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IS CO_2 AN ACTIVE SPECIES IN STIMULATING THE HILL REACTION IN THYLAKOID MEMBRANES?

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ABSTRACT

Addition of CO_2 to $CO_2(HCO_3^-)$ - depleted thylakoids at 4°C activates the ferricyanide Hill reaction in light within 2s in the presence or the absence of formate. This activation appears instantaneous after corrections for the instrumental lag. However, prior addition of carbonic anhydrase produces a lag of about 6s that approaches the lag (~6-8s) observed by HCO_3^- addition. These experiments suggest that CO_2 is an active species involved in the stimulation of the Hill reaction. Upon CO_2 injection, the final rate of O_2 evolution begins instantaneously when formate is absent from the reaction medium. However, when formate is present, the initial rate is lower than the final rate. These results suggest an interaction between CO_2 and formate; this needs to be further explored. The instantaneous increase in the Hill reaction rate upon CO_2 addition indicates that, at least, one location of the binding site is on the external thylakoid surface. In agreement with the recent data of R. Khanna, K. Pfister and J.J.S. Van Rensen, we speculate that CO_2 binds to a component on the external side of the thylakoid membrane, perhaps, to a protein close to the "B" region.

INTRODUCTION

CO_2 is the source of fixed carbon in photosynthetic carbon assimilation [1]. $CO_2(HCO_3^-)$ also plays an important role in the activation (or stimulation) of reaction center II complex [2], RUBP carboxylase [3], photophosphorylation [4], and the electron flow between the two photoreactions [5,6,7]. Good [8] showed the stimulation of electron transport by $CO_2(HCO_3^-)$ at pH 6.5; he also found that $CO_2(HCO_3^-)$ dependence was greater in the presence of high concentrations of NaCl and Na formate in the $CO_2(HCO_3^-)$ depletion medium. On the basis of these and other results, he implied that HCO_3^- may be the active species involved in the stimulation of Hill reaction. Stemler and Govindjee [9] observed the stimulation of Hill reaction by HCO_3^- to be greater at pH 6.8 than at pH 5.8. Khanna et al. [10] found the greatest stimulation of Hill reaction by sub-saturating amounts of HCO_3^- in the pH range of 6 to 7. Since in this pH range, HCO_3^- is the predominant species, the possibility of it being the active species was discussed.

However, the stimulatory effect of $CO_2(HCO_3^-)$ declines dramatically at pH
8.0 even though HCO_3^- ion remains the predominating species at that pH.
Whether CO_2 or HCO_3^- is the active species involved in the stimulation
of Hill reaction is an open question. In this communication, we report
data which suggests that CO_2 must be an active species involved in the
$CO_2(HCO_3^-)$ stimulation of the Hill reaction. Whether HCO_3^- is also involved
in it cannot be answered from the present data.

THE STRATEGY

The strategy of our method to study the nature of the active species
involved in the $CO_2(HCO_3^-)$ stimulation of the Hill reaction was (a) to use
a low assay temperature (5°C) to slow down the interconversion of CO_2 and
HCO_3^- and an alkaline pH (7.3) to obtain a high (>10) ratio of HCO_3^-/CO_2 at
equilibrium; and (b) to see the effect of the prior addition of carbonic
anhydrase that is known to increase the rate of equilibration between CO_2
and HCO_3^-. In (a), an injection of CO_2 (or HCO_3^-) provides an instantaneous
supply of CO_2 (or HCO_3^-) that is expected to last long enough to show its
effect. Thus, if there is any difference in the activation of Hill reac-
tion by CO_2 and HCO_3^-, it should be observed in the kinetics of O_2 evolution
under this experimental condition. However, in (b), carbonic anhydrase
will reduce the differences between CO_2 and HCO_3^- by causing a faster equil-
ibration between the two species. Of course, no differences are expected
for (a) or (b) at longer times because the addition of low concentrations
of CO_2 or HCO_3^- does not affect the steady state pH of the buffered reaction
medium. Our experimental results show that the above strategy indeed works
and that CO_2 must be an active species in the stimulation of the Hill reac-
tion.

KINETICS OF OXYGEN EVOLUTION WITH CO_2 OR HCO_3^- [11]

Preparation of Samples

Pea thylakoids, isolated in phosphate buffer containing sucrose and
NaCl (pH 6.8), were osmotically shocked in the same buffer without sucrose,
and then $CO_2(HCO_3^-)$ was removed by suspending these thylakoids in a phos-
phate – NaCl buffer containing 100 mM Na formate (pH 5.2) at 5°C. The low
temperature was used to reduce the loss of activity during the depletion
process. For details of the procedure, see refs. [11,12]. The depleted
sample was finally suspended in a buffer containing 50 mM Na phosphate
(pH 7.2), 100 mM Na formate and 100 mM NaCl. In experiments with formate-
free samples, the buffer was the same as above except that formate was
left out in the suspension medium.

Experiments

1.7 ml of $CO_2(HCO_3^-)$ - depleted thylakoid suspension containing 2 mM ferricyanide was placed in the chamber of a Clark electrode maintained at 4-5°C, and illuminated with saturating orange light (100 mW cm^{-2}). There was no O_2 evolution in these samples.

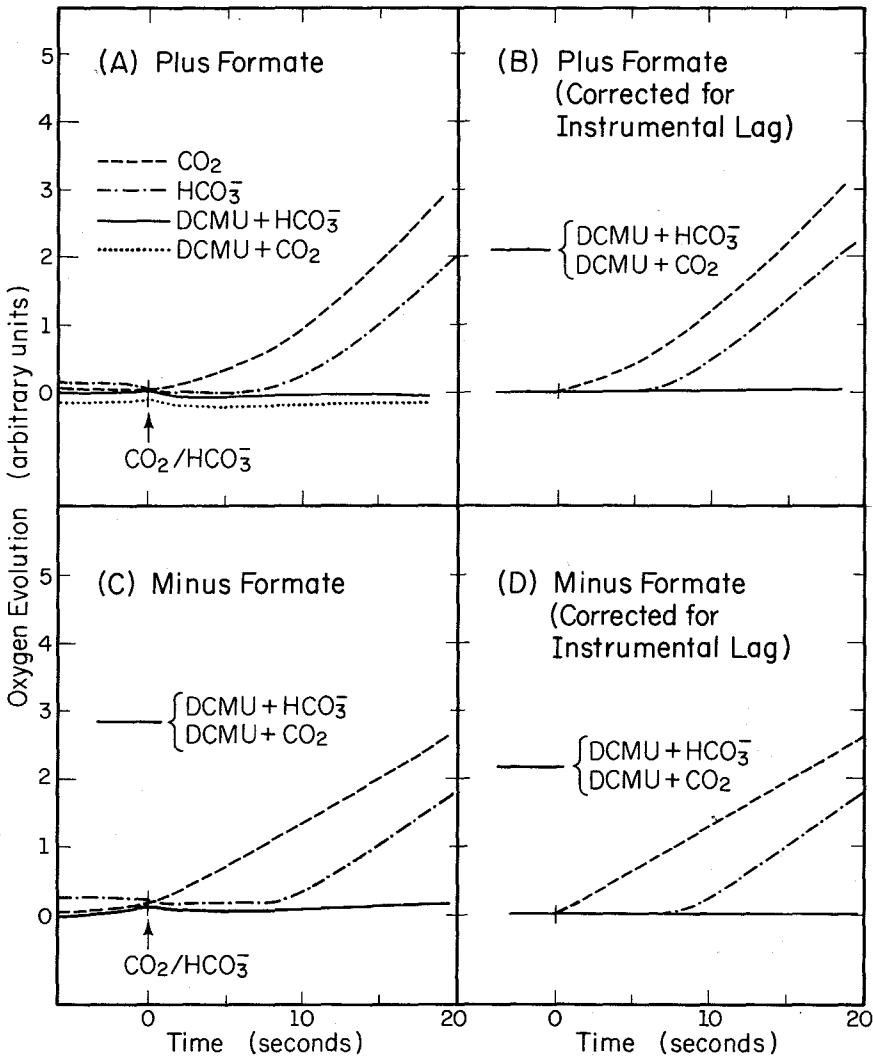After thylakoids were illuminated for 1-2 minutes, a mixture of 17 µl of 200 mM NaHCO$_3$ and 7 µl of H_2O (pH 8.6) was injected to provide 2 mM

Fig. 1. Rate of O_2 Evolution in Ferricyanide Hill Reaction as a Function of Time in $CO_2(HCO_3^-)$ - Depleted Pea Thylakoids and Those Supplied with CO_2 or HCO_3^- in the Presence (A,B) or the Absence (C,D) of Formate in the Reaction Mixture at 5°C. Controls with thylakoids treated with diuron (DCMU) are also shown.

HCO_3^-. There was a clear lag for 6-9 seconds before O_2 evolution started whether Na formate was present or absent (Fig. 1). However, a mixture of 17 μl of 200 mM $NaHCO_3$ and 7 μl of 0.55N HCl (pH 2.0), providing 2 mM CO_2, produced O_2 evolution within 2s. When corrections are made for the instrumental lag (also observed with ferricyanide injection to control chloroplasts or by exposure of control chloroplasts containing ferricyanide to light), CO_2 is shown to stimulate O_2 evolution instantaneously. When Na formate is present, the initial rate of O_2 evolution is in the range of 4 to 5 μ moles O_2/mg chl/hr. However, when equilibrium is reached, a 3 to 4 fold higher rate (~15-20 μ moles O_2/mg chl/hr) of Hill reaction is observed whether CO_2 or HCO_3^- is the injected species. When Na formate is absent, CO_2 injection leads to a steady increase in O_2 evolution. The initial rate of O_2 evolution is 10-18 μ moles O_2/mg chl/hr.

It was important to establish that the above observed differences between CO_2 and HCO_3^- injections are not artifacts of injection. The best controls were thylakoids treated with 5 μM diuron (DCMU). Figure 1 shows that injection of CO_2 or HCO_3^- to diuron treated thylakoids produced no significant artifacts under our experimental conditions.

EFFECTS OF CARBONIC ANHYDRASE [11]

If the injection of CO_2, but not HCO_3^-, stimulates the Hill reaction instantaneously, then the addition of carbonic anhydrase should change the kinetics of the observed reaction because it accelerates the equilibration of CO_2 and HCO_3^- ($CO_2 + H_2O \rightleftharpoons H_2CO_3 \rightleftharpoons H^+ + HCO_3^-$). When 210 units of carbonic anhydrase is added at 5°C, 2 minutes prior to the injection of CO_2, to the illuminated $CO_2(HCO_3^-)$ - depleted thylakoids, the observed lag time increases from 2 to 6-7s (Table 1). Conversion of CO_2 to HCO_3^- must have decreased the availability of CO_2 for the initial stimulation of Hill reaction. At 25°C, the equilibrium between CO_2 and HCO_3^- is reached at a faster rate in the presence as well as in the absence of carbonic anhydrase and thus no significant difference between CO_2 and HCO_3^- injections are observed here (Table 1).

THE ACTIVE SPECIES; COMPETITION WITH FORMATE

The above results clearly suggest that CO_2 is the active species involved in the stimulation of Hill reaction in $CO_2(HCO_3^-)$ - depleted thylakoids. Whether HCO_3^- is also involved in the stimulation process cannot be answered from the present data.

The biphasic kinetics observed upon CO_2 injection in the presence of formate may be due to competition between CO_2 and formate for the same

TABLE 1. EFFECT OF CARBONIC ANHYDRASE ON THE LAG TIME OF CARBON DIOXIDE
          OR BICARBONATE STIMULATION OF FERRICYANIDE HILL REACTION (AFTER
          SAROJINI AND GOVINDJEE [11]

| TEMPERATURE, °C | ADDITIONS | | LAG TIME* |
	CO_2/HCO_3^-, 2 mM	Carbonic Anhydrase, units	seconds
25	$+CO_2$	0	1.9 ± 0.1
25	$+ HCO_3^-$	0	2.2 ± 0.2
25	$+ CO_2$	210	2.1 ± 0.3
25	$+ HCO_3^-$	210	3.1 ± 0.1
5	$+ CO_2$	0	1.6 ± 1.2
5	$+ HCO_3^-$	0	8.1 ± 1.4
5	$+ CO_2$	210	6.3 ± 1.0
5	$+ HCO_3^-$	210	5.9 ± 0.7

* This is the time it takes for the O_2 evolution rate to rise above the
  zero line on the sensitivity scale measurements were made.

binding site because in the absence of formate only a monophasic kinetics
is observed. Further work is in progress to understand the implications
of the kinetics observed here.

MECHANISM OF CO_2 ACTION ON HILL REACTION

   We do not address ourselves to the question whether CO_2 plays any role
in the O_2 evolving mechanism (water oxidation) of photosynthesis. We do
not have any results that suggests that this happens [5,6,7,12]. We do,
however, have data suggesting that there is an effect on the donor side of
the photosystem II [2,12-14]. This can be explained by an effect of CO_2
on the reaction center II complex II [2] or on the electron donation from
one of the two electron donors (Z_1 or Z_2) donating electrons in parallel
to the reaction center II [15] without invoking any effect of CO_2 on the
actual O_2 evolving apparatus. We are, however, keeping an open mind to-
ward future discoveries in this area.

   Most of our experiments suggest that a major action of $CO_2 (HCO_3^-)$ is in

stimulating the electron flow from the two electron acceptor B (or R) to the plastoquinone (PQ) pool [14,16]. In CO_2 depleted thylakoids, the half time of electron flow from B^{2-} to PQ is 100-200 ms, and this is the bottle neck reaction. However, in control thylakoids and depleted thylakoids re-supplied with CO_2, the bottleneck reaction has a half time of 20-25 ms located in the re-oxidation of the reduced plastoquinone pool. These results certainly provide an explanation of the discovery that CO_2 stimulates Hill reaction in saturating light by a factor of 4 to 10 at room temperature. We speculate that CO_2 binds to a protein covering the Q-B-PQ region, and, its effect may extend to an action on the reaction center II complex that may include one of the two Zs. This binding may place "B" in an appropri-ate conformation such that B^{2-} can efficiently donate electrons to PQ and that B can efficiently accept electrons from Q to explain the data in ref-erences [2,14,16]. Since this CO_2-binding protein is suggested to be on the outerside of the thylakoids membrane, binding of CO_2 to it is expected to elicit an instantaneous response, as observed here. If the site was buried in the membrane, it may have taken a longer time to elicit the CO_2 effect as the half time of CO_2 to cross the thylakoids membrane at 20°C is 15s (D. Ort, personal communication), and this time may even be longer at 5°C. Recent experiments by Khanna and coworkers [12,17] support the con-clusion of a binding in the B-region: (a) the binding of a herbicide atra-zine, suggested to bind to the B-region, is altered in CO_2-depleted thyla-koids; (b) there is a difference in the ability of the thylakoids to lose their CO_2 (HCO_3^-) when prepared from atrazine resistant plants as opposed to those from atrazine - susceptible plants; (c) addition of trypsin, which is suggested to affect a protein in the B-region, leads to a decrease in the CO_2 - enhancement effect and a decrease in the Hill reaction. None of the above results prove that CO_2 binds to a protein in the B-region, but, are certainly suggestive of this possibility. Further work is in progress to clarify the mechanism(s) of CO_2 action on the Hill reaction.

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