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A study of the specific effect of bicarbonate on photosynthetic electron transport in the presence of methyl viologen

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Summary

In view of the contradictory results in the literature regarding the specific role of HCO_3^- in photosynthetic electron flow, we have investigated the action of HCO_3^- in the Hill reaction supported by methyl viologen. (1) The $H_2O \rightarrow$ methyl viologen reaction under aerobic conditions exhibits a 6–7-fold stimulation following the addition of HCO_3^- to HCO_3^--depleted samples, in contrast to a previous conclusion obtained under anaerobic conditions (Fischer, K. and Metzner, H. (1981) Photobiochem. Photobiophys. *2*, 133–140). (2) Artificial electron donors (hydroxylamine and benzidine) to Photosystem (PS) II exhibit a rate-limiting step of donation of the order of the rate-limiting step due to HCO_3^--depletion. However, the oxidation of the primary quinone acceptor (Q_A) of PS II is dramatically inhibited in these artificial donor systems. (3) A fully reversible HCO_3^- effect on the oxidation of Q_A^- occurs even when the formate anion, previously regarded as an essential factor for the HCO_3^- effect to be observed, is omitted from both the depletion and reaction media. (4) The dibromothymoquinone-sensitive partial reaction, duroquinol $\rightarrow$ methyl viologen, is insensitive to HCO_3^-, thus firmly indicating that the site of HCO_3^- action is *before* the site of plastoquinol oxidation. (5) The rate-limiting step introduced by HCO_3^--depletion under our experimental conditions is of the order of 30 ms per electron, in contrast to ~ 5 ms in control and HCO_3^--restored samples, in

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Abbreviations: Chl, chlorophyll; MV, methyl viologen; PQH_2, plastoquinol; PS I, Photosystem I; PS II, Photosystem II; Q_A, primary quinone acceptor of PS II; Q_B, secondary quinone acceptor of PS II.

the steady-state. Contrary to a previous claim that the major site of HCO_3^- action was on the donor side of PS II, our findings are consistent with the idea that the major site of action in the Hill reaction supported by methyl viologen is at the level of the exchange reactions of plastoquinone and plastoquinol at the two-electron gate between PS II and PS I. This work also demonstrates that there is a specific HCO_3^- effect, which is not due to the presence of formate in the reaction medium.

bicarbonate effect; fluorescence; formate; methyl viologen; oxygen evolution; Photosystem II

Introduction

Bicarbonate (HCO_3^-) is believed to be an activator of Photosystem II (PS II) [1,2].* A site of HCO_3^- action has been established at the level of reoxidation of Q_A^- [3,4], where Q_A is the primary quinone electron acceptor of PS II. However, a major inhibitory effect of HCO_3^--depletion may be to retard the plastoquinone/plastoquinol exchange reactions at the two-electron gate involving a secondary quinone electron acceptor Q_B [5–8].

Measurements of ferricyanide-supported oxygen evolution, in formate-containing thylakoid membranes, have been used routinely to observe an 8–10-fold stimulation of electron transport rates following the addition of millimolar amounts of HCO_3^- to previously HCO_3^--depleted thylakoids [see, e.g., 9,10]. Using methyl viologen as the electron acceptor in an anaerobic system, it was, however, reported [10] that the addition of HCO_3^- to HCO_3^--depleted thylakoids was able to produce only a 2-fold stimulation. This observation, coupled with other similar observations using artificial electron donors, has been used as evidence for an additional PS II donor side site of action for HCO_3^- [10]. Given the possibility of an involvement of HCO_3^- in the chemistry of oxygen evolution [11] and that such measurements are quite incompatible with the existing HCO_3^- literature [1,2] we undertook a further study of the stimulation of the $H_2O \rightarrow$ methyl viologen reaction by HCO_3^-.

Materials and Methods

Thylakoid membranes were isolated from spinach leaves by first grinding leaf segments in a medium containing 400 mM sorbitol, 50 mM NaCl, 1 mM EDTA (ethylenediaminetetraacetic acid) and 50 mM Tricine (pH 7.8) for 10 s in a Waring blendor. The resultant homogenate was filtered through 4 and then 12 layers of cheesecloth. The filtrate was then spun at $5000 \times g$ for 45 s and the supernatant collected and spun at $5000 \times g$ for 10 min. After discarding the last supernatant, the pellet was resuspended and osmotically shocked in a medium containing 50 mM

* As the active species is still unknown this paper does not distinguish between CO_2 and HCO_3^-.

NaCl, 5 mM $MgCl_2$ and 10 mM Tricine (pH 7.8). The thylakoids were then again spun at 5000 × *g* for 10 min and the pellet resuspended in 300 M sorbitol, 10 mM NaCl, 5 mM $MgCl_2$ and 10 mM Tricine (pH 7.8) at a concentration of 2.0 mg Chl/ml. Chlorophyll (Chl) concentrations were determined as described by Arnon [12]. Where used, pea thylakoid suspensions were prepared as in [13].

HCO_3^--depletion was performed by a method developed by J.F.H. Snel, W.F.J. Vermaas and J.J.S. van Rensen (personal communication). Thylakoids were suspended in 300 mM sorbitol, 25 mM sodium formate, 10 mM NaCl, 5 mM $MgCl_2$, 10 mM sodium phosphate (pH 5.8) and at a Chl concentration of 250 μg/ml. The thylakoids were then immediately collected by centrifugation at 2500 × *g* for 10 min and then depleted of HCO_3^- by resuspension, at a [Chl] of 250 μg/ml, in the above medium under a constant stream of N_2 gas. The gas was passed over the solution for 60–90 min. The above medium, called the depletion medium, was boiled and allowed to cool for at least 30 min before each experiment while being constantly bubbled with N_2 gas to remove HCO_3^-.

The reaction medium contained 300 mM sorbitol, 10 mM NaCl, 5 mM $MgCl_2$, 25 mM sodium phosphate (pH 6.5), 0.25 mM methyl viologen (MV), 1 mM sodium azide and 200 units/ml of superoxide dismutase. To obtain maximum rates of electron flow and to avoid complications due to the possible effects of HCO_3^- on photophosphorylation [14], 10 mM ammonium chloride and 0.01 μM gramicidin D were also present to uncouple the electron flow from photophosphorylation (see [15]). HCO_3^- was removed from the assay medium by bubbling with either N_2 gas or a mixture of N_2(80%) and O_2(20%) for 45 min prior to any measurement. All gasses for the removal of HCO_3^- were passed through a column of soda-lime and ascarite to facilitate the removal of any trace of CO_2, and through a water column to prevent evaporation of the sample.

When experiments were conducted on the HCO_3^- effect in the absence of formate, the latter was omitted from both the depletion and reaction media.

Steady-state measurements were made with a Clark-type oxygen electrode (Hansatech, U.K.). Saturating orange light was obtained from white light supplied by a high-intensity slide projector (Kodak Carrousel 4200 projector) which was passed through a 2 mm thick Corning colored glass filter, CS3-68, and a 2-inch water filter containing 1% $CuSO_4$. The intensity of the visible light (400–700 nm) on the surface of the temperature-controlled, water-jacketed electrode assembly was 225 $mW \cdot cm^{-2}$ as measured by a Lambda Instruments LI-185 radiometer. The reaction volume (1.25 ml) was comprised of 100 μl of HCO_3^--depleted thylakoids added to 1.15 ml of the reaction medium. Thus, the thylakoid suspension in the reaction chamber was at a Chl concentration of 20 μg/ml. The O_2 evolution was recorded on an Esterline-Angus recorder (model E1101S). This apparatus was calibrated by adding sodium dithionite crystals to stirred water in the reaction chamber as described in [16]. The reaction chamber was flushed with N_2 or the 80% N_2/20% O_2 mixture before each measurement.

Measurements of the decay of Q_A^- were made using the double-flash method monitoring the decay of Chl *a* variable fluorescence. The instrument and the specific technique have already been described [8,13].

Addition of 10 mM HCO_3^- to depleted thylakoids was made in the dark and followed by an incubation for 2 min as described earlier [9]. Hydroxylamine [17–19] and benzidine [20,21] were used as electron donors to PS II. Details regarding the electron donor systems are given in the appropriate legends.

Results and Discussion

Steady-state electron transport from water to methyl viologen

Electron flow from $H_2O \rightarrow$ methyl viologen was measured, as O_2 uptake, by the Clark electrode. The stimulation of electron transport rates in HCO_3^--depleted thylakoids containing 25 mM formate, following an addition of 10 mM HCO_3^-, is depicted in Fig. 1. These data were obtained under both anaerobic (Fig. 1a) and aerobic (Fig. 1b) conditions. When HCO_3^- had been removed in the reaction medium by bubbling only with N_2 gas, regeneration of the $H_2O \rightarrow$ methyl viologen reaction was severely inhibited. This is in agreement with the report of Fischer and Metzner [10] where argon gas was used to keep the reaction medium HCO_3^--free. It was under these conditions that the low (i.e., only 2-fold) maximal stimulation of electron flow by HCO_3^- was inferred from a plot of the methyl viologen Hill reaction as a function of the concentration of added HCO_3^-. Under our anaerobic conditions, we were able to reproduce the published observations (data not shown). However, a quite different result was obtained when we removed HCO_3^- from our reaction medium using a mixture of 80% N_2 and 20% O_2. Under these aerobic conditions, regeneration of electron transport rates following the addition of 10 mM HCO_3^-

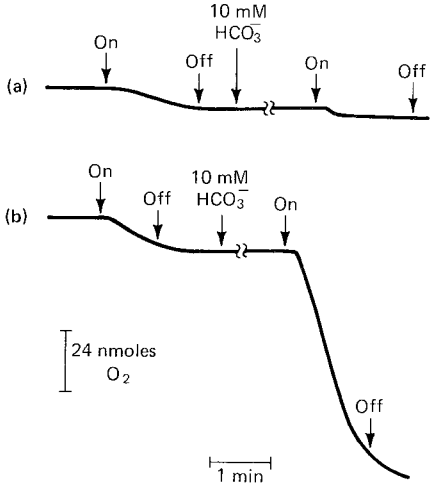

Fig. 1. Bicarbonate stimulation of photosynthetic electron transport supported by methyl viologen. (a) Anaerobic conditions (initial rate, in μequiv./mg chlorophyll per h, is 100, and after the addition of 10 mM HCO_3^- it cannot be measured); (b) aerobic conditions (initial rate, in μequiv./mg chlorophyll per h, is 145 and after the addition of 10 mM HCO_3^-, 1074). The spinach thylakoids used in this experiment had been stored in liquid nitrogen. For other details see Materials and Methods.

resulted in a 7-fold stimulation. In fact, as can be seen from Table I, the HCO_3^--restored rate in the presence of methyl viologen was identical to that observed for non-depleted thylakoids measured in the same reaction medium. Thus, when there is no rate-limitation due to the experimental conditions used, a normal HCO_3^- effect is indeed observed in the $H_2O \rightarrow$ methyl viologen electron flow. The argument [10] for a HCO_3^- effect on the electron donor side of PS II, based on a comparison of this effect with ferricyanide from that with methyl viologen as electron acceptor, cannot be sustained.

Steady-state electron transport to methyl viologen from artificial electron donors

As noted in the Introduction, there is now considerable evidence that HCO_3^--depletion in the presence of formate results in a rate-limiting step at the level of the plastoquinone/plastoquinol exchange reactions with the plastoquinone pool [5–8]. However, there exists considerable interest in a possible site of HCO_3^- action on the donor side of PS II [11]. Fischer and Metzner [10] found that in the presence of artificial electron donors (notably, hydroxylamine), addition of HCO_3^- to HCO_3^--depleted samples produced only a slight stimulation of photosynthetic electron transport. This observation was suggested to support the claim that the major site of HCO_3^- action was before the site of hydroxylamine donation.

Table I shows the results obtained for three artificial electron donor systems using our aerobic conditions. Although it is clear in the cases of hydroxylamine and benzidine (both PS II donors) that the addition of HCO_3^- produces little or no stimulation in electron transport rates, the key observation is contained in the electron transport rates for the non-depleted controls. Using artificial electron donors to PS II, the electron transport rates in non-depleted controls are not significantly different from those obtained in HCO_3^--depleted samples. An identical result was also obtained when we used catechol as an electron donor (data not shown). Thus, the rate-limiting step in our electron transport systems employing artificial donors to PS II is of the order of the rate-limiting step introduced by

TABLE I

EFFECT OF BICARBONATE ON VARIOUS ELECTRON TRANSPORT SYSTEMS SUPPORTED BY METHYL VIOLOGEN

In (2) 15 mM hydroxylamine was used. In (3) the thylakoids were treated in 0.8 M Tris (pH 8.0) at 250 μg chlorophyll/ml for 30 min on ice in diffuse room light; the donor system had 1 mM benzidine and 1 mM ascorbate. In (4) 1 mM duroquinol, prepared as in [15], and 5 μM DCMU (3-(3,4-dichlorophenyl)1,1-dimethylurea) were also present. Data are average values of three measurements. Other experimental conditions are as described in Materials and Methods.

System	Electron transport (μequiv./mg chlorophyll per h)		
	$-HCO_3^-$	+10 mM HCO_3^-	Control
(1) $H_2O \rightarrow$ MV	210 ± 14	1455 ± 7	1428 ± 20
(2) Hydroxylamine → MV	112 ± 8	118 ± 6	128 ± 8
(3) Benzidine → MV	128 ± 2	223 ± 5	222 ± 5
(4) Duroquinol → MV	1656 ± 9	1662 ± 17	1657 ± 18

HCO_3^--depletion and therefore no further conclusions can be drawn regarding the site of HCO_3^- action from this approach.

On the other hand, Fig. 2 demonstrates the existence of the HCO_3^--dependent site on the acceptor side of PS II when electrons are supplied by the artificial donor hydroxylamine. These data show the decay of Q_A^-, as monitored by the decay of chlorophyll *a* variable fluorescence yield, after the third of a series of actinic flashes spaced 1 s apart. The half-time for the HCO_3^--depleted sample is ~ 10 ms and for the HCO_3^--resupplied sample the half-time is ~ 350 μs, which is the same as for the non-depleted control thylakoids. The half-time for this decay with the intact oxygen-evolving system (with water acting as the natural donor), under the experimental conditions used here, has recently been reported to be ~ 350 μs [8]. Thus, there is a complete agreement for these decays whether H_2O or hydroxylamine is the electron donor to PS II.

Table I also shows that the partial reaction of duroquinol (tetramethyl-*p*-hydroquinol) → methyl viologen is insensitive to HCO_3^-. Here, there is no rate-limitation in the electron donation by duroquinol. This result has been obtained independently by S. Izawa (personal communication). It has already been shown, using reduced diaminodurene, that PS I is unaffected by HCO_3^--depletion [22]. However, duroquinol donates at the level of the plastoquinone pool and in a dibromothymoquinone (DBMIB)-sensitive fashion [23]. Therefore, the insensitivity of this reaction to HCO_3^--depletion suggests that the site of HCO_3^- action is before the plastoquinol oxidation on the plastoquinol-plastocyanin oxidoreductase. It has already been shown that the H_2O → silicomolybdate partial reaction and the H_2O → ferricyanide reaction, following a treatment with trypsin that enables ferricyanide to

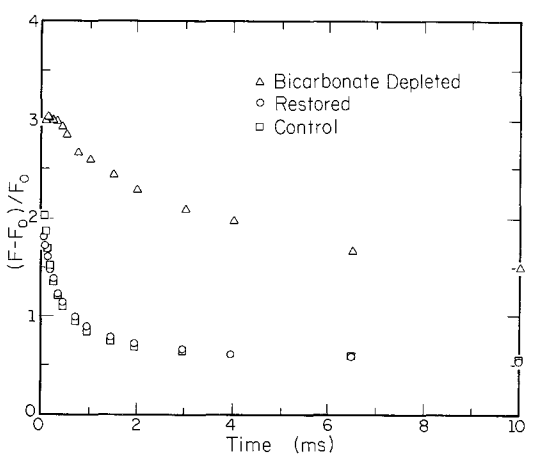

Fig. 2. Decay of the variable chlorophyll *a* fluorescence after the third actinic flash in a series of flashes spaced 1 s apart with 15 mM hydroxylamine acting as the electron donor to PS II. F_0 is the chlorophyll *a* fluorescence yield from the measuring flash when all Q_A is oxidized, and F is the yield at the indicated time after the actinic flash. The reaction medium (containing formate; see Materials and Methods) was supplemented with 0.1 mM methyl viologen, and 0.1 μM gramicidin D. Half-times were determined as in [8].

accept electrons directly from Q_A^-, are both unaffected by HCO_3^--depletion [22,24]. Therefore the result with duroquinol confirms the notion that the site of HCO_3^- action is at the level of the plastoquinone/plastoquinol exchange reactions on the quinone acceptor complex.

The bicarbonate effect in the absence of formate

It has recently been claimed [25–27] that the HCO_3^- effect in formate-containing thylakoids does not result from the removal of HCO_3^- from the system, but from the presence of the formate anion. That is, the restoration of control levels of electron transport in HCO_3^--resupplied thylakoids only arises from HCO_3^- displacing the inhibitory formate from an anion binding site. Fig. 3 demonstrates that this is not the case. Here, the decay of Chl *a* variable fluorescence after the third of a series of actinic flashes spaced 1 s apart is again shown, but formate was omitted from both the depletion medium and the reaction medium. A fully reversible HCO_3^- effect is demonstrated. The possibility that other anions in our experimental media may have produced this inhibition can be discounted. The chloride concentration used here is optimal for PS II activity [28,29], while a study of the effects of various buffers found that no inhibition could be attributed to the phosphate buffer system (Robinson and Eaton-Rye, unpublished observations). As in the formate-containing system [6,8] we obtained maximal inhibition after the third flash. However, in the absence of formate we found that the overall half-time of Q_A^- reoxidation after the third and subsequent actinic flashes remained in the range of 2.0–2.5 ms. This compares with an overall half-time of ~ 10–12 ms in the formate-containing system

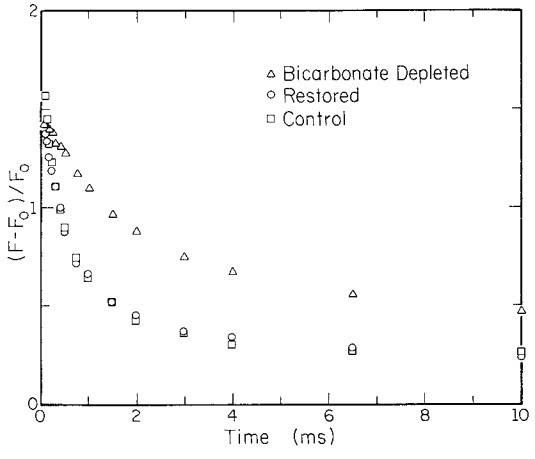

Fig. 3. Decay of variable chlorophyll *a* fluorescence, in formate-free samples, after the third actinic flash in a series of flashes spaced 1 s apart with water acting as the natural electron donor to PS II. F_0 is the chlorophyll *a* fluorescence yield from the measuring flash when all Q_A is oxidized, and F is the yield at the indicated time after the actinic flash. Pea thylakoids were used in this experiment and prepared as described in [13]. Formate was omitted from both the depletion and reaction media (see Materials and Methods). The reaction medium was supplemented with 0.1 mM methyl viologen, and 0.1 μM gramicidin D. Half-times were determined as in [8].

[8]. A binding constant of 80 μM for HCO_3^- has recently been reported [26]. As noted by these authors, this would suggest that the HCO_3^- effect may not be physiological. However, the ease and effectiveness of HCO_3^--depletion is highly dependent on experimental conditions [c.f. 4,8,26,30] and the in vivo HCO_3^- binding constant may not be as large as 80 μM.

A study of the HCO_3^- effect in the absence of formate is in progress in our laboratory and will appear as a separate publication.

Rate-limitation in HCO_3^--depleted and restored samples

If we assume a molecular weight of 1000 for Chl, a photosynthetic unit size of 600 Chl molecules per reaction center II [31], and an average value of 1400 μequiv./mg chlorophyll per h for our steady-state rates of electron transport in non-depleted and HCO_3^--resupplied thylakoids (Table I), then we obtain an average rate of transport of 200 electrons per reaction center per second for our methyl viologen system. This suggests the rate-limiting step in our HCO_3^--resupplied samples and controls is of the order of 4 or 5 ms per electron which is in agreement with the data of Whitmarsh and Cramer [32] on control thylakoids. These calculations do not, however, take into account any heterogeneity in PS II [see e.g., 33]. However, accepting an average rate of 200 μequiv./mg chlorophyll per h (Table I) as the rate of electron transport in depleted thylakoids, the above assumptions would predict that the rate-limiting step as a result of HCO_3^--depletion is of the order of 30 ms per electron.

These calculations should not cause earlier measurements of the decay of Chl *a* variable fluorescence [6] and of plastoquinol oxidation [5], indicating a $t_{1/2}$ of the order of 200 ms for plastoquinol oxidation in HCO_3^--depleted samples, to be discounted. As noted in [8] the overall half-times for the oxidation of Q_A^- reported here yield several exponential decay components upon analysis. Previous experimental conditions may have resulted in changes being measured for a component with a half-time of the order of 200 ms [see 8]. Additionally, control measurements for the ferricyanide-supported Hill reaction in earlier studies [see e.g. 9,22] resulted in rates 7–10-fold lower than reported here. Thus, a $t_{1/2}$ of ~ 200 ms [5] and of ~ 120–160 ms [6] for the rate-limiting step in HCO_3-depleted thylakoids reported under different experimental conditions (such as [5,6,9,22]), is qualitatively very similar to a value of $t_{1/2}$ ~ 30 ms reported here for the steady-state and a $t_{1/2}$ of ~ 10–12 ms for the oxidation of Q_A^-. The variability in our values for the overall half-time of the rate-limitation introduced by HCO_3^--depletion must depend, in part, on the assumptions chosen for our steady-state estimation and also the very different nature of the two measurements. For example, our measurements of Q_A^- oxidation were made with an oxidized plastoquinone pool. Therefore, one would expect a higher probability for a plastoquinone to occupy the Q_B binding site in this measurement than in the steady-state.

Concluding remarks

The Chl *a* fluorescence decays presented here (Figs. 2 and 3) and in [8] are clearly biphasic. We suggest that the observed large slow component in HCO_3^--depleted

samples results from an altered equilibrium of Q_A^- with plastoquinone and/or plastoquinol at the Q_B binding site on the quinone acceptor complex of PS II. This conclusion is consistent with our hypothesis that removal of HCO_3^- results in a retardation of the plastoquinone/plastoquinol exchange reactions of the two-electron gate. We suggest that this may be a consequence of altering the association constants for one or more of the plastoquinone/plastoquinol species and/or by affecting the protonation reactions of the partially reduced plastosemiquinone anion or the doubly reduced plastoquinol. Thus, dark adaptation prior to the first flash may enable a large number of centers in HCO_3^--depleted thylakoids to bind plastoquinone for electron transfer from Q_A^-, and consequently it is not until the first full turnover of the two-electron gate that the full effect of HCO_3^--depletion can be observed. This would explain the observation [6,8] that the decay of Chl *a* variable fluorescence after the first actinic flash is less inhibited than after the third and subsequent actinic flashes, but intermediate after the second flash.

In addition to the biphasic decays discussed above additional Chl *a* fluorescence decay components exist in the 0.1–10 s range [4,8]. These components may reflect the population of longer-lived Q_A^- reducing as yet unspecified chemical species in the membrane, or particularly in the case of components in the > 1 s range, centers undergoing a back reaction.

Finally, we conclude from this study that: (i) under the experimental conditions used, HCO_3^--depletion increases the $t_{1/2}$ of the bottleneck reaction from ~ 4 ms to ~ 30 ms per electron transferred, (ii) our results are consistent with the concept that this rate-limiting step, introduced by HCO_3^--depletion, is at the level of the plastoquinone/plastoquinol exchange reactions and not plastoquinol oxidation, and (iii) that we are unable to find any evidence to support an additional site of HCO_3^- action on the donor side of PS II, or to sustain the notion that all of the HCO_3^- effect is simply a reversal of formate inhibition. The present authors, however, do not have any comment on the mass spectrometric data of Metzner and coworkers [34] which suggest that bicarbonate may function on the water oxidation process of photosynthesis (see also Ref. 11).

Acknowledgements

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