EFFECTS OF CO_2-DEPLETION ON PROTON UPTAKE AND RELEASE IN THYLAKOID MEMBRANES

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1. Introduction

The ability of CO_2 to stimulate the Hill reaction in thylakoid membrane is well established [1]. When $NaHCO_3$ is added to CO_2-depleted thylakoids, a large (4–6-fold) increase in oxygen evolution is observed. Recent experiments [2–6] have demonstrated that a major site of inhibition of Hill reaction by CO_2 depletion is between electron acceptor Q of photosystem II (PS II) and the plastoquinone (PQ) pool, and more specifically between the two electron acceptor R (or B) and PQ [4,5]. Although a major site of bicarbonate action has been clearly established, and recent experiments suggest that CO_2 is the species that diffuses and may initially bind to the membrane [7], the mechanism of its action is not known.

During illumination of thylakoids there is an uptake of protons from the external phase and a release of protons into the interior space [8–10]. There are two proton uptake sites on the outer side of the membrane [11] and two sites of proton release on the inner side [12]. One of the sites on the outer surface is attributable to the reduction of the electron acceptor used. The second site of proton uptake is associated with the reduction of plastoquinone ($PQ + 2\,H^+ + 2\,e^- \rightarrow PQH_2$) which acts as a shuttle such that its oxidation releases protons at the inner side of the membrane. The other site of proton release into the inner phase is attributed to the oxidation of water ($1/2\,H_2O \rightarrow 1/4\,O_2 + H^+ + e^-$). The sites of proton binding from the outer phase and proton release into the inner aqueous volume have been studied with pH indicating dyes by flash spectrophotometry [12,13].

Thylakoid membranes depleted of bicarbonate show an increased $t_{1/2}$ for electron flow from R (or B) to PQ, i.e., the formation of plastohydroquinone is slowed down [4,5,14]. This suggested to us that the absence of bicarbonate could affect proton uptake and release at the plastoquinone level. Here, we present measurements on the proton uptake and release in control and bicarbonate depleted thylakoids confirming a significant role of the CO_2 depletion at the PQ level.

2. Materials and methods

Thylakoid membranes were isolated from leaves of fresh market spinach (*Spinacea oleracea*) and depleted of bicarbonate by a modification of the method in [15].

For the measurements of internal pH changes, thylakoids were suspended at a 10 μg chl/ml in a medium (15 ml) containing 20 mM KCl, 2 mM $MgCl_2$, 0.5 mM ferricyanide, 0.3 μM non-actin and 10 μM neutral red. The absorption changes of neutral red were measured at 524 nm as in [16]. Addition of nonactin accelerated the intrinsic electrochromic carotenoid changes so that the observed changes are mainly due to neutral red [17]. Neutral red distributes itself in the external medium as well as in the internal space occupied by thylakoids. Bovine serum albumin (BSA) (1.3 mg/ml) was then added to buffer away pH changes in the external space such that the absorption changes of neutral red are then entirely due to the pH changes in the internal space. The pH_{in} indicating absorption changes of neutral red were obtained as the difference between signals measured in the absence

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and in the presence of the permeating buffer, as in [16].

For measurement of external pH changes, the suspension medium (15 ml) contained 20 mM KCl, 2 mM $MgCl_2$, 0.5 mM ferricyanide, 20 μM bromocresol purple (BCP) and thylakoids at 10 μg chl/ml. The absorption change of BCP were measured at 574 nm as in [18].

Short ($t_{1/2}$ = 15 μs) saturating flashes of light were used for excitation. Transient absorption changes were recorded and the signal to noise ratio was improved by averaging over 10 transients induced by repetitive flashes (dark time between flashes, 10 s).

3. Results and discussion

Fig.1 shows pH_{in} indicating absorption changes of neutral red at 524 nm at pH 7.0. In control thylakoids, the absorption changes show biphasic kinetics for the release of protons into the internal phase. The more rapid phase is attributable to the protons released from the water oxidising system, and the slower phase to the protons released from the oxidation of plastohydroquinone [16]. In bicarbonate-depleted thylakoids, only the fast phase due to the water oxidizing system is seen which appears consistent with the conclusion that bicarbonate depletion mainly affects the reducing side of PS II [1]; however, the amplitude of the rapid phase is smaller by 55% indicating a partial inactivation of photosystem II complex. In contrast, the slow proton release by PQH_2 oxidation is apparently fully abolished. In these experiments, measurements on the bicarbonate reconstituted samples were not possible as addition of 2–10 mM bicarbonate (needed to obtain reconstitution) buffered away the pH changes. The pK of CO_2 is 6.38, and the buffering capacity of 2–10 mM CO_2 at pH 7.0 is calculated to be 0.24–1.20 mM; this is to be compared with the intrinsic buffering capacity of a chloroplast suspension which is ~1.25 mM for the internal phase [13]. The buffering capacity for the external phase is of the same order of magnitude (W. J., unpublished). Thus, the above analysis explains our inability to make measurements with CO_2-depleted thylakoids reconstituted with CO_2.

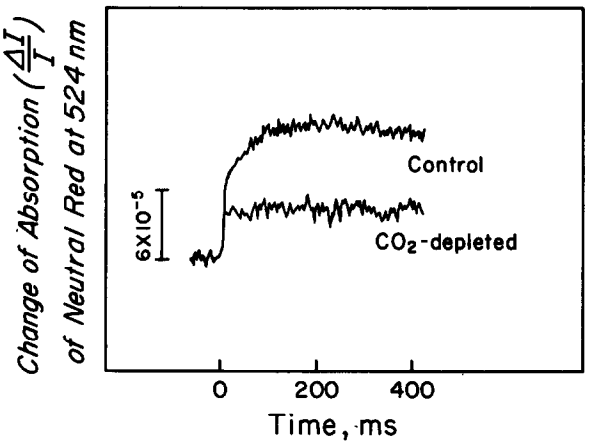

Fig.1. Kinetics of proton release into the internal phase of spinach thylakoids as indicated by absorption change of neutral red at 524 nm. The signals shown in the figure represent a difference between two transient signals (signal in the presence of BSA and neutral red minus signal in the presence of BSA, neutral red and imidazole). Thylakoids were suspended at 10 μg chl/ml, in a medium containing 20 mM KCl, 2 mM $MgCl_2$, 0.5 mM $[Fe(CN)_6]^{3-}$, 0.3 μM nonactin, 10 μM neutral red and 1.3 mg/ml BSA. The pH of the medium was 7.0. Saturating single-turnover flashes ($t_{1/2}$ = 15 μs) were used; dark time between flashes, 10 s. Signal was averaged over 10 flashes. Other details were as in section 2.

pH changes in the outer aqueous phase of the thylakoid membrane were measured with the pH indicating dye BCP. The absorption changes of BCP are proportional to the changes in proton concentration in the outer phase of thylakoids. The pH indicating absorption change of BCP at 574 nm at pH 6.4 is shown in fig.2. The absorption changes shown have been corrected for the contribution of the intrinsic

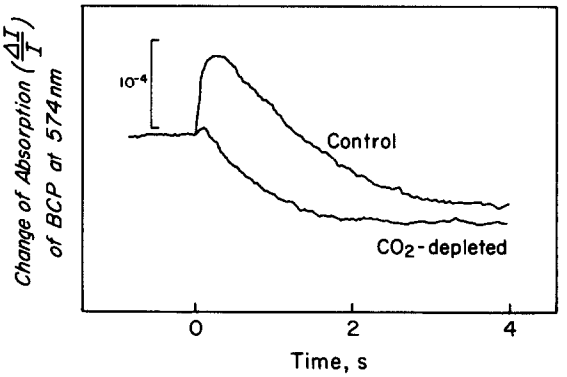

Fig.2. Absorption changes of bromocresol purple at 574 nm induced by a single turnover flash in control and CO_2-depleted spinach thylakoids. Reaction mixture (15 ml) contained 20 mM KCl, 2 mM $MgCl_2$, 0.5 mM ferricyanide, 20 μM bromocresol purple, and spinach thylakoids at 10 μg chl/ml. The pH of the medium was 6.4. Signal was averaged over 10 flashes ($t_{1/2}$ = 15 μs); dark time between flashes, 10 s. For other details see section 2.

absorption changes of thylakoid membranes by subtracting the background signal obtained in the presence of the buffer. In the control thylakoid trace, there is an initial alkalization due to the proton uptake by the plastoquinone pool. This is followed by a slow acidification due to the release of internal protons (from plastohydroquinone oxidation and water oxidation) to the exterior of the thylakoids. In the bicarbonate depleted thylakoids, there is apparently no proton uptake by the PQ pool at the outer side of the membrane. The final irreversible acidification level in bicarbonate-depleted thylakoids is about the same as in the control. It was not possible to make measurements with CO_2-reconstituted thylakoids because the buffering capacity of 2–10 mM CO_2 at pH 6.4 is high (0.5–2.5 mM). Neither the proton release nor the proton uptake measurements were made at pH 8.0, where the buffering capacity of CO_2 is low, because the CO_2 effect is almost absent at that pH [6].

Our results reveal that CO_2 depletion:

(i) Decreases by ~50% proton release by the water oxidizing system;

(ii) Causes an apparent decrease in the proton uptake from outside and the proton release inside, associated with the reduction and the oxidation of plastoquinone, respectively.

This is indicated from the recordings of the pH changes in the internal space (fig.1) and in the external space (fig.2). It is not yet clear whether the effect on proton release during water oxidation is due to a partial inactivation of reaction center II or of the water splitting enzyme complex itself. The apparent abolition of proton uptake and release associated with plastoquinone can be interpreted as follows: The rate constant for proton leakage in CO_2-depleted thylakoids is accelerated by a factor of two (k = 1.46 s^{-1} as compared to 0.73 s^{-1} in the control, calculated by replotting data of fig.2, not shown). On the other hand, the reduction of PQ is slowed down by a factor of 20 (k = 3.5 s^{-1} as compared to 70 s^{-1} in the control) as known from [5]. If the reduction of PQ were slowed down at the reaction step ($R^{2-} + PQ \rightarrow R + PQ^{2-}$) which is believed to be associated with protonation, then the observed time course of proton uptake shown in fig.2 can be easily mimicked by a kinetic calculation with the above parameter (not shown), the rationale being that the rate constants for proton uptake (outside) and proton release (inside) is close to the rate constant for proton leakage across the membrane. Another effect decreasing the proton uptake and release associated with PQ is the improved competition of ferricyanide with PQ as an electron acceptor for PS II under conditions where the reduction of PQ is slowed down. In conclusion, the protolytic reactions in CO_2-depleted chloroplasts can be explained by the effects of depletion on electron transport without the need of assuming a particular action on protolytic reaction steps. Further experiments are needed to test our suggestions.

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