Biochimica et Biophysica Acta, 936 (1988) 208–214
Elsevier

BBA 42867

Kinetics of the bicarbonate effect and the number of bicarbonate-binding sites in thylakoid membranes

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(Received 15 July 1988)

Key words: Photosystem II; Bicarbonate effect; Carbon dioxide; Electron transport; Binding site; Thylakoid; (Spinach)

In HCO_3^--depleted thylakoids in which the basal activity was less than 7% of the fully restored activity after readdition of HCO_3^-, the restored activity at a half-saturating HCO_3^- concentration was non-linear with the chlorophyll concentration. This indicates that there was still some endogenous HCO_3^- remaining in these thylakoids, even though they appeared to be well depleted of HCO_3^-. A kinetic analysis of the activity curve for these thylakoids, as a function of HCO_3^- concentration, indicates that there are at least two high-affinity sites of HCO_3^- in Photosystem II (PS II), apparently with cooperative binding. An additional low-affinity site has been shown to exist earlier (Stemler, A. (1977) Biochim. Biophys. Acta 460, 511–522; Blubaugh, D.J. and Govindjee (1984) Z. Naturforsch. 39c, 378–381). Illumination was shown to convert non-exchanging membrane HCO_3^- to an exchanging one. It is suggested that HCO_3^- is an essential activator for PS II electron transport, and that a complete removal of HCO_3^- would result in zero activity.

Introduction

In addition to the role of CO_2 as the ultimate electron acceptor in photosynthesis, bicarbonate (HCO_3^-) has been shown to stimulate electron flow at the electron-acceptor side of Photosystem II (PS II) [1–4]. Little is known about its mechanism of action, although a detailed model has been presented [4]. Recently, we showed that the active species is HCO_3^-, not CO_2 [5]. Until recently, it seems to have been tacitly assumed that there is a single major HCO_3^--binding site involved in the regulation of electron flow though the plastoquinones of PS II. In this paper, we present a kinetic analysis of a velocity versus $[HCO_3^-]$ curve. Our analysis suggests: (1) an endogenous tightly bound $[HCO_3^-]$ exists in HCO_3^--depleted membranes; (2) at least two high-affinity HCO_3^--binding sites exist in thylakoids; and (3) preillumination causes a conformational change that converts non-exchanging membrane HCO_3^- to an exchanging one.

Abbreviations: Chl, chlorophyll; DCIP, 2,6-dichlorophenolindophenol; K_d, dissociation constant for the HCO_3^-·PS II complex; PQ, plastoquinone; PS II, Photosystem II; Q_A, primary plastoquinone electron acceptor of PS II; Q_B, secondary plastoquinone electron acceptor of PS II.

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Materials and Methods

Thylakoids were obtained from market spinach, as described in Ref. 6. The Chl concentration was determined by the spectrophotometric method of MacKinney [7].

N_2 gas, used to purge containers and solutions of CO_2, passed through a drying column of $CaCl_2$ and ascarite to remove any residual CO_2, and then was hydrated by bubbling through distilled H_2O. All solutions were bubbled for at least 10 min before use. The thylakoids were resuspended in a depletion medium (CO_2-depleted 50 mM Na phosphate/100 mM NaCl/100 mM $NaHCO_2$/5 mM $MgCl_2$ (pH 5.5)) to a [Chl] of 40–80 μg/ml, then incubated with gentle shaking for 5 min at room temperature in the dark. The depleted thylakoids were pelleted at $3500 \times g$ for 7 min at 7°C, then resuspended under N_2 to a [Chl] of 12 μg/ml in the reaction medium (the same as the depletion medium, but with pH 6.5 and only 5 mM $NaHCO_2$). After restoration of the Hill activity with a saturating [HCO_3^-], the electron transport rates were, typically, around 400–600 μeqv./mg Chl per h, using DCIP or ferricyanide as electron acceptor, and were about 60–80% of the original activity. Rates of electron transport from H_2O to DCIP or ferricyanide were measured either polarographically as O_2 evolution on a Clark electrode or spectrophotometrically, follwing the procedure in Ref. 5, with one exception: although DCIP is itself an uncoupler, 10 mM methylamine and 100 nM gramicidin D were also added, as they appeared somewhat to stabilize the initial rate of DCIP reduction. Other details are reported in the figure legends.

Results

A double-reciprocal plot of the velocity curve (rate of Hill reaction as a function of [HCO_3^-] at equilibrium) obtained from HCO_3^--depleted thylakoids, in which the basal rate of electron transport was less than 7% of the fully restored rate, was found to be linear, as has been shown previously [8,9]. We questioned, however, whether the 7% residual activity might be due to some endogenous HCO_3^- not removed during the depletion procedure.

In an enzyme preparation, endogenous substrate can be detected by measuring the velocity as a function of enzyme concentration, at a constant but subsaturating concentration of added substrate. If endogenous substrate is present, the curve deviates from linearity, since adding more enzyme also adds more substrate [10]. For example, the Michaelis-Menten equation,

$$V = \frac{k_p[S]}{K_S + [S]}[E]_t \qquad (1)$$

is linear if [S] is constant, but since the endogenous substrate concentration must be equal to some fraction, c, of the total enzyme concentration, Eqn. 1 becomes

$$V = \frac{k_p([S]_{added} + c[E]_t)}{K_S + ([S]_{added} + c[E]_t)}[E]_t \qquad (2)$$

which is no longer linear for $c \neq 0$. Here, K_S is the dissociation constant for the enzyme/substrate complex, k_p is a forward rate constant for the formation of product P, E is free enzyme, S is unbound substrate, and $[E]_t$ is total enzyme concentration. The above argument applied whether HCO_3^- is a substrate or an activator. Whatever form the velocity equation takes, V is proportional to $[E]_t$ as long as both substrate and activator concentrations are constant. However, if either one increases with increasing $[E]_t$, then a plot of V vs. $[E]_t$ will not be linear.

Fig. 1 shows a plot of rate of DCIP reduction versus [Chl] (closed symbols). All of the points were made at a [Chl] at which the light is saturating; in non-HCO_3^--depleted control thylakoids, the velocity as a function of [Chl] was linear up to a [Chl] of 25 μg/ml (data not shown). The deviation from linearity, when a half-saturating [HCO_3^-] is added, is evident. Assuming the thylakoid volume to be 1 ml per 8 mg Chl (the approximate volume of pelleted thylakoids), we have calculated that the maximum deviation due to a decreasing solution volume with increasing [Chl] would only be 0.3%, whereas the observed deviation is of the order of 25%. Therefore, the existence of endogenous HCO_3^- in these membranes is certain. However, the residual activity of the HCO_3^--depleted thylakoids in the absence of added HCO_3^- appears to be linear with [Chl], whereas it should have also deviated from linearity, according to Eqn. 2 (with $[S]_{added} = 0$). A possible explanation is that the endogenously bound HCO_3^- was not exchangeable with the bulk solution until after the addition of exogenous HCO_3^- and/or preil-

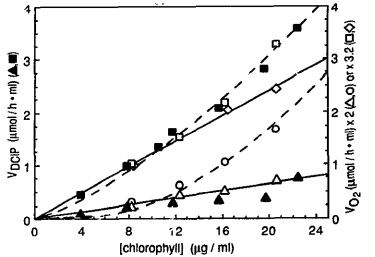

Fig. 1. The rate of DCIP reduction (V_{DCIP}; closed symbols) or of O_2 evolution (V_{O_2}; open symbols) in HCO_3^--depleted thylakoids, as a function of the [Chl]. The reduction of DCIP was calculated from the decrease in absorbance at 600 nm. The pH was 6.52. The data are from two separate experiments: Expt. 1 (closed symbols): The HCO_3^--depleted sample used was the same one used to obtain the data in Fig. 2. At each [Chl], the residual rate (▲) was measured during a 20 s illumination, then the restored rate (■) was measured during a second illumination beginning 3.0 min after the addition of 0.27 mM $NaHCO_3$ (0.16 mM HCO_3^- after equilibrium). Expt. 2 (open symbols): The residual rates were measured with, (○), or without, (△), a 20 s preillumination given 3.0 min before the measurement. The restored rates were likewise measured with, (□), or without, (◇), the preillumination. For comparison, the residual and restored rates of Expt. 2 are separately normalized to the rates of Expt. 1; the ratio of restored to residual rates in the two preparations differed. The drawn curves are from two theoretical predictions of the data, based on a model of two cooperative HCO_3^--binding sites and an endogenous $[HCO_3^-]$ of 0.1 mM. The velocity equation is

$$V = \frac{k_p\left([HCO_3^-]_{added} + [HCO_3^-]_{endog}\right)^2}{K' + \left([HCO_3^-]_{added} + [HCO_3^-]_{endog}\right)^2}[Chl]$$

with $k_p = 230$ μmol DCIP reduced/mg Chl per h, $K' = 0.0613$ mM^2, and $[HCO_3^-]_{added} = 0.16$ mM (top pair of curves) or $[HCO_3^-]_{added} = 0$ (lower pair of curves). Solid lines: The membrane HCO_3^- is assumed to be non-exchangeable with the bulk medium, so $[HCO_3^-]_{endog}$ is constant at 0.1 mM. Broken lines: The membrane HCO_3^- is assumed to freely exchange with the bulk medium, so that $[HCO_3^-]_{endog} = c[Chl]$, with $c = 0.1$ mM/0.0436 mg Chl (the constant amount of Chl in Fig. 2, from which the value of 0.1 mM endogenous HCO_3^- was estimated).

lumination. In other words, each binding site would initially only see the HCO_3^- accessible within the membrane, regardless of the presence of other membranes ($[S]_{endog} \neq c[E]_t$, but rather $[S]_{endog}$ = constant). This might suggest a sequestered pool of HCO_3^- within the membrane. Further experiments suggest that this is indeed the case.

First, we confirmed that the rate of O_2 evolution with 2 μM ferricyanide as an electron acceptor gave the same results, with increasing [Chl], as DCIP photoreduction (Fig. 1, open symbols, normalized to the DCIP rates). In addition, in HCO_3^--depleted samples, a non-linear curve was obtained if the samples were first preilluminated (open circles). On the other hand, in the absence of preillumination, the curve with half-saturating HCO_3^- became linear (open diamonds). Thus, the existence of an endogenous HCO_3^- pool can only be observed by this method after brief preillumination, after which this HCO_3^- can apparently exchange with the HCO_3^- in the medium.

Less than 7% of the fully restored activity remained in these thylakoids after HCO_3^--depletion (Fig. 2). It appears likely then, that had all of the endogenously bound HCO_3^- been removed, the activity would have been zero, suggesting that HCO_3^- may be an essential activator. A double-reciprocal plot of the data in Fig. 2, without any correction for the endogenous HCO_3^- (not shown), is linear, as observed by others [8,9]. From this, it would erroneously appear that the HCO_3^- site follows Michaelis-Menten kinetics. However, after a conservative correction for the endogenous HCO_3^- (assumed to be 0.02 mM after exchange with the bulk solution, estimated by extrapolation of the curve in Fig. 2 to zero activity), the deviation of the double-reciprocal plot (Hill activity^{-1} vs. $[HCO_3^-]^{-1}$ from linearity became evident (data not shown). However, a plot against $[HCO_3^-]^{-1.4}$ is linear (Fig. 3), suggesting that the number of binding sites is at least 1.4; this means the minimum number of binding sites is 2.

Given the heterogeneity of PS II (for a review, see Black et al. [11]), it is conceivable that HCO_3^- binds to a single site, but with different affinities in the different types of center. This would be analogous to a multiple-enzyme system, in which each enzyme catalyzes formation of the same product. A double-reciprocal plot for such a system is not linear. However, the deviation from linearity is in the direction opposite to that observed here (for a discussion of the kinetics of

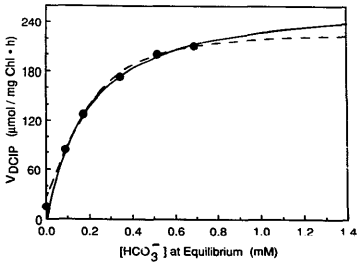

Fig. 2. The rate of DCIP reduction (V_{DCIP}) in HCO_3^--depleted thylakoids, as a function of the equilibrium HCO_3^- concentration. The reduction of DCIP was calculated from the decrease in absorbance at 600 nm. After a 20 s illumination of the depleted thylakoids, $NaHCO_3$ was added. The rates were measured during a second illumination beginning 3.0 min later. The equilibrium HCO_3^- concentrations were calculated according to Ref. 5. The pH was 6.52, and the [Chl] was 10.9 μg/ml. The curves are from two theoretical predictions of the data: Solid line: A single HCO_3^--binding site, with no endogenous HCO_3^-, is assumed. The velocity equation is

$$V = \frac{k_p[HCO_3^-]}{K_S + [HCO_3^-]}[Chl]$$

with $k_p = 273$ μmol DCIP reduced/mg Chl per h and $K_S = 197$ μM. Broken line: Two cooperative HCO_3^--binding sites and an endogenous [HCO_3^-] of 0.1 mM are assumed. The velocity equation is

$$V = \frac{k_p[HCO_3^-]^2}{K' + [HCO_3^-]^2}[Chl]$$

with $k_p = 230$ μmol DCIP reduced/mg Chl per h and $K' = 0.0613$ mM^2.

such a system, see Ref. 10, pp. 64–71). While HCO_3^- may psosibly bind with different affinities to the different types of PS II center, such a model is not sufficient to explain the data presented here.

Discussion

The Hill equation for an enzyme with multiple substrate sites is

$$V = \frac{V_{max}[S]^{n_{app}}}{K' + [S]^{n_{app}}} \quad (3)$$

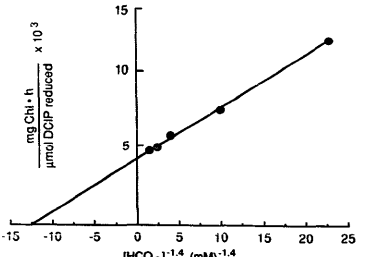

Fig. 3. A double-reciprocal plot of the rate of DCIP reduction in HCO_3^--depleted thylakoids as a function of the equilibrium $[HCO_3^-]^{-1.4}$, corrected for an assumed endogenous [HCO_3^-] of 0.02 mM. The data are from Fig. 2.

where n_{app} is the apparent number of sites, and K' is a function of the dissociation constants for each site, as well as of the cooperativity between them (see Ref. 10, pp. 355–361). For multiple essential activation sites, the velocity equation is functionally identical (with [A] in place of [S], where A = activator; see Ref. 10, pp. 403–404). A functionally equivalent velocity equation can also be shown to apply to mixed sites, where the same compound is both substrate and activator [12]. Since this is the case, it is not necessary to distinguish between models in which HCO_3^- is an activator or a substrate. The presence of an inhibitor (e.g., 5 mM formate), assuming that it binds with much lower affinity than the substrate/activator, increases the apparent value of K', without altering the form of the equation [12].

The value of n_{app} can be obtained by plotting $1/V$ vs. $1/[S]^{n_{app}}$ for various values of n_{app} until a linear plot is obtained. The value for n_{app} must be between 1 and the true number of sites, with n_{app} approaching the true number with increasing cooperativity between the sites (with no cooperativity, $n_{app} = 1$). The data of Fig. 2, after a conservative correction for endogenous HCO_3^-, yield a linear plot with $n_{app} = 1.4$ (Fig. 3), suggesting at least two HCO_3^- sites with some degree of cooperative binding.

Cooperativity will cause the low end of the velocity vs. [HCO_3^-] curve to become sigmoidal. If

sigmoidicity is imposed on the data of Fig. 2, then the estimate of endogenous [HCO_3^-] is larger than the 0.02 mM correction applied in Fig. 3, and n_{app} is larger. The degree of cooperativity between the HCO_3^--binding sites could, therefore, be high. A value of 2 is obtained for n_{app} if an endogenous [HCO_3^-] of 0.1 mM is assumed.

Fig. 2, in addition to the data discussed earlier, also shows the theoretical curve for a two-site, cooperative model with an effective endogenous [HCO_3^-] of 0.1 mM and the predicted curve for a Michaelis-Menten one-site model with no endogenous HCO_3^-. It is clear that with only a few points, clustered around the middle of the curve (see also Ref. 8, 9), it is impossible to discriminate between the two models, unless it is known whether or not there is endogenously bound HCO_3^-.

Since Figs. 1 (closed symbols) and 2 were both obtained from the same depleted thylakoids, it is necessary that any model chosen be able to predict both sets of data. As discussed previously, a Michaelis Menten model, while consistent with Fig. 2, requires the assumption of no endogenous HCO_3^- and is therefore inconsistent with Fig. 1. On the other hand, the two-site model is consistent with both sets of data. As shown in Fig. 2, it is consistent with the velocity curve. The prediction by the two-site model of the activity vs. [Chl] curve is shown in fig. 1. The broken lines are the predictions based on the assumption that the endogenous HCO_3^- is freely exchangeable with the bulk medium. The solid lines are the predictions based on a non-exchangeable pool of HCO_3^-. The model predicts the data fairly well if it is assumed that there was no exchange until after the addition of HCO_3^-. As discussed earlier, this appears to have been due to the preillumination.

Although a value of 0.1 mM for the endogenous [HCO_3^-] permits a good fit of the data of Figs. 1 and 2, this value is quite large in relation to the concentration of PS II reaction centers; it requires several thousand HCO_3^- molecules per PS II. Although quite large, such a number is not without precedent. Stemler [13] demonstrated, by $H^{14}CO_3^-$ labelling of chloroplasts, the existence of a low-affinity pool of HCO_3^-, with a concentration at least as high as the bulk [Chl]. The concentration of this pool may be much higher, since he reported encountering solubility problems with the HCO_3^- before the low-affinity pool was saturated. Similarly, we showed previously [14] the effects of added HCO_3^- on Chl *a* fluorescence induction which did not saturate until 60 mM HCO_3^- and we concluded that there was a pool of very-low-affinity sites. Given the size of the endogenous pool of HCO_3^- suggested in this paper, it is likely that it is this same low-affinity pool.

Although consisting of low-affinity sites, there appears to be some mechanism capable of preventing the exchange of membrane HCO_3^- with the bulk solution (Fig. 1, lower curve, without preillumination). One possibility is a sequestered pool of HCO_3^- buried within the membrane, with a hydrophobic lipid/protein barrier preventing diffusion of the HCO_3^-. Although CO_2 could diffuse across such a barrier, the exchange rate could be very slow due to limited availability of H^+, necessary for conversion of HCO_3 to CO_2, within this sequestered space (see for example, Ref. 15). Stemler [13] showed that binding of HCO_3^- to the low-affinity sites in the dark required 30 min to reach equilibrium, which is an order of magnitude longer than reported for the high-affinity sites [5,9,16]. As discussed earlier, preillumination appears to induce changes which expose the low-affinity sites to the bulk medium (Fig. 1, lower curves).

The two cooperative sites of HCO_3^- binding suggested here are separate from the low-affinity pool since they are detected kinetically under conditions in which the low-affinity sites are exposed to the bulk solution and are largely empty. A very low percentage of these low-affinity sites would be filled at the [HCO_3^-] at which V_{max} was reached in these experiments.

On the other hand, the low-affinity pool may serve an important function in vivo by buffering the [HCO_3^-] in the vicinity of the high-affinity sites: if one or both of the HCO_3^- groups is involved in protonating Q_B^- and/or Q_B^{2-}, with release of CO_3^{2-}, the low-affinity pool would provide a ready source of new HCO_3^- molecules for rapid binding to the high-affinity site(s). The sequestering of this low-affinity pool might also provide a concentrating mechanism, as follows: during illumination, the consumption of H^+ within this sequestered space by PQ reduction would drive the CO_2/HCO_3^- equilibrium towards

HCO_3^-, increasing logarithmically as the pH increases ($[CO_2]$ would remain constant, since CO_2 would be free to diffuse into this sequestered space). With a large number of low-affinity sites in equilibrium with this $[HCO_3]$, a large number of HCO_3^- molecules could be stored. When the intracellular $[CO_2]$ drops during photosynthesis, the efflux of HCO_3^- from this pool would be very slow due to the limited availability of H^+ ions to convert HCO_3^- to CO_2. Thus, the low-affinity pool could provide a mechanism for keeping the high-affinity sites loaded during rapid turnover of the reaction centers, even when the intracellular $[CO_2]$ is low.

It has long been known that low pH and high salt concentrations facilitate HCO_3^- depletion of thylakoids. We speculate that they may do this, in part, by causing the loss of the stored pool. Protonation of an ionizable group and/or charge shielding of this same group could induce a conformational change that exposes the low-affinity sites to the bulk phase, with consequent unloading of the pool. Consistent with this model is the observation by Stemler [13] that $H^{14}CO_3^-$ bound to the high-affinity site(s) does not exchange readily with the bulk solution, except at low pH.

The pH optimum for the HCO_3^- requirement is 6.5 [17,18]. The rising arm of this pH profile is undoubtedly due to the pK_a of CO_2/HCO_3^-. The declining arm, on the other hand, may reflect the pK_a of the ionizable group just proposed. At the pH of these experiments (6.5), the integrity of the sequestered pool was perhaps tenous, so that structural changes during illumination were sufficient to expose the low-affinity sites to the bulk liquid.

The two most likely candidates for the high-affinity sites are: a species liganded to Fe^{2+}, as previously proposed by Michel and Deisenhofer [19], and the other complexed with an arginine residue, as suggested by Shipman [20]. The ligand bound to Fe^{2+} would account for several observations: the large increase in the light-induced EPR signal at $g = 1.82$, attributed to the $Q_A^- \cdot Fe^{2+}$ complex, upon HCO_3^- depletion [21]; the inability of high-potential quinones to oxidize the Fe^{2+} in the presence, but not in the absence, of formate [22]; and the apparent lack of a homologous sequence in PS II which would correlate with a region of the bacterial reaction center subunit M that carried a glutamate residue liganded to the Fe^{2+} [19]. The possibility that HCO_3^- binds to arginine-257 of the Q_B protein was discussed previously by us [4,5] and reveales a viable candidate for the second high-affinity site.

We have proposed a detailed working model [4] incorporating the ideas reported here. In this model, the HCO_3^- bound to Fe^{2+} is structural only, providing a salt bridge between the Fe^{2+} and the D_2 protein of the PS II reaction center, analogous to the glutamate ligand to Fe^{2+} in the bacterial reaction center [19]. This salt bridge may be disrupted by low pH and high ionic strength to expose the low-affinity sites to the bulk solution. The HCO_3^- bound to arginine is suspected by us to be the HCO_3^- involved in the protonation of Q_B^- and/or Q_B^{2-} (see for example, Refs. 23, 24). The two sites appear to be cooperative. Release of CO_3^{2-} from the arginine during illumination (see Refs. 4, 5) may, therefore, weaken the binding of HCO_3^- at Fe^{2+} sufficiently at pH 6.5 to account for the effect of preillumination in exposing the low-affinity sites to the bulk phase.

Acknowledgement

We thank the National Science Foundation (PCM 83-06061) for support.

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