Effect of Hydroxylamine Derivatives on Photorespiration in the Tobacco Aurea Mutant *Nicotiana tabacum* Su/su

G. H. Schmid, K. P. Bader, A. Radunz, C. J. van Assche*, N. Reinier*, and B. Courtiade*

Universität Bielefeld, Fakultät für Biologie, Lehrstuhl Zellphysiologie, D-4800 Bielefeld 1, Bundesrepublik Deutschland

* CRBA Division Scientifique, Procida-Roussel UCLAF, B.P. 1, F-13367 Marseille Cédex 11, France

Z. Naturforsch. **42c**, 965–969 (1987); received April 6/May 4, 1987

Hydroxylamine Derivatives, Photorespiration, Enzyme Conformation, Warburg Effect, Tobacco Mutant

O-(*p*-nitrophenyl)-hydroxylamine affects the size of the Warburg effect measured in leaves of the tobacco aurea mutant Su/su. If leaves are treated with a 10^{-5} M aqueous solution of the hydroxylamine derivative the Warburg effect which is usually very high in this mutant, is practically reduced to zero. Thus, if in the control (the untreated leaf) the Warburg effect consists in an enhancement of CO_2-fixation from 270 μmol CO_2 fixed mg·chlorophyll^{-1}·h^{-1} under 21% oxygen partial pressure to 530 μmol CO_2 fixed·mg Chl^{-1}·h^{-1} under 3% oxygen, the treated sample yields the maximal rate of photosynthesis already under 21% oxygen partial pressure. At concentrations around 10^{-4} M the compound is toxic to the plants and inhibits CO_2 fixation substantially. A second hydroxylamine derivative used in this study has an additional methyl group in the ortho position to the $-O-NH_2$ group. This compound is already toxic to the plant at concentrations around 10^{-5} M at which O-(*p*-nitrophenyl)-hydroxylamine optimally enhances CO_2 fixation at normal oxygen partial pressure. From our studies it appears that O-(*p*-nitrophenyl)-hydroxylamine binds to ribulose-1,5-bisphosphate carboxylase inducing a conformational change of the enzyme. This is concluded from the observation that a monospecific antiserum to ribulose-1,5-bisphosphate carboxylase is exhausted at considerably higher enzyme concentrations when the enzyme has been treated with the hydroxylamine derivative prior to the antiserum addition. The observation is interpreted as being due to a conformational change induced by the compound, leaving less antigenic determinants accessible in the enzyme surface, when compared to the untreated control. The inhibition of photosynthetic CO_2 fixation at high concentrations of both hydroxylamine derivative studied is due to the inhibition of photosynthetic electron transport on the donor side of photosystem II, a phenomenon which is known already for unsubstituted hydroxylamine from the literature. The inhibition of electron transport on the donor side is deduced from an analysis of fluorescence rise kinetics at room temperature.

Introduction

Photorespiration is a wasteful process occurring in C_3 plants in which freshly fixed CO_2 is given back to the ambient atmosphere and at the same time oxygen is taken up. The phenomenon is caused by the oxygenase function of the CO_2-fixing enzyme ribulose-1,5-bisphosphate carboxylase. It is common knowledge that photorespiration appears suppressed at high CO_2 partial pressure or at low O_2 partial pressure, a fact which shows that the photorespiration is regulated by the affinity of this bifunctional enzyme towards its substrates CO_2 and O_2. As the affinity of a given enzyme towards its substrate depends on the enzyme's conformation, we have studied changes in substrate affinity of the enzyme "Rubisco" towards its substrates CO_2 and O_2, in the presence of artificial effector molecules. More than seven years ago Okabe, Codd and Stewart have been able to demonstrate that hydroxylamine enhances the carboxylating activity and inhibits the oxygenase activity of a cyanobacterial Rubisco [1]. As plant material for the studies in the present paper we have chosen the tobacco aurea mutant Su/su [2–5] which, as shown by a number of laboratories, exhibits an especially high activity of photorespiration, when compared with the wild type [6–9]. With these tobacco plants and ribulose-1,5-bisphosphate carboxylase/oxygenase preparations from this mutant [10] we have made an analysis of substrate affinity changes in the presence of hydroxylamine derivatives.

Abbreviations: Compound 368, O-(*p*-nitrophenyl)-hydroxylamine; compound 378, O-(*p*-nitro-*o*-methylphenyl)-hydroxylamine; Rubisco, ribulose-1,5-bisphosphate carboxylase/oxygenase.

Reprint requests to Prof. Dr. Georg H. Schmid.

Verlag der Zeitschrift für Naturforschung, D-7400 Tübingen
0341–0382/87/0700–0965 $ 01.30/0

Materials and Methods

Plant material: The tobacco aurea mutant *N. tabacum* Su/su used in the present study has been described first in 1964 by Burk and Menser [2].

Chloroplast preparations were made according to Homann and Schmid [11].

Warburg effect measurements were carried out as described earlier [12] using an IR-CO_2 Analyzer (Hartmann, Germany).

Activity of ribulose-1,5-bisphosphate carboxylase was measured according to Lorimer *et al.* [17]. The enzyme was activated also according to Lorimer *et al.* [18] by incubation for 5 min at 30 °C in Tris HCl at pH 8.

The antiserum to ribulose-1,5-bisphosphate carboxylase was prepared according to the methods described earlier [13, 14].

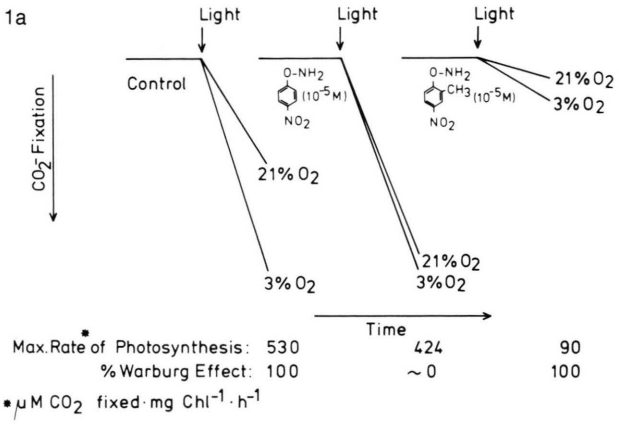
The monospecificity of the antiserum was verified as described by Radunz [15].

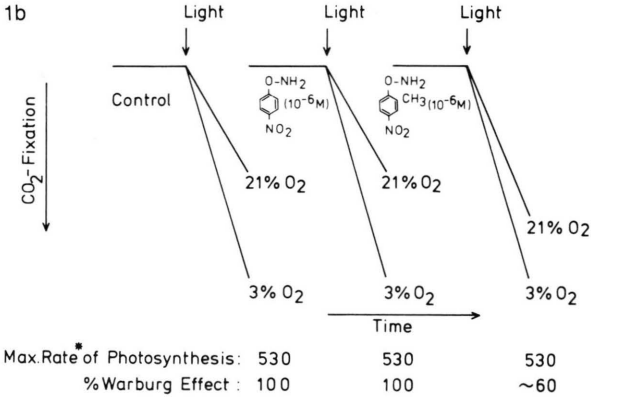
O-(*p*-nitrophenyl)-hydroxylamine and O-(*p*-nitro-*o*-methylphenyl)-hydroxylamine have been put at the disposition of the present study by Roussel Uclaf [21].

Fluorescence induction kinetics at room temperature have been analyzed with a home-made device [19] set-up according to the principles described by Joliot *et al.* [20].

Results

In the first set of experiments we have sprayed a tobacco mutant plant with 10^{-5} M aqueous solution of O-(*p*-nitrophenyl)-hydroxylamine "product 368" [16] or O-(*p*-nitro-*o*-methylphenyl)-hydroxylamine "product 378". The quantity absorbed by the plant is not defined but we have wetted the plant twice within a 2-h interval. The next day we have measured the Warburg effect in leaf sections of the thus treated plant (Fig. 1a). It is clearly seen that with product





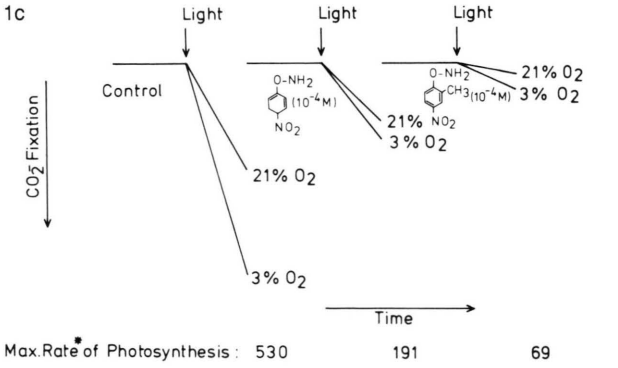


Fig. 1. Rate of photosynthetic CO_2 fixation in dependence on time recorded with an IR-CO_2 analyzer in leaves of the tobacco aurea mutant *Nicotiana tabacum* var. Su/su. a) Warburg effect *i.e.* rate of photosynthesis in 21% O_2 and in 3% O_2. The control rate at 3% O_2 partial pressure of the untreated leaf is 530 µmol CO_2 fixed · mg chlorophyll^{-1} · h^1. First curve: Control, untreated leaf; second curve: leaf treated with 10^{-5} M O-(*p*-nitrophenyl)-hydroxylamine; third curve: leaf treated with 10^{-5} M O-(*p*-nitro-*o*-methylphenyl)-hydroxylamine; b) same experiment as in a) but with leaves treated with a 10^{-6} M solution of the respective hydroxylamine derivative; c) same experiment as in a) and b) but with a 10^{-4} M solution of the respective hydroxylamine derivative. The uncertainty caused by the introduction of substances into the plant by spraying the leaves does not easily permit the analysis of the dependence of the Warburg effect on a wide range of compound concentrations. On the other hand high concentrations of both analyzed compounds are toxic and inhibit the control rate (Fig. 1c). The present figure is a typical experimental series of a total of 17 experiments. The plant material used was 6 weeks old.

368, photosynthesis, that is the CO_2 fixing capacity under 21% oxygen, is enhanced to the value of the untreated plant at 3% oxygen partial pressure. This means that the Warburg effect by addition of product 368, is reduced to practically 0%. As van Assche *et al.* [21] have recently shown, O-(*p*-nitrophenyl)-hydroxylamine changes the conformation of the fully activated spinach enzyme in the sense that in the presence of the compound the number of accessible sulfhydryl groups is diminished from 12 or 13 SH groups to 8. If the result in Fig. 1a is to be interpreted in this way, this would indeed mean that the hydroxylamine derivative changes the enzyme's function, thus yielding higher affinity towards CO_2 and diminishing at the same time the oxygenase function. If, however, product 368 is used at higher concentrations (10^{-4} M) the overall effect is an inhibition of photosynthesis, as shown in Fig. 1c. It seems as if a certain threshold value is not to be exceeded otherwise the effect of product 368 is generally toxic to the plant. A second product which we call in the following product 378 is a hydroxylamine derivative which differs from 368 by substitution of a methyl group in the ortho position to the hydroxylamine residue. This product has at the concentration of 10^{-5} M, at which product 368 acts optimally, already an inhibitory effect on the overall rate of photosynthesis, hence is clearly toxic to the plant (Fig. 1a). However, at a concentration of 10^{-6} M at which 368 is still fully ineffective this product shows a 40% reduction of the Warburg effect (Fig. 1b). If the observation with product 368 and 378 was due to a conformational change of the enzyme, induced by the binding of these "effector" molecules onto the enzyme, this should be easily verifiable: We have prepared Rubisco from Su/su-leaves and have treated the activated enzyme with product 368 and have determined with the thus treated enzyme the titer of a monospecific antiserum to ribulose-1,5-bisphosphate carboxylase/oxygenase. The experiment was carried out by incubating 1 ml antiserum with increasing amounts of Rubisco. The precipitation resulting from the antiserum-antigene reaction is separated from the supernatant by centrifugation. The supernatant is then tested for the remaining antiserum activity by measuring the per cent inhibition of CO_2 fixation caused upon a Rubisco assay.

Fig. 2 clearly shows that the number of accessible antigenic determinants is reduced in the presence of product 368 which finds its expression in the fact that

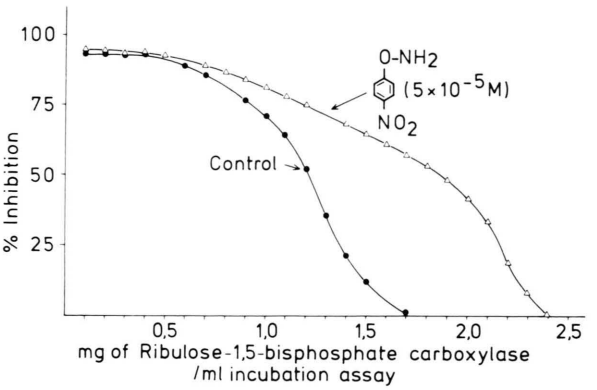


Fig. 2. Remaining antiserum activity after incubation of 1 ml antiserum with increasing amounts of ribulose-1,5-bisphosphate carboxylase. Rubisco treated with 10^{-5} M O-(*p*-nitrophenyl)-hydroxylamine exhausts the antiserum at much higher concentrations than the control.

in the presence of product 368 more enzyme is necessary to exhaust the antiserum than in its absence. This fully confirms the observation by van Assche *et al.* [21] in which the authors conclude that the product induces a conformational change, since in the presence of the product nearly half of the otherwise detectable SH-groups become inaccessible to the test reagent [21]. At high product concentrations (see Fig. 1c) photosynthesis is strongly inhibited. This inhibition is apparently due to an inhibition of photosynthetic electron transport on the donor side of photosystem II. This is verified by measuring the induction of room temperature fluorescence with chloroplast preparations of Su/su (Fig. 3). In the presence of product 368 and even more pronounced with product 378 the fluorescence rise kinetics is substantially slowed down in comparison to the control. This is interpreted as being due to the fact that the quencher Q stays preponderantly in the oxidized condition (in which fluorescence is low), because electrons from the donor side of PS II arrive too slowly and do not compensate electron removal from Q by photosystem I. The inhibitory effect of hydroxylamine on photosynthetic electron transport is long known from the literature. According to the literature hydroxylamine in suitable concentrations inhibits on the donor side of photosystem II [22, 23]. At certain high concentrations hydroxylamine acts as a water-analogue and is electron donor for photosystem II [22]. This clearly explains the dual effect of

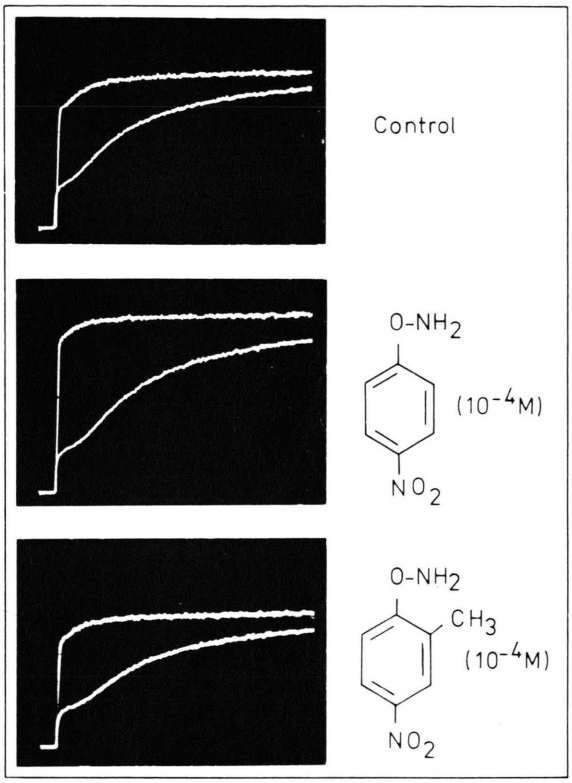


Fig. 3. Room temperature fluorescence induction in chloroplasts of *Nicotiana tabacum* var. Su/su. a) Control: Lower curve, rise kinetics after 5 min dark adaptation; upper curve, without dark adaptation, sweep speed 100 msec/cm; b) chloroplasts treated with 10^{-4} M O-(*p*-nitrophenyl)-hydroxylamine; c) chloroplasts treated with 10^{-4} M O-(*p*-nitro-*o*-methylphenyl)-hydroxylamine.

product 368 and 378 on the CO_2 fixation of leaf sections in Fig. 1.

Discussion

In the present paper we demonstrate that photorespiration is reduced by certain hydroxylamine derivatives. As plant material for this demonstration we used the tobacco aurea mutant Su/su which as shown by the literature [4, 6] is a plant with an especially high photorespiration. In particular Okabe [24] and Okabe and Schmid [8] have shown that Rubisco isolated from this plant had an increased affinity towards oxygen and a decreased one towards CO_2 when compared to the green control. On the other hand, the plant exhibits under conditions, such as high CO_2 partial pressure or low O_2 partial pressure, which exclude the functioning of photorespiration, maximal photosynthetic rates which are manyfold higher than those of any green plant [3]. With this plant material we observe that leaves treated with aqueous solutions of O-(*p*-nitrophenyl)-hydroxylamine exhibit less photorespiration than untreated leaves. It appears that the compound readily penetrates into tobacco leaves, being after a very few hours at its site of action, leading to a reduced photorespiration evidenced by a substantial enhancement of CO_2-fixation rates at the normal O_2 partial pressure of air (Fig. 1). Whether the effect is caused by the compound as such or a metabolite, produced from it in the plant, is not entirely clear. It appears, however, that the hydroxylamine derivative acts with isolated Rubisco from Su/su but also with the commercially available spinach enzyme from Sigma, inducing an alteration of the enzyme conformation. This conformational change is deduced from the observation that the number of SH groups accessible to the test reagent diminishes in the presence of the hydroxylamine derivative [21] and that the number of antigenic determinants accessible in the enzyme surface to a monospecific antiserum to Rubisco is reduced in the presence of the compound (Fig. 2). This seems to be at least valid for the activated enzyme. It is difficult to imagine that in this isolated system an alteration of the hydroxylamine derivative other than the binding onto the enzyme would occur. Hence, the hydroxylamine derivative bound onto the enzyme might play the role of an effector molecule that exerts its effect on the enzyme's conformation.

[1] K. Okabe, G. A. Codd, and W. D. P. Stewart, Nature **279,** 525 (1979).
[2] L. Burk and H. A. Menser, Tobacco Sci. **8,** 101 (1964).
[3] G. H. Schmid, Planta **77,** 77 (1967).
[4] K. Okabe, G. H. Schmid, and J. Straub, Plant Physiol. **60,** 150 (1977).
[5] O. Canaani, Z. Motzan, and S. Malkin, Planta **164,** 480 (1985).
[6] I. Zelitch and P. R. Day, Plant Physiol. **43,** 1838 (1968).
[7] M. L. Salin and P. H. Homann, Plant Physiol. **48,** 193 (1971).
[8] K. Okabe and G. H. Schmid, in: Chloroplast Development (G. Akoyunoglou and J. H. Argyroudi-Akoyunoglou, eds.), p. 501, Elsevier Ass. Scientific Publishers, Amsterdam 1979.
[9] R. Ishii and G. H. Schmid, Z. Naturforsch. **37c,** 93 (1981).
[10] A. Radunz and G. H. Schmid, in: Progress in Photosynthesis Research, **Vol. 3** (J. Biggins, ed.), p. 617, Martinus Nijhoff Publishers, Dordrecht, Boston, Lancaster 1987.
[11] P. H. Homann and G. H. Schmid, Plant Physiol. **42,** 1619 (1967).
[12] R. Ishii and G. H. Schmid, Plant Cell Physiol. **24,** 1525 (1983).
[13] G. H. Schmid and A. Radunz, Z. Naturforsch. **29c,** 384 (1974).
[14] G. H. Schmid, A. Radunz, and W. Menke, Z. Naturforsch. **32c,** 201 (1975).
[15] A. Radunz, Z. Naturforsch. **33c,** 731 (1978).
[16] European Patents n° 85587 and 119892.
[17] G. H. Lorimer, M. R. Badger, and T. J. Andrews, Anal. Biochem. **78,** 66 (1977).
[18] G. H. Lorimer, M. R. Badger, and R. J. Andrews, Biochem. **15,** 529 (1976).
[19] G. A. Codd and G. H. Schmid, Z. Naturforsch. **35c,** 649 (1980).
[20] P. Joliot, P. Bennoun, and A. Joliot, Biochim. Biophys. Acta **305,** 317 (1972).
[21] C. J. van Assche, N. Reinier, B. Courtiade, A. Chancel, and S. Huber, Z. Naturforsch. **42c,** in press (1987).
[22] G. A. Den Haan, H. Garter de Vries, and L. N. M. Duysens, Biochim. Biophys. Acta **430,** 265 (1976).
[23] G. H. Cheniae and I. F. Martin, Plant Physiol. **47,** 568 (1971).
[24] K. Okabe, Z. Naturforsch. **32c,** 781 (1977).