

# Chemical Control of Photorespiration: Steady-State Kinetic and Conformational Changes of Ribulose-1,5-bisphosphate Carboxylase/Oxygenase Obtained with O-*p*-Nitrophenylhydroxylamine

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Spinach RuBP carboxylase-oxygenase (Rubisco) purified from the commercially available enzyme, underwent kinetic changes when micromolar concentrations of O-*p*-nitrophenylhydroxylamine (NPHA) were applied on fully activated enzyme. Under steady-state conditions, a decrease of  $K_m$   $\text{CO}_2$  was observed without changes in  $V_{max}$ . Double reciprocal plots showed a competitive stimulation of carboxylase function. The substrate specificity factor  $k = V_c K_o / V_o K_c$  was increased from approximatively 15–20% upon treatment with NPHA, using both parallel and simultaneous determinations of RuBP-carboxylase and oxygenase activities.

Such a limited change in partition between Rubisco functions actually resulted into an increase in  $\text{CO}_2$  fixation of intact soybeans leaves treated with NPHA, arising from a substantial alleviation of  $\text{O}_2$ -inhibition of photosynthesis.

From spectrophotometric and sulphydryl group titration data, evidence was accumulated for a conformational change of the native enzyme, induced by NPHA, bringing about an increased ratio of carboxylase to oxygenase activities. Direct interaction of NPHA with a cysteine residue of Rubisco near the catalytic site, appears as a candidate for such chemically-induced conformational modification.

## Introduction

In the crops possessing the  $\text{C}_3$  photosynthesis pathway, a substantial amount of the carbon fixed is diverted to an oxidative cycle: photorespiration, a process which is interlocked with the carbon reduction cycle. Control of photorespiration has appeared as a potential mean for increasing crop productivity. Attempts have been made to either chemically or genetically alter this process. Various chemicals have been offered for decreasing the magnitude of photorespiration: hydroxy-pyridine methanesulfonate derivatives [1], aminoethane sulfonates [2], INH [3], 8-

hydroxy-3-butynoate [4], glycine and serine derivatives [5], glycidate [6],  $\text{VO SO}_4$  [7]. However, these chemicals interfered more or less specifically with one or several steps of the photorespiratory glycolate pathway, which resulted in alterations which were demonstrated deleterious to the plant growth [8].

Photorespiration is initiated by the oxygenase activity of the bifunctional enzyme RuBP-carboxylase/oxygenase or Rubisco [9];  $\text{CO}_2$  and  $\text{O}_2$  are competitive substrates for RuBP. Relative magnitude of oxygenase (photorespiration) and carboxylase (photosynthesis) functions can be kinetically described by the following equation:

$$\frac{\text{Photosynthesis}}{\text{Photorespiration}} = \frac{V_c K_o [\text{CO}_2]}{t V_o K_c [\text{O}_2]} \quad [10]. \quad (1)$$

$V_c$  and  $V_o$  are the  $V_{max}$  values of the two activities,  $K_c$  and  $K_o$  are the  $K_m$  values for  $\text{CO}_2$  and  $\text{O}_2$  and  $t$ , the stoichiometry between  $\text{O}_2$  taken up and the  $\text{CO}_2$  released in photorespiration. Any attempts for chemically or mutagenically decrease photorespiration would involve a significant change in this ratio.

**Abbreviations:** C.E.R.,  $\text{CO}_2$  exchange rate; DTNB, 5,5'-dithio, bis-2-nitrobenzoate; DTT, dithiothreitol; HA, hydroxylamine; INH, isonicotylhydrazine;  $K_c = K_m \text{CO}_2$ ;  $K_o = K_m \text{O}_2$ ; NPHA, O-*p*-nitrophenylhydroxylamine; PGA, phosphoglyceric acid; RuBP, ribulose 1,5-bisphosphate;  $V_c = V_{max} \text{CO}_2$ ;  $V_o = V_{max} \text{O}_2$ .

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Compounds proposed to differentially alter one of the Rubisco functions include sugar phosphates [11], transition state analogs [12, 13], tetrinitromethane [14], aminoacid reagents such as pyridoxal phosphate [15, 16], diethylpyrocarbonate, rose bengal [17], glyoxylate [18], hydroxylamine [19, 20], glycidate and divalent metal cations such as  $\text{Cu}^{2+}$  [21] or  $\text{Mn}^{2+}$  [22].

The Rubisco oxygenase activity is expected to be chemically "unavoidable" and would coincide with carboxylase activity. This "inevability" does not necessarily imply that the velocity ratio cannot be changed, since the above mentioned chemicals (except  $\text{Mn}^{2+}$ ) were ineffective in such a change. The substrate specificity factor, representing these variations in terms of ratio between oxygenase to carboxylase velocities, has been indeed shown to vary among species (e.g. [23]).

This result was made possible by using an accurate and simultaneous determination of both activities.

We report the effect of a newly discovered chemical able to increase carboxylation reaction and thereby change the substrate specificity factor.

## Materials and Methods

**Enzyme.** Commercially available Rubisco was purchased from Sigma; PGA phosphatase-free enzyme was prepared as previously described [24]. Phosphoglycolate-phosphatase was purified by using a modified procedure [24] as described earlier [27].

**Chemicals.** O-*p*-nitrophenylhydroxylamine (NPHA) was synthesized according to [25] and dissolved into ethanol-water solution (ketones and ethers solvent must be avoided). [ $1^3\text{H}$ ]RuBP was prepared by using pentose phosphate pathway with [ $2^3\text{H}$ ]glucose as starting material, as previously described [24, 26].

### Separate determination of Rubisco activities

**Carboxylase.** Enzymatic activity was determined by measuring [ $^{14}\text{C}$ ]Na H  $\text{CO}_3$  incorporation into acid-stable fraction [28] after reaction with enzyme fully-activated [29] by incubation for 5 min at 30 °C in Tris HCl, pH 8.50 mM, Mg Cl<sub>2</sub> 20 mM, [ $^{14}\text{C}$ ]Na H  $\text{CO}_3$  10 mM (18.5 GBq/mol).  $\text{CO}_2$  concentrations were calculated from added bicarbonate using a pKa value of 6.3. Special care was taken to ensure steady-state kinetics, non-limiting substrate conditions, and a lack of interaction between NPHA and ingredients

of activation/reaction media, as well as maintaining the activated state of Rubisco. In this respect, DTT was not included in any medium (see Fig. 3a).

**Oxygenase.**  $\text{O}_2$ -consumption was measured using a Clark-type electrode [29] and the fully activated enzyme.  $\text{CO}_2$ -free buffers (0.1 M Tris-HCl, pH 8.6, MgCl<sub>2</sub> 20 mM) were bubbled either with  $\text{CO}_2$ -free nitrogen or  $\text{CO}_2$ -free  $\text{O}_2$ . Different  $\text{O}_2$  concentrations were obtained by mixing the two buffers [30].

**Simultaneous determinations of RuBP-carboxylase and oxygenase activities.** Assays were carried out by using [ $^{14}\text{C}$ ]Na H  $\text{CO}_3$  (specific activity: 11.1 kBq/ $\mu\text{mol}$ , [ $1^3\text{H}$ ]RuBP (specific activity: 4.07 kBq/ $\mu\text{mol}$ ) with activated, PGA-phosphatase-free enzyme, according to a modified procedure [24] described in [31]. Carboxylase activity was measured by counting radioactivity of the acid stable [ $^{14}\text{C}$ ]PGA, whereas oxygenase was measured by counting  $^3\text{H}$  incorporated into glycolate which was enzymatically derived from  $^3\text{H}$ -phosphoglycolate by using P-glycolate phosphatase.

**Kinetics.**  $K_m$  and  $V_{\max}$  values were determined using a Woolf plot. Substrate specificity factor (SFF) was either calculated from E (1) or obtained from the slope of the relationship between reaction velocities ratio  $v_c/v_o$  versus  $\text{CO}_2$  to  $\text{O}_2$  concentration ratio:

$$\text{SFF} = k = \frac{V_c K_o}{K_c V_o}.$$

**Titration of SH-groups by DTNB.** Free SH-groups of activated and non-activated native Rubisco were titrated with Ellman's reagent (5,5'-dithio, bis-2-nitrobenzoate or DTNB) [32]. A non-limiting concentration of DTNB was ensured by using a DTNB-to protein ratio of 500 to 700 (mol/mol of protein), and tested with a cysteine solution [33].

**$\text{CO}_2$  exchange rates.** CER were measured with a differential IR-gas analyzer ANARAD model AR-600, equipped with a Plexiglas clamp-on cuvette, that enclosed a 5 cm<sup>2</sup>-leaf area. Desired  $\text{O}_2$  concentrations were delivered by gas tanks containing  $\text{N}_2$ ,  $\text{O}_2$ , 355 ppm  $\text{CO}_2$ .

## Results and Discussion

### RuBP-carboxylase kinetics

$V_{\max}$  and  $K_m$  values were determined from data obtained by varying the relative ratios between spinach enzyme, RuBP,  $\text{CO}_2$  and NPHA concentra-

Table I. Spinach RuBP-carboxylase kinetic parameters.

Ratio Enzyme/RuBP	NPHA [ $\mu$ M]	$V_{max}$		$K_m \text{ CO}_2$	
		[nKat/mg]	% Control	[ $\mu$ M]	% Control
$0.47 \times 10^{-3}$	0	10.6	100	33.4	100
	50	10.8	101	35.8*	107
$0.94 \times 10^{-3}$	0	9.8	100	34.6	100
	10	10.1	103	31.2*	90
	100	9.9	101	28.8*	83
$1.89 \times 10^{-3}$	0	9.4	100	39.8	100
	50	9.1	96	32.4**	81
$3.78 \times 10^{-3}$	0	4.5	100	13.6	100
	50	4.2	93	7.8**	57

Reaction mixture = Tris-HCl 50 mM pH 8.0, Mg Cl<sub>2</sub> 20 mM, RuBP 250 or 500  $\mu$ M, Rubisco: 0.235  $\mu$ M and 0.945  $\mu$ M, NPHA as indicated, temperature = 30 °C.

\* Not statistically significant.

\*\* Statistically significant difference from the control value.

tions and calculated from a Woolf plot. Data are summarized in Table I. The values presented herein represent the average of replicates of the same assay with the same enzyme batch. Therefore, the differences between  $K_m \text{ CO}_2$ , experienced in presence of 50  $\mu$ mol of NPHA, are highly significant. In other

words, this means that the affinity of Rubisco for CO<sub>2</sub> almost doubled with the chemical treatment.

The experimental conditions are employed so that both substrates and NPHA concentrations are not limiting, and an absence of interaction between NPHA and RuBP is verified, which is a prerequisite for a correct estimation of inhibitors effect [38]. Consequently, it appears that, under a steady state kinetic situation, only  $K_m$  s vary with NPHA without changes in  $V_{max}$  values, as shown with double reciprocal plots (Fig. 1).

This result suggests that NPHA treatment brings about a competitive stimulation of carboxylase activity. The performance of NPHA is dependant upon the ratio between enzyme and RuBP. Since the reagents addition were properly ordered to prevent deactivation of Rubisco, influence of RuBP concentration on activity had to be considered only in terms of the relative amounts of enzyme and substrate.

There is an optimal substrate concentration, which is pH-dependant [34], which brings about a regulation [35] through a mechanism involving protein conformational changes [35]. Since it was demonstrated that varying O<sub>2</sub> concentration induced Rubisco conformational changes [37] as did RuBP [50] it can be expected that a defined enzyme/RuBP ratio results in a specific enzyme conformation, leading in turn to a shift between oxygenase to carboxylase activity in the presence of NPHA. However, such a speculative mechanism needs to be further clarified.

In any case, such a ratio seems to correspond with the situation *in vivo* [39, 40]. Also, conditions in

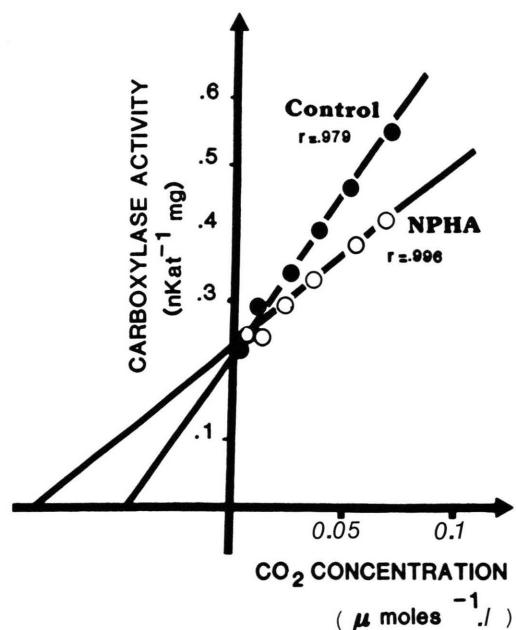


Fig. 1. Lineweaver-Burk plot of spinach RuBP-carboxylase activity as a function of CO<sub>2</sub> concentration, in the presence or the absence of 50  $\mu$ M NPHA.  $r$  = correlation coefficients.

which RuBP/CO<sub>2</sub> ratio could become limiting [41] have been avoided in this study. However, a determination of carboxylase data obviously requires a careful evaluation of oxygenase kinetics.

*Separate measurement of RuBP-carboxylase/oxygenase activities*

Using the methodology mentioned in Material and Methods carboxylase and oxygenase activities and kinetic parameters were separately measured and results are presented in Table II. Furthermore, the experiment was designed to check the possibility of an interaction of NPHA with activation state of the enzyme; consequently, 50 μM NPHA were added prior to activation, leading to a final concentration of 3.3 μM during catalysis. Under these conditions, with a enzyme/RuBP ratio similar to that of RuBP-carboxylase assays (Table I) *i.e.* 3.8 × 10<sup>-3</sup> an increase

in  $K_m$  O<sub>2</sub> ( $K_o$ ) and a decrease in  $K_m$  CO<sub>2</sub> ( $K_c$ ) was observed, in the presence of NPHA, without substantial changes in  $V_{max}$ . As a consequence the substrate specificity factor  $k = V_c \times K_o / V_o \times K_c$  shifted from 53 to 63.5, corresponding to an increase of 19%, in favor of the carboxylase reaction. However, these results must be interpreted with care: as a matter of fact, consistent  $K_m$  O<sub>2</sub> are difficult to obtain, whereas oxygenase velocities does not seem to follow Michaelis kinetics, as shown by non-saturating rates at 100% O<sub>2</sub> [42]. Moreover, although steady state requirements are met, experimental constraints imposed by a separate measurement of both activities implies different reaction conditions. Also, the results presented in this case correspond only to a single experiment. Consequently, a reliable evaluation of changes imposed to Rubisco function by NPHA needs to be carried out with a simultaneous measurement of carboxylase and oxygenase activities.

Table II. Separate determination of RuBP-carboxylase and oxygenase activities.

NPHA [μM]	Carboxylase $K_c$ [μM]	Carboxylase $V_c$ [nKat/mg]	Oxygenase $K_o$ [μM]	Oxygenase $V_o$ [nKat/mg]	$k = \frac{V_c \cdot K_o}{V_o \cdot K_c}$
0	16.4	4.5	700	3.6	53
3.3	14.6	4.4	780	3.7	63.5
% Control	89	98	111	102	119

Activation: the same as described in Material and Methods, except 50 μM NPHA were added prior to activation. Reaction media: carboxylase: Tris HCl 50 mM, MgCl<sub>2</sub> 20 mM, enzyme 0.97 μM, RuBP 250 μM. Oxygenase: as for carboxylase except NaHCO<sub>3</sub> 0.67 mM, pH 8.6, temperature 30 °C.

Table III. Simultaneous determination of oxygenase and carboxylase activities of PGA phosphatase free Rubisco.

	% O <sub>2</sub>	Control $v$ [nKat/mg]	% Activity at 40% over 20%	3.3 μM NPHA $v$ [nKat/mg]	% Activity at 40% over 20%	% v NPHA over Control
Oxygenase	20	0.308 ± 0.01	100	0.32 ± 0.01	100	103
	40	0.60 ± 0.02	194	0.54 ± 0.015	169	90
Carboxylase	20	3.18 ± 0.11	100	3.28 ± 0.1	100	103
	40	2.84 ± 0.13	89	3.22 ± 0.12	98	113.4

Reaction medium: Tris-HCl 50 mM, pH 8.6, MgCl<sub>2</sub> 20 mM, NaHCO<sub>3</sub> 6.3 mM, Enzyme 0.94 μM, RuBP 250 μM, temperature 30 °C.

### Simultaneous determination of PGA-phosphatase-free RubP carboxylase/oxygenase

By using [ $1^3\text{H}$ ]RuBP and  $^{14}\text{CO}_2$  as substrates for Rubisco, simultaneous velocities were measured in the presence and absence of NPHA; results are summarized in Table III. In comparing carboxylase and oxygenase activities, NPHA strongly alleviates  $\text{O}_2$ -inhibition of carboxylase whereas the product partly decreases  $\text{O}_2$ -enhancement of oxygenase. However, experimental conditions were such that carboxylase activity was in fact relatively favored as compared to oxygenase. First, pH and  $\text{Na H CO}_3$  concentration promote more carboxylase activity than oxygenase, as evidenced by a ratio of 10:1, whereas under normal atmospheric conditions at 20%  $\text{O}_2$ , 25 °C, this ratio was reported to be approximatively 4:1 [10]. In these conditions, NPHA performs less as compared to data shown in Table I. Second, since 50  $\mu\text{M}$  NPHA was introduced in the activation medium, the product is diluted in the reaction medium. At the final concentration of 3.3  $\mu\text{M}$  in this experiment, the NPHA had only a limited effect on the carboxylase activity (Table II).

Equation (1) suggests that reducing photorespiration to increase photosynthesis should be done either by increasing  $V_c$ ,  $K_o$  or  $\text{CO}_2$ , or by decreasing  $V_o$ ,  $K_o$  or  $\text{O}_2$ , in order to alter the relationship between the two processes; this relationship is conveniently expressed by the substrate specificity factor (SSF), and given by the slope  $k$  of the relation:

$$v_c/v_o = k [\text{CO}_2]/[\text{O}_2].$$

The results presented in Table IV, indicate that NPHA induces a shift of the  $k$  value from 71.3 to 82.7, thus increasing carboxylation reaction as compared to the oxygenase reaction. Furthermore, Table III emphasizes  $\text{O}_2$  effects on carboxylation (inhibition) and oxygenase (stimulation): NPHA clearly suppresses  $\text{O}_2$  toxicity towards carboxylase whereas it decreases  $\text{O}_2$ -induced oxygenase stimulation.

Table IV. Substrate specificity factor  $k$  in the presence or absence of NPHA, evaluated from the slope of the relationship between  $v_c/v_o$  and  $\text{CO}_2/\text{O}_2$ .

NPHA [ $\mu\text{M}$ ]	$v_c/v_o$	$k$	% Control	$r^*$
0	4.7	71.3	100	0.969
3.3	5.9	82.7	116	0.998

\*  $r$  = linreg correlation coefficient.

*Relief by NPHA of  $\text{O}_2$  inhibition of photosynthesis in intact soybean leaves.* Greenhouse-grown Ranson 2 soybean plants, sprayed over-the-top with an ethanol  $\text{H}_2\text{O}$  (50:50 v/v) solution of NPHA, corresponding to a rate of 60 g active ingredient/ha, were treated at the early-flowering stage. 24 h after treatment, CER were measured on different trifoliates, with a gas containing various  $\text{O}_2$  concentrations, flushed into the leaf cuvette and corresponding to 2%, 21% and 41%, respectively. Typical CER measurement is exemplified on Fig. 2 for one of the treated intact leaves, and expressed as a mean of 5 replications (insert). Dixon plot presented on Fig. 2 shows that at 2%  $\text{O}_2$ , no differences can be seen between treated and control leaves, whereas at 20 and 40%  $\text{O}_2$ , a stimulation of CER, increasing with  $\text{O}_2$  concentration, is experimented. Conceptually, a Dixon's plot should display a competition between the two phenomena, when the straightlines intersect behind the ordinate axis; this is not the case here, simply because at 2%  $\text{O}_2$ , there is no differences among CER values between treated and untreated leaves. However, the results presented herein, strongly support that  $\text{O}_2$  inhibition imposed on photosynthesis is largely released by NPHA, giving further support for a *in vivo* reduction of photorespiration.

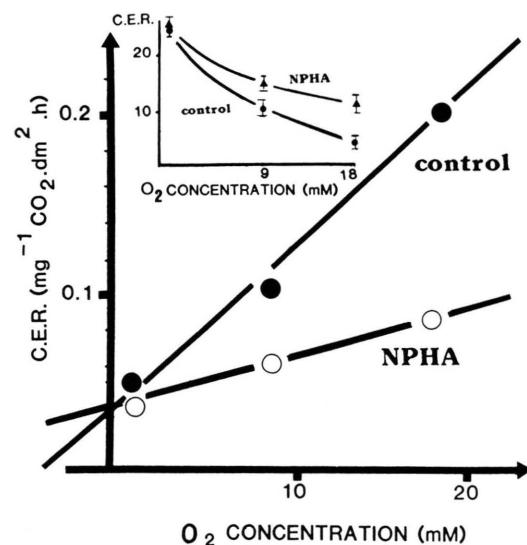


Fig. 2. Dixon plot of CERs measured at 2% (0.89 mM) 21% (9.4 mM) and 41% (18 mM)  $\text{O}_2$ , on NPHA-treated or untreated soybean trifoliolate leaves. Insert: Relationship between CER and  $\text{O}_2$  concentration.  $\text{CO}_2$  355 vpm, temperature 30 °C, PPF = 800  $\mu\text{Einstein dm}^{-2}/\text{h}^{-1}$ .

### Determination of SH groups on native Rubisco

In preliminary experiments, it was demonstrated that DTT can interact with NPHA (Fig. 3), bringing about an increase in absorbance at 400 nm and a decrease at 317 nm, as did cysteine but not cystine. This result suggests an interaction of NPHA with free SH-groups. When 50  $\mu$ M NPHA is added to Rubisco, the same change is observed (Fig. 3b), indicating a possible reaction of the chemical with the enzyme. Consequently, free SH-groups of the non-activated and activated Rubisco were titrated with DTNB in the presence and absence of NPHA; results are presented in Table V. The number of accessible SH-group per mol of Rubisco shifts from 12.63 (non activated) to 10.1 (activated) indicating conformational changes [37]. On the other hand, addition of NPHA decreases this number for both activated and non activated enzyme. Because NPHA

clearly reacts with SH groups of either cysteine or Rubisco, and a strict stoichiometry cannot be calculated between NPHA, free-SH groups of Rubisco and enzyme concentration, the binding of NPHA with a cysteine residue of the enzyme, together with chemically-induced conformation changes can presumably account for the observed effects. The importance of SH groups for Rubisco activation and catalysis has been suggested [37] and also proposed

Table V. Determination of the number of native accessible SH-groups in Rubisco expressed per enzyme molecule, titrated by DTNB.

Enzyme State	Control	NPHA	
		50 $\mu$ M	500 $\mu$ M
Non activated	12.63	11.28	8.2
Activated	10.09	8.9	7.16

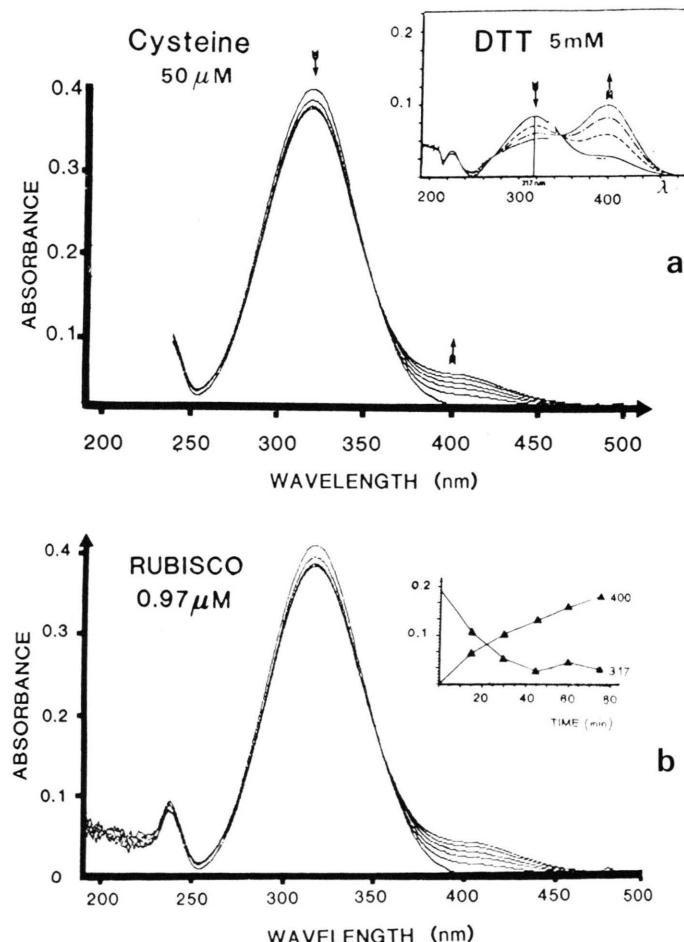


Fig. 3. Evolution of NPHA 50  $\mu$ M in the presence of: 3a: 50  $\mu$ M cysteine: time-span: 0 to 120 min, insert: DTT 5 mM, time: 0–60 min 3b: Rubisco, insert: time course of changes of optical density at 317 and 400 nm.

as a probe for Rubisco conformational variations [33, 43]. On the other hand, cysteine [44] and methionine [14] seemed to be involved at the active site [45].

## Conclusion

O-*p*-nitrophenylhydroxylamine (NPHA) increased Rubisco affinity to CO<sub>2</sub>, as shown by a decreased  $K_m$  CO<sub>2</sub>, without significantly increasing and even by decreasing the affinity for O<sub>2</sub>. As a consequence, the ratio between carboxylase to oxygenase activity was changed in favor of CO<sub>2</sub> uptake, as demonstrated with substrate specificity factors. The impact of Rubisco deactivation during the time course of catalysis, a notorious pitfall in the field, can be conveniently minimized by measuring simultaneously carboxylase/oxygenase activities, using [1-<sup>3</sup>H]RuBP and <sup>14</sup>CO<sub>2</sub> as substrates, leading to a correct evaluation of the velocity ratio and subsequent substrate specificity factor.

Hydroxylamine (HA) has been shown to interfere with Rubisco [19, 20] although specific inhibition of RuBP-oxygenase was questioned [46]. HA was shown as a O<sub>2</sub>-radical scavenger [47], thus suggesting the involvement of monovalent reduced oxygen in Rubisco activation. Also, HA blocks photosynthetic O<sub>2</sub> evolution by chelating Mn<sup>2+</sup>, and probably other divalent metallic cations [48]. NPHA, a hydroxylamine derivative, effective at micromolar concentrations, appears therefore to be a more specific and more active compound, as compared to HA.

Reactivity of NPHA with SH-groups indicated that the compound may act either through direct interaction with some Rubisco free SH-group, or by inducing conformational changes, or both. The presence of a cysteine residue near the catalytic center

suggests its involvement in the mode of action of NPHA, as proposed for glycidate or iodoacetamide [22], or for the role of sulfhydryl groups in activation by O<sub>2</sub> [49].

Reactivity of NPHA towards SH-group may throw some doubt on the specificity of this product due to potential interferences with other SH-enzymes. Also, the relative instability of hydroxylamine derivatives and their sensitivity to various groups, such as ketones, ethers, carbonyles, etc..., and dependence on specific RuBP/enzyme ratios, would probably limit their use under *in vivo* situations.

Limited but significant effects of NPHA on carboxylase/oxygenase activity ratio, together with conformational changes induced by the product, demonstrate that control of photorespiration is indeed feasible. Furthermore, an *in vivo* relief of photosynthesis O<sub>2</sub> inhibition strongly support evidence for a potential reduction of photorespiration as a mean for increasing CER. More information will be required regarding the exact mode of action of NPHA, including characterization of the putative specific binding, conformational changes of Rubisco as well as the region of interaction on the enzyme, role on sub-units, etc... In turn, this approach will give some clues to chemically or genetically manipulating oxygenase/carboxylase ratio.

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