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INHIBITION OF PHOTOSYSTEM II BY FORMATE

POSSIBLE EVIDENCE FOR A DIRECT ROLE OF BICARBONATE IN PHOTOSYNTHETIC OXYGEN EVOLUTION

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Summary

In broken chloroplasts the presence of 100 mM sodium formate at pH 8.2 will specifically lengthen the Photosystem II relaxation times of the reactions $S_2' \rightarrow S_3$ and $S_3' \rightarrow S_0$. Rates of reactions $S_0' \rightarrow S_1$ and $S_1' \rightarrow S_2$ remain unaffected. Evidence is presented which indicates the discrimination among S-states by formate cannot be attributed to a block imposed on the reducing side of Photosystem II. The results are interpreted in context of the known interaction of formate and CO_2 which is bound to the Photosystem II reaction center complex. It is proposed that those S-state transitions which show extended relaxation times in the presence of formate must result in the momentary release and rebinding of CO_2. Furthermore since formate is acting on the oxygen-evolving side of Photosystem II, it would seem that CO_2 is released in reactions that occur there. A chemical model of oxygen evolution is presented. It is based on the hypothesis that hydrated CO_2 is the immediate source of photosynthetically evolved oxygen and explains why, under certain conditions formate slows only the S-state transitions $S_2' \rightarrow S_3$ and $S_3' \rightarrow S_0$.

Introduction

The small amount of carbon dioxide (HCO_3^-) which is required for Photosystem II activity [1] is bound to the reaction center complex [2]. The ligand appears alternately hydrated in a 'dark' reaction and then light dehydrated [3].

Abbreviation: PS II, Photosystem II.

One hypothesis formulated to explain this cyclic behavior is that hydrated CO_2 is the immediate source of photosynthetically evolved O_2.

An important tool in the study of the HCO_3^--effect has been the use of high (100 mM) concentrations of formate and acetate. These ions can retard both the binding of exogenous $^{14}CO_2$ [3] and the rebinding of light-released endogenous CO_2 [3,4]. The present study makes use of high formate concentrations and flashing light to determine which light driven reactions result in the release of bound CO_2. Results indicate that CO_2 is released by reactions which occur on the oxygen-evolving side of Photosystem II.

Materials and Methods

Maize (*Zea mays* L.) plants were grown and chloroplasts isolated from them as described previously [3]. Only chloroplasts ruptured by osmotic shock during isolation were used in these experiments. It should be noted that in these studies, the grana were not washed to remove endogenously bound HCO_3^- [2] before experiments were carried out. Therefore, the effects of formate on 'normal' chloroplasts were observed.

The apparatus used to measure oxygen evolution in response to light flashes was similar to that described by Joliot and Joliot [5]. Signals were recorded on a Hewlett-Packard oscillographic recorder (model 74024). Flash illumination was from 2 Xenon lamps (General Radio Stroboslave types 15 39-A) which could be triggered sequentially with variable delay time. Light was focused with large condensing lenses. Each lamp was tested individually to assure light saturation. Inserting a Balzers 80 percent transmission neutral density filter between the lamp and the sample caused no change in the oscillatory pattern or in the steady-state flash yield of oxygen.

Rates of dark relaxation or 'turnover' reactions which occur between photoacts can be determined by varying the time between two light flashes and measuring the effect on the final yield of O_2. The methods and notation used were described in detail by Bouges-Bocquet [6]. In addition, small corrections for double hits which gave a yield on the second flash were carried out. Data were normalized to the same additive yield of flashes 20—25. Charge accumulation parameters, misses, single and double hits as well as initial S-state populations (S_0/S_1) were estimated with the matrix multiplication technique described by Thibault [7,8].

Before chloroplasts were placed on the platinum electrode, they were suspended in reaction mixture which contained 0.05 M Tricine buffer, pH 8.2 and either 0.2 M NaCl for the controls or 0.1 M NaCl plus 0.1 M sodium formate for the experimental samples. The chlorophyll concentration was approx. 0.25 $mg \cdot ml^{-1}$. Samples were placed on the electrode and dark-adapted for 15 min. The time permitted both settling of the chloroplasts and equilibration of chloride and formate across the thylakoid membrane. The high pH, 8.2, is of critical importance in these experiments. Such high pH insures that the inorganic carbon bound to the PS II center will, in the dark, have a >98 percent probability at any given time of being in the form of HCO_3^- (see Ref. 3 for the influence of pH on CO_2/HCO_3^- binding). As HCO_3^-, the ligand cannot be rapidly removed from the reaction center and formate cannot, therefore, have any

effect. Only when the ligand is dehydrated in the light will it take the form of CO_2 and be momentarily liberated. In this form, ions such as formate, acetate or even bicarbonate [3] can interfere with rebinding. Thus with the use of high pH, light reactions which convert HCO_3^- to CO_2 can be distinguished. At low pH, on the other hand, the CO_2-HCO_3^- equilibrium is shifted toward CO_2 in the dark and the effect of light alone is thus obscured.

Results

Oxygen evolution in flashing light with and without formate

When chloroplasts are given saturating flashes of light after a 10 min dark period, they evolve oxygen as shown in Fig. 1. The observed oscillations in oxygen yield are typical of those which formed the basis for the current four-step model for O_2 evolution [9]. There are no obvious pattern differences in the normalized yields with or without 0.1 M formate.

The data presented in Fig. 1 was used to calculate the transition parameters which determine the observed yields. These are shown in Table I. No significant differences occur in the frequency of misses, single or double hits with or without formate (lines 1, 2 and 3). Formate may cause a slight change in the initial relative populations of S_0 and S_1 (lines 4 and 5). In addition, the steady-state yields were reduced about 23 percent in the presence of formate (line 6). Probably under these conditions formate can remove this percentage of bound CO_2/HCO_3^- in the dark-adaptation period. Differential S-state susceptibility of bound CO_2/HCO_3^- to removal by formate may account for the apparent shift in the S_0 and S_1 populations. This possibility requires further study. The primary conclusion to be drawn from these data is that the presence of formate causes

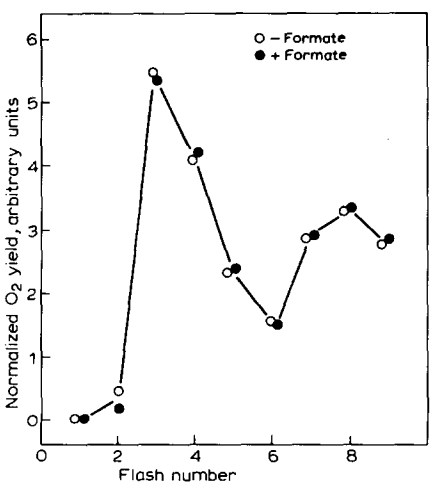


Fig. 1. Initial flash yields of oxygen from maize chloroplasts with or without formate. Reaction mixtures contained 0.05 M Tricine, pH 8.2, and either 0.2 M NaCl or 0.1 M NaCl plus 0.1 M sodium formate. The chloroplasts were dark adapted for 10 ms before measurements were taken. Each point represents an average of six measurements. Calculated standard errors fell within the point markers. Measurements were at room temperature. The time between flashes was 1.2 s.

TABLE I

OXYGEN EVOLUTION TRANSITION PARAMETERS FOR CHLOROPLASTS WITH OR WITHOUT FORMATE

Calculated values were derived (see Methods) from the oxygen flash-yield patterns shown in Fig. 1. Relative steady-state yields were each derived from 25 separate measurements.

Parameter	Without formate	With formate
Misses	0.171 ± 0.009	0.169 ± 0.011
Single hits	0.788 ± 0.007	0.786 ± 0.009
Double hits	0.041 ± 0.003	0.045 ± 0.005
S_0 (0)	0.25 ± 0.02	0.29 ± 0.04
S_1 (0)	0.75 ± 0.02	0.71 ± 0.06
Steady-state yield, arb. units	100 ± 3.13	77.3 ± 2.3

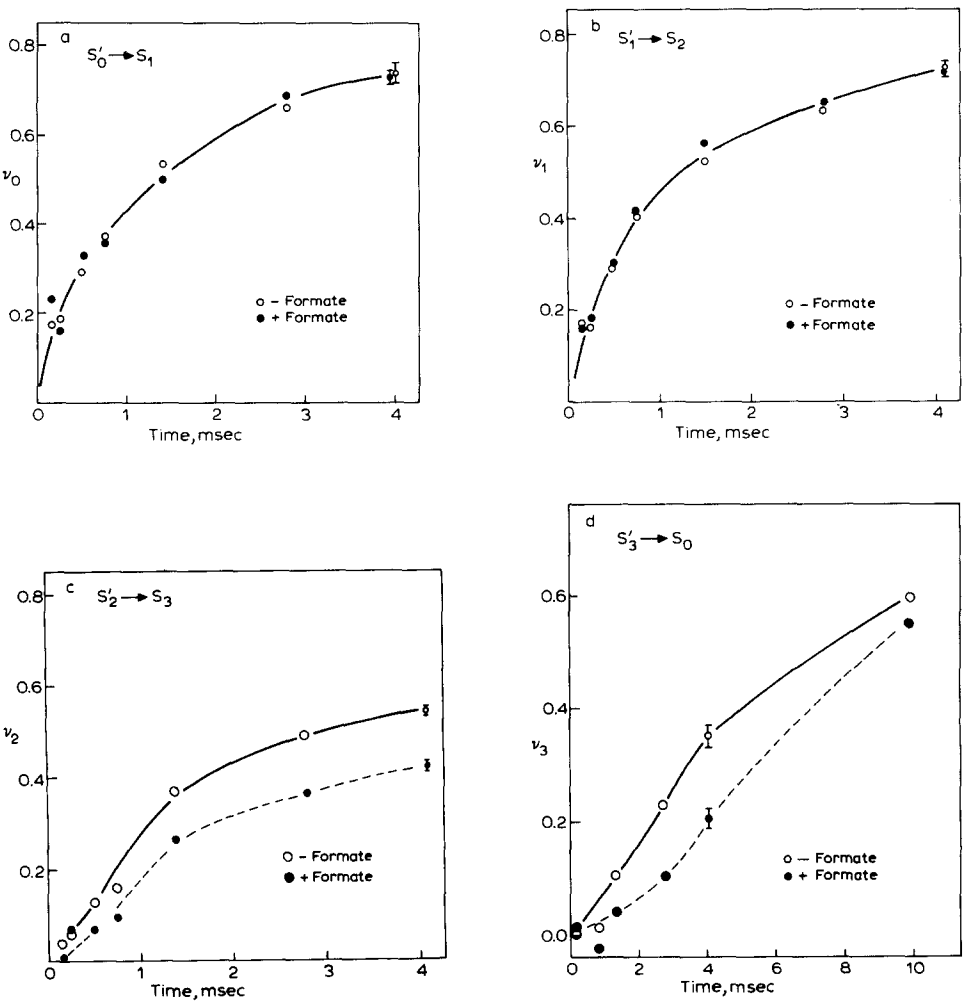


Fig. 2. Turnover times in maize chloroplasts with or without formate. Reaction mixtures were described in Fig. 1. Protocol for measurements and calculations were as described by Bouges-Bocquet [6]. Error bars for each of the 4 ms points were calculated from five separate measurements.

no change in the oscillatory pattern of oxygen evolution nor a dramatic change in any of the transition parameters.

Relaxation rates, $S'_n \rightarrow S_{n+1}$ with and without formate

$S'_0 \rightarrow S_1$. In chloroplasts the extent of the reaction $S'_0 \rightarrow S_1$ with time, denoted γ_0, is shown in Fig. 2a. Substituting 0.1 M sodium formate for an equal amount of NaCl causes no detectable difference in the rate of this reaction.

$S'_1 \rightarrow S_2$. The presence of formate causes no change in the rate of the $S'_1 \rightarrow S_2$ reaction (Fig. 2b). Clearly the first two S-state transitions are unaffected by this ion.

$S'_2 \rightarrow S_3$. The rate of the recovery reaction $S'_2 \rightarrow S_3$ is measurably slower in the presence of formate compared to formate-free controls (Fig. 2c). In the absence of formate, 54 percent of the reaction centers make the transition within 4 ms compared to 43 percent in the presence of formate.

$S'_3 \rightarrow S_0$. According to the current model of oxygen evolution, the reaction $S'_3 \rightarrow S_0$ results in the evolution of an oxygen molecule and a return of the system to its initial state. The rate of this reaction, or sequence of reactions, is found as with the $S'_2 \rightarrow S_3$ to be slower in the presence of formate compared to the control (Fig. 2d). Whereas in the control 35 percent of the reaction centers make the $S'_3 \rightarrow S_0$ transition in 4 ms, only 20 percent do so when formate is present.

Relaxation rates $S'_n \rightarrow S_{n+1}$ at steady state with and without formate

The foregoing reaction rates were measured by varying the time between either flashes 1-2, 2-3 or 3-4. It is possible that transient effects which occur on the reducing side of PS II can account for the slower recovery rates after the second and third flash. If so, discrimination among S-states by formate should disappear at steady-state. That this is not the case is shown by the following experiment: Chloroplasts were given flashes until steady-state oxygen yields were achieved (>30 flashes). A double flash was then given with a Δt of 4 ms and single flashes thereafter at 1.2 s intervals. The double flash introduces a perturbation from steady-state which continues in the following single flash yields. The yields will reflect the relaxation rates of all S'-states in the 4 ms time interval between the double flash. The perturbations seen with and without formate are compared in Fig. 3.

When a double flash is given to chloroplasts, the total yield (1 + 2) is seen as a single spike on the chart paper since Δt, 4 ms, is near the response time of the instrument. The total yield is the sum of reactions $S'_3 \rightarrow S_0$ induced by the first flash of the doublet plus the $S'_2 \rightarrow S_3$ reactions completed in the interval between flashes (1 + 2). Double hits and misses contribute to the total yield but these are assumed to be the same in the presence and absence of formate (Table I) and can be discounted for the purpose of comparison here. It is clear from the data presented in Fig. 3 that the yield of a double flash (1 + 2) is greater in the absence of formate than in its presence. With formate, fewer $S'_2 \rightarrow S_3$ transitions occurred in 4 ms, consistent with the results shown in Fig. 2c.

The yield of flash 3 will reflect those reaction centers which complete an

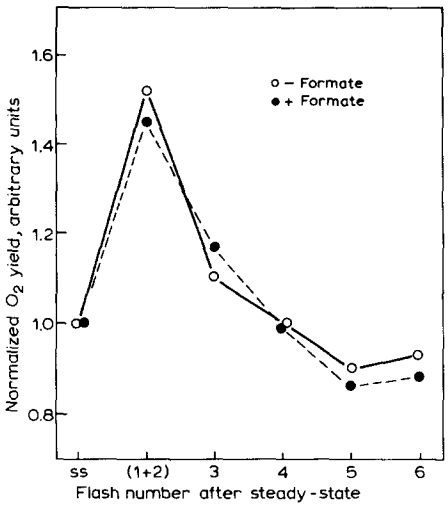


Fig. 3. Double-flash perturbation of steady-state oxygen yields from chloroplasts with or without formate. Light flashes were given at 1.2 s intervals until all oscillations in O_2 yield were damped (>30 flashes). A double flash (1 + 2) was applied, Δt 4 ms, and single flashes thereafter. Reaction mixtures were described in Fig. 1. Each point represents an average of at least twelve measurements. Calculated standard errors fell within the point markers. Data were all normalized to the steady-state flash yield just before the double flash.

$S_1' \rightarrow S_2$ transition between flashes (1 + 2) plus those centers failing to complete an $S_2' \rightarrow S_3$ transition during that time. Compared to the control, the O_2 yield of flash 3 in the presence of formate is enriched by exactly the amount that the (1 + 2) yield is reduced. The greater number of centers failing to complete an $S_2' \rightarrow S_3$ transition between flashes (1 + 2) in the presence of formate enhance the yield of flash 3. By implication, the transition $S_1' \rightarrow S_2$ must have occurred at the same rate with or without formate.

The yield of flash 4 is a function of those reaction centers completing an $S_0' \rightarrow S_1$ transition between flashes (1 + 2) plus those centers failing to complete an $S_1' \rightarrow S_2$ transition during that time. The yield of flash 4 is the same with or without formate which indicates, like Fig. 2a and b, that the rates of these reactions are independent of this ion.

The yield of flash 5 is a function of those reaction centers completing an $S_3' \rightarrow S_0$ transition between flashes (1 + 2) plus those centers which fail to complete an $S_0' \rightarrow S_1$ transition during that time. The yield is lower in the presence of formate compared to the control. Since the rate of $S_0' \rightarrow S_1$ must be the same with and without formate (to explain the flash 4 yield) it follows that the rate $S_3' \rightarrow S_0$ was slower in the presence of formate.

The data presented in Fig. 3 indicate clearly that even under steady-state flash conditions, formate can discriminate among S-states. The rates of reactions $S_2' \rightarrow S_3$ and $S_3' \rightarrow S_0$ are slowed while the rates of reactions $S_0' \rightarrow S_1$ and $S_1' \rightarrow S_2$ remain as in the controls. If the presence of formate introduced a rate limiting step on the reducing side of Photosystem II all of the S-state transitions should be slowed uniformly at steady-state, or at least the impediment should be most obvious in those transitions showing the fastest rates in the con-

trols i.e. $S_0' \rightarrow S_1$ and $S_1' \rightarrow S_2$. One observes quite the opposite. Those transitions which have the slowest rate in the controls, $S_2' \rightarrow S_3$ and $S_3' \rightarrow S_0$ are slowed even further in the presence of formate. The compelling conclusion is that formate must be operating directly on the oxygen evolving mechanism.

Discussion

The action of formate shown in these experiments can be understood in reference to the known interaction of formate and the CO_2/HCO_3^- which is bound to PS II. In a previous work [3] formate was shown to slow the binding of exogenous $^{14}CO_2$ to the reaction center complex. In another publication [4] formate was shown to inhibit the rebinding of endogenous CO_2 which is released as PS II cycles in the light. From past work, however, it was not clear if CO_2 were released in the light in consequence to reactions which take place on the oxidizing or reducing side of PS II. In this work, formate is shown to slow specific reactions on the oxidizing side of PS II. If it does so by slowing the rebinding of endogenous CO_2 released in the light, it would suggest that CO_2 is released in reactions that take place as part of the oxygen-evolving mechanism. Specifically, those reactions involved in the transitions $S_2' \rightarrow S_3$ and $S_3' \rightarrow S_0$.

If the interpretations presented thus far are valid, these experiments allow new inferences as to the site and mode of action of bicarbonate in PS II. Arguments have been made that HCO_3^- acts primarily on the reducing side of Photosystem II [10,11,12—15]. These arguments are based on chlorophyll fluorescence measurements and other techniques which show that electron transfer from both the primary electron acceptor Q to the secondary acceptor, B [16], and from B to plastoquinone is slowed down in HCO_3^--depleted chloroplasts [11—14]. Clearly electrons will not leave the reaction center complex at normal rates unless CO_2/HCO_3^- is bound, or rebound, to the complex. There is no compelling reason to believe, however, that the binding site is, in fact, a component on the reducing side of Photosystem II. The binding of exogenous $^{14}CO_2$ is controlled by the internal thylakoid pH [3], which implies that the ligand binds to the inside surface. One can imagine that from here, the ionic forms of the ligand, HCO_3^- or CO_3^{2-}, can help neutralize positive charges near *P*-680. This electrostatic effect could increase the tendency for electrons to leave reduced Q following a charge separation. Depleting reaction centers of negatively-charged bound HCO_3^-, then, may deny electrons on Q the 'push' that they need to speed transfer in the forward direction. In this way the absence of HCO_3^- bound to the oxygen-evolving mechanism would appear to impose some sort of block on the reducing side of the PS II reaction center. While by no means precluding other explanations, this proposed mechanism is consistent with all the results which indicate that CO_2/HCO_3^- acts on the reducing side of PS II.

The most important implication of the present work concerns the mode of action of HCO_3^-. In the opinion of the author, the reason for the HCO_3^- requirement in Photosystem II is now clear. HCO_3^- is almost certainly the immediate source of photosynthetically evolved oxygen. The following (circumstantial) evidence supports this view:

1. The binding site for HCO_3^- has been shown to be on the inside surface of the thylakoid membrane [3] where it is believed oxygen is evolved [17].

2. As shown here, a site of action of HCO_3^- appears to be the oxygen evolving mechanism.

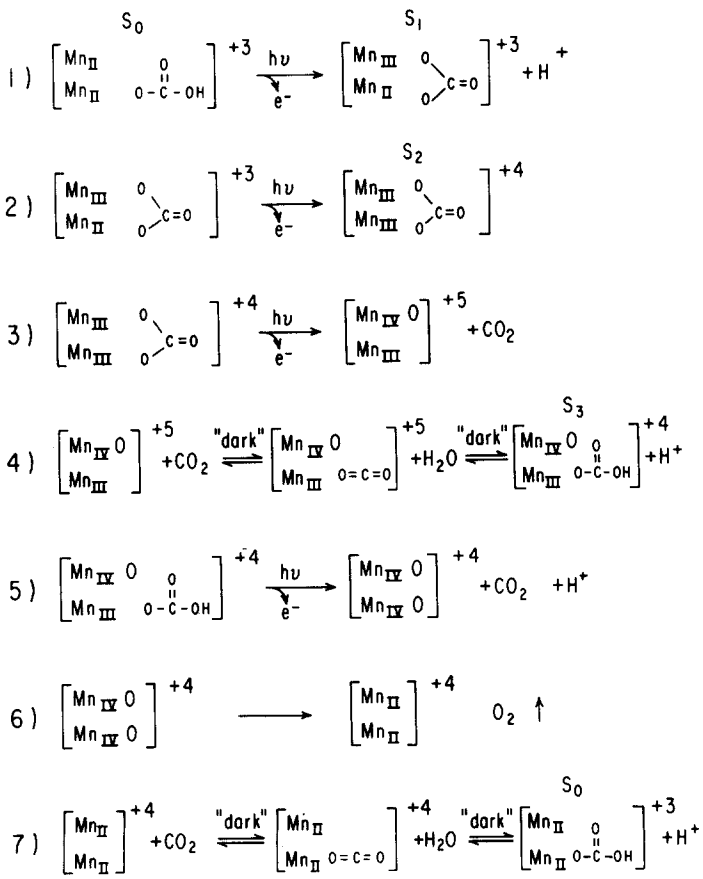
3. CO_2 appears to be alternately hydrated in a 'dark' reaction reaction and dehydrated in the light [3].

4. Exactly two out of four S-state transitions appear to allow the release of CO_2 in the light. This is expected if CO_2 must be sequentially hydrated and dehydrated twice to evolve an O_2 molecule.

5. Stable isotope studies question the notion that the O-H bonds of the H_2O are directly cleaved to evolve oxygen [18]. Other anomalies in isotope measurements which appear to conflict with the idea that O_2 comes directly from H_2O have been discussed by Metzner [19].

Given the probability that HCO_3^- is the immediate source of evolved oxygen, the following model was constructed as a working hypothesis. It is consistent with the 4-step model of oxygen evolution [9] and also includes, with some modification, the model recently developed by Wydrzynski and Sauer [20] suggesting a likely role for manganese in oxygen evolution (Scheme I).

SCHEME I



Reactions 1 and 2 are light driven and result in an increase in oxidation states of 2 Mn(II) ions bound to the reaction center complex. A proton is also released. The oxygen-evolving complex also contains one inorganic carbon ligand [2] which can change sequentially from CO_2 to HCO_3^- to CO_3^{2-}.

Reactions 3 and 4 depict S-state transition $S_2 \rightarrow S_3$. Here, with the removal of an electron, Mn(IV) is produced. Mn(IV) immediately extracts O^{2-} from CO_3^{2-} resulting in the production of $[Mn(IV)O]^{2+}$ and free CO_2. The CO_2 recombines with the Mn(III) still present, is hydrated, and forms HCO_3^- with the release of a proton.

Reactions 5, 6 and 7 depict transition $S_3 \rightarrow S_0$. In 5, Mn(III) is converted to Mn(IV). Mn(IV) extracts an O^{2-} from HCO_3^- or CO_3^{2-} resulting in the production of a second $[Mn(IV)O]^{2+}$, free CO_2 and a proton. In 6, the two $[Mn(IV)O]^{2+}$ react to form 2 Mn(II) plus free O_2. In 7, the reaction center complex, now with 2 Mn(II), combines with free CO_2. The CO_2 is hydrated and $HCO_3^- + H^+$ are formed. The cycle can thus repeat.

The above scheme shows CO_2 release during transitions $S_2' \rightarrow S_3$ and $S_3' \rightarrow S_0$. Formate is proposed to slow reactions 4 and 7 in the forward direction by competing with CO_2 for a binding site. This would explain the results presented here. The positive charges on the reaction center which are not neutralized by HCO_3^- or $CO_3^=$ may be neutralized by other anions such as chloride. The scheme is also formulated to show proton evolution following S-state transitions in a 1, 0, 1, 2 sequence. There is still some disagreement as to which of the S-state transitions release protons [21—23]. Perhaps the actual proton evolution sequence is itself a function of pH and may even be predicted in some instances from the p*K* values of carbonic acid. In any case, the scheme can be modified, within limits, to accommodate other proton evolution sequences. For example, at high pH, a proton may be given off as shown in reaction 1, whereas at low pH, that proton may be given off in reaction 2 instead.

This model differs from that of Wydrzynski and Sauer [20] in proposing the formation of quadrivalent Mn. Wydrzynski and Sauer propose instead that Mn(III) is the highest oxidation state reached by the metal. However, these authors state explicitly that their data do not rule out the possibility that oxidation states of manganese higher than +3 may be involved in oxygen evolution. If $[Mn(IV)O]^{2+}$ is formed, as proposed here, it is likely to be extremely reactive when released from the membrane by heat treatment (used routinely by Wydrzynski and Sauer [20]). Electron paramagnetic resonance-detectable Mn(II), produced by the reduction of heat solubilized Mn(IV) (as $[Mn(IV)O]^{2+}$) would be indistinguishable from Mn(II) released directly. Thus the scheme for oxygen evolution presented here is compatible with the observations of Wydrzynski and Sauer.

The scheme also explains why past experiments which used stable isotopes, particularly ^{18}O, to label oxygen evolving precursors invariably yielded results in which evolved oxygen had nearly the same isotopic composition as the medium water [24—26]. Dark reactions 4 and 7 in the scheme are reversible. Moreover, the hydration of bound CO_2 would have to be catalyzed by the reaction center itself, otherwise this normally slow reaction would be rate-limiting. A catalyzed hydration-dehydration of bound CO_2, however, would quickly, in the dark, produce bound CO_2/HCO_3^- with an isotope composition similar to

medium water. Thus the stable isotope labelling experiments done to date, do not rule out hydrated CO_2 as the immediate source of oxygen as proposed in the model. Indeed recent isotope studies done by Metzner et al. [27], while not definitive, suggest that something other than water may be the immediate source of photosynthetic oxygen.

Because of our yet limited knowledge of the chemistry of oxygen evolution, the constraints on any model are still far from strict: many plausible interpretations of existing data are possible. However, any future model should take into consideration the very strong likelihood that dissolved CO_2 (in its various forms) plays a direct role in oxygen evolution.

Acknowledgements

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References

1 Warburg, O. and Krippahl, G. (1960) Z. Naturforsch. 156, 367—369
2 Stemler, A. (1977) Biochim. Biophys. Acta 460, 511—522
3 Stemler, A. (1980) Plant Physiol. 65, 1160—1165
4 Stemler, A. (1979) Biochim. Biophys. Acta 545, 36—45
5 Joliot, P. and Joliot, A. (1968) Biochim. Biophys. Acta 153, 625—634
6 Bouges-Bocquet, B. (1973) Biochim. Biophys. Acta 292, 772—785
7 Thibault, P. (1973) J. Theor. Biol. 73, 271—284
8 Thibault, P. (1978) C.R. Acad. Sci. Paris 287, 725—728
9 Forbush, B., Kok, B. and McGloin, M.P. (1971) Photochem. Photobiol. 14, 307—321
10 Wydrzynski, T. and Govindjee (1975) Biochim. Biophys. Acta 387, 403—408
11 Jursinic, P., Warden, J. and Govindjee (1976) Biochim. Biophys. Acta 440, 322—330
12 Siggel, U., Khanna, R., Renger, G. and Govindjee (1977) Biochim. Biophys. Acta 462, 196—207
13 Khanna, R., Govindjee and Wydrzynski, T. (1977) Biochim. Biophys. Acta 462, 208—214
14 Govindjee, Pulles, M.P.J., Govindjee, R., van Gorkom, H.J. and Duysens, L.N.M. (1976) Biochim. Biophys. Acta 449, 602—605
15 Govindjee and van Rensen, J.J.S. (1978) Biochim. Biophys. Acta 55, 183—213
16 Bouges-Bocquet, B. (1973) Biochim. Biophys. Acta 314, 250—256
17 Diner, B.A. and Joliot, P. (1977) Encyclopedia of Plant Physiology, New Series, Vol. 5, (Trebst, A. and Avron, M., eds.), pp. 187—205, Springer-Verlag, Berlin
18 Sinclair, J., Sarai, A., Arnason, T. and Garland, S. (1978) in Photosynthetic Oxygen Evolution (Metzner, H., ed.), pp. 295—320, Academic Press, London
19 Metzner, H. (1975) J. Theor. Biol. 51, 201—231
20 Wydrzynski, T. and Sauer, K. (1980) Biochim. Biophys. Acta 589, 56—70
21 Junge, W. and Ausländer, W. (1978) in Photosynthetic Oxygen Evolution (Metzner, H., ed.), pp. 213—228, Academic Press, London
22 Saphon, S. and Crofts, A.R. (1977) Z. Naturforsch. 32C, 617—626
23 Fowler, C.F. (1977) Biochim. Biophys. Acta 462, 414—421
24 Ruben, S., Randall, M., Kamen, M. and Hyde, J.L. (1941) J. Am. Chem. Soc. 61, 877—879
25 Stemler, A. and Radmer, R. (1975) Science 180, 457—458
26 Radmer, R. and Ollinger, O. (1980) FEBS Lett. 110, 57—61
27 Metzner, H., Fischer, K. and Bazlen, O. (1979) Biochim. Biophys. Acta 548, 287—295