Redox interaction of Mn–bicarbonate complexes with reaction centres of purple bacteria

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It is found that dark reduction of photooxidized primary electron donor P_{870}^+ in reaction centres from purple anoxygenic bacteria (two non-sulphur Fe-oxidizing *Rhodovulum iodosum* and *Rhodovulum robiginosum*, *Rhodobacter sphaeroides* R-26 and sulphur alkaliphilic *Thiorhodospira sibirica*) is accelerated upon the addition of Mn^{2+} jointly with bicarbonate (30–75 mM). The effect is not observed if Mn^{2+} and HCO_3^- have been replaced by Mg^{2+} and HCO_2^- , respectively. The dependence of the effect on bicarbonate concentration suggests that formation of Mn^{2+} –bicarbonate complexes, $Mn(HCO_3)^+$ and/or $Mn(HCO_3)_2$, is required for re-reduction of P_{870}^+ with Mn^{2+} . The results are considered as experimental evidence for a hypothesis on possible participation of Mn–bicarbonate complexes in the evolutionary origin of oxygenic photosynthesis in the Archean era.

Keywords: manganese–bicarbonate complexes; reaction centres; purple bacteria

1. INTRODUCTION

It is known that bicarbonate is required for maximal activity of both electron acceptor and electron donor sides of photosystem II (PSII; for recent review, see Van Rensen & Klimov (2005)). The stimulating effect of bicarbonate on electron transfer on the donor side of PSII is especially pronounced during reassembly of the inorganic core of the Mn-containing water-oxidizing complex (WOC; Klimov *et al.* 1995a,b, 1997; Allakhverdiev *et al.* 1997; Baranov *et al.* 2000, 2004; Klimov & Baranov 2001). It was shown that after the removal of all four manganese ions from subchloroplast PSII preparations, an effective reactivation of electron transfer (Allakhverdiev *et al.* 1997; Klimov *et al.* 1995a,b, 1997; Hulsebosch *et al.* 1998) and oxygen evolution (Allakhverdiev *et al.* 1997; Baranov *et al.* 2000, 2004) was observed only if a nearly stoichiometric amount of Mn^{2+} (2–4 atoms of Mn^{2+} per PSII reaction centre) was added together with bicarbonate. Changes in redox properties of manganese ions upon formation of complexes with bicarbonate evidently play a key role in the effect described above. In fact, upon the addition of $NaHCO_3$ to an aqueous solution of Mn^{2+} , the potential for oxidation of Mn^{2+} to Mn^{3+} was shifted from 1.18 to 0.52–0.68 V at pH 8.3 as a result of formation of bi(carbonate) complexes (Kozlov *et al.* 1997, 2004; Dasgupta *et al.* 2006). Formation of Mn complex with carboxylates (formate, acetate) induced similar lowering of the oxidation potential; however, only bicarbonate stimulated the electron transfer from Mn^{2+} to PSII reaction centres (RCs; Kozlov *et al.* 2004). On the basis of the electrochemical and electron paramagnetic resonance (EPR) data, it was proposed

that the unique capability of Mn^{2+} –bicarbonate complexes to be photooxidized by PSII could be due to four possible reasons: (i) significantly larger decrease in the oxidation potential of Mn^{2+} (down to 0.52 V), (ii) electroneutrality of the functional electron transfer complex, (iii) the more favourable energetics reflected in the two pK_a values for H_2CO_3/HCO_3^- and HCO_3^-/CO_3^{2-} and greater number of proton transfer sites, and (iv) multiple composition possibilities for the Mn^{3+} photoproduct such as $Mn^{3+}(HCO_3^-)_3$, $Mn^{3+}(HCO_3^-)(CO_3^{2-})$ and $Mn^{3+}(HCO_3^-)_2(OH^-)$ (due to the high Lewis acidity of Mn^{3+} ; $pK_a < 1$; Kozlov *et al.* 2004; Dasgupta *et al.* 2006).

The oxidation potential of Mn–bicarbonate complex (0.52–0.67 V) is close to the midpoint redox potential of the primary electron donor in the RCs of anoxygenic bacteria (Kozlov *et al.* 2004) and therefore it has been suggested (Dismukes *et al.* 2001) that the capability of bicarbonate to form easily oxidizable complexes with manganese ions might be critical to the evolutionary origin of the first O_2 -evolving cyanobacteria from a non-oxygenic bacterial precursor in the Archean period (>2.2 Gyr ago) when the concentration of bicarbonate dissolved in water was 30–30 000 times greater than today.

Recently, it has been shown that as a result of a set of single mutations of amino acid residues near P_{870} , the primary electron donor in the RCs of purple anoxygenic bacterium *Rhodobacter sphaeroides* R-26, the P^+/P midpoint potential (equal to 0.5 V in the initial strain) was increased to 0.58–0.765 V, and exogenous Mn^{2+} added together with bicarbonate enabled electron donation to P^+ in RCs isolated from these mutants (Kalman *et al.* 2003). In the present work, we investigated the possibility of electron donation from Mn^{2+} –bicarbonate complexes to oxidized P in wild-types of anoxygenic photosynthesizing bacteria. Three types of purple bacteria (belonging to the class of

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Proteobacteria containing type II of RCs; Gupta *et al.* 1999) were used: (i) two non-sulphur Fe-oxidizing bacteria *Rhodovulum iodosum* and *Rhodovulum robiginosum* (isolated from sediment of the North Sea, Germany) that use Fe^{2+} as an electron donor for anoxygenic photosynthesis (Straub *et al.* 1999) and so can be good candidates as possible users of the easily oxidizable Mn^{2+} –bicarbonate complexes as electron donors, (ii) the purple sulphur alkaliphilic bacterium *Thiorhodospira sibirica* (received from the collection of Prof. V.M. Gorlenko, Institute of Microbiology, RAS, Moscow), found in 'soda' lake Malyi Kasitui (pH of water is 9.5); its optimal growth is at pH 9.0, at a carbonate concentration of 45 mM; the bacterium is resistant to high concentrations of NaCl (up to 1 M) and Na_2CO_3 up to 740 mM (Bryantseva *et al.* 1999), and (iii) the widely used non-sulphur bacterium *Rh. sphaeroides* R-26.

The results presented in this work show that the formation of Mn^{2+} –bicarbonate complexes favours electron donation from Mn^{2+} to type II RCs from at least four contemporary purple bacteria.

2. MATERIAL AND METHODS

(a) Isolation of pigment–protein complexes B890

Cell cultures of *Rh. iodosum* and *Rh. robiginosum*, and *Th. sibirica* were grown as described earlier (Straub *et al.* 1999; Bryantseva *et al.* 1999; respectively) at 25–30°C and continuous irradiance of 2000 lux. The cells were washed twice in distilled water, suspended in 10 mM HEPES (pH 7.8) and after sonication pigment–protein complexes B890 were isolated as described earlier (Qian *et al.* 2000) with some modifications. The chromatophore membranes ($\text{OD}_{890} = 50 \text{ cm}^{-1}$) were incubated in 10 mM HEPES buffer (pH 7.8) with 2% dodecyl- β -D-maltoside (DM) for 30 min at 4°C in the dark. The suspension was centrifuged (10 000g), and the supernatant was loaded onto a stepwise sucrose density gradient (0.3, 0.5, 0.6 and 1.2 M) and centrifuged (200 000g) at 4°C for 20 h. The pigment–protein complexes B890 appearing between the 0.5 and 0.6 M sucrose layers were collected and loaded onto a 20 mm $\times$ 200 mm DEAE 650s column that had been equilibrated with 10 mM HEPES (pH 7.8) containing 0.1% DM (eluent). The crude pigment–protein complexes B890 were applied to the column, washed with the eluent without and then with 0.05 M NaCl to remove free pigments. The salt concentration was then increased to 0.25 M to remove the LH2 component. Finally, the remaining pigment–protein complexes B890 were eluted with the eluent containing 0.5 M NaCl. The pigment–protein complexes B890 were stored at –20°C. The RCs from *Rh. sphaeroides* R-26 were isolated as described earlier (Shuvalov & Duysens 1986). The concentration of RCs was determined as described previously (Clayton & Wang 1971).

(b) Measurements of absorbance changes related to reversible photooxidation of P_{870}

Kinetics of photoinduced absorbance changes, related to reversible photooxidation of P_{870} , were measured in a 10 mm cuvette at 20°C using a phosphorescopic set-up (Klimov *et al.* 1982). Accumulation of the oxidized primary electron donor, P_{870}^+ , was reached by illumination of pigment–protein complexes B890 with red light ($\lambda > 600 \text{ nm}$, $1900 \mu\text{mol photon s}^{-1} \text{ m}^{-2}$) for 30 s. The difference 'light–dark' absorption spectrum was measured by a spectrophotometer (Hitachi-U-2800, Japan).

(c) Removal of bicarbonate from complexes B890

Partial removal of bicarbonate from pigment–protein complexes was achieved by a 50-fold dilution of the samples in the medium depleted of the endogenous bicarbonate by 60 min flushing with air freed of CO_2 by passage through a solution of 50% NaOH and a 20 cm layer of ascarite.

3. RESULTS

To study the possibility of redox interaction of Mn^{2+} –bicarbonate complexes with RCs of purple bacteria, we used the pigment–protein complexes B890 isolated from *Rh. iodosum*, *Rh. robiginosum* and *Th. sibirica*. The complex B890 from purple bacteria is known to be the light-harvesting complex (that is termed LH1) tightly associated with the RC in fixed stoichiometry 1 : 1 (Law *et al.* 2004). Figure 1a illustrates the absorption spectrum of the pigment–protein complexes B890 isolated from *Rh. iodosum* which is similar to that isolated previously from other types of purple bacteria (Law *et al.* 2004; Moskalenko *et al.* 2005).

Kinetics of photoaccumulation of the primary electron donor P_{870} in the oxidized form P^+ and its dark re-reduction, measured at 790 nm (ΔA_{790} ; corresponding to the electrochromic blue shift of absorption band at 800 nm due to oxidation of P_{870}), are shown in figure 1b. After a 30 s illumination at pH 8.2, nearly 50% of RCs remain in a 'long-lived' oxidized state: their re-reduction occurs in minute range. Using redox titration of the amplitude of the photoinduced ΔA_{790} (upon the addition of $\text{K}_3[\text{Fe}(\text{CN})_6]$ and $\text{K}_4[\text{Fe}(\text{CN})_6]$), the midpoint redox potential (E_m) of the pair P^+/P in *Rh. iodosum*, *Rh. robiginosum* and *Th. sibirica* was found to be equal to $450 \pm 30 \text{ mV}$ (which is close to E_m of P in other purple bacteria; Prince *et al.* 1976; Klimov *et al.* 1977; Lin *et al.* 1994).

The dark reduction of P^+ is considerably accelerated if 0.1 mM $\text{K}_4[\text{Fe}(\text{CN})_6]$ (a known artificial electron donor to P^+) is present in the medium so that only 15% of P were in the oxidized state after 30 s of darkness (figure 1b, curve 2). Interestingly, an acceleration of P^+ reduction is also observed upon the addition of 0.5 mM MnCl_2 together with 50 mM NaHCO_3 (figure 1b, curve 3). The effect was clearly seen for the long-lived component of P^+ relaxation: a 50% decay of this component was at 110, 63 and 5 s for the samples without additions, in the presence of 0.5 mM MnCl_2 together with 50 mM NaHCO_3 , and in the presence of 0.1 mM $\text{K}_4[\text{Fe}(\text{CN})_6]$, respectively. In further experiments on the effect of Mn^{2+} on the redox state of P^+ , the difference light–dark absorption spectra of P^+ corresponding to the long-lived state of photooxidized P_{870} were measured. A similar approach for revealing the redox interaction of Mn^{2+} with bacterial RCs was used earlier (Kalman *et al.* 2003). The spectra were measured after a 2.5 min dark incubation of the samples illuminated with red light ($\lambda > 600 \text{ nm}$, $1900 \mu\text{mol photon s}^{-1} \text{ m}^{-2}$) for 30 s.

The spectrum (figure 1c) is typical of photooxidation of P in pigment–protein complexes B890 of purple bacteria (Shuvalov & Klimov 1976; Moskalenko *et al.* 2005). It includes the bleaching of a broad absorption band of P with maximum at 870 nm accompanied by a

Figure 1. (a) Absorption spectrum, kinetics of photoinduced absorbance changes at 790 nm (ΔA_{790}) (b) related to photooxidation of the primary electron donor P_{870} in pigment–protein complexes B890 isolated from the non-sulphur Fe-oxidizing purple bacterium *Rh. iodolum* and (c) difference light–dark absorption spectra. (c) Spectra were measured after a 30 s illumination with actinic light ($\lambda > 600$ nm, $1900 \mu\text{mol photon s}^{-1} \text{m}^{-2}$) and 2.5 min incubation in the dark. The measurements of ΔA_{790} and difference light–dark absorption spectra were made without additions (1) and after the addition of 0.1 mM $\text{K}_4[\text{Fe}(\text{CN})_6]$ (2), 0.5 mM MnCl_2 and 50 mM NaHCO_3 (3). Up and down arrows indicate actinic light on and off, respectively. The measurements were done in the medium containing 50 mM HEPES (pH 8.2) depleted of $\text{CO}_2/\text{HCO}_3^-$ (see §2).

characteristic shift of the absorption band at 800 nm. The amplitudes of both the bleaching at 870 nm and the shift at 800 nm are decreased upon the addition of either 0.1 mM ferricyanide (figure 1c, curve 2) or 0.5 mM MnCl_2 added jointly with 50 mM NaHCO_3

(figure 1c, curve 3) indicating the acceleration of dark re-reduction of P^+ . For a detailed investigation of the specificity of the pair Mn^{2+} + bicarbonate in the acceleration of re-reduction of P^+ in purple bacteria, the difference $\Delta A_{790} - \Delta A_{810}$ ($\Delta A_{790-810}$; corresponding to the blue shift of the absorption band at 800 nm due to oxidation of P) was used as a measure of the fraction of RCs with oxidized P_{870} (to avoid possible absorbance changes related to photobleaching of the antenna BChl).

Figure 2a shows that the addition of 0.5 mM MnCl_2 together with 50 mM NaHCO_3 to pigment–protein complex B890 from *Rh. iodolum* accelerates re-reduction of P^+ (closed circles), while 0.5 mM MnCl_2 or bicarbonate added alone does not increase the rate of dark relaxation of $\Delta A_{790-810}$ (filled inverted triangles and filled squares in figure 2a, respectively). The bicarbonate requirement for reduction of P^+ with added Mn^{2+} is in agreement with the data reported earlier (Kozlov et al. 2004) on lowering of the oxidation potential of Mn^{2+} upon formation of complexes with bicarbonate.

Mg^{2+} (in contrast to Mn^{2+}) added jointly with bicarbonate does not increase the rate of re-reduction of P^+ (figure 2d). Similarly, replacement of bicarbonate by formate (without change in Mn^{2+} concentration) also leads to the loss of the effect observed in the presence of Mn^{2+} and bicarbonate (figure 2d). This clearly demonstrates that Mn^{2+} –bicarbonate complexes are important for the acceleration of P^+ re-reduction.

A similar effect of the increase of the rate of dark reduction of P^+ upon addition of the Mn^{2+} jointly with bicarbonate is observed in pigment–protein complex B890 isolated from two other purple bacteria: Fe-oxidizing *Rh. rubiginosum* (figure 2b) and sulphur alkaliphilic *Th. sibirica* (figure 2c).

The acceleration of dark reduction of P^+ upon the addition of Mn^{2+} depended on the concentration of added bicarbonate: at an Mn^{2+} concentration equal to 0.5 mM, the effect was not observed at bicarbonate concentrations of 10 and 15 mM; it is clearly seen starting from a bicarbonate concentration of 30 mM and is saturated at 50–70 mM (figure 3a). The dependence of the effect on Mn^{2+} concentration shows that in the presence of 50 mM NaHCO_3 , the acceleration of reduction of P^+ is observed already at Mn^{2+} concentration of 10 μM , and the effect is maximal at 0.3–0.5 mM MnCl_2 (figure 3b).

It has been shown earlier (Kalman et al. 2003) that in RCs isolated from the initial strain of *Rh. sphaeroides* R-26 (the P^+/P midpoint potential = 0.5 V), Mn^{2+} added jointly with 15 mM bicarbonate is not able to accelerate the dark reduction of P_{870}^+ . Figure 4 demonstrates that under the conditions used in our experiments (photoaccumulation of P^+ at pH 8.2, addition of 0.5 mM MnCl_2 together with 50 mM NaHCO_3), the rate of re-reduction of P^+ in RCs isolated from the initial strain of *Rh. sphaeroides* is considerably increased upon the addition of the pair Mn^{2+} + bicarbonate so that in their presence the concentration of P^+ remaining after a 30 s incubation of illuminated samples in the dark becomes 3.5 times lower.

Figure 2. Kinetics of the long-lived component of dark relaxation of photoinduced ΔA related to the reversible photooxidation of P_{870} in pigment–protein complexes isolated from (a) *Rh. iodosum*, (b) *Rh. rubiginosum*, (c) *Th. sibirica* in the absence of additions (open squares) and in the presence of 0.1 mM $K_4Fe(CN)_6$ (open triangles), 0.5 mM $MnCl_2$ (filled inverted triangles), 50 mM $NaHCO_3$ (filled squares), 0.5 mM $MnCl_2$ and 50 mM $NaHCO_3$ (filled circles). The difference $\Delta A_{790} - \Delta A_{810}$ (corresponding to the blue shift of absorption band at 800 nm due to oxidation of P_{870}) taken from difference light–dark absorption spectra measured after a 30 s illumination (for details see figure 1) was used as a measure of the fraction of RCs with oxidized P_{870} . The first spectrum was measured 30 s after switching the actinic light off. (d) Kinetics of the long-lived component of dark relaxation of photoinduced ΔA related to the reversible photoreduction of P_{870} in pigment–protein complexes isolated from *Rh. iodosum* in the absence of additions (open squares) and presence of 0.5 mM $MgCl_2$ and 50 mM $NaHCO_3$ (filled diamond); 0.5 mM $MnCl_2$ and 50 mM $NaHCO_2$ (right-faced filled triangle). The results are an average of three experiments.

4. DISCUSSION

Dismukes *et al.* (2001) put forward a hypothesis that Mn^{2+} –bicarbonate complexes could play a key role in the evolutionary origins of the WOC due to the use of Mn complexes as transient electron donors (and ‘building blocks’) for anoxygenic photosynthetic bacteria in the Archean Ocean when the content of CO_2 and HCO_3^- was high enough (a few orders higher than nowadays) to produce easily oxidizable Mn^{2+} complexes.

The results obtained in our work show that the formation of Mn^{2+} –bicarbonate complexes favours electron donation from Mn^{2+} to the RC of purple bacteria. Such a conclusion has been made on the basis of spectrophotometric measurements of ΔA related to reversible photooxidation of the primary electron donor, P. As in the case with ferrocyanide (a known exogenous electron donor to P^+), Mn^{2+} in the presence of bicarbonate accelerated the re-reduction of P^+ . The

midpoint potential for the P^+/P of anoxygenic purple bacteria is 0.45–0.50 V (Prince *et al.* 1976; Klimov *et al.* 1977; Lin *et al.* 1994) and a similar E_m is found in our work for the pair P^+/P in RCs isolated from *Rh. iodosum*. Therefore, the RC of these bacteria cannot act as an oxidant for Mn^{2+} (the oxidation potential for aqua ions of Mn^{2+} is 1.18 V). On the basis of electrochemical measurements, it was demonstrated that the formation of complexes of Mn^{2+} with bicarbonate greatly favours the electrochemical oxidation of Mn^{2+} to Mn^{3+} so that the oxidation potential of Mn^{2+} becomes 0.52–0.67 V (Kozlov *et al.* 2004). Therefore, in response to the formation of Mn^{2+} –bicarbonate complexes, the oxidation potential of Mn^{2+} becomes so low that it is possible to expect the oxidation of Mn^{2+} by RCs of anoxygenic purple bacteria, and that is what we see in our experiments. The replacement of Mn^{2+} by Mg^{2+} or bicarbonate with formate leading to the loss of the effect observed with the Mn–bicarbonate system confirms the

Figure 3. Effect of Mn^{2+} –bicarbonate complexes on the re-reduction of P^+ in the pigment–protein complexes B890 from *Rh. iodosum* at various concentrations of (a) bicarbonate and (b) MnCl_2 . The experiments shown in (a) were done in the presence of 0.5 mM MnCl_2 without other additions (filled squares) and after the addition of 30 (open triangles), 40 (open diamond), 50 (filled circles) and 75 mM (open inverted triangles) of NaHCO_3 . The experiments shown in (b) were done in the presence of 50 mM NaHCO_3 without other additions (filled squares) and after the addition of 0.01 (open triangles), 0.1 (open diamond), 0.3 (filled circles) and 0.5 mM (open inverted triangles) of MnCl_2 . The results are an average of three experiments.

specificity of Mn–bicarbonate complexes in this reaction (figure 3d). Recently, it has been found that carboxylates (formate and acetate) are also capable of producing complexes with Mn^{2+} that lead to a lowering of the oxidation potential of Mn^{2+} ; the oxidation potential of Mn^{2+} –formate complexes is approximately 0.76 V (Kozlov *et al.* 2004). The relatively high oxidation potential of Mn^{2+} –formate complexes (in comparison with Mn^{2+} –bicarbonate complexes) can explain the inability of the pair Mn^{2+} + formate to accelerate re-reduction of P^+ in purple bacteria. In addition, bicarbonate (in contrast to formate) can form electro-neutral complexes with Mn^{2+} and Mn^{3+} ($\text{Mn}^{\text{II}}(\text{HCO}_3)_2$ and $\text{Mn}^{\text{III}}(\text{HCO}_3)_3$ (Kozlov *et al.* 2004) that can also favour the redox interaction of Mn^{2+} with the RC of purple bacteria. It is interesting that PSII RCs are also unable to oxidize Mn–formate complexes (Kozlov *et al.* 2004) though the oxidation potential of P_{680}^+ (>1.1 V) is enough for their oxidation. Evidently, the multiple composition possibilities for the Mn^{3+} photoproduct, such as $\text{Mn}^{3+}(\text{HCO}_3^-)_3$, $\text{Mn}^{3+}(\text{HCO}_3^-)(\text{CO}_3^{2-})$ and $\text{Mn}^{3+}(\text{HCO}_3^-)_2(\text{OH}^-)$ (Kozlov *et al.* 2004), are important for the redox interaction of Mn–bicarbonate complex with RCs of both PSII and anoxygenic bacteria.

The acceleration of re-reduction of P^+ in the presence of Mn^{2+} begins from 30 mM of added bicarbonate and the effect is maximal at 50 mM. Electrochemical and EPR measurements (Kozlov *et al.* 2004; Dasgupta *et al.* 2006) revealed the presence of 1 : 1 ($[\text{Mn}(\text{HCO}_3)]^+$) and 1 : 2 ($\text{Mn}(\text{HCO}_3)_2$) complexes in water solution of Mn^{2+} and bicarbonate. The oxidation potentials for the 1 : 1 and 1 : 2 Mn^{2+} –bicarbonate complexes (0.61 and 0.52 V, respectively; Kozlov *et al.* 2004) are much lower than those for the aqua complex of Mn^{2+} (1.18 V). Using the equilibrium constants of Mn^{2+} –bicarbonate complexes presented earlier (Kozlov *et al.* 2004;

Figure 4. Difference light–dark absorption spectra of isolated RCs from *Rh. sphaeroides* R-26 measured after a 30 s illumination with actinic light ($\lambda > 600$ nm, $1900 \mu\text{mol photon s}^{-1} \text{m}^{-2}$) and a 30 s incubation in the dark in the absence (1) and presence (2) of 0.5 mM MnCl_2 and 50 mM NaHCO_3 .

Dasgupta *et al.* 2006), we estimated the ratio of Mn^{2+} –bicarbonate complexes in the solution at different concentrations of added bicarbonate. In the presence of 15 mM bicarbonate at pH 8.2, the total Mn^{2+} in the solution is represented by a mixture of 69% of ions Mn^{2+} aqua, approximately 23% of $[\text{Mn}(\text{HCO}_3)]^+$ and 8% of $\text{Mn}(\text{HCO}_3)_2$. The content of $\text{Mn}(\text{HCO}_3)_2$ increases with increase in the bicarbonate concentration and at 50 mM the mixture of total Mn^{2+} is 29% of Mn^{2+} aqua, approximately 31% of $[\text{Mn}(\text{HCO}_3)]^+$ and 40% of $\text{Mn}(\text{HCO}_3)_2$. Most probably, the bicarbonate concentration dependence of the acceleration of re-reduction of P^+ with Mn^{2+} (figure 3a) reflects the increase in the content of the complex $\text{Mn}(\text{HCO}_3)_2$ most active in the redox interaction with P^+ . It was shown (Kalman *et al.* 2003) that only RCs from the mutant cells of *Rh. sphaeroides* in which the P^+/P

midpoint potential was increased to 0.58–0.765 V were able to oxidize Mn^{2+} in the presence of 15 mM bicarbonate, while oxidation of Mn^{2+} by RCs from the initial strain (where the P^+/P midpoint potential was 0.45–0.5 V) was not observed. (Our experiments confirm that if the concentration of added bicarbonate is lower than 30 mM, Mn^{2+} does not accelerate the reduction of P^+ .) One can suggest that the domination of the complex $[\text{Mn}(\text{HCO}_3)]^+$ and, as a consequence, the low the concentration of the complex $\text{Mn}(\text{HCO}_3)_2$, were responsible for the lack of redox interaction of Mn–bicarbonate complex with the RCs of *Rh. sphaeroides* in the experiments reported earlier (Kalman *et al.* 2003).

The effect of Mn^{2+} –bicarbonate complexes on the redox state of P^+ was also obtained in pigment–protein B890 isolated from another non-sulphur Fe-oxidizing purple bacterium *Rhodovulum robiginosum* and from a purple sulphur alkaliphilic bacterium *Th. sibirica* (figure 2*b,c*). Thus, the results demonstrate that even without pre-modifications of the redox properties of the RCs, at least four contemporary purple bacteria (containing type II RCs) are capable of using Mn–bicarbonate complexes as electron donor for oxidized P and this capability could be typical of most purple bacteria with a P^+/P midpoint potential near 0.45–0.5 V. Therefore, under certain conditions (appropriate pH, enhanced concentration of bicarbonate) favourable for the formation of the easily oxidizable Mn^{2+} –bicarbonate complexes, the purple bacteria could use the Mn^{2+} –bicarbonate complexes as a source of electrons; according to the hypothesis (Dismukes *et al.* 2001) this eventually could have led to the origin of the WOC in the Archean era.

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- ### Discussion
- V. Batista (Yale University, USA). What is the experimental evidence that bicarbonate would be involved in the proton exit channel?
- V. V. Klimov. Our data on the increase of the stimulating effect of bicarbonate on both oxygen evolution and variable fluorescence upon the addition of PSII-associated carbonic anhydrase CAH3 to PSII preparations isolated from a CAH3 lacking mutant of *Chlamydomonas reinhardtii* and similar stimulation of the activities upon the addition of an amphiphilic buffer neutral red to these preparations allow us to suggest that bicarbonate and CAH3 can be involved in the process of removal of protons released during water oxidation (Shutova et al. Submitted). There is no direct evidence for involvement of bicarbonate in the proton exit channel.
- A. W. Rutherford (CEA Saclay, France). How close is carbonic anhydrase to PSII? Is it bound and if so where?
- V. V. Klimov. In *Chlamydomonas reinhardtii*, PSII-associated carbonic anhydrase CAH3 is located on the luminal side of PSII (judging from the amino acid sequence characteristics of other luminal PSII proteins), and upon isolation of PSII core complex from PSII particles, it remains bound to PSII and its content (per Chl basis) increases (Villarejo et al. 2002).
- H. Dau (Freie University, Berlin, Germany). I have a comment. Tatiana Shutova (Laboratory of Klimov, Pushinov, Russia, and G. Samuelsson, Umeå, Sweden) carried out measurements in our laboratory using a time-resolved delayed fluorescence approach (Grabolle & Dau 2005; Buchta et al. 2007) to study the donor-side processes in PSII preparations from wild-type cells and mutants lacking the PSII-associated carbonic anhydrase (CA; Villarejo et al. 2002). Interestingly, the results obtained for the CA-free mutant suggest that proton release in the $S_2 \rightarrow S_3$ and $S_3 \rightarrow S_4$ transition is indeed accelerated by bicarbonate addition. This finding supports that bicarbonate facilitates more efficient proton removal from the Mn complex.
- W. Hillier (Australian National University, Australia). I would like to comment. PSII is often found to contain carbonic anhydrase activity. We have quantified this activity in a number of different samples, and surprisingly for the PSII from *Th. elongatus* there is practically no carbonic anhydrase activity associated with PSII.
- J. Barber (Imperial College London). I would like to point out that the electron density tentatively assigned to carbonate in the OEC by Ferreira et al. (2004) is in part occupied by D1Asp170 in the Loll et al. model (2005).
- S. Styring (Uppsala, Sweden). I have two questions. Was the effect of carbonic anhydrase specific to the enzyme from *Chlamydomonas* and can you relate any role of bicarbonate to a specific S-state transition?
- V. V. Klimov. Yes. The effect of carbonic anhydrase was specific for the enzyme from *Chlamydomonas*; the effect was not seen upon the addition of a bovine carbonic anhydrase. We consider bicarbonate as an available base with an appropriate pK_a (6.4) which can be used as an acceptor for protons released from the water oxidation centre (especially for the $S_3 \rightarrow S_4 \rightarrow S_0$ transition; Shutova et al. Submitted).