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INFLUENCE OF OXYGEN ON THE LEVEL OF RIBULOSE-1,5-BISPHOSPHATE CARBOXYLASE/OXYGENASE (RUBISCO): ONLY A SUBSTRATE OR ALSO AN INHIBITOR?

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Abstract. Influence of oxygen upon the carboxylation of ribulose-1,5-bisphosphate (RuBP) in wheat (*Triticum aestivum*) leaves was studied at the steady state photosynthesis and at sharp transfers to a higher or to a lower O_2 concentration. In leaves exhibiting a 26% Warburg effect at the steady state photosynthesis in the saturating light, no inhibition of carboxylation by oxygen was observed when the rate was measured by means of labelling during a 0.2-s exposure. Linearity of the measured carboxylation rate to the CO_2 concentration over the range from 0 to 100% showed that the uptake represented a bimolecular reaction. The rate estimated by the method applied was a characteristic of the primary binding of CO_2, a partial reaction of carboxylation, not of the total turnover of the catalytic site. Independence of this partial reaction of $[O_2]$ indicated that oxygen did not inhibit carboxylation, neither did it cause any significant reduction in the pool of the immediate CO_2 acceptor, the Rubisco–RuBP complex, despite its role as an alternative substrate.

Key words: carboxylation, O_2 concentration, inhibition.

Oxygen decreases the rate of photosynthesis and simultaneously enhances the photorespiratory efflux of CO_2. Both phenomena are due to the same reason: bifunctionality of Rubisco catalyzing the alternative binding of CO_2 or O_2 to RuBP (see review [1]). This creates competition between the two substrates for the acceptor. The observation that not only the net rate of photosynthesis (representing the difference of influx and efflux of CO_2 of the leaf), but also gross photosynthesis (defined as the CO_2 flux into the leaf) is decreased by oxygen, has been interpreted as an evidence of depletion of the acceptor pool and/or as an evidence of

competitive inhibition of Rubisco by oxygen. Very often the gross CO_2 uptake has been taken as an equivalent of the carboxylation rate. However, this is not always correct. Under conditions where the stomatal resistance is significant, an essential proportion of carboxylation proceeds as the intrafoliar reassimilation of (photo)respiratory CO_2 and is not measured as the CO_2 flux into the leaf from the ambient atmosphere. Therefore the real carboxylation rate may be markedly higher than the flux of CO_2 into the leaf [2]. Occasionally, the intrafoliar reassimilation may represent 30 to 40% carboxylation. When this proportion has been taken into account, it has occurred that in the saturating light the atmospheric oxygen concentration does not decrease the rate of carboxylation [3]. Oxygen as a competitive substrate is capable of reducing the acceptor pool and the CO_2 binding only in the low-intensity light, where the production of RuBP limits carboxylation. In those experiments, no inhibition of carboxylation by oxygen could be observed. In the saturating light, the consumption of RuBP in oxygenation was completely compensated by its enhanced production.

In the experiments referred to above, the carboxylation rate was estimated in the steady state of photosynthesis. The possible inhibition by oxygen on the level of carboxylation might have been disguised by an increased supply of RuBP due to different statuses of the RuBP cycle under the normal and a reduced oxygen concentration. In the present work, it is demonstrated that, at the light saturation, the carboxylation rate is not influenced by oxygen either at its application during preillumination or at a sharp change in its concentration. The rate of the primary CO_2 binding is linear to its concentration over the whole range from 0 to 100%. O_2 and CO_2 have no direct influence upon the binding of each other if the acceptor pool remains saturating.

MATERIALS AND METHODS

Plants of wheat (*Triticum aestivum* L. cv. Saratovskaya-29, hexaploid) were grown in pots with soil at an irradiance of 450 $\mu mol \cdot m^{-2} \cdot s^{-1}$ and a 16-h photoperiod. The middle sections of a 6.5 cm cut from the first leaf of plants were sampled 9 d after sowing. Each sample consisted of six leaf sections, which were placed with their lower ends into a plastic bag containing water. The total area of the sections above the bag edge, exposed to the ambient gas phase, was about 12 cm^2 per sample.

The samples were inserted into the exposure chamber described earlier [4], preilluminated at an irradiance of 1600 $\mu mol \cdot m^{-2} \cdot s^{-1}$ flushing with N_2 containing $^{12}CO_2$ (300 to 3000 ppm) and O_2 (1.5%). The temperature of the chamber was 30°C. The CO_2 uptake during preillumination was monitored by the infrared gas analyser (Infralyt-4; Junkalor, GDR). The transpiration rate indicating the status of stomata was monitored by a micro-psychrometer (according to [5]).

When a steady rate of photosynthesis had been achieved, the chamber was transferred to the gas system with $^{14}CO_2$ of the same or a varied concentration and O_2 of 0, 1, or 21%. In some experiments, the preilluminated leaves were flushed with the CO_2-free gas phase for 5 to 6 s to allow them to accumulate RuBP and to saturate the active centres of Rubisco. The leaves prepared in this way were transferred to $^{14}CO_2$ (concentrations shown in tables and figures) and fed with the tracer for 0.2, 0.3, 0.4 or 0.6 s. After the labelling, the leaves were dropped into boiling 80% ethanol for killing. Thereafter the samples were extracted with acidified 96% ethanol.

The radioactivity of the extracts and of the residue was measured by means of a scintillation counter. The RuBP content of leaves was estimated by the postillumination tracer uptake at the exposure to 10 000 ppm $^{14}CO_2$ as described earlier [6].

The experimental data in the figures and the tables are averages of three to five samples, six leaf sections each.

RESULTS

The leaves preilluminated in 1% and 21% O_2 had a slightly different steady state concentration of RuBP (Table 1). The net photosynthesis rate in 21% O_2 was also somewhat lower, the Warburg effect making up 26%, which is an ordinary value for the C_3 plants. However, the rate of carboxylation measured by the 0.2-s exposure to $^{14}CO_2$ of the same concentration as during preillumination, was not different at the two O_2 concentrations (set 1 vs. 4). The transfer from low to high [O_2] (set 1 vs. 2) and vice versa (set 3 vs. 4) did not cause any decrease or increase in the carboxylation rate.

Table 1

Dependence of the net rate of photosynthesis and carboxylation on the concentration of oxygen in the ambient gas phase

Set No.	Preillumination				Exposure to $^{14}CO_2$	
	[O_2]	[RuBP]	Net photosynthesis		[O_2]	Carboxylation
	%	nmol · dm^{-2}	nmol · dm^{-2} · s^{-1}	%	%	nmol · dm^{-2} · s^{-1}
1.	1	729 ± 65	265 ± 9	100	1	246 ± 3
2.	1	729 ± 65	265 ± 9	100	16	274 ± 2
3.	21	566 ± 60	195 ± 6	74	5	223 ± 5
4.	21	566 ± 60	195 ± 6	74	21	229 ± 6

When the leaves were preilluminated in 300 ppm $^{12}CO_2$, 1 or 21% O_2, and thereafter illuminated for 5 s in the CO_2- and O_2-free atmosphere, the subsequent carboxylation rates were as given in Table 2. When the leaves were preilluminated at 1% O_2, the rate measured after illumination in the absence of CO_2 was equal to that at the preceding steady state. In the case of preillumination at 21% O_2 the uptake rate after illumination in the absence of CO_2 and O_2 was significantly greater.

Table 2

The rate of carboxylation during the steady state photosynthesis and after a 6-s illumination in the absence of CO_2 and O_2 depending on the O_2 concentration during preillumination

Steady state		After 6-s absence of CO_2 and O_2	
$[O_2]$, %	Carboxylation, $nmol \cdot dm^{-2} \cdot s^{-1}$	$[O_2]$, %	Carboxylation, $nmol \cdot dm^{-2} \cdot s^{-1}$
1	258 ± 7	0.5	229 ± 5
21	255 ± 8	0.5	392 ± 70

When gross or net photosynthesis is measured in experiments of the time order of several seconds or more, the CO_2 dependence of the process shows the Michaelis-type kinetics with K_M of about 7 to 25 μM depending on the plant species [7–10]. From the double reciprocal plot of the photosynthesis rate to $[CO_2]$ (Fig. 1, curve 4) $(1/C_w)_{y=0}$ and $(1/v)_{x=0}$ of 0.15 and 2.08×10^{-3} are obtained, respectively, corresponding to $K_M(CO_2)$ of 6.6 μM and V_{max} of 480 $nmol \cdot dm^{-2} \cdot s^{-1}$. However, if we plot in the same way the CO_2 binding rate estimated by the 0.2-s exposure to different concentrations of $^{14}CO_2$, the concentration dependence has quite a different nature (Fig. 1, curves 1, 2, 3). Leaves preilluminated at 300, 1000, or 3000 ppm $^{12}CO_2$, 1% O_2, followed by a 6-s illumination in the absence of CO_2 and O_2, were exposed to $^{14}CO_2$ of varied concentration. To eliminate the influence of differences in [RuBP] created due to different rates of carboxylation during the exposure, the rates obtained for the first 0.2-s exposure were normalized to the zero time by multiplying the rate by the factor $[RuBP_0]/[RuBP_n]$, where $[RuBP_0]$ and $[RuBP_n]$ are respectively the concentrations of RuBP at the zero time and at the 0.1-s point. The latter is calculated as $[RuBP_n] = [RuBP_0] - 0.5 \times c$, where c is the amount of carbon bound during 0.2 s, equivalent to the amount of consumed RuBP. From curves 1, 2, and 3 in Fig. 1 it may be calculated that in leaves preilluminated in 300, 1000, and 3000 ppm CO_2 the values of $1/V_{max}$ were respectively -0.1×10^{-3}, -0.1×10^{-3}, and 0.02×10^{-3}. We interpret that as an evidence of the lines passing zero and

of the linearity of the CO_2 binding measured during 0.2 s to the CO_2 concentration over the whole range from 0 to 100%.

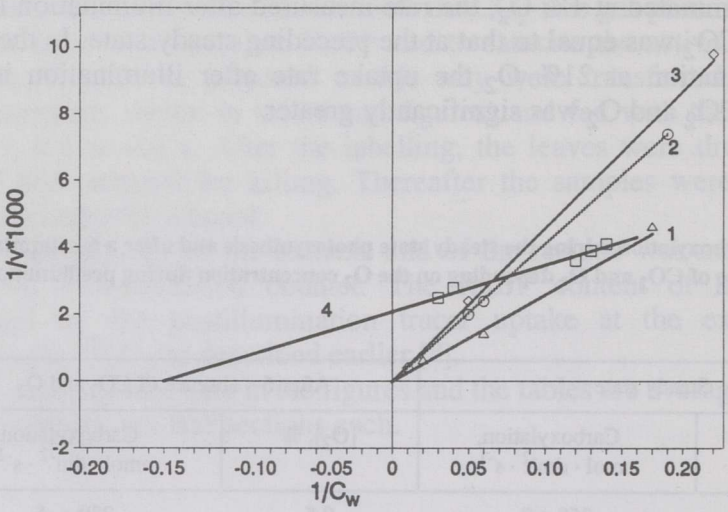

Fig. 1. Double reciprocal plot of the carboxylation rate (v, normalized to the initial concentration of RuBP) vs. intracellular CO_2 concentration (C_w) after preillumination at 300 ppm (1), 1000 ppm (2), or 3000 ppm (3) CO_2, and at the steady state photosynthesis in 300 ppm CO_2 (4).

The linearity of the carboxylation rate to $[CO_2]$ at short-term labelling could be deduced also from the kinetics of the carboxylation efficiency (ε) defined as the ratio of the CO_2 binding rate to the intracellular $[CO_2]$. After preillumination in the saturating light at a reduced O_2 concentration, the leaves were illuminated for 6 s in the absence of CO_2 and O_2 and thereafter exposured to different $[^{14}CO_2]$ for 0.2, 0.3, and 0.4 s. From the kinetics of the labelling (Fig. 2*A*, curves 1, 2, 3, 4), the kinetics of the carboxylation rate was obtained (Fig. 2*B*). From the profiles of CO_2 in the chamber during the labelling (Fig. 2*A*, curves 1*a*, 2*a*, 3*a*, and 4*a*, respectively) and the transpiration rate, the intracellular $[CO_2]$ (C_w) was calculated ([5]; data not shown here). The kinetics of ε obtained from these parameters is shown in Fig. 3. At higher $[CO_2]$, applied after illumination without CO_2, the carboxylation efficiency declined faster due to a higher rate of the consumption of RuBP. However, it is remarkable that with all $[CO_2]$ values applied the lines extrapolated to the same initial value of ε (ε_0). This value depended on the CO_2 concentration during preillumination, obviously due to the different RuBP concentration at the zero time, but not on $[CO_2]$ at the labelling.

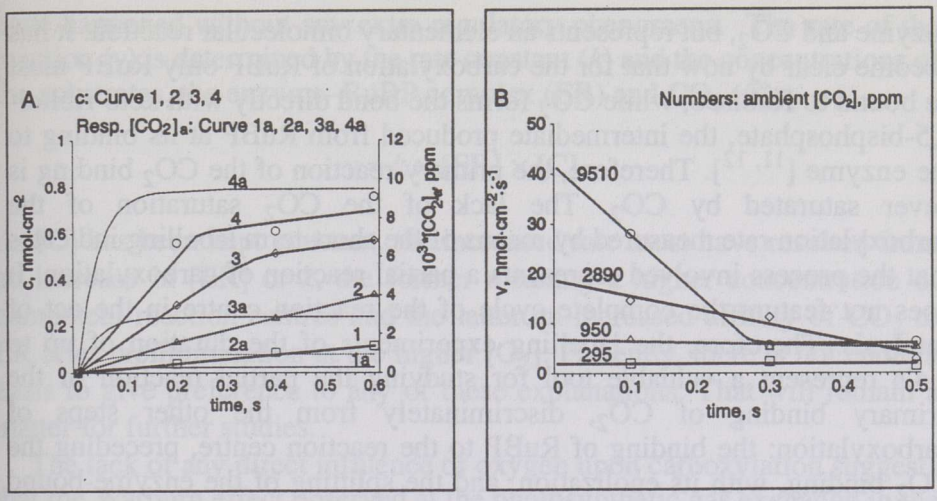

Fig. 2. Kinetics of the CO_2 uptake after a 6-s illumination without CO_2. *A*. Amount of assimilated carbon (*c*); concentration of the ambient CO_2 ($[CO_2]_a$). *B*. CO_2 uptake rate (*v*).

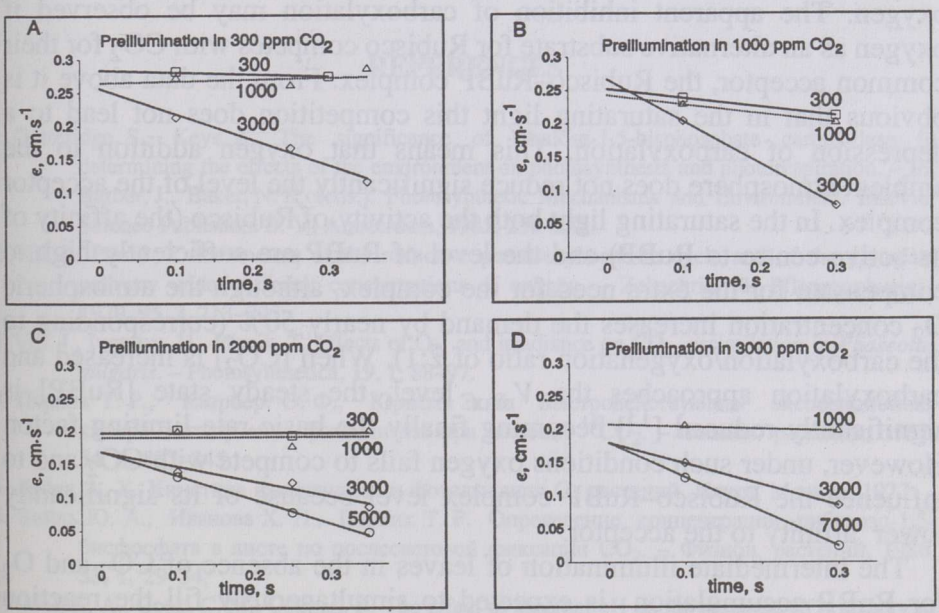

Fig. 3. Kinetics of the carboxylation efficiency (ε) after a 6-s illumination without CO_2. Numbers at curves: $[CO_2]$ at the exposure.

DISCUSSION

Linearity of the rate of carboxylation to the CO_2 concentration observed at the short-term labelling shows that the reaction involved does not require any formation of a Michaelis-type intermediate between the

enzyme and CO_2, but represents an elementary bimolecular reaction. It has become clear by now that for the carboxylation of RuBP only RuBP must be bound to Rubisco, while CO_2 forms the bond directly with keto-ribitol-1,5-bisphosphate, the intermediate produced from RuBP at its binding to the enzyme [11, 12]. Therefore, the primary reaction of the CO_2 binding is never saturated by CO_2. The lack of the CO_2 saturation of the carboxylation rate measured by means of the short-term labelling indicates that the process involved represents a partial reaction of carboxylation. It does not feature the complete cycle of the reaction centre in the act of catalysis. Therefore, the labelling experiments of the duration of up to 0.4 s represent a valuable tool for studying the partial reaction of the primary binding of CO_2, discriminately from the other steps of carboxylation: the binding of RuBP to the reaction centre, preceding the CO_2 binding, with its enolization, and the splitting of the enzyme-bound 6C intermediate into two 3C molecules with the release of the catalytic centre which follows the step of the primary CO_2 binding.

The data in Table 1 show that the primary CO_2 binding is not sensitive to $[O_2]$, independent of whether applied during preillumination or labelling. This rules out any real direct inhibition of the reaction by oxygen. The apparent inhibition of carboxylation may be observed if oxygen as an alternative substrate for Rubisco competes with CO_2 for their common acceptor, the Rubisco–RuBP complex. From the data above it is obvious that in the saturating light this competition does not lead to a depression of carboxylation. This means that oxygen addition to the ambient atmosphere does not reduce significantly the level of the acceptor complex. In the saturating light both the activity of Rubisco (the affinity of its active centre to RuBP) and the level of RuBP are sufficiently high to compensate for the extra need for the complex, although the atmospheric O_2 concentration increases the demand by nearly 50% (corresponding to the carboxylation/oxygenation ratio of 2:1). When $[CO_2]$ is increased and carboxylation approaches the V_{max} level, the steady state [RuBP] is significantly reduced [13] becoming finally the basic rate-limiting factor. However, under such conditions oxygen fails to compete with CO_2 and to influence the Rubisco–RuBP complex level because of its significantly lower affinity to the acceptor.

The intermediate illumination of leaves in the absence of CO_2 and O_2 for RuBP accumulation is expected to simultaneously fill the reaction centres of Rubisco. The subsequent exposure to CO_2 should result in the maximum rate of the uptake possible at the concentration applied. From the data above it may be seen that in the leaves preilluminated at a reduced O_2 concentration the intermediate illumination did not increase the subsequent uptake rate compared with the steady state. Obviously, the overwhelming proportion of the active centres were in the RuBP-saturated state already during the steady state photosynthesis at the atmospheric concentration of CO_2. Surprisingly, after preillumination in 21% O_2 the intermediate illumination in the absence of CO_2 markedly increased the subsequent uptake rate, which exceeded the suggested maximum rate measured in leaves preilluminated in 1% O_2. This could not

have happened without any extra regulatory phenomena. The rate of the reaction (v) is determined by the rate constant (k) and the concentrations of the substrates, the enzyme–RuBP complex (ER) and CO_2 (C):

$$v = k \times [ER] \times [C].$$

As [C] has been kept constant, the increased rate must have resulted from an increase in [ER] or k, the former meaning a higher concentration of competent reaction centres and the latter an increased affinity of CO_2 to ER after preillumination at the higher $[O_2]$. Presently, there is not enough basis to give preference to any of these explanations. That will remain a matter for further studies.

The lack of any direct influence of oxygen upon carboxylation suggests that the Warburg effect observed at the photosynthetic gas exchange in the saturating light is essentially the result of the photorespiratory decarboxylation. However, its component fluxes are modified by the state of stomata, which determines the ratio of the efflux and reassimilation of the released CO_2.

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HAPNIKU TOIME RIBULOOS-1,5-BISFOSFAADI KARBOKSÜLAAS/OKSÜGENAASILE (RUBISCOLE): KAS AINULT SUBSTRAAT VÕI KA INHIBIITOR?

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Uuriti hapniku toimet ribuloos-1,5-bisfosfaadi (RuBP) karboksüleerimisele nisu (*Triticum aestivum*) lehtedes statsionaarsel fotosünteesil ja järskudel üleminekutel kõrgemale või madalamale O_2 kontsentratsioonile. Lehtedes, mille statsionaarsel fotosünteesil esines Warburgi efekt suurusega 26%, ei täheldatud mingit hapniku inhibeerivat toimet karboksüleerimisele, kui viimase kiirus määrati 0,2-sekundiste ekspositsioonide järgi radioaktiivses CO_2-s. Karboksüleerimise kiirus oli lineaarne CO_2 kontsentratsiooni suhtes vahemikus 0–100%. See näitab, et tegemist oli bimolekulaarse reaktsiooniga. Rakendatud meetodi abil määratud kiirus iseloomustab üht karboksüleerimise osareaktsiooni: CO_2 primaarset sidumist, kuid mitte tervet katalüüsitsüklit. Selle osareaktsiooni sõltumatus hapniku kontsentratsioonist näitab, et hapnik ei ole karboksüleerimise inhibiitor ega kutsu esile CO_2 vahetu aktseptori, Rubisco–RuBP-kompleksi kontsentratsiooni langust.

ДЕЙСТВИЕ КИСЛОРОДА НА КАРБОКСИЛАЗУ/ОКСИГЕНАЗУ РИБУЛОЗО-1,5-БИСФОСФАТА (RUBISCO): ТОЛЬКО СУБСТРАТ ИЛИ ТАКЖЕ ИНГИБИТОР?

Юта ВИЙЛЬ, Хийе ИВАНОВА, Тийт ПЯРНИК

Изучено действие кислорода на карбоксилазу/оксигеназу рибулозо-1,5-бисфосфата в листьях пшеницы (*Triticum aestivum*) при стационарном фотосинтезе и при резких переходах на повышенную или на сниженную концентрацию O_2. В листьях, в которых наблюдался эффект Варбурга в 26%, не было обнаружено никакого ингибирования карбоксилирования кислородом, определенного по поглощению метки за 0,2 с. Скорость карбоксилирования была линейной в отношении концентрации CO_2 в диапазоне 0–100%. Это

показывает, что процесс представляет собой бимолекулярную реакцию. Скорость, определенная с помощью этого метода, характеризует одну из удельных реакций карбоксилирования, первичное связывание CO_2, но не целый каталитический цикл. Независимость этой удельной реакции от концентрации кислорода показывает, что кислород не является ингибитором карбоксилирования и на насыщающем свету не вызывает снижения концентрации непосредственного акцептора CO_2, комплекса фермента с рибулозобисфосфатом.