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A CRITICAL ROLE OF BICARBONATE IN THE RELAXATION OF REACTION CENTER II COMPLEX DURING OXYGEN EVOLUTION IN ISOLATED BROKEN CHLOROPLASTS

Govindjee*, A. J. Stemler* and G. T. Babcock†
*Departments of Botany and Physiology and Biophysics
University of Illinois, Urbana, Illinois 61801 USA
and †Department of Chemistry, Lawrence Berkeley Laboratory
University of California, Berkeley, California 94720 USA

Abstract

In bicarbonate (HCO_3^-) depleted chloroplasts exposed to brief saturating light flashes, period 4 oscillations in O_2 yield per flash are damped within 3 cycles. Readdition of HCO_3^- to these preparations restores the oscillatory pattern to higher flash numbers, indicating that HCO_3^- reduces the probability of "misses" in the photosystem II reaction center. To explain our data, we must also propose that a certain percentage of reaction centers are completely inactive in the absence of HCO_3^- and that this, even more than an increase in the "miss" rate, lowers the steady state yield about 50 percent. Furthermore, the rate of the dark relaxation reaction $S_n \dashrightarrow S_{n+1}$ (where S refers to the oxidation state of the oxygen evolving mechanism and n = 0, 1 or 2), following a photoact in the PS II reaction center, is retarded by about ten-fold in HCO_3^- depleted chloroplasts compared to the rate for this reaction in depleted chloroplasts to which HCO_3^- has been resupplied. However, HCO_3^- has no effect on the dark deactivation of the higher oxidation states (S_2 and S_3) of the positive charge accumulating system. We propose that the relaxation of reaction center II complex ($Z^{n+}Chla_{II}^{+}Q^-$, after a photoact, to $Z^{(n+1)+}Chla_2 \cdot Q$, where Z is the charge accumulating secondary electron donor, $Chla_{II}$ is primary electron donor and Q is primary electron acceptor) in dark requires HCO_3^-. This recovery reaction is affected either through the recovery of Q^- to Q or to the transfer of positive charges from $Chla_2$ to Z or both.

Abbreviations: Chl, chlorophyll; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethyl urea; DCPIP, dichlorophenol indophenol; DPC, diphenyl carbazide; PS II, photosystem II; Z Chl a_2 Q, reaction center of PS II, Z and Q being electron donor and acceptor, and Chl a_2 being the reaction center chlorophyll (the primary electron donor) of PS II.

Introduction

Recent investigation of the role of HCO_3^- in the Hill reaction indicates that this ion plays a critical role in the oxygen evolving mechanism[1-4]. Evidence is available which suggests[1] that HCO_3^- may act on the oxygen evolving side of photosystem (PS) II. Electron flow from the artificial electron donor diphenyl carbazide (DPC) to dichlorophenol indophenol (DCPIP) via PS II is insensitive[1] to HCO_3^-. Effects of HCO_3^- on chlorophyll (Chl) a fluorescence transients and on delayed light emission in the 0.5 - 5 sec time period were also interpreted to suggest a site of action of HCO_3^- on the oxygen evolving side of PS II . This latter work led Stemler and Govindjee[2] to speculate that HCO_3^- somehow stabilized higher oxidation states of the PS II reaction centers (referring to the kinetic model of oxygen evolution of Kok et al.[5]). We therefore studied[4] the effects of HCO_3^- on oxygen evolution in response to both continuous light and to brief light flashes. Our results[4] show that HCO_3^- reduces the frequency of reaction center "misses" and thus maintains oscillations in oxygen yield per flash for a much greater number of flashes. In addition, HCO_3^- was shown[4] to accelerate the relaxation reactions S_n--->S_{n+1}, where n = 0, 1 or 2) following a photoact. The present paper summarizes some of the results reported in reference 4.

Materials and Methods

Chloroplast Preparation. Maize (Zea mays) chloroplasts were obtained in a manner already described[1]. While even under optimum conditions maize chloroplasts usually do not perform the Hill reaction at very high rates compared to chloroplasts from other sources, we used maize to minimize precipitation of the chloroplasts during the HCO_3^- depletion procedure[3]. Bicarbonate depletion of pea (Pisum sativa) chloroplasts, under milder conditions, produced 4- 10-fold HCO_3^- stimulation of oxygen evolution with total yield equal to untreated controls (T. Wydrzynski and Govindjee, unpublished data). The HCO_3^- depletion procedure therefore does not necessarily result in gross chloroplast damage thereby accounting in some way for the HCO_3^- effect. To deplete them of HCO_3^- the chloroplasts were suspended in a solution containing 0.25 M NaCl, 0.04 M Na acetate, 0.05 M Na phosphate buffer at pH 5.0. The suspension was stirred slowly for 30 minutes at room temperature while the gas above the suspension was continuously flushed with nitrogen. After depleting the chloroplasts of HCO_3^- they were centrifuged in capped test tubes, previously flushed with N_2 and resuspended in reaction mixture. All vessels and reaction mixtures were carefully sealed or otherwise handled to avoid contamination with atmospheric CO_2 prior to deliberate addition of $NaHCO_3$.

O_2 Evolution in Flashing Light. The apparatus used for measuring oxygen evolution in response to brief light flashes was described by Weiss and Sauer[6] and

modified according to Babcock [7]. The xenon lamp pulses were 10 μsec in duration and were filtered through Corning 1-69 and 3-74 filters before being focused on the electrode surface. All flashes used in these experiments were of saturating intensity. The solution flowing above the membrane holding the chloroplasts to the surface of the platinum electrode contained 0.25 M NaCl, 0.04 M Na acetate, 0.05 M Na phosphate buffer at pH 6.8 and was either HCO_3^- free or supplied with 0.01 M $NaHCO_3$ in bicarbonate readdition experiments. The electrolyte was gassed continuously with 80 percent N_2, and 20 percent O_2.

Results

We have shown[4] that the stimulation of O_2 evolution caused by resupplying 0.01 M $NaHCO_3^-$ to the chloroplasts depends on light intensity. At saturating intensity stimulation is nearly 5-fold. This stimulation is clearly present at the lowest intensity used although it is reduced to about 2-fold.

1. Effects of HCO_3^- on Oscillations in Oxygen Yield. The kinetics of oxygen evolution in response to brief light flashes have already been described in detail for normal systems by Joliot et al.[8] and Kok et al.[5] (also see Mar and Govindjee[9]). From the evidence presented in Figure 1 (bottom), HCO_3^- depleted chloroplasts, when normalized to the same total oxygen yield (under steady state), show damped oscillations in oxygen evolution as a function of flash number compared to those chloroplasts resupplied with 0.01 M HCO_3^-. (In HCO_3^- depleted chloroplasts resupplied with HCO_3^-, oscillations were very similar to untreated controls; data not shown.) The damping of oscillations in the HCO_3^- depleted chloroplasts is suggested to be due to a greater number of "misses". Reducing the number of misses is not the only function of HCO_3^-, however, as will be seen from other results to be discussed below.

It is also evident from the recorder traces presented in Figure 1 (top) that total oxygen yield induced by light flashes spaced one second apart is nearly 2-fold greater in the presence of HCO_3^-. Under high intensity continuous light these same chloroplasts showed a 4- 5-fold greater rate of oxygen evolution. Thus brief flashes of high intensity light spaced one second apart produce the same reduced HCO_3^- stimulation as seen with low intensity continuous light. However, a 50 percent decrease in steady oxygen yield in the absence of HCO_3^- cannot be attributed to misses alone. If the miss rate were indeed 50 percent, the yield on the 3rd flash would be much less than that of the steady state yield. Since it is not, a miss rate of less than 20 percent is implied. We must therefore propose that a certain percentage of reaction centers are completely inactive in the absence of HCO_3^- and that this, even more than an increase in the miss rate, lowers the steady state yield about 50 percent.

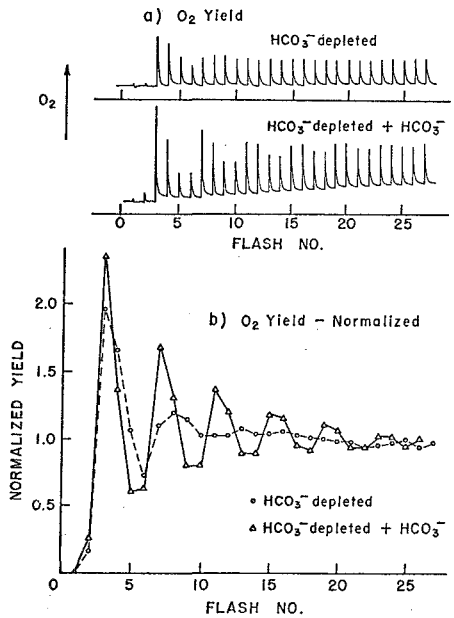

Fig. 1. Oxygen evolution in flashing light in the presence and absence of 0.01 M Na HCO_3^- following 5 minutes dark time. Top: recorder traces; saturating 10 μsec flashes spaced 1 sec apart were used to stimulate oxygen evolution. Bottom: oxygen yield as a function of flash number, from experimental traces in (a), normalized to the same total steady state yield of oxygen. The chloroplast suspension injected onto the platinum electrode contained 0.25 M NaCl, 0.04 M Na acetate, 0.05 M Na phosphate buffer pH 6.8, 20 μg ferredoxin ml^{-1}, 0.5 mM $NADP^+$ and 0.3 mg Chl ml^{-1} suspension. The chloroplasts used were previously depleted of HCO_3^-. To resupply HCO_3^- to the chloroplasts, HCO_3^- (to 10 mM) was added to the electrolyte flowing over the membrane holding the chloroplasts to the platinum. Other conditions are described in materials and methods (after Stemler et al.4).

2. Effect of HCO_3^- on Relaxation Reactions (S_n--->S_{n+1}). Studies on the rates of the dark relaxation reactions occurring between photoacts have been made by Kok et al.[5] and particularly by Bouges-Boucquet[10]. The rates are measured by varying the time between the flashes and measuring the effect on the final yield of O_2. The half-times of the reactions $S_0' \xrightarrow{dark} S_1$, $S_1' \xrightarrow{dark} S_2$ and $S_2' \xrightarrow{dark} S_3$ are all in the order of 200-600 μsec in normal chloroplasts[10]. In HCO_3^- depleted chloroplasts, however, the half-times of these reactions are dramatically extended, while resupplying HCO_3^- restores the normal rates.

Figure 2 shows the effect of HCO_3^- on the reaction S_2'--- S_3 (this process proceeds biphasically and may involve two or more reactions[10]). The half-time for this

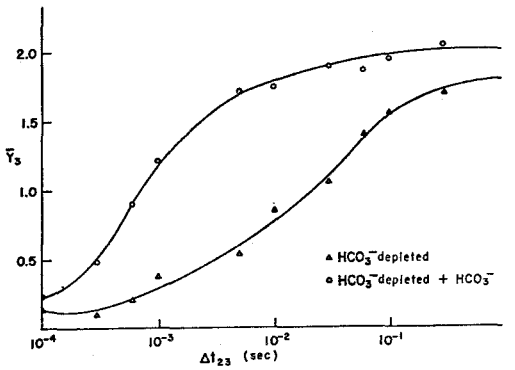

Fig. 2. Time course of the relaxation reaction $S_2' \longrightarrow S_3$; oxygen yield on the third flash, $\bar{Y}_3$, in the presence and absence of 0.01 M Na HCO_3 as a function of the time between the second and third flash (Δt_{23}). Y_3 is the yield normalized with respect to the steady state yield. Other flashes 1 sec apart. Other conditions as in Fig. 1 (after Stemler et al.[4]).

reaction is approximately 11 msec in HCO_3^- depleted chloroplasts and about 700 μsec in HCO_3^- depleted chloroplasts resupplied with HCO_3^-. Thus, HCO_3^- speeds the rate of this reaction, $S_2' \longrightarrow S_3$ by more than 10-fold. The rate of the reaction $S_1' \longrightarrow S_2$ was affected in a manner similar to that for $S_2' \longrightarrow S_3$ (not shown here). Again the half-time was approximately 10 msec in HCO_3^-depleted chloroplasts and about 600 μsec in chloroplasts resupplied with HCO_3^-. Likewise the rate of the reaction $S_0' \longrightarrow S_1$, calculated by the method of Bouges-Bocquet[10] is comparably reduced in HCO_3^- depleted chloroplasts (data not shown).

3. Effect of HCO_3^- on the Deactivation of the States S_2 and S_3. If the time between the first and second light flash, or between the second and third, is extended beyond about 1 sec, deactivation of the states S_2 or S_3, respectively, can be observed[11,12].

Figure 3 shows that the decay of the S_3 state in chloroplasts depleted of HCO_3^-, and those resupplied with HCO_3^-, follow the same time course, although there is a difference in the amount of O_2 evolved. Likewise the decay of the S_2 state (data not shown) is the same in the presence and absence of HCO_3^-. It is clear, therefore, that HCO_3^- has no effect on the stability of the higher oxidation states (i.e., S_2 and S_3) of the PS II positive charge accumulating system, but

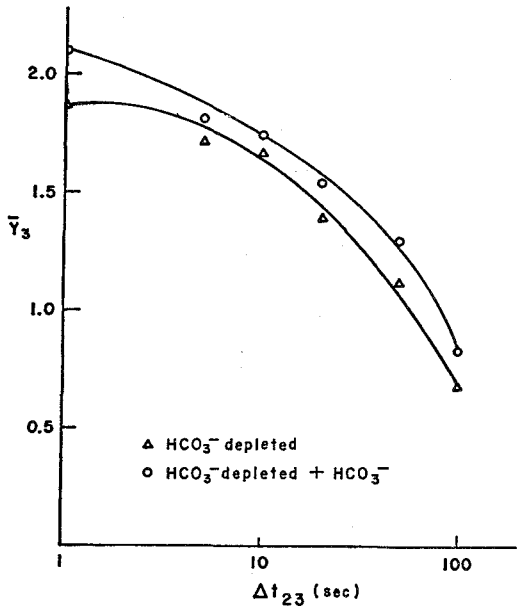

Fig. 3. Decay of the S_3 state; oxygen yield on the third flash $\bar{Y}_3$ in the presence and absence of 0.01 M Na HCO_3 as a function of the time between the second and third flash (Δt_{23}). Other conditions as in Fig. 2 (after Stemler et al.[4]).

it affects only on the rate of formation of these states following a photoact.

Discussion

The observation that HCO_3^- speeds about 10-fold the relaxation reactions between photoacts (Fig. 2) explains why less HCO_3^- stimulation is seen at low light intensity[4] and when saturating flashes are given spaced one second apart. Under these conditions the reaction centers have enough time to undergo relaxation (even at the lower rate imposed by HCO_3^- depletion) before another photon arrives. Thus HCO_3^- has less observed effect. The small stimulation in O_2 yield per flash (Fig. 1, top) that is still observed under these conditions must be due primarily to a greater number of active reaction centers and secondarily to the reduced number of "misses" that occur in the presence of HCO_3^-. Further quantitative analysis is needed to explain how the HCO_3^- effects reported here account for the 10-fold effect observed under saturating light intensities.

The ability of HCO_3^- to speed relaxation reactions can be interpreted to indicate either that this ion accelerates the reoxidation of the primary electron

acceptor for PS II (Q) by the pool of intersystem intermediates or that HCO_3^- is acting on the electron donor side of PS II. The first interpretation, as mentioned in the introduction, appears to be inconsistent with our previous work[1-3]. For example, if HCO_3^- depletion imposed a block between Q and A, one would predict that the chlorophyll a fluorescence transient would rise to maximum (F^∞) very fast, as it does in the presence of 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU). Although the fluorescence rise is, at first, faster, it slows down with time in the HCO_3^- depleted chloroplasts[2]. As HCO_3^- depletion may not cause a perfect block as does DCMU, new fluorescence experiments are being performed by T. Wydrzynski and Govindjee with different concentrations of DCMU to provide further data. Further suggestion that HCO_3^- may not be acting on the reducing side of PS II is provided by long-term delayed light emission, which is thought to reflect back reactions following light induced charge separation. If HCO_3^- accelerated the reoxidation of Q by A, less Q^- should be available to back react. Hence we would expect less delayed light emission in the presence of HCO_3^-. Instead, more delayed light emission is observed[2] in the presence of HCO_3^-. These results, the absence of an HCO_3^- effect when PS II electron flow is from DPC to DCPIP, and others already discussed[1-3] imply that HCO_3^- is acting on the electron donor side of PS II. However, since all our arguments which support this view are admittedly based on various assumptions, we plan to continue to test this hypothesis. New experiments are in progress in our laboratory (Urbana) to monitor the effect of HCO_3^- on membrane phenomena, on fluorescence decay after a flash of light, on the electron flow from H_2O to electron acceptors that may accept electrons directly from Q, on ΔpH, and on the $^{18}O/^{16}O$ ratio when chloroplasts are injected with $HC^{18}O_3^-$. Experiments are also planned to test the effect of HCO_3^- on the recovery of $Chla_2^+$ to $Chla_2$. Meanwhile it appears reasonable to consider how HCO_3^- may be influencing the oxygen evolving mechanism.

The kinetic model for oxygen evolution advanced by Kok et al.[5] (see ref. 13) can now be extended in several possible ways to include the action of HCO_3^-. Ignoring for our purposes the reducing (Q) side of PS II, we may represent a photoact II as: $Chla_2 \xrightarrow{h\nu} Chla_2^+$, where $Chla_2$ is the primary electron donor to Q[14]. $Chla_2^+$ undergoes reaction with the first secondary electron donor Z: $Chla_2^+ + Z \longrightarrow Chla_2 + Z^+$. Z^+ in turn undergoes the HCO_3^- mediated reaction: $Z^+ + S_n \xrightarrow{HCO_3^-} Z + S_{n+1}$, where S is the charge accumulating enzyme or system in the nth state (n = 0, 1 or 2) and $Z^+ + S_n$ corresponds to the S_n' state mentioned earlier in the results section. We can imagine that O_2 evolution occurs simply as: $Z^+ + S_3 \longrightarrow S_4$; $S_4 + O_2$ precursor(s) $\longrightarrow S_0 + O_2$. A second possibility is that Z^+ and S_3 cooperate as: $Z^+ + S_3 + O_2$ precursor(s) $\longrightarrow Z + S_0 + O_2$. In the above model, HCO_3^- may control the transfer of the first 3 electrons from the

positive charge accumulating mechanism to oxidized Z.

An alternative explanation of the HCO_3^- effect is also possible. In this second model Z is eliminated as an intermediate entirely, or rather it is equated with the positive charge accumulating system. Thus the reaction sequence can be written as: $Chla_2 \dashrightarrow Chla_2^+$; $Chla_2^+ + S \xrightarrow{HCO_3^-} Chla_2 + S_{n+1}$, where n is again equal to 0, 1 or 2. In this model HCO_3^- controls the rate of transfer of the first 3 electrons from the positive charge accumulating system (called S in the Kok et al. model) directly to the oxidized reaction center $Chla_2^+$. An entirely different view is to suggest that HCO_3^- accelerates electron flow from Q^- to the intersystem intermediate A. Experiments are in progress in our laboratory to decide between the models presented here.

Besides accelerating the relaxation reactions, HCO_3^- also reduces the number of misses which are apt to occur in reaction centers (Fig. 1). While these are clearly different effects, they are not necessarily independent. We propose that if the relaxation reactions following a photoact are accelerated by HCO_3^-, less time might be available for a back reaction of $Chla_2^+$ and Q^-. Such a back reaction, occurring in the msec time period or earlier after a flash could constitute a miss. It follows that this reaction would have less time to occur in the presence of HCO_3^-. If this is the case, we might expect greater amounts of delayed light emission (reflecting more misses) from HCO_3^- depleted systems in the msec and μsec time range following a flash.

The possible role of HCO_3^- on the actual O_2 evolving step must be seriously analyzed. Does it work as an allosteric effector on the O_2 evolving enzyme? New experiments are needed to understand how the absence of HCO_3^- inactivates reaction center II, increases "misses", and slows down the relaxation reactions of reaction center II -- all at the same time. Lastly, we wish to point out that the action of HCO_3^- described by us here and elsewhere[1-4,15] may have regulatory significance. When CO_2 (or HCO_3^-) is lacking in the microenvironment of chloroplasts so that they cannot manufacture carbohydrates, it would be unnecessary and perhaps harmful for the chloroplasts to produce the reducing power ($NADPH_2$) and ATP. Thus, nature may have evolved a system to "shut off" (or decrease drastically) the early reactions of photosynthesis -- the operation of reaction center II and consequently O_2 evolution. On the other hand, HCO_3^- may have some more direct role in the chemistry of O_2 evolution.

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