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Effect of bicarbonate on the water-oxidizing complex of photosystem II in the super-reduced S-states

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Abstract

It is shown that the hydrazine-induced transition of the water-oxidizing complex (WOC) to super-reduced S-states depends on the presence of bicarbonate in the medium so that after a 20 min treatment of isolated spinach thylakoids with 3 mM NH_2NH_2 at 20 °C in the CO_2/HCO_3^--depleted buffer the S-state populations are: 42% of S_{-3}, 42% of S_{-2}, 16% of S_{-1} and even formal S_{-4} state is reached, while in the presence of 2 mM $NaHCO_3$, the same treatment produces 30% of S_{-3}, 38% of S_{-2}, and 32% of S_{-1} and there is no indication of the S_{-4} state. Bicarbonate requirement for the oxygen-evolving activity, very low in untreated thylakoids, considerably increases upon the transition of the WOC to the super-reduced S-states, and the requirement becomes low again when the WOC returns back to the normal S-states using pre-illumination. The results are discussed as a possible indication of ligation of bicarbonate to manganese ions within the WOC.

Keywords: S-state; Bicarbonate; Water-oxidizing complex; Photosystem II

1. Introduction

Photosynthetic oxygen evolution is an unique fundamental biological process which occurs in the water-oxidizing complex (WOC) of the multi-component pigment–protein complex called photosystem II (PSII) (for recent reviews, see Ref. [1]). The active site of the WOC of PSII is thought to be a cluster of four Mn and one Ca ions (for reviews see Refs. [2–4]). From measurements of the flash-induced oxygen evolution patterns (FIOPs) [5], it was concluded [6] that the WOC can exist in five redox levels, termed S_n-states, where S_0 is the most reduced and S_4 the most oxidized states of the Mn-containing cluster. During sequential absorption of photons and charge separation in PSII the WOC undergoes four one-electron oxidation steps, $S_0 \rightarrow S_1 \rightarrow S_2 \rightarrow S_3 \rightarrow S_4$, coupled to oxidation of two H_2O molecules and release of O_2 on each fourth flash. After thorough dark-adaptation, almost all centers are in the initial 'dark-stable' S_1 state due to deactivation of all other states [7,8]. However, until recently, there is no clear agreement whether four Mn^{III} [9] or two Mn^{III} and two Mn^{IV} [10] are redox states of four manganese ions in the S_1 state. Limburg and co-workers [11] suggested that Mn^V can be involved in oxidation of water in PSII.

Besides the normal S-states of Kok cycle, the WOC can be reduced to redox states below S_0 by sample treatment with weak hydrophilic reductants such as NH_2NH_2, NH_2OH, H_2O_2 [12–14] or with such gases as H_2S [15] and $NO^{\cdot}$ [16,17]. The lowest super-reduced S-state which can be achieved is S_{-3} state that was shown using the treatment of membrane thylakoids with NH_2NH_2 [18]. However, there are first indications that S_{-4} and S_{-5} states also may exist [19]. As the transition of Mn_4Ca-cluster is approached to the lowest S_{-n} states, the fraction of Mn^{II} ions is increased [17] without leaving their binding sites [18]. On the other hand, some part of the Mn_4Ca-clasters can be deconstructed by the presence of NH_2NH_2 or NH_2OH and it is coupled with the release of Mn^{II}, and some inactivation of oxygen evolution is observed under this process [20–22]. In addition, there is also evidence that S_{-1}–S_{-2} states [23] and even S_{-3} state [24] can exist *in vivo* in the absence of exogenous reductants. At present, it is well known that S_{-n} states play important role in photoactivation [25].

Bicarbonate is well established to be required for maximal activity of PSII though the interpretation of the stimulating effect of bicarbonate remains controversial (for recent reviews, see Refs. [26–28] and references therein). In the early 1970s, the

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donor side of PSII was considered as possible acting site for bicarbonate [29,30]. However, later strong evidence for location of bicarbonate binding at the acceptor side of PSII, between Q_A and Q_B, the primary and secondary plastoquinone electron acceptors, was first presented by Wydrzynski and Govindjee [31]. This idea was supported by numerous experimental data, and the non-heme Fe acting between Q_A and Q_B has been shown to be the binding site for bicarbonate (for reviews, see Refs. [26–28] and references therein) that was clearly confirmed by recent X-ray analysis of the WOC structure [32].

Possible role of bicarbonate within the donor side of PSII has remained unclear for a long time (for reviews see Refs. [28,33]). Recently, it has been shown that bicarbonate ions are required for both maximal activity and stability of the WOC in PSII [34–37]. The stimulating effects of bicarbonate are especially pronounced during reactivation of the donor side of PSII with Mn^{II} ions added to Mn-depleted PSII preparations [38–39]. A range of suggestions concerning the possible role of bicarbonate within the WOC of PSII has been proposed (for review, see Refs. [28,40]). In particular, it has been suggested that bicarbonate can be considered as a direct ligand to the Mn_4Ca-cluster and its removal from the WOC makes Mn_4Ca-cluster unstable. In a recent publication on the X-ray structure of PSII core complex from *Synechococcus elongatus*, a bicarbonate molecule has been tentatively included as a non-protein ligand of the WOC [32]. Interesting results were obtained by studies of electrochemical and EPR characteristics of bicarbonate complexes with Mn^{II} and Mn^{III} [41–43] showing that the unique property of bicarbonate to initiate assembly of inorganic core of the WOC from apo-WOC and Mn^{II} can be explained by capability of bicarbonate (contrary to carboxylate anions, such as acetate and formate) to form electroneutral complexes with Mn^{II} and Mn^{III}. As follows from the electrochemical determination of redox potentials of Mn-bicarbonate complexes [41,42], the dissociation constant (K_d) for Mn^{III}–bicarbonate is nearly 10 orders lower than K_d for Mn^{II}–bicarbonate complexes.

However, in functionally active S_1–thylakoids or PSII membrane fragments (BBY-particles), the effect of bicarbonate on the WOC is relatively low [35,40], and evidently bicarbonate has no effect on the formate-induced shift of the WOC from S_1 to S_0 state [44] (though in an earlier publication [45] an increase in the S_0/S_1 state ratio was observed in HCO_3^--depleted chloroplasts). If the bicarbonate effects are related to direct ligation of bicarbonate to Mn within the WOC, then the low effect of bicarbonate in functionally 'healthy' PSII can be explained by difficulties in removal of bicarbonate ions from the WOC since Mn of the WOC in S_1 state is in the valency Mn^{III} and/or Mn^{IV}. Consequently, upon the transition of the WOC to the super-reduced (S_{-n}) states coupled with the reduction of Mn by NH_2NH_2 or other reductants, one may expect that the dependence of functional characteristics of the WOC on the presence of bicarbonate will be much higher, since Mn^{III} and/or Mn^{IV} are mainly converted to Mn^{II}.

In this connection, the aim of present work is to investigate the effect of bicarbonate on both flash-induced oxygen evolution patterns and O_2-evolving activity under continuous illumination of thylakoids with the WOC transferred to S_{-n} states.

2. Materials and methods

Thylakoid membranes from spinach leaves were isolated as described previously [46] with some modifications (20 mM HEPES–NaOH, pH 7.8, and 35 mM NaCl were used instead of 20 mM Tricine, pH 8.0, and 2 mM $MgCl_2$) in a medium containing 400 mM sucrose, 35 mM NaCl, 1 mM EDTA, and 20 mM HEPES–NaOH (pH 7.8). After centrifugation at 5000×*g* for 20 min, the pellet was twice washed in medium containing 35 mM NaCl, 5 mM $MgCl_2$ and 20 mM Tris–HCl (pH 7.5) and diluted in to final suspension medium containing 330 mM sucrose, 35 mM NaCl, 20 mM MES–NaOH (pH 6.5) and 10% glycerol. After isolation steps the thylakoids [*c*(Chl)=2.5 mg/mL] were frozen at −85 °C until used.

Bicarbonate removal from thylakoid membranes was achieved as described earlier [34–36], by a 200-fold diluting of concentrated preparations into medium A (150 mM MES–NaOH buffer, pH 6.3, 400 mM D-Mannit, 20 mM $CaCl_2$, 10 mM $MgCl_2$) depleted of endogenous CO_2/HCO_3^- by means of 60 min flushing with air depleted of CO_2 by passage through a solution of 50% NaOH and a 20-cm layer of ascarite. The sample was subsequently incubated in this medium for 10 min at 4 °C in the dark.

The treatment of thylakoid membranes with hydrazine was done according to the method described earlier [18] with some modifications. The thylakoids were washed in medium A and then diluted to a *c*(Chl) of 900 μg Chl/mL either with medium A or with 3 mM (or 10 mM) NH_2NH_2 dissolved in medium A. The NH_2NH_2 incubation was carried out in the dark at 20 °C. Before the incubation, all samples were enriched in S_1 by one saturating flash and subsequent 20-min dark incubation. In the case of hydrazine removal from the samples, the thylakoids diluted 10-fold in medium A were washed by centrifugation.

Polarographic detection of FIOPs was made using a laboratory-built, Clark-type Pt/Ir electrode (diameter 5.5 mm) that was equipped with a special polymer membrane which was stretched to a thickness of about 1 μm [47,48]. It prevented the interaction of added electron acceptors with the polarized electrode. The samples at Chl concentration of 900 μg/mL were layered into a home-built chamber [49] with 20 μL volume and 0.3 mm thick over the membrane. The electrode was operated at a polarization voltage of 700 mV. The measurements were performed in the presence of 0.5 mM ferricyanide as electron acceptor. Before measurements were started, the samples were dark-polarized for 6 min on covered electrode at 20 °C. For saturating light flashes (duration of 10 μs), a xenon flash lamp at a repetition rate of 0.5 Hz was used [47]. The O_2 signals were amplified by a laboratory-built-low-impedance circuit and integrated over a time of 0.1–0.5 s. All measurements were repeated at least three times.

The O_2-yields of the first 13 flashes were analyzed using Excel spreadsheet program based on extended Kok model for the super-reduced S_{-1}...S_{-5} states developed by J. Messinger and essentially described earlier [17,18,50]. Obtained data were calculated by two strategies: both excluding and including the lowest formal S_{-4} and S_{-5} states of the WOC (see Results). The option for a higher double hit parameter in the first flash (β_1) was included since ferricyanide was used as electron acceptor during our measurements. The fit quality (fq) of experimental data to theoretical was calculated in accordance with previous publications [17,18].

The rate of the photosynthetic oxygen evolution was measured by monitoring the concentration of oxygen with a Clark-type oxygen electrode for 60 s after the start of continuous actinic illumination. The measurements were carried out at 20 °C in the presence of 0.1 mM 2,6-dichloro-*p*-benzoquinone (DCBQ) plus 1 mM $K_3[Fe(CN)_6]$ as electron acceptors and 10 μM gramicidin added as uncoupler. The concentration of chlorophyll was assayed in 80% acetone [51].

3. Results

3.1. Flash-induced oxygen evolution patterns

Fig. 1A (curves 1 and 2) shows the flash-induced oxygen evolution patterns (FIOPs) with typical period of four oscillations with maxima on the third and seventh flashes obtained in control dark-adapted S_1-thylakoid membranes in the medium depleted of CO_2/HCO_3^- (see Materials and methods) in the absence (curve 1) and presence (curve 2) of 2 mM $NaHCO_3$. There is no significant differences between these two FIOPs

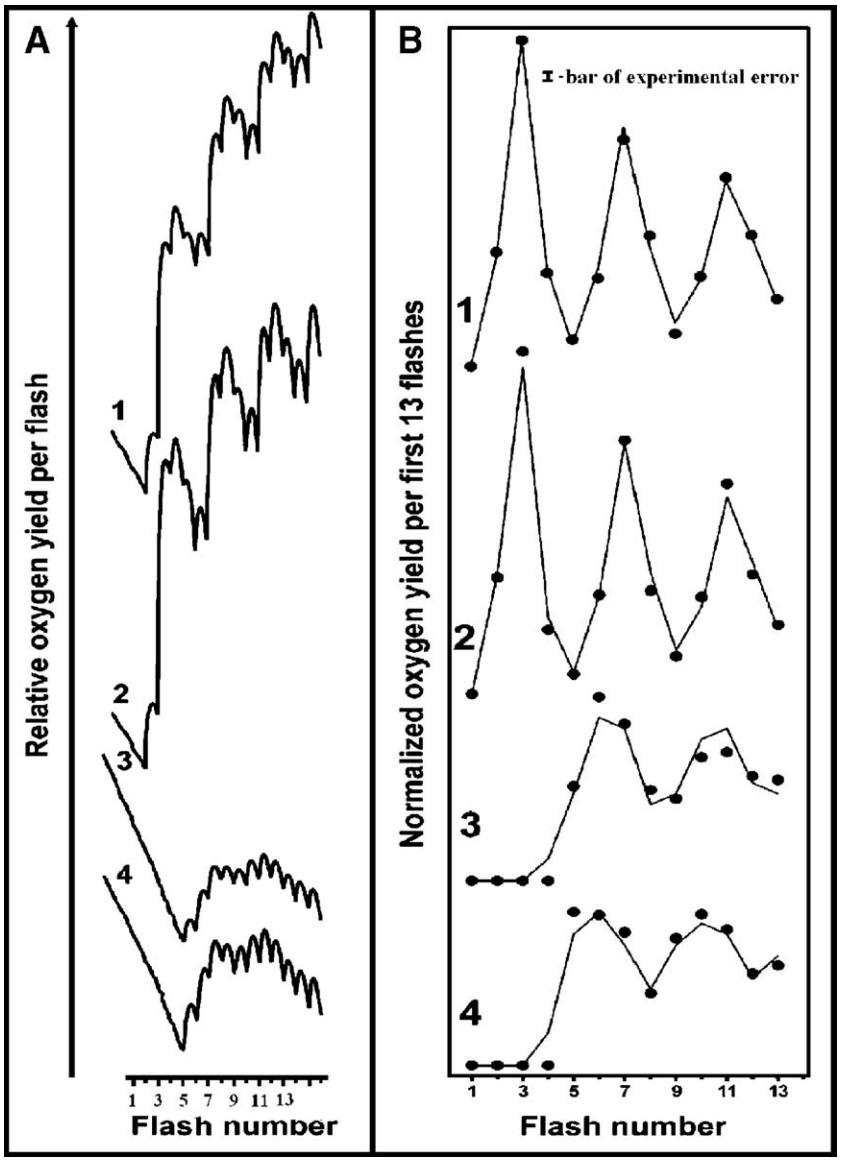

Fig. 1. Original traces of flash-induced oxygen evolution patterns (FIOPs) (A) and their calculated oxygen yields per flash (B) of dark-adapted spinach thylakoids in medium A depleted CO_2/HCO_3^-. Traces 1 and 2 were obtained in untreated (control) samples in the absence and presence of 2 mM $NaHCO_3$, respectively. While traces 3 and 4 were obtained after a 20-min treatment of the samples with 3 mM NH_2NH_2 at 20 °C in the absence and presence of 2 mM $NaHCO_3$, respectively. The measurements were done without removal of NH_2NH_2 from the treated samples. 'Calculated' curves (1–4) on B show the best fit lines in accordance with the extended Kok model (see fits A, B1 and C1 in Table 1) with misses, $\alpha = 0.07$, double hits, $\beta = 0.016$ and high double hit per first flash, $\beta_1 = 0.22$. Experimental data on B are represented by circles. Bar shows maximal possible experimental error which could be caused by noise level and it spreads on all flashes. The flash frequency was 0.5 Hz. All measurements were carried out in the presence of 0.5 mM $K_3[Fe(CN)_6]$ as electron acceptor. Chlorophyll concentration during treatment and measurements was 0.9 mg/mL.

except the amplitudes per flashes are slightly higher in the presence of bicarbonate. Rather high oxygen yield per 2nd flash is explained by the presence of ferricyanide (0.5 mM) as electron acceptor, which is known to lead to a high double hit probability (β_1) per first flash [52,53]. Visible shift of the first maximum of O_2 evolution from flash 3 to flash 6/7 that appears after a 20-min dark incubation at 20 °C of S_1–thylakoids in the presence of 3 mM NH_2NH_2 in the medium preliminary depleted of CO_2/HCO_3^- (pH 6.3) is shown in Fig. 1 (curve 3). The same NH_2NH_2-treatment in the presence of 2 mM $NaHCO_3$ leads to the shift of the first O_2 evolution maximum to flash 5/6 (curve 4).

Detailed analysis of these oscillation patterns was done by using an extended Kok model (see Materials and methods). A few approaches (data not shown) were used for computation of control oxygen oscillation patterns of dark-adapted thylakoids in order to obtain the best fit quality [17,18]. Perfect fit for obtained FIOPs in CO_2/HCO_3^--depleted thylakoids shown as fit A in Table 1 and curve 1 in Fig. 1B was achieved with the miss (α) of about 7%, double hit (β) of 1.6% and high double hit per first flash (β_1) of 22% when S_1 and S_0 state populations were allowed as free parameters. These parameters were practically unaffected by the presence of 2 mM $NaHCO_3$ during incubation (see fit a in Table 1 and curve 2 in Fig. 1B). However, some differences were observed in distribution of the initial S_1–S_0 states so that in the absence of bicarbonate the PSII centers were found in S_1 (92%) and S_0 (8%) while in the presence of 2 mM $NaHCO_3$ almost all

Table 1
Fits of oxygen oscillation patterns shown in Fig. 1

Fit	S_1 (%)	S_0 (%)	S_{-1} (%)	S_{-2} (%)	S_{-3} (%)	S_{-4} (%)	S_{-5} (%)	α (%)	β (%)	β_1 (%)	fq
a	98.0	2.0	–	–	–	–	–	6.8	1.7	21.9	0.00004
A	92.0	8.0	–	–	–	–	–	6.9	1.6	22.2	0.00003
B1	0.0	0.0	16.0	42.0	42.0	–	–	(6.9)	(1.6)	(22.2)	0.00023
B2	0.0	0.0	12.0	46.0	35.0	7.0	0.0	(6.9)	(1.6)	(22.2)	0.00027
C1	0.0	0.0	32.0	38.0	30.0	–	–	(6.9)	(1.6)	(22.2)	0.00024
C2	0.0	0.0	32.0	38.0	30.0	0.0	0.0	(6.9)	(1.6)	(22.2)	0.00032

Parameters of the extended Kok model and extensions outlined in the text were used to fit the oxygen oscillation patterns of dark-adapted spinach control thylakoids (fit A and Fig. 1B, curve 1) in medium A depleted of CO_2/HCO_3^- and hydrazine-reduced thylakoids incubated during 20 min both in the absence (fits B1–B2 and Fig. 1B, trace 3) and in the presence of 2 mM $NaHCO_3$ (fits C1–C2 and Fig. 1B, trace 4). Fit a - parameters obtained from oxygen oscillation pattern of dark-adapted spinach control thylakoids (Fig. 1B, curve 2) in medium A in the presence of 2 mM $NaHCO_3$. The parameters: S_1–S_{-5}, normalized S-states populations of the WOC; α, miss probability; β, double hit probability; β_1, high double hit probability per first flash; fq, fit quality. The first 13 flash-induced oxygen yields of each oscillation pattern have been analyzed. Numbers in parenthesis are fixed parameters given from control. –, parameters excluded from fit. The S_n-state transition probabilities α, β and β_1 were determined by a least-squares fit of the relative oxygen yields with theoretical sequence based on an extended Kok model [18].

centers were found in S_1 state (98%) and only about 2% in S_0 state (fits A and a in Table 1). Similar, but larger effect of bicarbonate on S_0/S_1-states distribution in HCO_3^--depleted chloroplasts was observed in an earlier work [45].

Two strategies were undertaken to analyze the FIOPs in NH_2NH_2-treated samples shown in Fig. 1A (curves 3 and 4): (i) assuming that S_{-3} state is the lowest possible oxidation state of the WOC (fits B1 and C1 in Table 1) since recently clear evidence has been provided (using NH_2NH_2 or NH_2OH as the reductant) that reduced PSII samples can attain a fairly stable S_{-3} state [18] and (ii) assuming that S_{-5} is the lowest oxidation state of the WOC (fits B2 and C2 in Table 1) because preliminary evidence for the existence of labile S_{-4} and S_{-5} states has been obtained from the analysis of FIOPs on *Synechococcus elongatus* thylakoids [19]. Therefore, S_1–S_{-3} and S_1–S_{-5} states populations were varied (Table 1, fits B1, C1 and B2, C2) but α, β and β_1-probabilities were fixed to the control values obtained in CO_2/HCO_3^--depleted samples (numbers in parenthesis in Table 1) as described in [17–19].

The presence of 2 mM $NaHCO_3$ during NH_2NH_2-treatment, as seen in Fig. 1B (curves 3 and 4) and in Table 1 (compare fits B1 and C1) obviously changes S_{-n}-state distribution of reduced WOC. Normalized S-state populations in the presence of 2 mM $NaHCO_3$ were the following: 32% of S_{-1}, 38% of S_{-2} and 30% of S_{-3}. In the absence of bicarbonate, only 16% of S_{-1} state still remains while S_{-2} and S_{-3} states dominated (42% and 42%, respectively). Remarkably, if we evaluate the S-state populations assuming that S_{-5} is the lowest S state of the WOC (fits C1 and C2 in Table 1) then S-state distribution in the presence of bicarbonate remains unchanged, while in the absence of bicarbonate, a quite different S-state distribution corresponding to 12% of S_{-1}, 46% of S_{-2}, 35% of S_{-3} and 7% of formal S_{-4} is obtained (fits B1 and B2). True, this approach is coupled with some impairment of fit qualities (Table 1). Nevertheless, these two approaches make it clear that the shift to the lowest super-reduced S-states is higher in the samples treated with hydrazine in the absence of bicarbonate.

Fig. 2 displays the calculated normalized S_{-n} state populations assuming that S_{-3} (A, B) or S_{-5} (C, D) states are the lowest reduced states of the WOC as a function of treatment time of S_1–thylakoids with 3 mM NH_2NH_2 at 20 °C in the absence (A, C) and in the presence (B, D) of 2 mM bicarbonate. The S-states that were found equal to zero (such as S_0, S_{-5} and S_1 in the case of absence of bicarbonate) are not presented. As indicated in Fig. 2A and B, the presence of 2 mM $NaHCO_3$ lowers the shift to S_{-3} state (by about 10%), that is coupled with conservation of a quite high S_{-1} state population (about 60% and 25% at 6 min and 25 min of incubation, respectively). By contrast, in the absence of bicarbonate, the S_{-1} state population was lower (about 40% and 17% at 6 min and 25 min of dark treatment with NH_2NH_2, respectively). The least differences between S-state populations in the presence and in the absence of bicarbonate are observed for S_{-2} state. Its formation is delayed in the presence of bicarbonate (by 7–10%) only at the initial incubation times (until 10 min) but becomes approximately equal to S_{-2} populations obtained in the absence of bicarbonate after 15–25 min of incubation (Fig. 2). Small amount of the initial S_1 state population (about 2%) still remains after a 6-min incubation of the samples with added bicarbonate and NH_2NH_2. Computation under the speculation that S_{-5} is the lowest oxidation state of the WOC does not result in apparent changes in the S-state distributions obtained in the presence of bicarbonate (Fig. 2, compare B and D) while fairly wide distinctions take place under this assumption (compare A and C) in the case of the absence of bicarbonate during NH_2NH_2 treatment. These differences are mainly characterized by the appearance and slow increase of formal S_{-4} state population (up to 15% at 25 min of incubation) along with the decrease of S_{-3} and, partially, S_{-1} state populations. Thus, the results obtained by two ways of calculation do not contradict each other and show that the shift of the WOC to the lowest reduced S-state (by NH_2NH_2) is retarded by the presence of 2 mM $NaHCO_3$ in the medium. The possible explanations of such an effect of bicarbonate are discussed below.

Fig. 3 represents the evaluation of suppression (which is known to be caused by NH_2NH_2 treatment [20,22]) of obtained FIOPs (as in Fig. 1A) in thylakoid membranes in the absence (curve 1) and in the presence (curve 2) of 2 mM $NaHCO_3$, where for criterion of relative O_2-evolving activity the sum of O_2-yields resulting from flashes 4–7 was used. Relevant sums of O_2-yields (4–7 flashes) obtained in control (untreated) S_1 thylakoids were taken as 100%. It is seen that the presence of bicarbonate during the dark treatment with 3 mM NH_2NH_2 efficiently prevents the loss of relative oxygen-evolving activity throughout the hydrazine treatment.

3.2. Oxygen evolution under continuous illumination

The measurements of the rate of oxygen evolution at continuous actinic illumination also showed that the presence of bicarbonate during the treatment of thylakoids by hydrazine slowed down the inactivation of the WOC (Fig. 4). If the hydrazine treatment of preparations was done in the CO_2/HCO_3^--

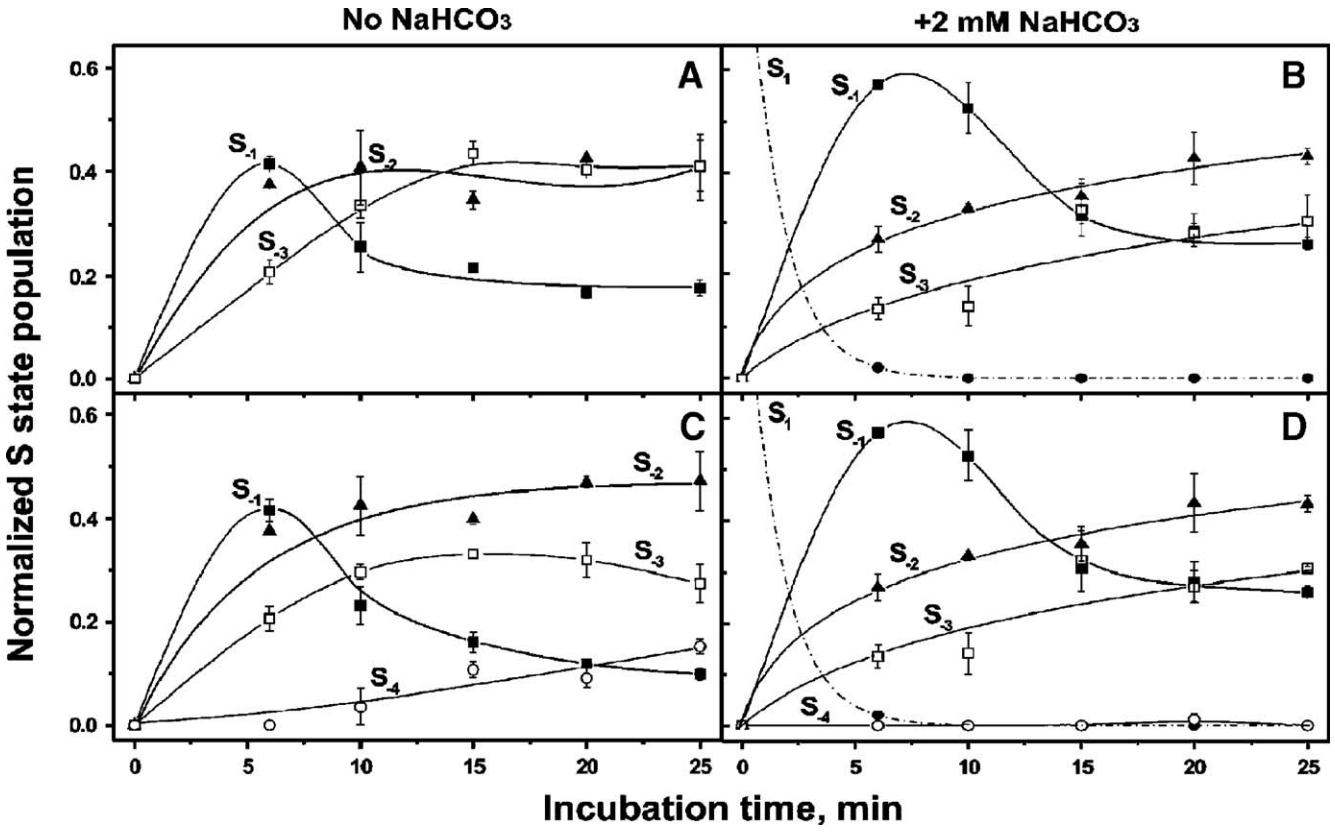

Fig. 2. Normalized S_i-state population of spinach thylakoids as a function of dark incubation time in the presence of 3 mM NH_2NH_2 in medium A depleted of CO_2/HCO_3^-. The incubation of the samples was carried out in the absence (A, C) and in the presence (B, D) of 2 mM $NaHCO_3$. The obtained data were calculated assuming that the lowest redox states of the WOC are S_{-3} (A, B) or S_{-5} (C, D) states. For the sake of clarity, S_{-5} state is not shown as no population of this state was obtained. In the case of incubation in the presence of $NaHCO_3$ (B, D) fast decay of S_1 population is also shown since after a 6-min incubation some population of this state still remains while in the absence of $NaHCO_3$ no population of S_1 state was obtained. The O_2 yields of the first 13 flashes were included in the fit. Misses, double hits and high double hits per first flash were kept at the values determined for the control thylakoids. The results are an average of 3 experiments. All measurements were done in the presence of 0.5 mM $K_3[Fe(CN)_6]$.

depleted medium, nearly 40% and 55% of the O_2-evolving activity was lost (curve 4), after 10 min and 20 min of dark incubation, respectively. However, if 2 mM $NaHCO_3$ was added to medium before the incubation with NH_2NH_2, the rate of inactivation was considerably decreased so that just nearly 15% and 32% of the activity was eliminated (curve 3) after incubation for 10 min and 20 min. The protective effect of bicarbonate was especially pronounced in the first minutes (1–3 min) of the incubation of thylakoids in the medium with hydrazine so that the loss of the O_2-evolving activity of thylakoids was 25% and 2–3% in the absence and presence of 2 mM $NaHCO_3$, respectively. In untreated thylakoids (S_1–thylakoids) the bicarbonate effect on the oxygen evolving activity during the incubation in CO_2/HCO_3^--depleted medium was insignificant (compare curves 1 and 2 in Fig. 4).

In order to investigate the effect of bicarbonate on the activity of thylakoid membranes transferred in the super-reduced S-states, the hydrazine-treated thylakoids were washed with hydrazine-free medium (to remove hydrazine from the samples) and resuspended in CO_2/HCO_3^--depleted medium both in the absence and presence of 2 mM $NaHCO_3$. Fig. 5 shows that the difference between the activities in the absence and presence of 2 mM $NaHCO_3$ is equal to about 30% (column 2) for the NH_2NH_2-treated thylakoids while for untreated thylakoids it is less than 10% (column 1). Thus, the transition of the WOC to super-reduced S-states by hydrazine leads to a higher dependence of oxygen evolution on the presence of bicarbonate in the medium.

To elucidate, whether there is a correlation between the transition of the Mn^{III}/Mn^{IV} to Mn^{II} within the WOC and the enhancement of the bicarbonate effect, the NH_2NH_2-treated thylakoid membranes were pre-illuminated by 20 saturating flashes (it is well known (for example, see Ref. [54]) that the illumination of the thylakoid membranes treated with hydrazine leads to re-transition of the WOC to normal S-states (S_0, S_1) without significant inhibition of the O_2-evolving activity). Before illumination, hydrazine-treated thylakoids were washed with hydrazine-free medium to remove hydrazine from the samples. Fig. 5 (column 3) shows that the pre-illumination with 20 saturating flashes results in a decrease of the bicarbonate effect on oxygen evolution in NH_2NH_2-treated thylakoids so that it becomes similar to that observed in untreated thylakoids. The pre-illumination of treated thylakoids also leads to some increase of the O_2-evolving activity (lost upon the NH_2NH_2 treatment).

So, the dependence of oxygen evolution on the presence of bicarbonate in the medium becomes higher upon a shift of S-states to super-reduced (S_{-2}, S_{-3}) states using hydrazine and the

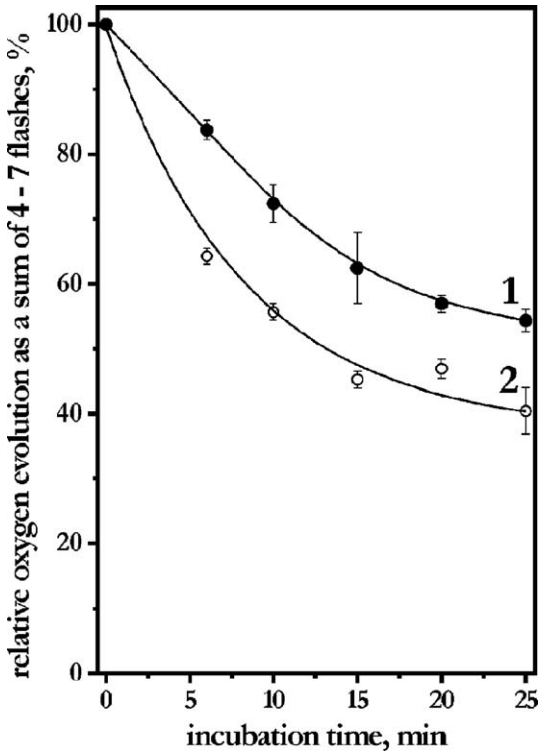

Fig. 3. Summarized O_2-yields for flashes 4–7 as a function of dark incubation time of spinach thylakoids in the presence of 3 mM NH_2NH_2 (20 °C) in medium A depleted of CO_2/HCO_3^-. Incubation of thylakoids was done in the absence (2) and in the presence (1) of 2 mM $NaHCO_3$. 100% is the O_2-yields due to flashes 4–7 taken from dark-adapted untreated (control) thylakoids in S_1 state. Chlorophyll concentration of the samples during the NH_2NH_2 treatment and measurements was 0.9 mg/mL. All measurements were carried out in the presence of 0.5 mM $K_3[Fe(CN)_6]$ as electron acceptor.

bicarbonate requirement is decreased when we return to the initial states using pre-illumination.

4. Discussion

Our results clearly demonstrate that the hydrazine-induced transition from the normal to super-reduced S-states depends on the presence of bicarbonate in the medium: in the CO_2/HCO_3^--depleted buffer the S_{-3} state (and even formal S_{-4} state) are easier reached as a result of treatment with 3 mM NH_2NH_2, than in medium containing bicarbonate (Fig. 2).

One of the possible explanations of the bicarbonate-induced retardation of the transition to S_{-n}-states is that bicarbonate binding to Mn ions changes the redox properties of the WOC. It is known [41,42] that formation of Mn–bicarbonate complexes in water solutions results in a considerable decrease of the oxidation potential of Mn. If for aquo complexes of Mn, the redox potential (E^0) of the pair Mn^{III}/Mn^{II} is equal to 1.18 V; for Mn–bicarbonate complexes, it lies between 0.51 V and 0.67 V [40]. So, it would be more difficult to reduce Mn^{III} (with hydrazine or any other reductants) when Mn^{III} (in a water solution or within the WOC) forms a complex with bicarbonate. This interpretation suggests that one of the roles of bicarbonate bound to the WOC is to slow down the decay of the normal S-states and to prevent the transition to super-reduced S-states.

On the other hand, we cannot exclude that a general destabilization of the WOC in the absence of bicarbonate, reported earlier [55,56] and confirmed in our paper (Figs. 3, 4 and 5), can lead to a better accessibility of reductants to the WOC resulting in an easier reduction of Mn^{III} and Mn^{IV}, and higher enrichment in the lowest S_{-n}-states in CO_2/HCO_3^--depleted samples (Fig. 2). The stabilizing effect of bicarbonate on the WOC can be related to bicarbonate binding to either Mn ions or other components of PSII complex.

An important result reported in this paper is the change in the effect of bicarbonate on the oxygen-evolving activity upon the reversible shift of the WOC to super-reduced S-states: the bicarbonate requirement, very low for the normal (S_0, S_1) S-states, considerably increases upon the hydrazine-induced transition to the S_{-n}-states and it becomes low again when the WOC returns back to the normal S-states using pre-illumination with 20 flashes (Fig. 5). These results are in agreement with the idea that bicarbonate can be a direct ligand to Mn within the WOC. Indeed, Mn ions of the WOC in the normal S-states are mainly in the redox states Mn^{III} and Mn^{IV} [9,10,57], and in case of bicarbonate binding to the inorganic core of the WOC, it would be very difficult to remove bicarbonate from the WOC (and subsequently to obtain a good response to bicarbonate re-addition) since according to Kozlov et al. [41,42] the stability constant (K_{st}) for

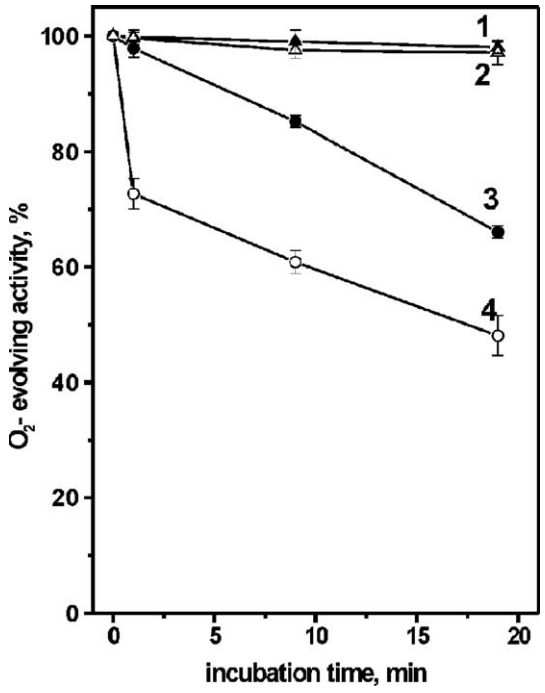

Fig. 4. Dependence of O_2-evolving activity of thylakoid membranes on incubation time at 20 °C in CO_2/HCO_3^--depleted medium A containing 3 mM NH_2NH_2 in the absence (4) and in the presence of 2 mM $NaHCO_3$ (3). After certain incubation time, NH_2NH_2 was not removed from the samples but it was diluted 90-fold in medium A in 1 mL-oxygen chamber. Untreated thylakoid membranes in medium A depleted of CO_2/HCO_3^- in the presence of 2 mM $NaHCO_3$ and in the absence of bicarbonate are presented by curves 1 and 2, respectively. The measurements were carried out at [Chl] = 10 μg/mL in the presence of 0.1 mM DCBQ and 1 mM ferricyanide as exogenous electron acceptors and 10 μM gramicidin as uncoupler. The activity of the control sample was 125 μmol O_2 (mg Chl)$^{-1}$ h^{-1} and this value was taken as 100%.

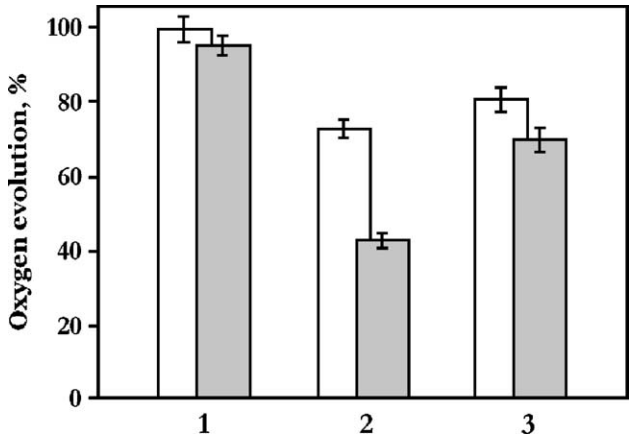

Fig. 5. The oxygen-evolving activity of untreated (1) and hydrazine-treated (2, 3) thylakoid membranes after 10-min incubation in the CO_2/HCO_3^--depleted medium in the absence (gray columns) and presence of 2 mM $NaHCO_3$ (white columns). The thylakoid membranes (0.9 mg Chl/mL) were pre-incubated for 10 min at 20 °C in the medium A containing 10 mM NH_2NH_2, and then were washed from hydrazine by centrifugation and resuspended in the medium A depleted of CO_2/HCO_3^- to Chl concentration of 10 μg/mL. (3)–NH_2NH_2-treated thylakoid membranes were pre-illuminated by 20 saturated flashes. The measurements were carried out at [Chl] = 10 μg/mL in the presence of 0.1 mM DCBQ and 1 mM ferricyanide as exogenous electron acceptors and 10 μM gramicidin as uncoupler. The activity of the control sample was 158 μmol O_2 (mg Chl)$^{-1}$ h^{-1} and this value was taken as 100%.

complex $Mn^{III}(HCO_3^-)_3$ is equal to 1.7×10^{14}. On the other hand, upon the shift to super-reduced S-states (using hydrazine reduction of both Mn^{III} and Mn^{IV} to Mn^{II}) the dependence of the O_2-evolving activity on the presence of bicarbonate in the medium becomes much higher since the K_{st} for complexes of Mn^{II} with bicarbonate is nearly 12 orders lower than that for Mn^{III}–bicarbonate complexes ([41,42] and references therein) and, therefore, bicarbonate is easily removed from the WOC. The return to the normal S-states (when Mn^{II} is converted to Mn^{III} and Mn^{IV}) results in a much lower dependence of the O_2-evolving activity on the presence of bicarbonate in the medium. The dependence of bicarbonate binding to Mn on the Mn valency is evidently responsible for a much better effect of bicarbonate on the donor side of PSII when Mn^{II} is used as an electron donor to PSII depleted of Mn (compared to the effect of bicarbonate on the functionally competent WOC when Mn is mainly in the valency Mn^{III} and Mn^{IV}) reported earlier [34–40].

Direct ligation of bicarbonate to the inorganic core of the WOC has been suggested earlier [58] on the basis of the results of investigation of bicarbonate effects on the difference light-induced Fourier-transform infrared difference absorption spectrum originating from the donor side of O_2-evolving PS II in submembrane PSII preparations. In spite of a contribution of absorbance bands of water to the spectrum, characteristic bands at 1589 cm^{-1} and 1365 cm^{-1} clearly responding to replacement of ^{12}C–bicarbonate by ^{13}C–bicarbonate and assigned to COO^- stretching modes of bicarbonate were revealed in the difference spectra. Yruela et al. [58] suggested that bicarbonate (rather than a carboxylic group of amino acid residues ligating the inorganic core of the WOC [59]) is a bridging ligand between a Mn-ion and Ca^{2+} within the WOC. Later, a similar suggestion that a bicarbonate (or carbonate) anion is located between Ca^{2+} and Mn has been made from the X-ray analysis of the WOC structure [32] (though the resolution of 3.5 Å is evidently not high enough to insist on this conclusion). On the other hand, bicarbonate as a ligand to Mn is not seen in a recent structure of the WOC obtained with the resolution of 3.0 Å [60] (that probably could be related to the loss of bicarbonate from the WOC due to reduction of Mn ions to Mn^{II} caused by X-ray irradiation as well as by the treatments required during the X-ray measurements).

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