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MECHANISM OF BICARBONATE ACTION ON PHOTOSYNTHETIC ELECTRON TRANSPORT IN BROKEN CHLOROPLASTS

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In CO_2-depleted chloroplasts electron transport between the Photosystem II electron acceptor Q and plastoquinone is largely suppressed. In the presence of a high concentration of sodium formate (greater than 10 mM), which probably binds to the bicarbonate site, addition of bicarbonate restores the ferricyanide Hill reaction only after incubation in the dark. With lower formate concentrations bicarbonate is able to restore electron transport in the light. The Hill reaction rate in CO_2-depleted chloroplasts after bicarbonate addition, divided by the rate in CO_2-depleted chloroplasts before bicarbonate addition, shows a sharp optimum at pH 6.5. Furthermore, the rate-limiting step in bicarbonate action is probably diffusion. The results are explained in terms of a hypothetical model: the bicarbonate-binding site is located at the outer side of the thylakoid membrane, but not directly accessible from the 'bulk'. To reach the site from the bulk, the molecule has to pass a channel with negatively charged groups on its side walls. In the light these groups are more negatively charged than in the dark. Therefore, the formate ion cannot exchange for bicarbonate in the light, and a dark period is necessary to enable exchange of formate for bicarbonate.

Introduction

CO_2 is not only required as a substrate for ribulosebisphosphate carboxylase, but is also indispensable for photosynthetic electron transport [1]. A major site of action is between the Photosystem II electron acceptor Q and plastoquinone [2–4]. (For a review see Ref. 5.)

It was not known whether CO_2 or HCO_3^- is the active species regulating electron flow until Good [6] presented evidence that HCO_3^- is involved in the bicarbonate effect. He showed that it was easier to lower the rate of the Hill reaction with ferricyanide by CO_2 depletion in the presence of HCO_3^--like anions (e.g., HCO_2^-, $CH_3CO_2^-$) than in their absence. These anions would displace HCO_3^- from the binding site, causing a lowering of the Hill reaction rate, since binding of HCO_3^- (or CO_2) is required for photosynthetic electron flow.

The mode of action of HCO_3^- is not yet understood, although it has been suggested that HCO_3^- forms a complex with the two-electron carrier R (or B) in the dark [7].

The ratio of the Hill reaction rate after the addition of HCO_3^- to that in CO_2-depleted chloroplasts has been shown to be strongly dependent on pH. At pH 5.8 the restoration of the Hill reaction in CO_2-depleted chloroplasts requires more HCO_3^- addition than at pH 6.8 [8]. Furthermore, a maximal effect has been observed at pH 6.5–7.0. At pH 8.0–8.5 the ratio of the Hill reaction rate of

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Abbreviations: DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; Mes, 4-morpholineethanesulfonic acid; simeton, 2-methoxy-4,6-bis(ethylamino)-1,3,5-triazine; Chl, chlorophyll.

$+HCO_3^-/-HCO_3^-$ is nearly 1.0 [3]. In this pH range the concentration of HCO_3^- is maximal in comparison to the concentrations of CO_2 and CO_3^{2-} (pK_a of H_2CO_3 is 6.4; pK_a of HCO_3^- is 10.2 at 25°C). This suggests that HCO_3^- is not the only important factor required in photosynthetic electron transport.

In CO_2-depleted chloroplasts, suspended in media containing 100 mM sodium formate, the Hill reaction has been shown to be restored by adding HCO_3^- followed by dark incubation for 1–2 min. Under these conditions no restoration is possible in the light [7,8]. However, we have been able to observe reactivation of the Hill reaction rate by addition of HCO_3^- to CO_2-depleted chloroplasts in HCO_2^--free media in the light. We present evidence based on this reactivation in the light and on the dependence of the bicarbonate effect on pH and on temperature that HCO_3^- and CO_2 are both involved in 'bicarbonate action'. We suggest that HCO_3^- is the species that binds at the thylakoid membrane, but that the molecule reaches its binding site mainly in CO_2 form. The HCO_3^--binding site is covered by the negatively charged membrane and therefore is not readily accessible for HCO_3^- from the bulk. However, the uncharged CO_2 can pass easily. The binding site is not specific for HCO_3^-. Other organic anions like HCO_2^-, $CH_3CO_2^-$ and the herbicide, 4,6-dinitro-*o*-cresol, can also bind to the site, but only after binding of HCO_3^- is electron transport possible.

A preliminary report of this work has been presented earlier [9].

Materials and Methods

Chloroplasts were isolated from 10-day-old peas (*Pisum sativum*, cv. Rondo) and broken, as described elsewhere [10]. The broken chloroplasts were suspended in a standard medium, containing 50 mM sodium phosphate, 100 mM NaCl, 100 mM sodium formate and 5 mM $MgCl_2$ (pH 5.0); chlorophyll concentration was 50 μg/ml. The suspension was shaken vigorously for 10 min at room temperature in the dark, while N_2 gas was bubbled through. The CO_2-depleted chloroplasts were pelleted and resuspended in the standard medium at pH 6.5, unless indicated otherwise. All centrifuge tubes and media were made CO_2-free before use.

Electron transport was measured as O_2 production as described earlier [11], using 0.5 mM ferricyanide as electron acceptor.

When HCO_2^--free CO_2-depleted chloroplasts were needed, the chloroplasts were washed after CO_2 depletion and resuspended in a medium containing 50 mM Mes, 0.4 M mannitol and 5 mM $MgCl_2$ (pH 6.5).

Results

Reactivation by bicarbonate in the light and in the dark. We have been able to obtain CO_2-depleted chloroplasts with a ferricyanide Hill reaction rate of less than 0.5 μmol O_2/mg Chl per h at pH 6.5 in the standard medium. Fig. 1 shows that maximal reactivation of the Hill reaction rate is observed after the adddition of 10 mM HCO_3^- (which does not cause a detectable pH change) and 2 min dark incubation. Then the ferricyanide Hill reaction is 20–30 μmol O_2/mg Chl per h. Half reactivation occurs at 1 mM HCO_3^-. Fig. 1 confirms the result obtained earlier by Khanna et al. [3]. Prolongation of the dark incubation period does not cause a further increase of the Hill reaction rate. Control chloroplasts, i.e., not CO_2-depleted and reactivated, show an uncoupled electron flow rate with ferricyanide in the standard medium of about 150 μmol O_2/mg Chl per h. The CO_2-depleted chloroplasts also appear to be

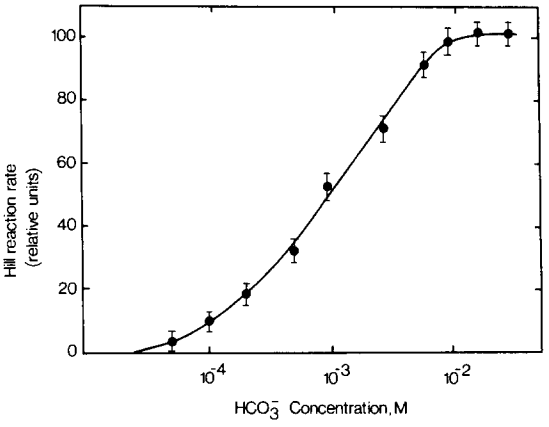

Fig. 1. Hill reaction rates in CO_2-depleted chloroplasts after 2-min dark incubation with various amounts of HCO_3^-. The measurements were performed in the standard medium at pH 6.5. 100 relative units are equivalent to 21.5 μmol O_2/mg Chl per h.

uncoupled, presumably due to the high salt concentration of the depletion and reaction medium [3].

The site which is irreversibly damaged by the depletion procedure appears to be located at or before the reoxidation of Q^-, because after trypsin treatment, which is known to make Q accessible for ferricyanide [12–14], or using silicomolybdate as an electron acceptor, CO_2-depleted chloroplasts yield a rate of the Hill reaction not very different from the rate in the presence of saturating amounts of HCO_3^- [3,15]. Addition of exogenous Photosystem II donors, such as 2 mM diphenylcarbazide or 70 μM benzidine, does not increase the dichlorophenolindophenol Hill reaction rate in CO_2-depleted chloroplasts to which HCO_3^- is added (Vermaas, W.F.J. and Van Rensen, J.J.S., unpublished observations), indicating that the site of the irreversible damage is situated after (or at) rereduction of Z^+. Therefore, we suggest that the irreversible damage is located between rereduction of Z^+ and reoxidation of Q^-.

In a completely different system, i.e., non-HCO_3^--depleted chloroplasts, Crane and Barr [16] found that addition of HCO_3^- inhibited the DCMU-insensitive silicomolybdate reduction by Photosystem II. (For a discussion of this phenomenon, see Ref. 5.)

In the standard medium, no increase of the Hill reaction rate is observed in CO_2-depleted chloroplasts after the addition of HCO_3^- in the light. A dark incubation period seems to be necessary, in agreement with Stemler [7]. However, in the absence of high concentrations of HCO_2^-, HCO_3^- is able to increase the Hill reaction rate in CO_2-depleted chloroplasts also in the light (Fig. 2). Addition of the other constituents of the standard medium (100 mM NaCl or 50 mM sodium phosphate, pH 6.5) does not prohibit reactivation in the light, only HCO_2^- does.

The CO_2-depleted chloroplasts suspended in the HCO_2^--free medium show a measurable O_2 production rate, presumably caused by the absence of the HCO_3^--binding inhibitor, HCO_2^-. The minimal O_2 production rate under these conditions is about 5 μmol O_2/mg Chl per h. After the addition of HCO_3^-, the ferricyanide Hill reaction rate is stimulated by a factor of 3–6.

Action of formate. It has been suggested that HCO_2^- binds to the same site as HCO_3^- does [3,6]. However, electron flow occurs only when HCO_3^-

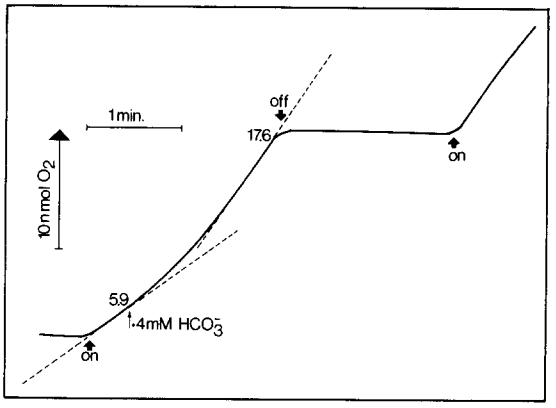

Fig. 2. Reactivation of CO_2-depleted chloroplasts by addition of 4 mM HCO_3^- in the light. Reaction medium: 4 mM Mes, 0.4 M mannitol, 5 mM $MgCl_2$, 0.5 mM ferricyanide and Chl concentration of 33 μg/ml; pH was 6.5. Numbers along trace: μmol O_2/mg Chl per h.

is bound to the site. To quantify the formate effect on HCO_3^- action, different HCO_2^- concentrations were added to CO_2-depleted HCO_2^--free chloroplasts. In the light, at time zero, 10 mM HCO_3^- was added and the reactivation as a function of time was measured (Fig. 3A). An initial increase in the ferricyanide Hill reaction is observed after the addition of HCO_3^-, followed by a decrease due to deactivation of the chloroplasts (e.g., by denaturation of proteins). When the Hill reaction rate is plotted on a logarithmic scale against the time on a linear scale, this deactivation appears to give a straight line. So, $d = k \cdot e^{-ct}$, in which d is deactivation, c and k are constants and t is time. Deactivation appears to be a (pseudo) first-order reaction. We assume that deactivation starts at time zero and follows a straight baseline. Now the differences between the baselines and the observed values were calculated as %Δlog(Hill reaction rate); this is the percentage of the difference between the baseline and observed values, both taken as their logarithms. The difference at time zero was taken as 100%. These values were plotted against time. Fig. 3B shows the results for various HCO_2^- concentrations. From this figure, it is easily seen that even 0.1 mM HCO_2^- has an appreciable effect in decelaration of bicarbonate action in the light. No exact quantitative data can be obtained from this figure, because it is not known how much HCO_2^- is left in the sample after washing and resuspension in HCO_2^--free medium.

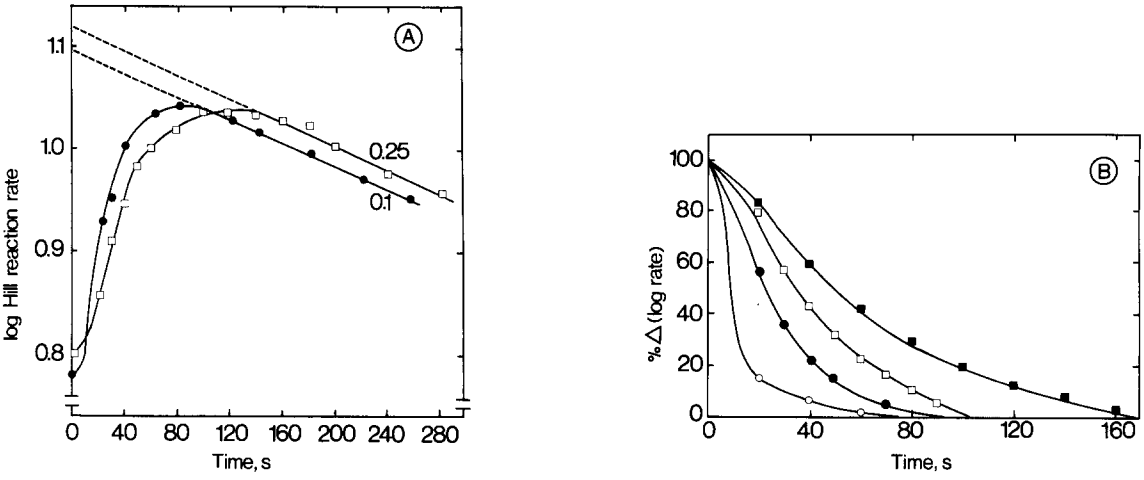

Fig. 3. (A) Reactivation of the Hill reaction in the light by addition of 10 mM HCO_3^- at time zero in the presence of 0.1 mM (•) or 0.25 mM (□) HCO_2^-. Reaction medium: 50 mM Mes, 0.4 M mannitol, 5 mM $MgCl_2$, 0.5 mM ferricyanide, 33 μg Chl/ml and pH 6.5. (B) Relative difference between baseline and measured values as shown in A. ○, without HCO_2^-; •, 0.1 mM; □, 0.25 mM; ▪, 10 mM HCO_2^- added. For explanation, see text.

From this plot we conclude that one cannot expect a measurable reactivation by adding HCO_3^- in the light to CO_2-depleted chloroplasts when 100 mM HCO_2^- is present, because in the presence of 10 mM HCO_2^- the reactivation is already very slow.

The dependence on pH. As mentioned in the Introduction, the ratio of the Hill reaction rate after the addition of HCO_3^- to that in its absence is strongly dependent on pH. To obtain a more precise value for the influence of pH on this ratio than that measured earlier [3], we have prepared partially CO_2-depleted chloroplasts. Their ferricyanide Hill reaction rate was about 3 μmol O_2/mg Chl per h at pH 6.5. These chloroplasts were suspended in CO_2-free media at a different pH, and the reactivation by addition of 10 mM HCO_3^- was measured (Fig. 4).

We have found a sharp pH optimum at pH 6.5. This is close to the pK_a of H_2CO_3, which is 6.37 at 25°C. Our result is a good indication that both HCO_3^- and H_2CO_3 or CO_2 are involved in the reactivation of CO_2-depleted chloroplasts. However, we have to realize that the pH very close to the negatively charged membrane surface may be lower, due to the ζ-potential [17].

The dependence on temperature. At 25°C, 2 min dark incubation of CO_2-depleted chloroplasts in standard medium with HCO_3^- is just enough for maximal restoration of electron transport.

We have investigated the influence of temperature on bicarbonate action by calculating the Q_{10} value (i.e., the Hill reaction rate at $T = t$°C over that at $T = (t - 10)$°C). For that purpose the ferricyanide Hill reaction rate in CO_2-depleted chloroplasts was measured after 2 min dark incubation with 1 or 10 mM HCO_3^- at different temperatures. From Fig. 5, a Q_{10} value of 2.0 ± 0.2 can be calculated ($0 \leqslant T < 25$°C). This is close to the value of 1.8 obtained

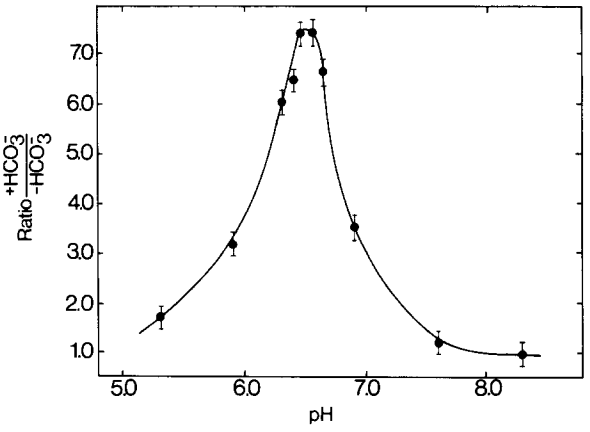

Fig. 4. Ratio of the Hill reaction rate in the presence of 10 mM HCO_3^- divided by that in its absence in CO_2-depleted chloroplasts at various pH values. Measurements were performed in the standard medium at various pH values.

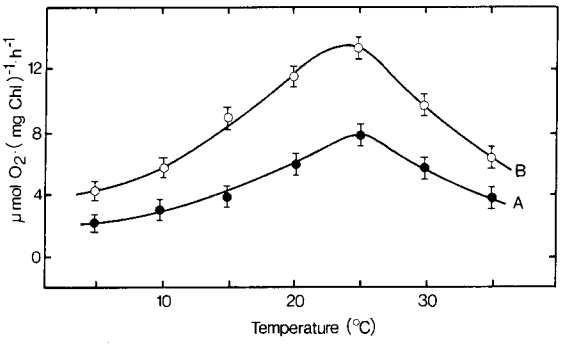

Fig. 5. Hill reaction rates in CO_2-depleted chloroplasts after 2-min incubation with 1 mM (A) or 10 mM (B) HCO_3^- at various temperatures. The measurements were performed in standard medium. Since the chloroplasts were very thoroughly depleted, the Hill reaction rates in the presence of HCO_3^- are rather low.

in normal (not CO_2-depleted) chloroplasts, suspended in the same standard medium (unpublished results). Because the Q_{10} values of 2.0 and 1.8 are almost the same, we conclude that bicarbonate action per se is quite insensitive to temperature. A low Q_{10} value might point to diffusion or to an enzymatic reaction as the rate-limiting step in bicarbonate action.

If an enzymatic reaction is involved in this rate-limiting step, it would probably be a carbonic anhydrase-catalyzed reaction. Therefore, we have investigated the influence of carbonic anhydrase on the bicarbonate reactivation of CO_2-depleted chloroplasts in the light in a medium containing little HCO_2^-. We have found no difference in the reactivation rate with or without carbonic anhydrase. For this reason, it is very probable that diffusion is the rate-limiting step in bicarbonate action. Sarojini and Govindjee (personal communication) have shown that carbonic anhydrase affects equilibrium at 5°C, but carbonic anhydrase has no effect at 25°C as we have found.

Discussion

Depleting broken chloroplasts of CO_2 causes a complete inhibition of electron transport. Although there is definitely a major effect on the reducing side of Photosystem II [2–5], which is the suppression of electron flow between the electron acceptor Q and plastoquinone in the absence of HCO_3^-, it has been argued that there should be a CO_2 effect on 'water-splitting', because CO_2 might be the immediate source of photosynthetically evolved O_2 [18–20]. We discuss our results in relation to the effects of CO_2 and HCO_3^- at the reducing side of Photosystem II.

The reactivation by HCO_3^- is affected by HCO_2^- [9,20–22]. We observed that in the presence of 100 mM HCO_2^-, half-maximal reactivation in CO_2-depleted chloroplasts is observed by incubation in the dark for 2 min in the presence of 1 mM HCO_3^- (Fig. 1). At the pH of the medium (6.5) nearly all added formate is in HCO_2^- form, since the pK_a of formate is 3.7. At pH 6.5 about half of the added HCO_3^- is in HCO_3^- form, the other half in the CO_2 form. This means that at half reactivation at pH 6.5, 0.5 mM HCO_3^- and 100 mM HCO_2^- are present. Thus, the dissociation constant of the HCO_2^--binding site complex is about 200-times higher than the dissociation constant of the HCO_3^--binding site complex, assuming that there is no cooperativity. Fig. 3B shows that the presence of 0.1 mM HCO_2^- already has a significant effect on bicarbonate action in the light. This would suggest that in fully CO_2-depleted chloroplasts in a medium without HCO_2^-, even 0.5 μM HCO_3^- will show a measurable effect on the Hill reaction rate (0.1 mM divided by 200). This might be the reason why it is almost impossible to prepare CO_2-depleted chloroplasts in media which do not contain HCO_2^-. Also, Khanna et al. [3] have observed that a comparable restoration of electron transport in CO_2-depleted chloroplasts occurs at lower concentrations of HCO_3^- in the absence than in the presence of 100 mM HCO_2^-.

We suggest that the ζ-potential, defined as the electrokinetic potential at the hydrodynamic plane of shear [17], plays an important role in the bicarbonate effect. The ζ-potential is more negative in the light than in the dark [23]. Figs. 2 and 3 show that HCO_3^- can restore the Hill reaction in CO_2-depleted chloroplasts in the light in the absence of HCO_2^-. Thus, bicarbonate action is possible even in the presence of a strong negatively charged membrane surface. We suggest that the uncharged CO_2 can pass this charged surface by diffusion. Also, Sarojini and Govindjee [24] considered it likely that CO_2 is the species that diffuses into the membrane.

Furthermore, Stemler [21] suggested that CO_2 is initially taken up by the thylakoid membrane. We propose that after the negative shield has been passed, CO_2 is converted into HCO_3^- which is able to bind to a site below the membrane surface (Fig. 6). This suggestion is in accordance with our conclusion that the rate-limiting step in bicarbonate action is diffusion and that the optimum for the bicarbonate effect is at pH 6.5, where comparable quantities of CO_2 and HCO_3^- are present in the bulk (Fig. 4). However, we cannot exclude the possibility that CO_2 can also bind to the site.

To explain our results we propose a hypothetical model, presented in Fig. 6. According to this model HCO_3^- binds to a site at A and B below the surface of the thylakoid membrane. Only when A and B are both occupied is electron transport between Q and plastoquinone possible. HCO_2^- can bind to the B part of the site, but then electron transport cannot proceed because A is unoccupied. To reach the HCO_3^--binding site, ions have to pass negative charges at the surface of the membrane and along the path from the surface to the site. In the light, the negatively charged HCO_2^- is not able to leave its binding site. In the dark, the ζ-potential is more positive [23] and, HCO_2^- can leave its site easier and exchange for HCO_3^-, thus inducing restoration of electron flow.

Our model also accommodates observations made by Stemler [25] that washing of chloroplasts with silicomolybdate removes HCO_3^- bound to the site and that DCMU, given prior to the silicomolybdate washing, strongly decreases HCO_3^- removal. Stemler concluded that DCMU 'overlays' the bound HCO_3^-, protecting it from silicomolybdate attack. Furthermore, Barr and Crane [26] have reported that addition of 20 mM HCO_3^- results in a full inhibition of the silicomolybdate Hill reaction at pH 8.0. At pH 6.0, however, where the HCO_3^-/CO_2 ratio is much lower, this amount of added HCO_3^- causes only a partial inhibition. This supports our hypothesis that silicomolybdate binds to the same site as HCO_3^-. Van Rensen and Vermaas [15] showed that DCMU and also the s-triazine herbicide simeton are partial competitive inhibitors of HCO_3^- binding. Both compounds decrease the affinity of the thylakoid membrane for HCO_3^-. Khanna et al. [27] have found that CO_2 depletion causes an altered binding of [^{14}C]-atrazine.

It appears that a number of anions can bind to the HCO_3^--binding site: HCO_3^-, HCO_2^-, silicomolybdate and the herbicide 4,6-dinitro-*o*-cresol [15].

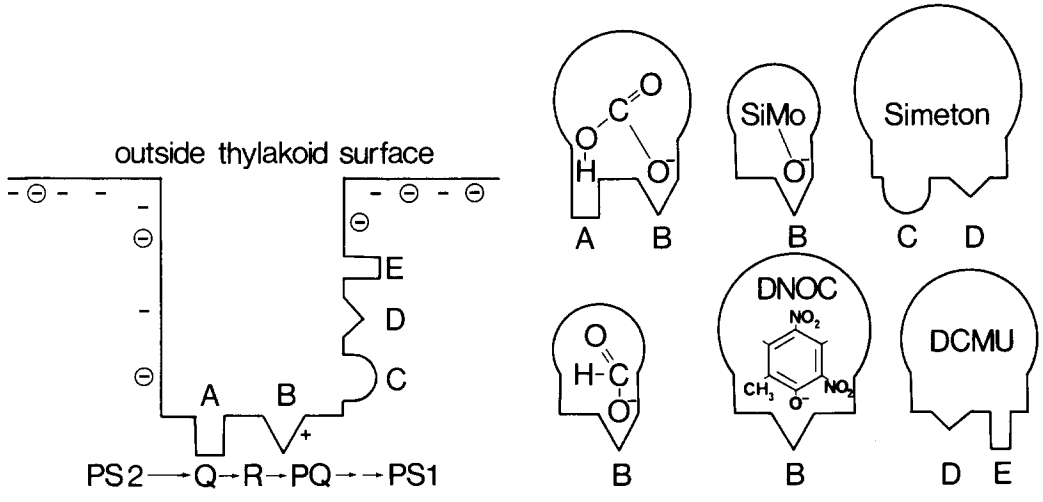

Fig. 6. A hypothetical scheme for the HCO_3^--binding site. –, charge existing in dark and light; ⊖, charge existing in the light only. B is a nonspecific binding site, to which molecules with a = C-O$^-$ group can bind. Electron transport can only proceed when there is also binding to site A; only HCO_3^- is able to do so. The herbicides, DCMU and simeton, bind to the side of the pathway from the bulk to the HCO_3^--binding side, thus covering part of the HCO_3^--binding site. This causes a lowering of the affinity of the site for HCO_3^-. SiMo, silicomolybdate; DNOC, 4,6-dinitro-*o*-cresol; PS, photosystem.

Thus, the binding site is nonspecific in binding anions, but is very specific in its requirement for HCO_3^- to restore electron transport. It may be that the binding site of these anions and herbicides is located within the Photosystem II shielding protein proposed by Renger [28] and that the shielding protein has partly different binding sites for various classes of Photosystem II inhibiting herbicides, as suggested by Trebst and Draber [29].

Acknowledgements

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