pH profile of HCO_3^- dehydration than observed in other known CAs. These findings indicate that the high CA activity, we have observed, belongs to BBYs, i.e., free of other CAs. At pH 6.5, CA activity of BBYs is shown to be directly correlated with that of photosynthetic O_2 evolution. We propose that the CA activity may accelerate the removal of H^+s during water oxidation.

Celebrating 80th birthday of Alan James Stemler, a pioneer on the role of bicarbonate on the electron donor side of Photosystem II. S.G. Vaklinova & associates (1982), and A.J. Stemler (1986) were the first who have measured carbonic anhydrase activity in Photosystem II preparations.

** BBYs stand for Photosystem II samples made by the procedure of Berthold (B), Babcock (B) and Yocum (Y); see Berthold et al. (1981).

1. Introduction

Photosystem II (PSII) is a unique pigment-protein complex, capable of using the energy of the sunlight for water oxidation accompanied by the release of molecular oxygen & protons and the uphill transfer of electrons to plastoquinones (Shevela et al., 2021, 2023; Wydrzynski and Satoh, 2005). It is well established that PSII requires bicarbonate (HCO_3^-) for high rates of photochemical reactions and photosynthetic O_2 evolution. The function of HCO_3^- in PSII has been extensively studied over the past five decades (see reviews: van Rensen et al., 1999a; van Rensen and Klimov, 2005; Shevela et al., 2012)), and it has been shown to be required for the electron transfer process on both the electron acceptor and the electron donor sides of PSII (Allakhverdiev et al., 1997; Blubaugh and Govindjee, 1988, 1986, 1984; Eaton-Rye and Govindjee, 1988a,b; Fantuzzi et al., 2023, 2022; Govindjee et al., 1976; Khanna et al., 1981; Klimov et al., 1995a,b, 1997a,b; Stemler and Govindjee, 1973; Stemler et al., 1974; Wydrzynski and Govindjee, 1975). Fig. 1 shows the scheme of PSII and the estimated sites where bicarbonate ions play key roles.

If HCO_3^- is important for the PSII function, then the acceleration of its interconversion to CO_2 and *vice versa* may be crucial for PSII efficiency (Stemler and Jursinic, 1983). In general, the following interconversion (Eq. (1))

$$CO_2 + H_2O \rightleftarrows HCO_3^- + H^+ \quad \text{(Eq. 1)}$$

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is known to be catalyzed, in different organisms, by different carbonic anhydrases (Silverman and Lindskog, 1988; Moroney et al., 2011). About 40 years ago, Stemler and Jursinic (1983) had proposed that the rate of CO_2 hydration, i.e., the forward reaction of Eq. (1) (shown below as Eq. (2), when the chemical equilibrium is shifted towards the formation of HCO_3^- and H^+ upon the addition of excess of CO_2 gas (which is dissolved in H_2O), we denote this shift by the right-sided arrow) must be high to provide enough bicarbonate to PSII.

$$CO_2 + H_2O \rightarrow HCO_3^- + H^+ \quad \text{(Eq. 2)}$$

We note that the acceleration of CO_2 hydration has been found to be associated with PSII, isolated from higher plants (Hillier et al., 2006; Ignatova et al., 2011; Lu et al., 2005; Moskvin et al., 2004; Pronina et al., 2002; Rudenko et al., 2007; Shitov et al., 2009; Stemler, 1986). However, Bricker & Frankel (2011) have suggested that the low measured CA activity of PSII was due to other CAs adhering to the photosystem during the preparation. We therefore ask whether the CA activity of PSII is indeed associated with its proteins and what is the nature of this activity. We consider three possibilities: (1) the CA in PSII is a constitutive, separate enzyme that is not yet known; (2) the CA activity is the second function of the known proteins of PSII; (3) PSII has CA activity due to other CAs ('admixture CAs'). If CA in PSII from higher plants is a separate enzyme, its gene should be present in the genome of plants among the genes of other CAs. However, as stated by Zhurikova et al. (2016), such genes (the products of which might be associated with PSII) had not been found. On the other hand, Rudenko with co-authors (Rudenko et al., 2020a) proposed that the so called α-CA4 may be the one associated with PSII. If this is the case, it is likely that α-CA4 may play a role in facilitating rapid electron transfer in PSII, given that a CA in PSII is '*connected*' to photosynthetic activity. On the other hand, the lack of this CA is expected to decrease the rate of electron transfer in PSII. However, the knockout of α-CA4 genes did not result in a decrease of electron transfer from water to plastoquinone in PSII (Rudenko et al., 2020a). In contrast, Shitov et al. (2011, 2018) have shown that lack of CA activity, in the presence of CA inhibitors, decreases the rate of electron transfer from water to plastoquinone in BBYs (Berthold-Babcock-Yocum particles –thylakoid membrane fragments enriched in PSII isolated by the method of Berthold et al. (1981)). These results suggest that CA activity found in BBYs in the work of Shitov et al. (2011, 2018) is not due to the activity of α-CA4, which implies that α-CA4 is not the functional CA of PSII. It is likely that α-CA4 might be located only near the PSII complex, but not inside it. Further, the products of other known genes of CAs in higher plants have been suggested to be independent of PSII (Ignatova et al., 2019; Rudenko et al., 2020b). Thus, the proposal that the CA in PSII is a separate enzyme has not been directly confirmed to date. Further, molecular biology approaches have not yet provided direct results on the presence of CAs in PSII from higher plants. Consequently, the first aim of this work is to test if the CA activity is associated with the known proteins of PSII or if it is an artefact due to other CAs present in the PSII samples.

If the CA activity is indeed associated with the proteins of PSII, we expect it to be clearly '*connected*' to the photosynthetic activity. It is obvious to us that to clarify this '*connection*', we should measure both activities under optimal conditions for PSII photosynthetic reactions, i.e. at pH 6.5 (as shown by Schiller and Dau (2000)). However, the activity of CA in PSII at pH 6.5 has never been measured before. The spectroscopic method of Shingles and Moroney (1997), using pH 6.0, which is close to pH 6.5, has the potential to measure CA activity in PSII. Thus, the second aim of our research is to adapt the pyranine method of Shingles and Moroney (1997) to the measurement of CA activity in PSII at pH 6.5. The adapted pyranine method will help us to investigate the '*connection*' between CA and photosynthetic activity in PSII. If the CA activity obtained by the adapted pyranine method is high and shown to be 'connected' to photosynthetic activity, it means that it is important to do further studies, since high CA activity would imply that it has an important role in the function of PSII.

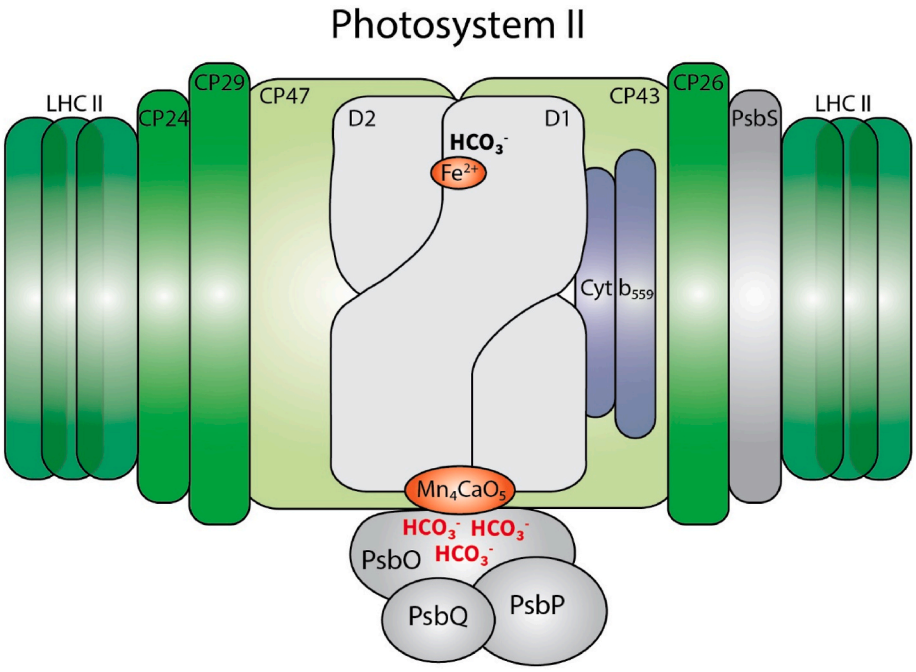

Fig. 1. The scheme of PSII (BBY) in higher plants.** D1 and D2 are the reaction center proteins, Cyt b_{559} is cytochrome b_{559}. CP43 and CP47 are 43 and 47 kDa Chl-protein complexes forming the inner antenna system of PSII. CP29, CP26 and CP24 are monomeric Chl-proteins of 29, 26 and 24 kDa, respectively. They belong to the outer antenna system of PSII. LHCIIs (light harvesting complex II) are trimers of LhcB 1–3 Chl-proteins, which are the components of the outer antenna of PSII. PsbS (22 kDa) is the protein involved in non-photochemical quenching of Chl fluorescence in PSII. PsbO (33 kDa), PsbP (23 kDa) and PsbQ (16 kDa) are extrinsic proteins of water oxidizing complex (WOC) that stabilize and optimize its function. Fe^{2+} is the non-heme Fe on the electron acceptor side of PSII. Mn_4CaO_5 is the manganese-oxygen-calcium cluster in WOC that is located on the electron donor side of PSII. Bicarbonate (HCO_3^-), shown in black, indicates its location near the non-heme Fe on the electron acceptor side of PSII. The red HCO_3^-s mark the site, on the electron donor side of PSII, where its function needs further investigation.

Based on the above, the absolute value of CA activity in PSII is a key for further studies. However, in previous work, the activity values showed significant heterogeneity, spanning several orders of magnitude (see section 3.5 for more details). McConnell et al. (2007), by directly measuring CA activity with the membrane inlet mass spectrometry (MIMS), found that this activity in BBY preps was almost two orders of magnitude lower than that of α-CAII from bovine erythrocytes (one of the most active CAs), which suggests a relatively low absolute value of CA activity in their BBYs. The low value of CA activity may provide an insufficient amount of bicarbonate to PSII, which seems to contradict Stemler and Jursinic (1983) who had proposed an important role for CA activity in PSII function. McConnell et al. (2007) had stated that MIMS is the most sensitive method for measuring CA activity, however, without directly comparing it with other methods under the same conditions. We question whether MIMS is sufficiently sensitive and appropriate to measure the actual CA activity in PSII. Remarkably, the CA activity of the α-CAII, measured by the highly sensitive spectroscopic method, with pyranine (8-hydroxy-pyrene-1,3,6-trisulfonate) as a fluorescent pH indicator (Shingles and Moroney, 1997), has been found to give a k_{cat} value 100 times higher than that obtained by MIMS (McConnell et al., 2007). Another highly sensitive spectroscopic method (see e.g., Kernohan, 1964, 1965; Pocker and Bjorkquist, 1977a, 1977b; Nielsen and Fago, 2015), which uses different pH indicators, also gave similar results to the pyranine method. Based on all the above, we conclude that the spectroscopic methods (using pH indicators) are much more appropriate than the MIMS method for α-CAII. Thus, one of the major aim of this work is to find the method that will give the higher absolute value of CA activity in PSII. Finally, we plan to test whether the 'connection' between CA and photosynthetic activity actually exists.

In this paper we have measured high CA activity in the BBYs from *Pisum sativum*. In comparison with all other methods, the modified method of Shingles and Moroney (1997) has been found to be more suitable for BBYs than the other methods used thus far, as it led to the detection of higher levels of CA activity. Further, we have found that the CA activity of BBYs has a unique pH profile within the pH range of 5.0 and 7.5, which, along with the unique lower sensitivity to some CA inhibitors, differs in its properties from those of other known CAs (Rowlett, 2010; Supuran, 2008). The unique properties of CA activity in PSII lead us to the conclusion that this high activity belongs only to CA (s) in BBYs, not to other CAs, located in thylakoids and in the stroma near the PSII. The high photosynthetic activity of BBYs has been shown to correlate well with high CA activity at pH 6.5. Therefore, our research demonstrates, for the first time, a clear connection between the activity of PSII and of CA at pH 6.5, which, in turn, shows that high CA activity plays a significant role in PSII. We present our results, in details, because of the fundamental nature of the issue involved; our results provide new direction in the study of the mechanism of PSII function since a complete investigation of CA activity in PSII would help not only to understand the mechanism of the function of HCO_3^- in PSII, but also the mechanism of the function of PSII, in general.

2. Materials and methods

2.1. Carbonic anhydrase

Purified CA from bovine erythrocytes (containing α-CAII isoform – carbonate hydrolase, EC 4.2.1.1, sequence accession number P00921, NCBI ID 9913) was obtained from Sigma (USA) Cat. N№ C3934.

2.2. Preparation of photosystem II (BBYs) with high activity

Photosystem II BBYs were prepared with modifications, as described below, from the leaves of *Pisum sativum*. The plants were grown for 14 days in the greenhouse. If CA activity is indeed related to photosynthetic activity, it is necessary to use BBYs with high electron transport activity. Thus, to make such BBYs, we have slightly modified the protocol of Schiller and Dau (2000), which yields high photosynthetic activity: BBYs from spinach have been shown to produce 1000–1450 μmol O_2·(mg Chl)$^{-1}$·h^{-1}. Thylakoid membranes were isolated as described in Berthold et al. (1981). Further, the membranes were resuspended in the buffer (25 mM HEPES-NaOH (pH 6.0), 15 mM NaCl and 10 mM $MgCl_2$ containing 1M of glycine-betaine) and were treated with 25% Triton X-100 for 20 min in the dark, kept in an ice bath. The Chl concentration during the treatment was 2 mg ml^{-1}, the ratio Chl (mg): Triton X-100 (mg) was 1 : 20. After treatment by Triton X-100, the suspension was centrifuged for 3 min at 1000g and for 17 min at 48 000g (2 ^{o} C). The dark-green upper layer of the pellet was taken to five cycles of resuspension and centrifugation (10 min at 48 000g) using the same buffer (but, without Triton X-100). Finally, BBYs were resuspended in the storage buffer (20 mM MES-NaOH (pH 6.5), 15 mM NaCl, 5 mM $MgCl_2$, 330 mM sucrose, containing up to 15% of glycerol) and kept at −70 ^{o} C until use.

Moskvin et al. (2004) had demonstrated that washing thylakoid membranes four or more times helps to obtain preparations free of stromal water-soluble CAs. Thus, the protocol of Schiller and Dau (2000) was modified by using 5 washings of BBYs to remove most of the water-soluble stromal and lumenal CAs, which may be the major contaminants of thylakoid membranes, according to Rudenko et al. (2020a). In addition, the detergent Triton X-100 (usually used to prepare BBY-particles (Berthold et al., 1981; Schiller and Dau, 2000)) is able to disrupt all the hydrophobic interactions between the BBY-particles and the water-soluble CAs. Thus, 5 washings of BBYs and the use of the detergent Triton X-100 are expected to significantly decrease (or completely remove) most of water-soluble CAs that could adhere to the BBYs. Thus, in this work, we have exploited the potential of the Schiller and Dau (2000) procedure, using several BBY preps.

2.3. Characterization of the BBYs

The photosynthetic activity of the BBYs (see above) was studied by two methods: (1) The measurement of oxygen evolution activity, as described by Shitov et al. (2018); (2) The maximal quantum yield of Photosystem II (inferred from F_v/F_m, which is the ratio of "variable" to "maximum" Chl *a* fluorescence (Kalaji et al., 2014, 2017)), as described in, e.g., Karacan et al. (2016), but by using a Multi-Color PAM fluorometer (Walz, Germany). The content of total Chl, in the preparations, was determined by the method of Porra et al. (1989). Chl *a* fluorescence spectra were recorded at 77K, as described by Cederstrand and Govindjee (1966), in the 600–750 nm range, using 435 nm as excitation light, by a Hitachi fluorescence spectrophotometer (Tokyo, Japan). The ratio of total Chl to photosynthetic reaction center of PSII (RCII) was determined according to the methods used by Kaminskaya et al. (2005) and Terentyev et al. (2020).

2.4. Preparation of pigment extracts from the BBYs

In order to test whether the photosynthetic pigments of BBYs have an effect on the CA activity in this complex, we extracted these pigments from our preps as follows. BBY preparations (with 250 μg of Chl) were sedimented at 6900 *g* for 3 min; the pellet was diluted with 600 μL 96% ethanol. This mixture was shaken and then centrifuged at 8200 *g* for 10 min, using a Mini Spin centrifuge (Eppendorf, Hamburg, Germany); the supernatant, containing the pigments, was then used for measurements (final Chl concentration, 4.2 μg mL^{-1}). The precipitate was colorless, without any green or yellow tint, assuring complete pigment extraction.

2.5. Measurements of carbonic anhydrase activity using the HCO_3^- dehydration method

2.5.1. Choosing a suitable method to measure CA activity

Based on the high sensitivity of the method of Shingles and Moroney (1997) for α-CAII (see INTRODUCTION) we chose this method to test its

sensitivity using BBYs. Shingles and Moroney (1997) had measured the dehydration activity of the most active α-CAs in combination with a stop-flow technique. We emphasize that the stop-flow method is an indispensable tool for accurately recording the rapid kinetics of enzymatic reactions. In addition, Shingles & Moroney have used the reverse of the reaction (Eq. (3)) shown above in Eq. (1). The right-sided arrow assumes that the chemical equilibrium in Eq. (1) is shifted towards the formation of CO_2 and H_2O upon the addition of excess HCO_3^-.

$$HCO_3^- + H^+ \rightarrow CO_2 + H_2O \quad \text{(Eq. 3)}$$

Therefore, in this study, we also employed the stop-flow to measure the dehydration of HCO_3^- in the BBYs from *Pisum sativum*. The following section describes the approach of Shingles and Moroney (1997) optimized to pH 6.5.

2.5.2. The equipment and the conditions for the measurement of HCO_3^- dehydration

Pyranine fluorescence was recorded by a Varian Cary Eclipse fluorescence spectrophotometer (Agilent Technologies, Santa Clara, USA), equipped with the "RX.2000 Stopped-Flow Mixing Accessory" (Applied Photophysics, Leatherhead, United Kingdom). This stopped-flow apparatus had the "Pneumatic drive DA.1" to ensure uniformity of the mixing process. The mixing time was 8 ms, and the HCO_3^- dehydration reaction was started after the solutions A and B were mixed in a ratio of 1: 1 at 3 Bars (43.5 psi) and at 25 °C. Solution A contained the substrate – $NaHCO_3$ that was ≥99.5% pure (Sigma, Saint Louis, USA) and suspended in 2.0 mL of buffer A (0.5 mM Tricine-KOH) at pH 8.0. Solution B contained α-CAII or BBY-particles in 2.0 mL of buffer B (5 mM HEPES-KOH, 5.5 μM pyranine) at pH of 6.5, unless otherwise stated. Pyranine fluorescence was measured at 512 nm (where changes are known to be pH dependent) by exciting the sample with 446 nm light (see Shingles and Moroney, 1997) at an intensity of 0.20 μmol photons·m^{-2} s^{-1}. The plots of the kinetics for pyranine fluorescence changes and the fitting of Lineweaver-Burk plots (Lineweaver and Burk, 1934), as well as statistical analyses, were carried out in Origin 6.5 software package (OriginLab, Northampton, USA).

2.5.3. Steady state initial rates of HCO_3^- dehydration

"Steady state initial rates" were calculated as published by others (Khalifah, 1971; Pocker and Bjorkquist, 1977a; Shingles and Moroney, 1997; Steiner et al., 1975) using the time interval 20–25 s (just after HCO_3^- addition). The obtained initial rates were expressed in three different units: "pH·s^{-1}" (see Shingles and Moroney, 1997); relative units of fluorescence per second (rel.un.fl.·s^{-1}), and mM H^+·s^{-1}. The rate, expressed in pH·s^{-1}, was used to compare our results with that obtained in the paper of Shingles and Moroney (1997). For further details, see section 1 and Fig. S1 under Supplementary Information (SI). The rate of the reaction, expressed in rel.un.fl.·s^{-1}, provides the initial value for the subsequent calculation of the rate expressed in mM H^+·s^{-1}; this is the best method for the determination of the kinetic parameters of the enzymatic reactions, since it uses the actual changes in H^+ concentrations.

To obtain the actual rates of the HCO_3^- dehydration, the initial rates of the steady state (i.e. the rate of absorption (or fluorescence) change of the pH indicator) were multiplied by Q, the "Buffer factor" (Khalifah, 1971; Pocker and Bjorkquist, 1977a; Shingles and Moroney, 1997; Steiner et al., 1975), which depends only on the properties of the buffer system used; it was calculated according to Eq. (4):

$$Q = \Delta pH \text{ (or } \Delta[H^+]) \,/\, \Delta F, \quad \text{(Eq. 4)}$$

where, ΔpH is the observed change of pH per second, $\Delta[H^+]$ is the actual change of the concentration of protons per second, and ΔF is the change in pyranine fluorescence per second. The ΔpH was measured directly by a pH-meter (Orion 420Aplus; Thermo Electron Corporation, Waltham, USA), whereas, $\Delta[H^+]$ was calculated as $\Delta[HCO_3^-]$, as in Pocker and Bjorkquist (1977a); the $\Delta[HCO_3^-]$ was obtained using the concentrations of HCO_3^- at the start and at the end of the reaction at equilibrium (Eq. (1)). These concentrations of HCO_3^- were calculated by the Henderson-Hasselbalch equation (pKa 6.48 at 25 °C (Nielsen and Fago, 2015)). From the above, we found Q to have a value 0.0109 ± 0.0001 pH·rel.un.fl.$^{-1}$, or 2.34 (±0.14)·10^{-2} mM (H^+) ·rel.un.fl.$^{-1}$- depending on the unit used. We note that the Q (for both ΔpH and $\Delta[H^+]$) remained constant over the entire range of HCO_3^- concentration used. Since the rate expressed in mM H^+·s^{-1} gives the actual rate of HCO_3^- dehydration (see section 1 in SI), we used Q for $\Delta[H^+]$ to determine the kinetic parameters of the enzymatic reactions.

The HCO_3^- dehydration activity of α-CAII and of BBYs was also expressed in Wilbur and Anderson units per mg of protein or of Chl, respectively (W-A un.·(mg protein or Chl)$^{-1}$), using the following relationship:

$$\left(tg_{enz.} \,/\, tg_{spont.} - 1\right) \,/\, m\,(v), \quad \text{(Eq. 5)}$$

where, $tg_{enz.}$ is the tangent of the line, which gives us the steady state initial rate, obtained in the presence of α-CAII or in the presence of BBY preps, i.e., when the HCO_3^- dehydration is accelerated by the enzyme; and $tg_{spont.}$ is the tangent of the line, which is the steady state initial rate, obtained in the absence of any biological sample, i.e. when HCO_3^- dehydration is spontaneous; and *m* is the amount of α-CAII or of BBYs (in terms of Chl), in milligrams. To express the activity in W-A un. per mole of α-CAII or per mole of RCII (W-A un.·(mole protein or mole RCII)$^{-1}$), *m* is replaced by *v* (in moles of protein or RCII).

2.5.4. Calculation of carbonic anhydrase kinetic parameters: V_{max}, K_m, k_{cat} and k_{cat}/K_m

Lineweaver and Burk (1934) plots (1/V versus 1/S) were used to determine V_{max} and K_m, where, V is the actual rate of HCO_3^- dehydration catalyzed by α-CAII, and S is the substrate concentration (here: $[HCO_3^-]$). From these plots, their equations were obtained, which were then used to calculate both the K_m and the V_{max} values. Finally, we calculated k_{cat} using Eq. (6):

$$k_{cat} = V_{max} \,/\, [E_t], \quad \text{(Eq. 6)}$$

where, V_{max} is the maximal rate expressed as pH·s^{-1} or as (mM H^+)·s^{-1}; $[E_t]$ – α-CAII concentration, thus obtained, was 1.725·10^{-5} mM (0.5 μg mL^{-1}).

2.6. Measurements of carbonic anhydrase activity using CO_2 hydration

The CO_2 hydration activity was measured at 1–2 °C, as described by Wilbur and Anderson (1948) with modifications (Karacan et al., 2016; Shitov et al., 2009). The initial pH of the buffer, prior to the addition of CO_2 saturated water, was 8.3. To calculate the activity, we measured the time taken for the pH to drop from 8.2 to 7.7 in the control (i.e., the buffer in the absence of the sample) and in the sample. For the analysis of the effect of sulfonamide inhibitors on the CA activity in BBYs, 10 μM ethoxyzolamide or acetazolamide was incubated with preps in the cell for 1 min before the measurements. The rate of CO_2 hydration was calculated in "W-A un.·(mg Chl)$^{-1}$)" or in "W-A un.·(mole RCII)$^{-1}$", as well as in "μmol H^+·(mg^{-1} of Chl)·min^{-1}", as was described in an earlier paper (Shitov et al., 2009).

2.7. SDS-PAGE electrophoresis

Proteins of the BBYs, isolated from *Pisum sativum*, were separated by electrophoresis under denaturing conditions in the 12.5% (w/v) polyacrylamide gel (Laemmli, 1970), using Mini-PROTEAN 3 Cell (Bio-Rad, Hercules, USA). BBY preps were diluted in 50 mM Tris-HCl buffer (pH 6.8) containing 8 M urea, 3% SDS, 10% sucrose, and 0.005% bromophenol blue, and then heated for 3 min at 100 °C. After electrophoresis,

polypeptides in the gel were stained, as described by Kang et al. (2002). Precision Plus Protein Standards (Bio-Rad, USA) were used as molecular mass markers.

3. Results

3.1. On the BBYs, with high photosynthetic activity

As mentioned in section 2.2, the first thing we did was to isolate BBYs with a high level of photosynthetic activity. Table 1 shows data for 3 BBY preps. The values of F_v/F_m (line 5) of our BBYs were quite high (0.69–0.77), but also the rates of oxygen evolution (line 6) were high, as they ranged from 510 to 729 μmol O_2·(mg Chl)$^{-1}$·h^{-1}. These results are characteristic of high photochemical activity of BBY (see e.g., Berthold et al., 1981). Thus, in this work, we have 3 BBY preps, all of which had high photosynthetic activities.

3.2. The test of contamination of BBYs by other CAs

As mentioned in the INTRODUCTION, some other CAs may be located not in, but near the PSII. Two possible sources of other CAs are: (1) Water-soluble CAs from the stroma of chloroplasts and from the lumen of thylakoids (Rudenko et al., 2020b), and to a lesser degree, water-soluble CAs from the cytoplasm of the cells. (2) CA, associated with Photosystem I (for a review see Rudenko et al. (2015)), may also contribute to the CA activity of PSII, since most thylakoid membrane preparations enriched in PSII usually contain small amounts of Photosystem I (PSI) (Ignatova et al., 2006).

Table 1
CA and photochemical activity of BBYs obtained at different times of the year.

Characteristics of preparations	Units	BBYs obtained at different times of the year (2018)			Average values ± standard deviations
		March	May	October	
Hydration CA activity	W-A un.·(mg Chl)$^{-1}$	30.8 ± 1.0	12 ± 0.7	20.4 ± 1.4	21.1 ± 9.4
	W-A un.·(mole RCII)$^{-1}$·10^9	6.20 ± 0.20	3.67 ± 0.21	3.36 ± 0.23	4.4 ± 1.6
Hydration CA activity + 10 μM EA	W-A un.·(mg Chl)$^{-1}$	2.2 ± 0.17	0.6 ± 0.05	2.1 ± 0.15	1.6 ± 0.9
Hydration CA activity + 10 μM AA	W-A un.·(mg Chl)$^{-1}$	15.5 ± 0.9	5.9 ± 0.4	10.2 ± 0.7	10.5 ± 4.8
F_v/F_m		0.74 ± 0.02	0.77 ± 0.02	0.69 ± 0.01	0.73 ± 0.04
O_2 evolution activity	μmol O_2·(mg Chl)$^{-1}$·h^{-1}	729 ± 21	610 ± 10	510 ± 16	616 ± 110
	μmol O_2·(mole RCII)$^{-1}$·h^{-1}·10^8	3.49 ± 0.1	4.44 ± 0.07	2.00 ± 0.06	3.31 ± 1.23
Chl/RCII ratio	mole/mole	223/1	339/1	183/1	(248 ± 81)/1
RCII concentration	mM·10^{-5}	2.087 ± 0.004	1.375 ± 0.003	2.550 ± 0.005	2.004 ± 0.590
Dehydration CA activity at pH 6.5	W-A un.·(mg Chl)$^{-1}$	193 ± 28	159 ± 13	133 ± 16	167 ± 33
	W-A un.·(mole RCII)$^{-1}$·10^{10}	3.8 ± 0.6	4.9 ± 0.6	2.2 ± 0.3	3.6 ± 1.1

Since the BBYs were added to the reaction mixture in amounts based on equal Chl concentration, and the Chl/RCII ratios were different, the RCII concentration was, thus, different for each sample. These concentrations are given in the above Table for the reaction mixtures containing 4.2 μg·Chl mL^{-1}.
The average values (column 6), expressed in W-A un.·(mg Chl)$^{-1}$ or μmol O_2·(mg Chl)$^{-1}$·h^{-1}, are given for the reference only since BBY preps differed in number (in mass) of Chl per one RCII. Thus, the use of the average values expressed in W-A un.·(mole RCII)$^{-1}$ or μmol O_2·(mole RCII)$^{-1}$·h^{-1} is considered to be a more correct way to present the above data.

On the contamination by water soluble CAs. A wide variation in the activity of different PSII preparations (PSII preps) or BBYs, isolated by the same method, would have indicated contamination by other CAs since the presence of even a small amount of other highly active CAs in the preparation would dramatically increase its CA activity. [In the following, the term "PSII preps" refers to thylakoid membrane preparations enriched in PSII, but isolated by methods other than that of the Berthold- Babcock-Yocum (BBY) technique.] The equal contamination of different preps by other soluble CAs (leading to the similar amount of the activity of PSII preps or BBYs) seems to be highly unlikely since it rather indicates that "the contaminating" CA(s) would be a constituent component of the preps. Thus, to test the absence of other water soluble CAs in our BBYs, we checked if 3 different BBYs had close values of activity (all BBYs isolated by the procedure of Schiller and Dau (2000) from leaves of pea plants grown in 3 different months (March, May, and October) in 2018).

We have found that CO_2 hydration activity varies from 12 to 31 W-A un.·(mg Chl)$^{-1}$ (Table 1, line 1), or from 222 to 574 μmol H^+·(mg^{-1} of Chl)·min^{-1}. While, when we expressed the activities in W-A un., relating it to the concentration of RCII (Table 1, line 2), two, out of three, preps showed no significant difference (according to the *T-Test*, $p = 0.16$). Nevertheless, a 2-fold difference in CO_2 hydration activity in the third BBY prep (see Table 1, line 2) requires additional approaches, which are described below, to exclude possible contamination by other types of CAs.

On the contamination by CAs of PSI. A strong contamination of BBYs by CA associated with PSI may take place if and when the detergents used leave significant amount of PSI in the preparation. To test the amount of PSI in our BBY preps, we used 77 K fluorescence spectra (Fig. S2 in SI); these spectra show that the BBY preps contained only 7–10% of PSI. Nevertheless, we ask: how does this low level of PSI affect the overall CA activity of the BBYs obtained? The CA activity of PSI preps is known to be completely inhibited by 10 μM ethoxzolamide (EA) and by 10 μM acetazolamide (AA) (Ignatova et al., 2006; Rudenko et al., 2007). In contrast, 10 μM EA almost completely inhibits CA activity of PSII preps and BBYs (obtained by different methods), while 10 μM AA suppresses it by only 50% (Ignatova et al., 2006; Rudenko et al., 2007; Shitov et al., 2009, 2011, 2018). This is the unique response of the CO_2 hydration activity of different PSII samples (including "PSII preps" and BBYs) to sulfonamides. If PSII samples have any significant contamination by CAs of PSI, then the CA activity of such preps is expected to be inhibited by 10 μM AA by more than 50%. This would also be true, if our BBYs were contaminated with water soluble α-CAs and β-CA from the stroma (the most likely candidates for the contamination of BBYs), since the CA activity of these CAs is also always suppressed by 10 μM EA and by 10 μM AA completely (Rowlett, 2010; Supuran, 2008; Terentyev et al., 2019). Our results, however, clearly show that 10 μM EA lowered CO_2 hydration by 90–95%, while 10 μM AA inhibited the activity only by 50% in all the BBYs, used here (Table 1, lines 3 and 4, respectively). These results indicate the unique response of the CA activity of the BBYs to sulfonamides showing that the CA activity of PSI (as well as the activity of other CAs) has no effect on the activity of the BBY preps used in our research. Thus, BBYs, used in this work, are free of water-soluble CAs and of CA associated with PSI.

The separation of proteins of the BBYs, used here, by SDS-electrophoresis. To further check the purity of our preps, we did SDS-PAGE of all the BBYs (Fig. 2). As seen in this figure, all the three BBYs had equal number of bands (each of them corresponding to a separate protein subunit), which indicates that these preps have identical composition of the proteins. Further, the protein composition of our preps (Fig. 2) was identical to that of the BBYs, used in our earlier research (Shitov et al., 2009) and to PSII preps, obtained by others, e.g., by Lu et al. (2005) [Also see Barera et al. (2012) for additional information on the mobility of some PSII proteins from peas in the SDS-PAGE gels.].

The identity of the protein composition show that all the observed

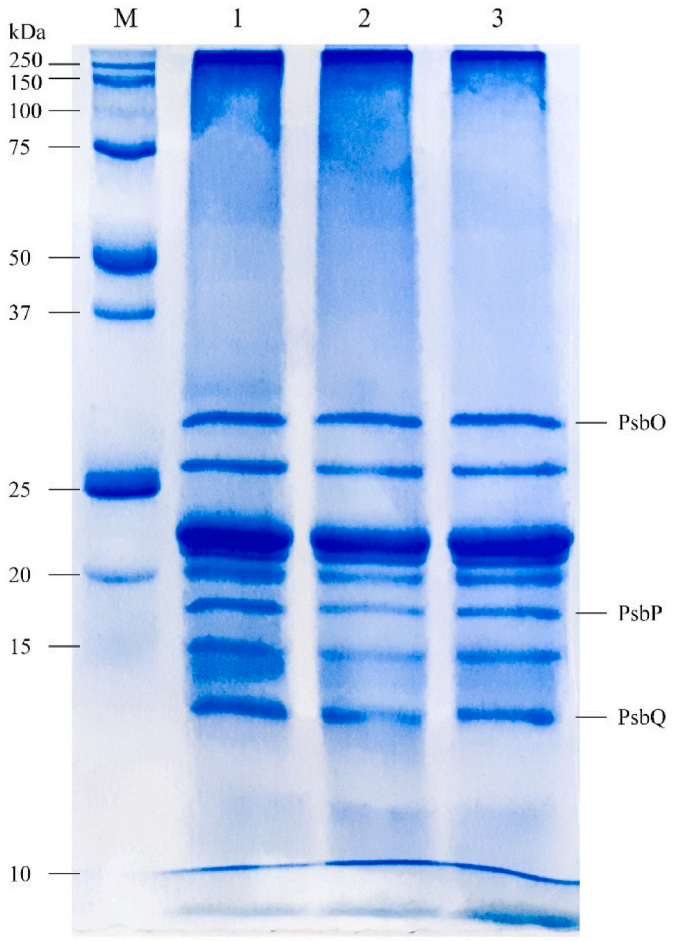

Fig. 2. SDS-PAGE of PSII (BBY) samples isolated from *Pisum sativum*. The lane labeled as "M" is for markers (5 μL) of known molecular masses. "1" is for the sample obtained in May 2018 (cf. Table 1), "2" – is for the October 2018 sample, and "3" – is for the March 2018 sample. The names of proteins of WOC are shown on the right; the relative electrophoretic mobility of these proteins was obtained from the results of the salt treatment of BBYs (for further information, see Fig. S3). BBYs were loaded on the gel at an equal amount of Chl (2 μg) per track.

bands are from PSII. It is well known that PSI proteins are usually seen in SDS-PAGE gels as bands in the region of ~50–70 kDa (Shao et al., 2011; Zolla et al., 2002, 2004). Fig. 2 shows that there are no bands in this area in all the three BBYs, used here, which establishes that the BBYs, used in this research, do not contain any significant amount of PSI; if any, it must be under the lower limit of the sensitivity of protein staining used in the gel. In addition, since no other bands were seen in the gel, no other peptides, including CAs, appear to be associated with our three BBYs. Thus, all our results, obtained by different biochemical and biophysical approaches, reliably demonstrate that the samples, used in the work presented here, are free from contamination by other CAs.

Considering the absence of the constitutive separate CA (see INTRODUCTION) and the absence of other CAs in the obtained BBYs, we suggest that the CA activity in PSII is associated with its known proteins as a second function of them. However, this statement needs to be corroborated by clarifying the 'connection' between the CA and photosynthetic activities at the same pH - 6.5. To do this, we should first measure the CA activity of BBYs at pH 6.5.

3.3. Investigation of the CA activity of the BBYs at pH 6.5

To investigate the CA activity of the BBYs at pH 6.5, we did the following: **(i)** modified the method, described by Shingles and Moroney, for pH 6.5; **(ii)** tested its sensitivity, using very active CAs (whose catalytic properties are well known); **(iii)** measured the CA activity of BBYs by our modified method; and **(iv)** determined the minimal concentration of BBY, which gave stable results. We describe below, step by step, the details of our results.

3.3.1. Adaption of the method described by Shingles and Moroney to pH 6.5

The resolution of spectroscopic methods, which use dyes (pH indicators), usually depends on the concentration of the dye used and of the substrate, which determines the concentration of the buffer to be used (Kernohan, 1965; Nielsen and Fago, 2015; Pocker and Bjorkquist, 1977a; Shingles and Moroney, 1997). However, we note that Shingles and Moroney (1997) used the substrate up to 10 mM, but the buffer (HEPES) at only 0.5 mM. In order to adapt their method to pH 6.5 and to our fluorescence spectrophotometer (for measuring pyranine fluorescence), we tested the resolution of our equipment by changing the concentration of the buffer (HEPES), from 2.5 to 10 mM; and of the pyranine, from 1.8 to 11 μM, while using spontaneous (without biological catalyst) reaction of HCO_3^- dehydration. We found that 5 mM HEPES and 5.5 μM pyranine gave the highest signal to noise ratio (i.e., highest resolution) in our experiments. The increase in resolution appears to be due to the increased buffering capacity (which decreases the value of the fluctuations in pH/in noise reflected in our measurement system by the pyranine fluorescence) when we increased the concentration of HEPES from 0.5 to 5 mM compared to the method of Shingles and Moroney (1997). Furthermore, in spectroscopic approaches, actually the high buffering capacity, we assume, enhances the linear dependence of the signal (absorbance, fluorescence) on CO_2 or HCO_3^- formed, as described by Kernohan (1964). This also improves the precision of our adapted method. The actual effect of the buffering capacity on our measurement system is explained in section 3.4. The increase in pyranine concentration from 0.1 to 5.5 μM also seems to be the reason for the decrease in noise. Then, to determine the highest precision of the adapted method, we tested the concentration of the substrate (HCO_3^-) in the range of 1–10 mM, and obtained highly reproducible results with 1.5–6 mM of HCO_3^-, which is close to that obtained by Shingles and Moroney (1997). Thus, by adapting the published method to pH 6.5 and to our equipment, we achieved the highest resolution and precision for the measurement of spontaneous HCO_3^- dehydration by using 5.5 μM pyranine, the substrate at 1.5–6 mM, and the buffer at 5 mM.

3.3.2. The test of the adapted method by α-CAII from bovine erythrocytes

α-CAII from bovine erythrocytes is an appropriate enzyme for testing the method used in our work, since this CA had also been used earlier by Shingles and Moroney (1997), and its catalytic properties are well described by others (Kernohan, 1964; Pocker and Bjorkquist, 1977a). To check how well the adapted method works at pH 6.5, we analyzed: (1) the rates of spontaneous and enzymatic reactions; (2) the sensitivity of the method; and (3) the kinetic parameters for the CAII. Below we describe the results and conclusions of the above-mentioned experiments.

(1) The rate of the enzymatic reaction, catalyzed by CA, must be significantly higher than the spontaneous one. We found that the time to reach the equilibrium of the reaction (Eq. (1)) was ~80 s for the spontaneous dehydration reaction (Fig. S4), while it was ~15 s for the reaction in the presence of the CAII (Fig. 3), which indicates the acceleration of the HCO_3^- dehydration by the CAII at pH 6.5. We note that, when the HCO_3^- concentration was increased, both the level of pyranine fluorescence at equilibrium (Eq. (1)) and the steady state of the initial rate were also increased (Fig. 3). Further, we found that kinetics of pyranine fluorescence (as shown in Fig. 3) was almost identical to those obtained for α-CAII at pH 6.0 by Shingles and Moroney (1997),

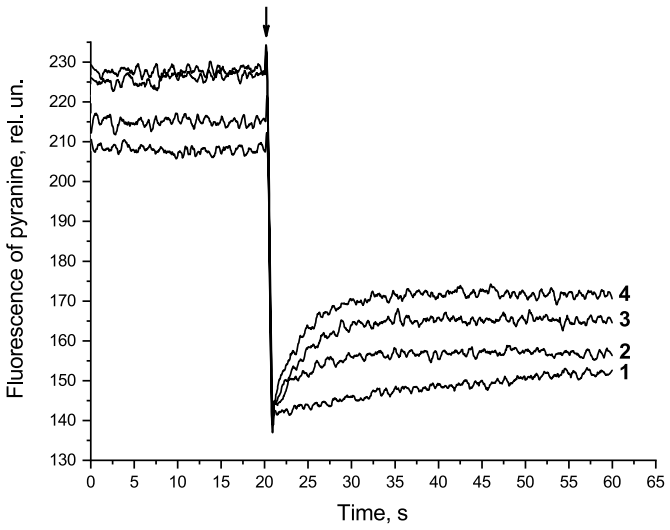

Fig. 3. Kinetics of pyranine fluorescence changes related to the HCO_3^- dehydration activity of bovine α-CAII in the presence of different concentrations of HCO_3^- (the substrate). Curve 1 is pyranine fluorescence after the addition of 2 mM $NaHCO_3$ (as the substrate) in the absence of CAII (spontaneous reaction). Curves 2–4 show changes in pyranine fluorescence after the addition of 2, 3 and 4 mM $NaHCO_3$ respectively in the presence of the CAII (catalyzed reactions) with the final protein concentration of 0.5 μg mL^{-1} ($1.725 \cdot 10^{-8}$ M). Note that traces of curves 3 and 4 were shifted upwards by 10 and 18 relative units, respectively, to align the initial fluorescence level with that of the other curves. The arrow indicates the time when HCO_3^- was added. The curves were smoothed, using algorithm of Savitzky-Golay 35 points.

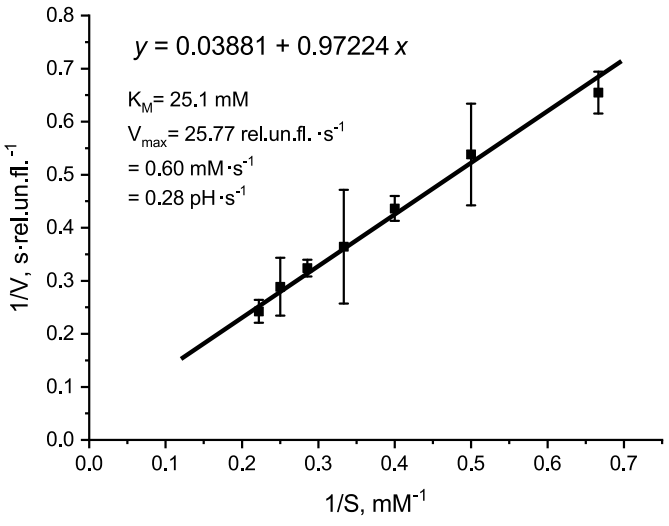

Fig. 4. Lineweaver-Burk plot (1/velocity V vs. 1/substrate S) for the initial rate of HCO_3^- dehydration reaction as a function of the substrate (HCO_3^-) concentration for bovine α-CAII. From this data, we obtain: K_m = 25.1 mM and V_{max} = 25.77 rel.un.fl.·s^{-1} = 0.60 mM s^{-1} = 0.28 pH·s^{-1} for the CAII. The concentrations of substrate (HCO_3^-) used were: 1.5, 2.0, 2.5, 3.0, 3.5, 4.0 and 4.5 mM. The final concentration of the CAII, used in the above experiment, was 0.5 μg mL^{-1}. Each point was an average of at least 5 separate experiments with calculated standard deviations (SDs). The Adjusted R-Squared (calculated using Origin program) for the curve fit was 0.978.

where authors have revealed the enzymatic acceleration of HCO_3^- dehydration. Thus, all these results clearly indicate the enzymatic acceleration of the reaction in the presence of the CAII at pH 6.5.

(2) Our results show that the rate of HCO_3^- dehydration for the CAII is 2.0192 (±0.083)·10^4 W-A un.·(mg protein)$^{-1}$ or 5.87 (±0.24)·10^{11} W-A un.·(mole protein)$^{-1}$. In order to test the sensitivity of our system, we measured the activity at different concentrations of the CAII (0.5, 0.25, and 0.125 μg mL^{-1}, i.e., $1.725 \cdot 10^{-8}$, $0.86 \cdot 10^{-8}$, and $0.43 \cdot 10^{-8}$ M), and obtained accurate and reliable results even at low concentration (0.125 μg mL^{-1} of the CAII (Fig. S5)). This low amount of the CA (with detectable CA activity) is essentially the same as that reported earlier by Shingles and Moroney (1997), showing that our adapted method has a high sensitivity at pH 6.5.

(3) Using the initial rates from the kinetics, shown in Fig. 3, we obtained a Lineweaver-Burk plot (Lineweaver and Burk, 1934) and derived its equation (see Fig. 4). Then, using the equation: $y = 0.03881 + 0.97224x$, we calculated the kinetic parameters K_m and V_{max} and, after that k_{cat}, as well as k_{cat}/K_m (see Table 2, column 1).

The K_m value (25.1 mM) for the CAII, obtained here, is close to that published by others (Kernohan, 1964; Pocker and Bjorkquist, 1977a); see Table 2, columns 3 and 5, respectively. Here, the V_{max} for the CAII is equal to 0.28 pH·s^{-1}, which is similar to that published by Shingles and Moroney (1997); see also Table 2, column 2. We conclude that the method used above is as useful as other spectroscopic methods, since the kinetic parameters of HCO_3^- dehydration, obtained here for the CAII, were found to be similar to those published in other investigations of the CAII (Table 2). Thus, the adapted method of Shingles and Moroney (1997) is certainly suitable for accurate measurements of high rates of HCO_3^- dehydration reaction at pH 6.5 and, we assume, has the potential to investigate CA activity in BBYs.

3.3.3. The investigation of HCO_3^- dehydration activity of the BBYs, using the method of Shingles & Moroney adapted for measurements at pH 6.5

The dehydration activity of the BBYs was found to be 167 ± 33 W-A un.·(mg Chl)$^{-1}$ (Table 1, line 10). The absolute value of the rate of HCO_3^- dehydration in BBYs will be presented in section 3.5. The relatively small variations in activities, expressed in W-A un.·(mole RCII)$^{-1}$ (Table 1, line 11), additionally indicate that the preps, used in our current research, are free of other CAs (for further information about variations and its apparent origin, see section 2 in SI).

We have found that the time (50–60 s) to reach the steady-state of pyranine fluorescence in the presence of BBYs (Fig. 5, curve 3 and Fig. S4) is lower than in the spontaneous reaction (~80 s, see Fig. S4, curve "Buffer"); and this time decreases as the concentrations of the substrate and/or BBYs are increased (see, e.g. Figs. S4 and S6, and the supplementary text in section 3 in SI). In addition, in the time range of 20–25 s, the steady state initial rate increases linearly with increasing concentrations of the BBYs (see inset in Fig. 5 and Fig. S7B) or of the substrate (Fig. S6A). Thus, all these observations clearly show that the BBYs accelerate the dehydration of HCO_3^-.

At 20 s, we observed a drop in the intensity of pyranine fluorescence (Figs. 3 and 5). Two separate experiments (Figs. S8 and S9) indicate that the reason of this drop is simply due to the dilution of the buffer B. Therefore, this drop has no effect on the dehydration of HCO_3^- (for more information on this drop see section 3 and Fig. S10 in SI).

Further, it is important to note that the photosynthetic pigments of BBYs (which reduce the fluorescence of pyranine) have no effect on the rate of HCO_3^- dehydration (also see: sections 4–5 and Figs. S11–S13, as well as Table S1 in SI).

Remarkably, the level of the steady-state of pyranine fluorescence, in the presence of BBY, exceeds that of α-CAII in the time range of 37–60s (Fig. 5, curve 3). The reason for this phenomenon is not yet clear, and, thus, needs further investigation.

3.3.4. The low concentration of BBYs gives stable results in the measurements of CA activity at pH 6.5

We note that the amount of Chl required to measure HCO_3^- dehydration is 38–74 times lower than that required to measure CO_2

Table 2
Comparison of bovine α-CAII kinetic parameters, obtained in the present work, by using the method of Shingles and Moroney (1997) adapted for measurements of HCO_3^- dehydration at pH 6.5, with those published earlier by others.

Kinetic parameters of dehydration reaction	Data source for bovine α-CAII						Human membrane bound CAIV (Baird et al., 1997)
	Present study	Shingles and Moroney (1997)	Kernohan (1964)	Kernohan (1965)	Pocker and Bjorkquist (1977a)	Nielsen and Fago (2015)	
α-CAII concentration, μg·mL^{-1}	0.5	1	–	–	–	–	–
α-CAII concentration, mM	$1.725{\cdot}10^{-5}$	–			$4.0{\cdot}10^{-5}$	$1{\cdot}10^{-3}$	$4.0{\cdot}10^{-5}$
K_m, mM	25.1	3.8	26–220	–	22	221	26
V_{max}, rel.un.fl.·s^{-1}	25.8	–	–	–	–	–	–
V_{max}, pH·s^{-1}	0.28[a]	0.33[a]	–			–	–
V_{max}, mM·s^{-1}	0.60[b]	–	1.3[b]			793[b]	$4.9{\cdot}10^{-2}$[b], $2.4{\cdot}10^{-2}$[b,c]
k_{cat}, s^{-1}	$1.6{\cdot}10^{4}$[a]	$8.3{\cdot}10^{5}$[a]	–	–	–	–	–
k_{cat}, s^{-1}	$3.5{\cdot}10^{4}$[b]	–		3.1–5.0·10^{6}[b]	$8.1{\cdot}10^{5}$[b]	$7.9{\cdot}10^{5}$[b]	$7{,}2{\cdot}10^{5}$[b], $5.5{\cdot}10^{4}$[b,c]
k_{cat}/K_m, M^{-1} s^{-1}	$6.5{\cdot}10^{5}$[a]	–	–	–	–	–	–
k_{cat}/K_m, M^{-1} s^{-1}	$1.4{\cdot}10^{6}$[b]				$1.7{\cdot}10^{7}$[b]	$3.6{\cdot}10^{6}$[b]	$2{,}8{\cdot}10^{7}$[b]

Data, presented without footnotes, based on V_{max}, expressed in rel.un.fl.·s^{-1}.
[a] Data based on V_{max}, expressed in pH·s^{-1}.
[b] Data based on V_{max}, expressed in mM·s^{-1}.
[c] Data obtained by Baird et al. (1997), using 5 mM HCO_3^-.

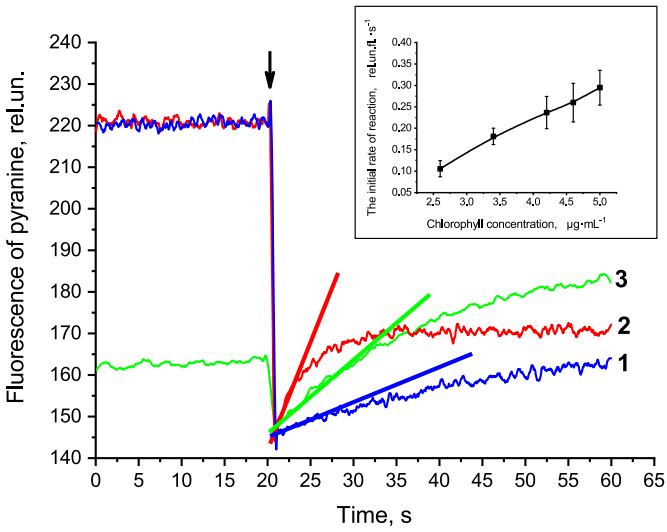

Fig. 5. Kinetics of pyranine fluorescence changes related to the HCO_3^- dehydration activity of BBY-particles and α-CAII. Here, BBY preparations obtained in March 2018 were used. Curve 1 (blue) shows the pyranine fluorescence changes in the buffer in the absence of biological sample (spontaneous reaction); curve 2 (red) shows these changes in the presence of bovine α-CAII (CAII catalyzed reaction) - final protein concentration: 0.5 μg mL^{-1} ($1.725{\cdot}10^{-8}$ M); and curve 3 (green) is for fluorescence changes in the presence of BBY particles (4.2 (μg Chl)·mL^{-1}). Note that the trace of curve 3 was shifted upwards by 32 relative units to align the initial fluorescence level with that of the other curves. Straight lines represent the initial rates of the reaction of HCO_3^- dehydration. The arrow indicates the time when HCO_3^- was added. The concentration of the substrate (HCO_3^-) was 2 mM. The curves were smoothed, by the algorithm of Savitzky-Golay, for 35 points. **The inset** (top right) displays the dependence of the initial rate of pyranine fluorescence changes on the amount of BBY preps, given in terms of their chlorophyll concentration. [Examples of the corresponding kinetics are available in Fig. S7.] The concentration of bicarbonate was 4 mM; the final concentrations of BBYs had 2.6, 3.4, 4.2, 4.6 and 5.0 (μg Chl)·mL^{-1}. Each point is an average of at least 7 separate experiments with calculated SD.

hydration: for HCO_3^- dehydration, we had used 1.36–2.63 μg of Chl (see the inset in Fig. 5), whereas for CO_2 hydration we had to use a sample with 100 μg of Chl. This finding implies that the method (using HCO_3^- dehydration) is much more sensitive than the electrometric method measuring CO_2 hydration. Furthermore, we observed stable results even at a minimal concentration of BBYs, 2.6 (μg Chl)·mL^{-1}: SD for BBYs is in the range of SDs for α-CAII (cf. the inset in Fig. 5 and Fig. S5). Further, the contribution of temperature to the properties of the method seems to be negligible (for information see section 6 in SI). The linear dependence, shown in the inset of Fig. 5, which was observed even at very low concentrations of the preps (from 2.6 to 5.0 (μg Chl)·mL^{-1}), indicates a high accuracy of this method for our BBYs. Thus, considering all of the above observations, and taking into account section 3.3.2, we have concluded that the method, we have adapted from Shingles and Moroney (1997), which is new for BBYs, is sensitive and accurate for PSII samples.

3.4. *The pH profile for HCO_3^- dehydration in the BBYs*

We emphasize that the kinetics of HCO_3^- dehydration in the BBYs (Fig. 5, curve 3) is similar to that of α-CAII (Fig. 5, curve 2) and, especially, to that of the CAII published by Shingles and Moroney (1997), who had described its kinetics as an "[enzymatically] catalyzed reaction [which] follows first-order kinetics". The facts described above imply that (1) a first-order reaction (Eq. (3)) is involved in both α-CAII and in CA of PSII; and (2) the mechanism of this reaction is the same in both the cases. However, this conclusion needs further investigation by a direct search of the mechanism of CA reactions in BBYs. The study of the dependence of HCO_3^- dehydration on pH in BBYs is the first step to understand the mechanism of CA activity in PSII.

The dependence of activity on pH is an important characteristic of many enzymes, including the CAs, the working of which is accompanied by the release/uptake of H^+s. The CA activity of PSII preps and of BBYs has been determined at specific pH values, but by different methods, using different PSII samples, not just one (we will discuss this further in section 3.6 and in section 4.1). It is obvious that it is incorrect to compare CA activities observed in that works, since they may have different sensitivity to changes in CO_2 (HCO_3^-) concentration. To obtain the correct pH profile of CA activity in BBY preps, it is imperative that we must use the same method for measurements at different pHs. Here, for the first time, this was done by the modified approach of Shingles and Moroney (1997) using HCO_3^- dehydration (Fig. 6).

The obtained pH profile can be approximated by a simple titration curve with a pK_a near 6.75, and a maximal activity of ~323 W-A un.·(mg Chl)$^{-1}$) at pH above 7.25. At pH below 6.0, the activity was found to be minimal (~136 W-A un.·(mg Chl)$^{-1}$), which is 70% of that measured at pH 6.5. The HCO_3^- dehydration of CAs is known to decrease as the pH is increased from 6.0 to 7.5 (Kernohan, 1965; Steiner et al., 1975). In contrast, the activity of the BBYs increases in this pH range (Fig. 6). Therefore, we conclude that the BBYs have a unique pH dependence for HCO_3^- dehydration. In addition, this unique property further confirms

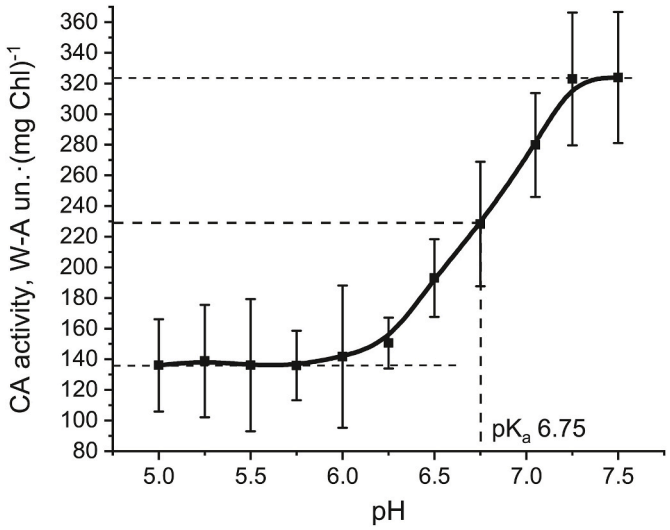

Fig. 6. Dependence of HCO_3^- dehydration activity (measured as carbonic anhydrase activity in W-A un.·(mg Chl)$^{-1}$) of the BBY preps on pH (from 5.0 to 7.5). The indicated pHs refer to the pH in buffer B (for further information, see section 2.5.2 under "MATERIALS AND METHODS"). The concentration of the substrate (HCO_3^-) was 4 mM, and the final concentration of BBY particles, in terms of chlorophyll, was 4.2 (μg Chl)·mL^{-1}. Each point is an average of at least 10 separate experiments with calculated SD.

that our preps are free of other known CAs, having a different pH profile for its activity.

3.5. The absolute value of the CA activity and the most appropriate method for measuring it in the BBYs

Comparison of pyranine method with MIMS, electrometric and radiotracer methods. Only three methods have been previously used to measure HCO_3^- dehydration in PSII samples: (i) membrane inlet mass spectrometry (McConnell et al., 2007), (ii) the electrometric method (Moskvin et al., 2004), and (iii) the radiotracer method (Lu et al., 2005). We note that k_{cat} calculated here for the α-CAII (Table 2, column 1) is ~5 times higher than that determined by MIMS in the paper by McConnell et al. (2007), which indicates the higher absolute value of the rate of HCO_3^- dehydration measured by our adapted method compared to the MIMS. The rate of HCO_3^- dehydration in PSII preps has never been directly compared with that of the CAII using electrometric and radiotracer methods. Which method gives the maximal value of CA activity? Moskvin et al. (2004) reported that the HCO_3^- dehydration in PSII preps was ~33.3 μmol H^+·(mg^{-1} of Chl)·min^{-1} using the electrometric method at pH 6.7. In contrast, the BBYs using the pyranine method-gave us a value of 4070 μmol H^+·(mg^{-1} of Chl)·min^{-1} at pH 6.7, which is higher than in the work by Moskvin et al. (2004). [Note that this value was calculated based on the fact that 21.1 W-A un.·(mg Chl)$^{-1}$ corresponds to a value of 390 μmol H^+·(mg^{-1} of Chl)·min^{-1} (cf. section 3.2), and that at pH 6.7 the activity was 220 W-A un.·(mg Chl)$^{-1}$ (as shown in Fig. 6)]. Thus, the activity measured by the adapted pyranine method is 123 times higher than that measured by the electrometric method. Further, Lu et al. (2005) have found that the HCO_3^- dehydration activity of PSII preps is ~23.5 nmol $^{14}CO_2$·(mg^{-1} of Chl) by the radiotracer method at pH 5.5. We note that this rate was obtained at 12 μM added $H^{14}CO_3^-$, and at higher concentrations of HCO_3^- we expect the rate of HCO_3^- dehydration to be higher. However, this assumption requires further study. On the other hand, BBYs, used in this research, have the activity of 140 W-A un.·(mg Chl)$^{-1}$) (see Fig. 6) at pH 5.5, which is 2590 μmol H^+·(mg^{-1} of Chl)·min^{-1}; this is ~5 orders of magnitude higher than that measured by the radiotracer method. Therefore, the value of CA activity obtained by different methods is rated as follows: the best is the method of Shingles & Moroney, as adapted here; then is MIMS, which is followed by the electrometric method; and the last is the radiotracer method. Thus, the adapted Shingles & Moroney method gives a higher value of CA activity of PSII samples compared to all the other methods used to date.

The absolute value of the CA activity in BBYs. It is obvious that the highest activity of BBYs is obtained when there is the lowest difference in the activity between the BBYs and the α-CAII. We have used, in this paper, bovine α-CAII as a reference since (1) its activity has been measured by almost all the methods designed for the determination of CA activity; (2) bovine α-CAII is one of the most active enzymes among all the known CAs, implying that its CA activity must be higher than that of the PSII samples in all the methods used. To appropriately compare the HCO_3^- dehydration activity of BBYs with that of the CAII (Fig. 5), we used equivalent concentrations of CAII and of BBYs: 1.725·10^{-8} M (0.5 μg mL^{-1}) for the CAII, as was described by Shingles and Moroney (1997); and, ~2·10^{-8} M (corresponding to 4.2 (μg Chl)·mL^{-1}) for the BBY, which was equal to that of the RCII in these BBYs). It is obvious that we must compare all the previous results with ours, obtained in the same pH. Here, the pH profile of HCO_3^- dehydration in BBYs (see Fig. 6) allowed us to provide the correct comparison, for the first time. At pH 6.5, the HCO_3^- dehydration of CA in the BBYs ((3.6 ± 1.1)·10^{10} W-A un.·(mole RCII)$^{-1}$ (Table 1, line 11)) was ~16 times lower than that of the CAII (see section 3.3.2). This difference in activity between the CAII and the BBYs is much lower than that obtained by MIMS (where, this difference was found to be ~82 times, see McConnell et al. (2007)). Therefore, all of the above shows that the method of Shingles and Moroney (1997), for the BBYs, as adapted in this work, is capable of measuring higher absolute value of CA activity than does the MIMS method.

Since the modified pyranine method gives the higher activity than the MIMS both in CAII and in BBYs, we conclude that the high CA activity (up to 324 W-A un.·(mg Chl)$^{-1}$ or up to 6.1·10^{10} W-A un.·(mole RCII)$^{-1}$) seems to be the actual activity of BBYs.

3.6. An interconnection between CA and photosynthetic activities at pH 6.5

Considering the connection between the CO_2 hydration activity and O_2 evolution, described by Shitov et al. (2011, 2018), we have tested if the correlation between the values of these activities actually exists by examining the results presented in Table 1 (see lines 1 or 2 and lines 6 or 7, respectively). We observe only a weak correlation, but this is because the O_2 evolution was measured at pH 6.5, while the CO_2 hydration was measured at pH 8.0–8.3. Obviously, we must compare CA and photosynthetic activities only at the same pH – pH 6.5, which is optimal for O_2 evolution. However, this had not been done before because there was no method that had been experimentally proven to correctly measure the CA activity of PSII at pH 6.5. The CA activity was measured at the following other pHs by different methods: the hydration activity was measured at pH 8.0–8.3 by the radiotracer method (Lu and Stemler, 2002) and then by the electrometric method (Khristin et al., 2004; Ignatova et al., 2006, 2011; Rudenko et al., 2007; Shitov et al., 2009, 2011, 2018; Fedorchuk et al., 2014), or at pH 7.5 by MIMS (both the hydration and the dehydration (McConnell et al., 2007)), the dehydration activity at pH 5.5 by the radiotracer method (Lu and Stemler, 2002) and at pH 6.8 by the electrometric method (Moskvin et al., 2004). The high sensitivity of the adapted method for BBYs (see section 3.3.4), at pH 6.5, showing the high CA activity (see the above section), has allowed us to correctly compare O_2 evolution (Table 1, line 7) and HCO_3^- dehydration (Table 1, line 11) at the same pH (6.5), for the first time (also, see Fig. 7).

Fig. 7 clearly shows a direct (strong and positive) correlation between these activities in the BBYs. Considering the nonlinear character of the curves in Fig. 7, the Spearman correlation analysis was made. The correlation coefficient was found to be 0.94 (p-value being 5.3·10^{-10}),

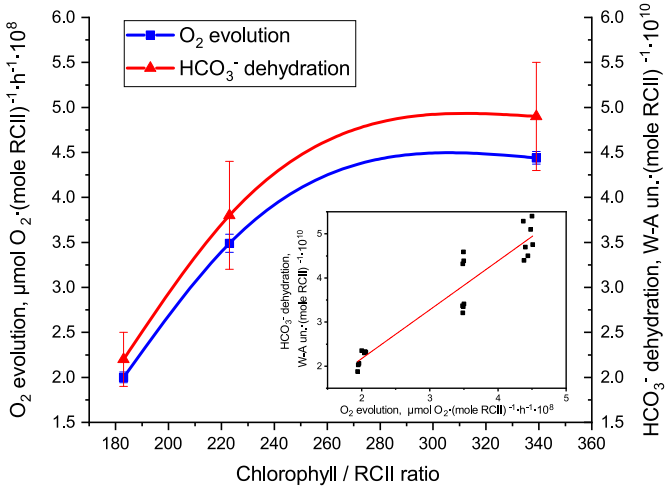

Fig. 7. Rate of oxygen evolution as a function of chlorophyll concentration showing an interconnection of the HCO_3^- dehydration CA activity with the photosynthetic O_2 evolution activity at pH 6.5. Data plotted from the values in Table 1. Different BBY preparations, obtained in March, May, and October 2018, were used. (■) shows the dependence of O_2 evolution on the ratio of chlorophyll/RCII in different BBY preps; (▲) shows the dependence of HCO_3^- dehydration on the ratio of chlorophyll/RCII in the same preps. The procedures to measure both the activities and the ratio of chlorophyll/RCII are described under "MATERIALS AND METHODS". When measuring HCO_3^- dehydration, the concentration of bicarbonate was 4 mM; the final concentration of BBYs had 4.2 (μg Chl)·mL^{-1}. Each point is an average of at least 7 separate experiments, with calculated SD. **The inset** (bottom right) shows the correlation plot of O_2 evolution vs. HCO_3^- dehydration. The Pearson correlation coefficient was found to be 0.95 (p-value was $9.9{\cdot}10^{-11}$). To draw the correlation plot, we used seven separate measurements of O_2 evolution and HCO_3^- dehydration for each BBY preparation.

which clearly indicates a strong and positive correlation between photosynthetic O_2 evolution and HCO_3^- dehydration activities. We suppose that this relationship was not previously clearly seen because non-optimal conditions for PSII had been used. Thus, at pH 6.5, which is optimal for photosynthetic reactions in PSII, we show a clear connection between CA and O_2 evolution activities in BBYs. This connection indicates an important role of CA activity in PSII function.

We now ask: on which side of PSII is this interconnection between the CA activity [bicarbonate] and the oxygen evolution? We show, in Fig. S14, that 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) has no effect on the fluorescence of pyranine as well as on the rates of HCO_3^- dehydration, and this is so for both the BBY catalyzed and the non-catalyzed reaction. Since DCMU only blocks electron transfer on the electron acceptor side of PSII (Wydrzynski and Govindjee, 1975; Blubaugh and Govindjee, 1984; Klimov et al., 1995a), we propose that HCO_3^- dehydration proceeds on the electron donor side of PSII, which is not blocked by DCMU.

We now return to a discussion about the presence of other CAs in our preps. Since the presence of other CAs has been excluded in these BBYs (see section 3.2), we assume that the difference in HCO_3^- dehydration activity of different BBY preps (Table 1, line 11), may be only due to different O_2 evolution activities of these preps (Fig. 7).

In summary, we have demonstrated, in this paper, high (up to 324 W-A un.·(mg Chl)$^{-1}$) CA activity in BBYs, prepared from the leaves of *Pisum sativum*. The unique properties of this activity (see sections 3.2 and 3.4) indicate that we have observed the own CA activity of BBYs, which is associated with its proteins, as a second function of some of them. Considering that this high activity correlates with the photosynthetic activity of PSII (see Fig. 7), we conclude that CA activity indeed plays an important role in the mechanism of PSII function.

4. DISCUSSION

The work, presented in this paper, is the first successful attempt to find whether CA activity in PSII from higher plants is high enough to be of any significance. By analyzing different approaches to measure CA activity used before – not only for the PSIIs, but also for other CAs, we have found the method, which is new for BBYs, and has allowed us to discover the high CA activity (HCO_3^- dehydration) in our preps (see section 3.5 and Fig. 5). Moreover, we suggest that, under specific conditions (e.g., pH 7.5), the absolute value of the CA activity in the BBYs may be closer to the activity of the known highly active CAs. We note that the pH profiles of the highly active CAs, from chloroplasts, have rarely been measured before, including one of the β-CAs (Johansson and Forsman, 1993), for which the pH profile has been investigated only to a limited extent. Therefore, we compare the pH profile of the BBYs with those of the known α-CAs, although unrelated to the thylakoid CAs. Given that the MIMS study was conducted at pH 7.5 using both BBYs and the CAII (see McConnell et al., 2007) and that at this pH the activity of BBYs is maximal (Fig. 6), it is of interest to ascertain the difference between the BBY and the CAII activities at pH 7.5. Kernohan (1964) reported that when the pH is raised from 6.5 to 7.5, the activity of the CAII decreases by a factor of four; from this, we expect that the activity of the CAII at pH 7.5 would be $1.5{\cdot}10^{11}$ W-A un.·(mole protein)$^{-1}$. On the other hand, the activity of BBYs was found to increase by a factor of 1.7 when the pH was increased from 6.5 to 7.5 (see Fig. 6), i.e., the activity would be $6.1{\cdot}10^{10}$ W-A un.·(mole RCII)$^{-1}$. From this, we conclude that in the current work, the difference in the activity between the CAII and the BBYs at pH 7.5 is approximately 2.3 times; this is in contrast with those by others (e.g., ~82 times in McConnell et al.). In other words, at pH 7.5, the CA activity of BBYs seems to be very close to the activity of the highly active α-CAII from bovine erythrocytes. Further, this high CA activity is shown here to be correlated with photosynthetic O_2 evolution at pH 6.5 (the condition for optimal O_2 evolution), see Fig. 7. This result is a critical and crucial improvement over the earlier research, as it illustrates the importance of CA activity for the function of PSII, including oxygen evolution, under optimal conditions.

The results obtained here, in this paper, may be applicable to the research of other CAs, since we have presented evidence that the measurement system using pyranine and 5 mM HEPES-buffer is applicable to the study of catalytic properties of highly active CAII (see section 3.3.2). The working pH range for this system (5.0–7.5, see Fig. 6), obtained in the present study, may also be useful for the investigation of other CAs.

In view of the prospects for further use of this method and the possibility of improving it, it would be interesting to know the reasons for the constant rate of HCO_3^- dehydration at pH 5.0–5.75 and at pH 7.25–7.5 (Fig. 6). We propose that the reason may be the buffering capacity of HEPES as well as the chemical properties of the dye used (fluorescence of which depends on pH). Remarkably, the HEPES-buffer used in our work, with a pK_a of 3.0 and 7.5, has maxima of buffering capacity at pHs close to 3.0 and to 7.5, whereas the buffering capacity is expected to be lower at pHs from ~4.5 to ~6.8 (considering the titration curve for HEPES). We suggest that the lower buffering capacity allows us to observe the change in the HCO_3^- dehydration rate at pH from 5.75 to 7.25, whereas the higher buffering capacity (at pH below 5.0 and above 7.5) seems to be the reason for the unchanged rate. We speculate that the chemical properties of HEPES and of the dye, not the properties of the BBYs or the CAs, determine the useful pH range in our experiments, which indicate the suitability of this measurement system for other CAs (for additional information about the suitability of the used method for other CAs, see section 7 in SI).

4.1. *The comprehensive approach to study the CA activity in the BBYs*

Previous studies had shown (by different approaches) the existence of CA activity in PSII and, thus, emphasizing the need for further research. However, the earlier investigations were incomplete because

of the following.

(i) The PSII preps and BBYs, that were used, were obtained by different methods; for further information see: Hillier et al. (2006); Ignatova et al. (2011); Lu et al. (2005); Moskvin et al. (2004); Pronina et al. (2002); Rudenko et al. (2007); Shitov et al. (2009); and Stemler (1986); these different methods may have led to different composition of proteins in the preps. Meanwhile, often the composition of the samples was not fully characterized by SDS-PAGE (in particular, in the papers by: Stemler (1986); Pronina et al. (2002); Moskvin et al. (2004); Hillier et al. (2006); Ignatova et al. (2006, 2011); Lu and Stemler (2007); McConnell et al. (2007); Rudenko et al. (2007)); this led us to question whether the preps were free of other CAs; also see a review by Bricker and Frankel (2011). Some of the above papers have presented variations (standard deviations of the measurements in per cents) in CA activity of the same preparation: up to 125% in the research by Ignatova et al. (2006) and by Rudenko et al. (2007) in peas; up to 80% in papers by Ignatova et al. (2011, 2019) in Arabidopsis; and one order of magnitude in the study by McConnell et al. (2007) in spinach. These relatively high variations may additionally indicate the presence of other CAs in those preps. We note that in five cases showing high variation in CA activity, PSII preparations and BBY were washed only once to eliminate Triton X-100. In the current work, we have slightly modified the method of Schiller and Dau (2000), by using 5 washings from the detergent (for details, see section 2.2), to isolate preps having relatively lower variations, up to 36% (see Table 1), in the CA activity. This is an indication of the absence of other CAs in the preps. Further, comprehensive characterization of the obtained BBYs, including SDS-PAGE, showing identical composition of proteins (Figs. 2), and 77 K fluorescence spectra, showing insignificant presence of PSI in our BBYs, as well as an inhibitor analysis, have additionally indicated the absence of other CAs in our preps (see section 3.2). Therefore, we have established here that the slightly modified protocol from that of Schiller and Dau (2000) provides BBYs, which are free of other CAs. This finding may be helpful for future investigations of CA activity in BBYs.

(ii) Lu and Stemler (2002, 2007), and Lu et al. (2005) have presented CA activities in specific units, which does not allow us to compare directly these activities with those obtained by others in PSII preps and in BBYs or in highly active CAs. Here, we have presented the CA activity of BBYs by different units, which would allow others to compare our results with those in previous studies not only on different PSII preps and BBYs, but also on other CAs.

We suggest that the incomplete research in previous studies may have resulted in erroneous conclusions. For example, McConnell et al. (2007) had stated that CA activity is not important for PSII function. This statement is erroneous because of the following: (i) McConnell et al. (2007) had used pH 7.5, which is not optimal for O_2 evolution activity; on the other hand, the optimal conditions for O_2 evolution (pH 6.5), using other pH for CA activity (pH 8.0–8.3), in research by Shitov et al. (2011, 2018), had revealed only weak correlation between CA and photosynthetic activities. However, as we have found here that to observe a clear correlation between these two activities, we must use the same pH for both activities and its value (pH 6.5) must be optimal for photosynthetic activity. (ii) The authors of earlier research had used unsuitable methods to measure CA activity in PSII, as stated in section 3.5, which prevented them to measure actual CA activities in the PSII preps and in the BBYs. And only here, in this paper, when we employed the pyranine method, the high absolute value of CA activity allowed us to observe clear correlation between the two activities. (iii) In most cases, the heterogeneity of PSII samples, in terms of the number of Chls per RCII, was not considered; further, the CA activity was provided only per mg of Chl or per mg of protein (the exception is the work of McConnell et al. (2007) and of Shitov et al. (2009)). Lastly, the calculation of activities per mole of RCII, rather than per mg of Chl, has played an important role in finding the correlation between the CA and the photosynthetic activity in our work.

Thus, by the comprehensive approach, used in this work, we have overcome all the disadvantages observed in earlier research. High CA activity of PSII (see section 3.5), which is important for photosynthetic O_2 evolution in this complex (see section 3.6), is expected to prompt further studies on the role of HCO_3^- and of high CA activity in the mechanism of PSII function, particularly on the water oxidation side.

4.2. Possible mechanism of the connection between CA and photosynthetic activities

A model describing the relationship between CA and O_2 evolution activities has not yet been proposed, since this relationship had previously been questioned in the papers of McConnell et al. (2007) and of Bricker and Frankel (2011). Given the importance of CA activity in the function of PSII, we now propose a model that elucidates the correlation between the CA and the O_2 evolution activities (see Fig. 8).

A pH of 6.5, which provides optimal conditions for photosynthetic O_2 evolution (Schiller and Dau, 2000), seems to be the key condition to observe the clear relationship between CA and photosynthetic activities (see above). This means that pH, i.e. the concentration of H^+s, may be crucial for both the CA activity and the O_2 evolution. We note that the rise (1.36 times, see Fig. 6) of HCO_3^- dehydration rate is very similar to the rise of O_2 evolution rate (1.25 times, see: Schiller and Dau (2000)) when the pH is increased from 6.0 to 6.5. It is known that the dependence of O_2 evolution on pH can be related to the function of Cl^- ions on the electron donor side of PSII (Commet et al., 2012). This similar increase in these activities may indicate that **(i)** both these activities may be located on the electron donor side of PSII since the effect of pH on O_2 evolution has been proposed to be associated with the electron donor side, i.e., the OEC (Oxygen Evolving Complex) side of PSII (Commet et al., 2012); and **(ii)** that there is a common event affecting both the activities.

We now ask: which process could affect CA and photosynthetic activity in the same way? We note that the pK_a for HCO_3^- dehydration in BBYs (6.75) is very similar (~7.0) to that of α-CAs (Kernohan, 1965; Steiner et al., 1975; Silverman and Lindskog, 1988), as well as to that of activation of O_2 evolution (close to 6.5), as mentioned by Commet et al. (2012). Obviously, the pK_a would indicate certain physicochemical properties of the amino acid side groups of the proteins, which are directly involved in the catalysis events. In the CAs, some side amino groups, usually His (having pK_a from 6.0 to 7.0), have been suggested to be the most important one for H^+ transfer from the metal-containing active center to the bulk solution (and *vice versa*), which is the rate-limiting step of catalysis (for further information, see Fig. 5 in Silverman and Lindskog (1988) and Fig. 8 in this paper). Similarly, in PSII, a pK_a of 6.5 for the activation of O_2 evolution has been suggested to indicate deprotonation of one or more amino acid residues involved in the transfer of H^+s from the Mn_4CaO_5-cluster to the lumen (Commet et al., 2012), which is also the rate-limiting step in O_2 evolution (Shutova et al., 2008). His 92(D1) and His 337(D1), located near the Mn_4CaO_5-cluster, have been proposed to be involved in the transfer of H^+s playing a crucial role in the O_2 evolution process (for details see Fig. 1 in Commet et al. (2012)). Here, we propose that the CA reaction in PSII may also involve His 92(D1) and His 337(D1) and/or other His residues. This idea is supported by the following: **(i)** the pK_as for CA activities in BBYs and in α-CAs, as well as for the activation of O_2 evolution in PSII are in the range from 6.5 to 7.0, which is within the pK_a range characteristic of His bound to other proteins (Commet et al., 2012); and **(ii)** His residues are known to play a key function in H^+ transfer from the active center to the bulk solution in both α-CAs (Silverman and Lindskog, 1988) as well as in photosynthetic O_2

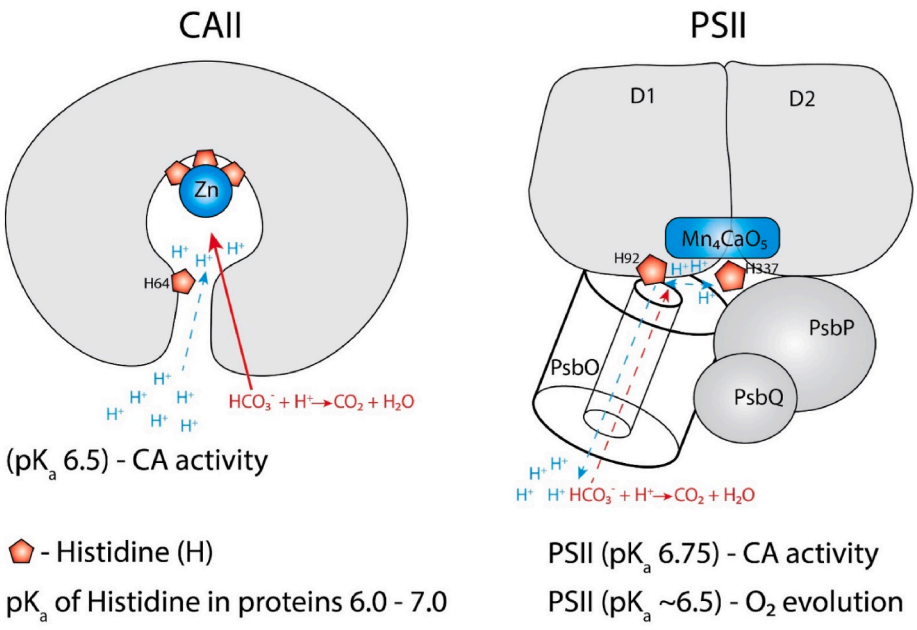

Fig. 8. A scheme for comparison of CA activity in Zn-CAII with that in PSII. It shows the connection between pK_a for CA and O_2 evolution activities in PSII, considering the close value of pK_a for CA activity in α-CAs and, considering the key role of histidine residues in reactions of H^+ transfer. Left: A diagram that schematically shows the active center of α-CAs, and the transfer of H^+s via His64 during the HCO_3^- dehydration catalysis (see the red arrow). **Right:** A diagram that schematically shows the WOC of PSII from higher plants, which includes a possible way of H^+ transfer, involving His92 and His337 of the D1 protein. We suggest that the transfer of H^+s may occur both near the Mn_4CaO_5-cluster and through the PsbO protein during photosynthetic O_2 evolution. The red pentagons indicate the approximate locations of His residues in α-CAs and in PSII complex. In the CAII, Zn is the catalytic center of α-CAs, at which CA reactions occur; it is coordinated by 3 His residues. In PSII, D1 and D2 are the reaction center proteins; PsbO (33 kDa), PsbP (23 kDa) and PsbQ (16 kDa) are extrinsic proteins that stabilize the water-oxidizing complex and optimize its function. Mn_4CaO_5 is the manganese-oxygen-calcium cluster. The light-blue dashed arrows show the H^+ movement, which is regulated by His residues. Red dashed arrow indicates the assumed movement of HCO_3^- ions, which may move from the lumen to the Mn_4CaO_5-cluster through the PsbO protein. Lastly, the tip of the solid red arrow marks the site of the HCO_3^- dehydration in the CAII; tips of dashed arrows (on the Right) mark two possible sites of the HCO_3^- dehydration in PSII (for further information see "DISCUSSION", section 4.2).

evolution in PSII (Commet et al., 2012). Considering the possible involvement of His residues in HCO_3^- dehydration, we conclude that the removal of H^+s from the Mn_4CaO_5-cluster to the lumen is the common event affecting both CA and photosynthetic activity in PSII (see Fig. 8).

We propose that the O_2 evolution, considering the involvement in it of the HCO_3^- dehydration, may be described by the following biochemical processes:

1) Water oxidation

$$2H_2O \rightarrow O_2 + 4\,H^+ \qquad \text{(Eq. 7)}$$

2) Transfer of H^+s released (possible ways of the transfer of H^+s are shown in Fig. 8); and,
3) "Capturing" of H^+s by HCO_3^- dehydration: $H^+ + HCO_3^- \rightarrow CO_2 + H_2O$ (Eq. (3)).

To remove H^+s released during water oxidation, they should be transferred to the active center of CA in PSII, where they will be captured by HCO_3^- dehydration. The accumulation of protons near the Mn_4CaO_5-cluster is known to inhibit photosynthetic water oxidation (Shutova et al., 2008), i.e., slowing down of the second process (see above) which would lead to the inhibition of the first process. It is obvious to us that the slowing down of the H^+ transfer will also slow down the rate of H^+ capture in the third process, due to the lack of the substrate. Therefore, a high rate of the second process is crucial for both the first reaction and the third. In nature, the second process is integral and the rate limiting part of the catalytic mechanism in all CAs, and this determines the overall rate of the reaction; thus, the last two processes can be considered as one common stage. Therefore, catalyzing the transfer of H^+s, i.e. its removal from the Mn_4CaO_5-cluster, CA in PSII supports, in our view, photosynthetic O_2 evolution. The proposed sites of the third process are marked by the tips of the dashed arrows in Fig. 8 (on the right side). Further, we note that the third reaction is reversible; however, it proceeds in the direction shown in Eq. (3), which is due to the excess of H^+s being constantly released during water oxidation.

It is well known that effective water oxidation (as measured by photosynthetic O_2 evolution) has an impact on photosynthetic productivity. Given the direct correlation between O_2 evolution and HCO_3^- dehydration (Fig. 7), and considering that O_2 evolution has an impact on plant productivity, we suggest that the HCO_3^- dehydration plays an important role in the overall productivity of plants in the same way as the water oxidation does. This means that when investigating the conditions for maximum water oxidation efficiency, one should also consider whether the conditions found are also optimal for CA activity, i.e. the factors that affect CA activity will also affect O_2 evolution and productivity. Hence, the main question is: to what extent? Finding the kinetic parameters of HCO_3^- dehydration (especially k_{cat}) and comparison of k_{cat} with that for photosynthetic O_2 evolution is expected to provide the answer to this question by providing an insight into the bottleneck of the above processes. A smaller k_{cat} would indicate the limiting process. The knowledge of the rate-limiting step has already led to predict how these two reactions may be affected by changes in environmental conditions (for example, the change of pH near the PSII). We assume that the change in pH affect these two biochemical reactions in two ways: (1) If the water oxidation is the rate limiting step of the whole process, then this whole process should be pH dependent in accordance with the pH dependence of O_2 evolution (see Schiller and Dau (2000)). (2) If the transfer of H^+s is rate limiting, then the whole process should depend on the pH just as HCO_3^- dehydration does (see Fig. 6). Even if the water oxidation will be found to be the limiting

factor, dehydration activity will still be an important part of the overall productivity, as inhibitors of CA activity and other inhibitory conditions (e.g. temperature) can make this activity limiting (making the rate constant of the HCO_3^- dehydration lower than that of water oxidation). If HCO_3^- dehydration turns out to be the rate-limiting step, it will be even more important than water oxidation for plant productivity.

Another question is: which of the known factors suppress PSII by inhibiting the activity of CA and at the same time reduce productivity and inhibit O_2evolution? This is an issue that needs to be addressed. It is therefore important to identify the inhibitors of CA activity (both natural and artificial) in order to prevent the associated loss of productivity. It is also important to look for the specific activators of HCO_3^- dehydration or conditions that favor its high rate. If HCO_3^- dehydration is the rate-limiting, the search for inhibitors and activators of CA activity is even more relevant for future studies.

Our preliminary data (forthcoming) seem to indicate that the rate of the HCO_3^- dehydration in BBYs is three times higher than the rate of proton release generated during photosynthetic water oxidation. When finalized, it would suggest that water oxidation is the limiting step of the whole process. But these data need to be further verified.

It is known that CAs can enhance photosynthesis in PSII (Kandoi et al., 2022). The similar function of CA, CAH3, has been proposed for photosynthetic reactions in PSII samples from *Chlamydomonas reinhardtii* (for a review, see Terentyev and Shukshina (2024)). CAH3 is the only CA that has been found to be associated directly with PSII in *Chlamydomonas*. This CA supports photosynthetic O_2 evolution (Shutova et al., 2008; Terentyev et al., 2019) by the acceleration of H^+ removal (also due to chemical equilibrium, as shown in Eq. (1)) from water oxidizing complex (also referred to as OEC). However, to verify this idea, both in *Chlamydomonas* and in higher plants, we still need to determine the mechanism of the CA reaction and its kinetic parameters in the BBYs, which is in our plan for future studies.

In addition, the site of HCO_3^- dehydration is not yet clear. However, in Fig. 8 we suggest two possible sites: one near the Mn_4CaO_5-cluster, and the other on or near the lumenal surface of the PsbO protein.

The similarity of pK_a for HCO_3^- dehydration and for O_2 evolution may indicate that both these activities are associated with the Mn_4CaO_5-cluster, i.e., inside the WOC, since His 92(D1) and His 337(D1), which play crucial roles in water oxidation, are located near this cluster. This assumption is also supported by the recovery of the electron transfer rate being suppressed by sulfonamide inhibitors of CAs upon the addition of the artificial electron donors ferrocyanide and N,N,N′,N′-tetramethyl-p-phenylenediamine (Shitov et al., 2011, 2018). There is further evidence to suggest that the active center of CA is located within the PSII complex on its electron donor side. Removal of WOC proteins by salt treatment has been shown to increase the sensitivity of the CA activity of BBYs to the hydrophilic sulfonamide inhibitor AA (Shitov et al., 2009), suggesting that the WOC proteins act as barriers to the entry of hydrophilic sulfonamides into the active center of CA in PSII. Thus, based on a number of experiments, we conclude that HCO_3^- dehydration may, indeed, be associated with the Mn_4CaO_5-cluster.

On the other hand, we cannot exclude that HCO_3^- dehydration may also occur on the lumen-facing surface of the WOC proteins. We note that PsbO, PsbP and PsbQ have been found to exhibit CA activity (Lu et al., 2005); Lu and Stemler, 2007; Shitov et al., 2009)). Also, CA activity may occur even in the lumen, which is supported by the presence of β-CA in the lumenal space (Fedorchuk et al., 2014)). It is obvious that the site of this reaction needs further investigation.

Furthermore, it is known that oxygen evolution requires the functioning not only of the electron donor side of PSII, but also of the electron acceptor side (Schiller and Dau, 2000; Shevela et al., 2023). Extensive studies in several laboratories have shown that HCO_3^- is involved in electron transfer on the electron acceptor side of PS II from the reduced Q_A to Q_B, facilitating the protonation of Q_B (Govindjee et al., 1976; Khanna et al., 1981; Blubaugh and Govindjee, 1984, 1986, 1988; Eaton-Rye and Govindjee, 1988a,b). Moreover, the processes involving HCO_3^- on this side were found to be a "bottleneck" in the overall electron transfer through the PSII (Eaton-Rye and Govindjee, 1988b; Shitov, 2022). Considering HCO_3^- as a crucial factor for the fast electron transfer on the electron acceptor side, we propose that the catalyzed interconversions of inorganic carbon forms (Eq. (1)), i.e. the CA activity, may also be crucial for the function of bicarbonate on the Q_A Q_B side. If the fast electron flow through the acceptor side provides high rates of water oxidation, then the CA activity on the acceptor side may also affect O_2 evolution. Therefore, we cannot exclude that the observed correlation between HCO_3^- dehydration and O_2 evolution (Fig. 7) may be indirectly or even directly affected by the electron acceptor side. However, the CA activity on the Q_A Q_B side has not been studied, which is an interesting topic for further research.

5. Conclusions and future perspective

In this paper, by using pyranine fluorescence, we have presented a sensitive, accurate and simple method for the measurement of HCO_3^- dehydration activity in BBYs; this method is new for the BBYs and is much more appropriate than the others used earlier. We note that this method can be used for the study of CA activity in a wide pH range, from 5.0 to 7.5, not only for the PSII samples but also for other CAs. Using this method, a high HCO_3^- dehydration activity of PSII has been demonstrated in this paper. Further, the observed correlation between CA and photosynthetic activities at pH 6.5 (Fig. 7) indicates the importance of CA activity for the PSII functions (on its electron donor side).

The knowledge of how CA and photosynthetic activities are connected in PSII is important; it will, in the future, significantly complement our understanding of the mechanism of PSII function in general. The high CA activity of BBYs at pH 6.5, when the rates of photosynthetic reactions were maximal, raises the question: How fast is the HCO_3^- dehydration in comparison with the rate of photosynthetic water oxidation? To answer this question, we should, in the future, investigate the kinetic parameters of HCO_3^- dehydration in PSII in order to compare k_{cat} for HCO_3^- dehydration with that for O_2 evolution (for the rate of H^+ release). The knowledge of this slower process is expected to reveal the "bottle neck" of the resulting process, which, in turn, will make it possible to establish the actual order of reactions in the mechanism of water oxidation, where the CA activity is involved. Further, the elucidation of the mechanism of the HCO_3^- dehydration will reveal how it fits into the current picture of the overall mechanism of water oxidation, and, further, it will also allow us to determine the precise mechanism of bicarbonate function on the electron donor side of PSII. The last aspect remains to be answered; for some novel but unproven ideas, see e.g., Stemler and Castelfranco (2023) as well as a review by Swain et al. (2023). In addition, available ideas about the bicarbonate function in the PSII have been presented in a minireview by Vinyard and Govindjee (2024). Given the direct connection of CA activity with O_2 evolution and, thus, with plant productivity, it is important to search for inhibitors and activators of CA activity in PSII. In addition, the search for the CA activity of the PSII components (including isolated separate proteins) is expected to open a new page in the understanding of where precisely the above-mentioned reactions take place. Our current findings, presented here, make the future investigation of the CA activity of PSII possible, although it remains a challenge.

Author statement

Alexandr Shitov: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Resources, Writing - Original Draft, Visualization. **Vasily Terentyev**: Formal analysis, Investigation. **Govindjee Govindjee**: Writing - Original Draft, Writing - Review & Editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.plaphy.2025.109516.

Data availability

No data was used for the research described in the article.

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