Properties of Ribulose Diphosphate Carboxylase/Oxygenase in the Tobacco Aurea Mutant Su/su var. Aurea

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The activity of ribulose 1,5-diphosphate (RuDP)-carboxylase and RuDP-oxygenase was measured in crude leaf extracts of the tobacco *(N. tabacum)* phenotypes which differed with respect to their gene constitution and with respect to their photosynthetic and photorespiratory activity. The green wild type (JWB) which carried the discussed two nuclear factors *su* and *aur* in the condition su/su Aur/Aur and su/su Aur/aur exhibits normal photosynthetic activity and low photorespiratory activity. A yellow-green chlorophyll-deficient phenotype (Su/su) carrying Su/su Aur/Aur has high photosynthetic activity on a chlorophyll basis but also high photorespiratory activity. A new yellow phenotype (Su/su var. Aurea) carrying both nuclear factors in a heterozygous condition Su/su Aur/aur has high photosynthetic activity on the basis of chlorophyll and low photorespiratory activity. The comparison of the RuDP-carboxylase/oxygenase activity in these three phenotypes shows that in the yellow-green phenotype Su/su the affinity of the RuDP-oxygenase towards oxygen is higher than in the green phenotype JWB and in the yellow phenotype Su/su var. Aurea. The $K_m(O_2)$ values for the RuDP-oxygenase activity are 890 μM for JWB, 630 μM for Su/su and 940 μM for Su/su var. Aurea and the corresponding $K_i(CO_2)$ values are 7.4 μM for JWB, 14.9 μM for Su/su and 5.8 μM for Su/su var. Aurea at pH 8.34. On the other hand, the affinity of the carboxylating activity of the enzyme towards CO_2 shows no difference between JWB and Su/su var. Aurea, but a lower affinity in Su/su. This is expressed by the $K_m(CO_2)$ values which are 107 μM for JWB, 143 μM for Su/su and 96 μM for Su/su var. Aurea at pH 7.8. However, the affinity of the oxygenase function of the enzyme towards RuDP seems to be unchanged in all three tobaccos and is found to be around K_m(RuDP) 27 μM. From this result it appears that the nuclear factor *su* decreases in the condition Su/su Aur/Aur the affinity of the RuDP-carboxylase towards CO_2 and increases the affinity of the RuDP-oxygenase towards oxygen. On the other hand, the factor *aur* seems to suppress this gene expression in the condition Su/su Aur/aur whereas both factors do not affect the binding of RuDP onto the enzyme.

Recently, the tobacco aurea mutant, Su/su var. Aurea, has been genetically and physiologically characterized by Okabe *et al.* [1]. The mutation is due to two independent nuclear factors *su* and *aur* both of which have to be present in a heterozygous condition Su/su Aur/aur to give rise to the new aurea phenotype. Four types of plants with four different gene constitutions were observed in the seed population of the selfed aurea mutant among which the green type JWB carries su/su Aur/aur or su/su Aur/Aur and the yellow-green type Su/su carries Su/su Aur/Aur. The aurea mutant Su/su var. Aurea (with Su/su Aur/aur) has a reduced photosynthetic unit size which is approximately 1/8 of the wild type. Despite its chlorophyll deficiency the plant grows well and exhibits maximal rates of photosynthesis which on a chlorophyll basis are at least 7 times higher than those of the green wild type at optimum conditions. In contrast to the earlier described Su/su [2] the new mutant does not exhibit more photorespiration than the wild type. Thus, the nuclear gene factor *aur* appeared to cause either repression of photorespiration or an increase in the number of functioning photosynthetic units.

In the present paper a relationship is found between the role of the nuclear factor *aur* and the kinetical nature of the RuDP carboxylase/oxygenase activity. Kung and Marsho had reported previously on the properties of the RuDP carboxylase/oxygenase activity in the wild type tobacco JWB and the earlier described tobacco mutant Su/su [3] from which the new mutant Su/su var. Aurea has been derived. They suggested that the small subunit of RuDP-carboxylase which is nuclear encoded should modify or regulate the carboxylase and oxygenase function of the enzyme. Therefore, it appeared

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Abbreviations: JWB, green wild type tobacco John Williams Broadleaf; Su/su, yellow-green chlorophyll-deficient tobacco mutant; Su/su var. Aurea, yellow chlorophyll-deficient tobacco mutant; RuDP, ribulose 1,5-diphosphate.

worthwile to investigate whether the nuclear gene factor *aur* has anything to do with the RuDP carboxylase/oxygenase activity in the new tobacco mutant.

Materials and Methods

The tobacco varieties used were the green wild type, John Williams Broadleaf, the aurea mutant Su/su [4] and the aurea mutant Su/su var. Aurea which was characterized in a previous paper [1]. The plants used were all obtained by selfing Su/su var. Aurea. The plants were grown in soil in a green house. Fully expanded first leaves were used for the experiments in a growth stage in which the plants had 7 – 9 leaves. The preparation of the crude enzyme extracts was carried out as follows: 6 – 7 tobacco leaves corresponding to approx. 6 g of fresh weight (midribs removed) were ground with 1 g sea sand (Merck) in 8 ml of ice-cold extraction buffer solution, containing 100 mM HEPES-NaOH, pH 7.8, 25 mM $MgCl_2$, 5 mM dithioerythritol (Sigma), 2 mM ribose 5-phosphate (Sigma) and 3 mM ATP (Sigma) and 1 g of polyvinyl-polypyrrolidone (Sigma). The homogenate was filtered through eight layers of cheese cloth, centrifugated (25 000 × *g*, 5 min) and the supernatant was freed of low-molecular-weight substances by passage through a Sephadex G-25 column (1.8 × 25 cm), equilibrated with CO_2-freed buffer solution containing 20 mM HEPES-NaOH pH 8.3, 25 mM $MgCl_2$, 0.5 mM dithioerythritol. All procedures were carried out at 2 – 4 °C within 30 min. The crude extract was stored in ice until used for the assay. RuDP-oxygenase was assayed by measuring RuDP-dependent oxygen uptake at 25 °C with a Clark type oxygen electrode (Rank Brothers, Bottisham, UK). The assay solution contained in a final volume of 1.25 ml 100 mM Bicine-NaOH pH 8.34, 15 mM $MgCl_2$, 0.5 mM RuDP (Sigma) and extract corresponding to 0.7 – 2.5 mg protein. The reaction was started by the addition of RuDP. The required oxygen concentration was established by bubbling the oxygen or nitrogen with a microsyringe through the solution. Endogenous CO_2 in the assay solution was removed by flushing with nitrogen at pH 3.9 prior to the assay. Subsequently, the pH was adjusted to 8.34 with carbonate-free NaOH. The reaction rate was measured during the first 50 seconds after the start and corrected for the rate without RuDP.

RuDP carboxylase was assayed via the RuDP-dependent $H^{14}CO_3^-$ incorporation into acid stable material at 25 °C. The assay solution contained in a final volume of 0.40 ml, Bicine-NaOH 100 mM pH 7.80 or 7.98, 15 mM $MgCl_2$, 0.5 mM RuDP and 0.2 – 0.3 mg protein. The reaction atmosphere in the 10 ml vial was nitrogen. The vial was closed with a surgical rubber cap which allowed the addition of $NaH^{14}CO_3$ and RuDP through this cap using microliter syringes. The reaction was started by the addition of RuDP 8 min after the addition of $NaH^{14}CO_3$. The reaction was stopped 45 seconds later by the addition of 0.4 ml of a 2 N HCl-50% methanol solution. All solutions were transferred to scintillation vials and dryed up at 90 °C. After dissolving the residue with 0.5 ml of water, 10 ml of Bray solution were added and the radioactivity was measured in a scintillation spectrometer (Philips). Protein was measured according to Lowry *et al.* [5] using crystalline bovine serum albumin (Serva) as a standard.

Results and Discussion

Extracts preincubated with $MgCl_2$ (15 mM) showed rapid O_2-uptake after addition of RuDP. The initial rate of O_2-uptake was a linear function of the amount of extract added. Thereafter, about 50 – 60 sec after the addition of RuDP, the activity gradually decreased. This rapid decrease was not due to the exhaustion of RuDP, because the further addition of RuDP to the reaction mixture did not affect the rate of O_2-consumption. On the other hand, the rate of O_2-uptake depended also upon the initial concentration of RuDP. Double reciprocal plots of RuDP-dependent O_2-uptake versus RuDP-concentration at an oxygen concentration of 257 μM O_2 are shown in Fig. 1. All three tobacco varieties showed little difference with respect to their K_m values for RuDP which were found to be 25.9, 26.0, and 27.6 mM, for JWB, Su/su, and Su/su var. Aurea, respectively. The maximum velocities can not be compared because of the use of crude ex-

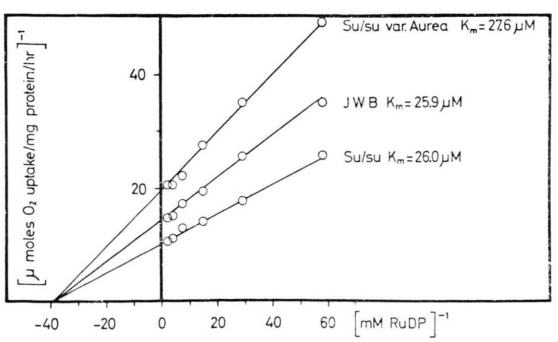

Fig. 1. Double reciprocal plots of the rate of RuDP dependent O_2-uptake versus the RuDP concentration at 257 μM O_2. The assay condition is given in Materials and Methods.

tracts, which led to variable V_{max} values in the individual experiments, in contrast to the fairly constant K_m values.

Our obtained K_m (RuDP) values for the oxygenase activity are of the same order of magnitude than that reported for the crystallized fraction 1 protein from tobacco (*N. tabacum*, cv. Turkish samsun) which was reported to be 22 μM by Marsho and Kung [6], and also agree with the K_m value for oxygenase from freshly lysed spinach chloroplasts according to Bahr and Jensen who reported on a K_m value of 45 μM [7].

Double reciprocal plots of the RuDP-oxygenase activity as a function of the oxygen concentration showed competitive inhibition by CO_2 with respect to oxygen in the three tobacco varieties (Figs 2 – 4). The results are similar to those reported in the literature [8–10]. The kinetic constants for CO_2 and O_2 for oxygenase, obtained from the experiments in Figs 2 – 4 and in additional independent experiments by linear regression are summarized in Table I. It should be noted that the mean K_m (O_2) value of Su/su var. Aurea is almost the same as that of the wild type JWB whereas the value of Su/su is distinctly smaller. The $K_i(CO_2)$ values were calculated to be 5.8 μM for Su/su var. Aurea, 7.4 μM for JWB and 14.9 μM for Su/su at pH 8.34. The CO_2 concentration was computed from the experimental pH and the used bicarbonate concentration, using the Henderson-Hasselbach equation with a value of 6.37 for the pK′, at 25 °C, of the CO_2-hydration reaction [11].

The K_m values of RuDP-carboxylase activity against CO_2 obtained by the linear regression of the reciprocal plots of the enzyme activity as a function of CO_2 were inversely related to the K_i of the RuDP-oxygenase activity against CO_2 and were found to be 96 μM for Su/su var. Aurea, 128 μM for JWB and 160 μM for Su/su at pH 7.98, and 96 μM for Su/su var. Aurea, 107 μM for JWB and 143 μM for Su/su at pH 7.80, supporting the notion of Andrews *et al.* that carboxylase and oxygenase reactions are catalyzed by the same active site of the same protein [12] (Table I), although it should be noted that the K_m values obtained were constantly higher than those reported as high affinity form [9, 13]. The present carboxylase assay system, using crude extracts, seems to have changed the enzyme to the "high K_m form" which leads to significant differences with Su/su extracts

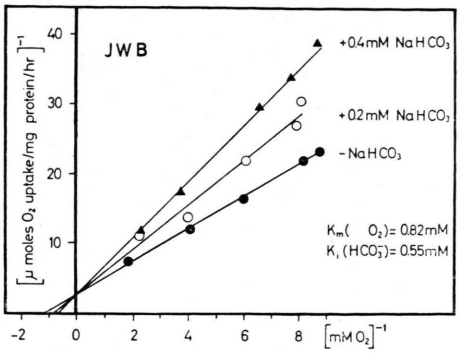

Fig. 2

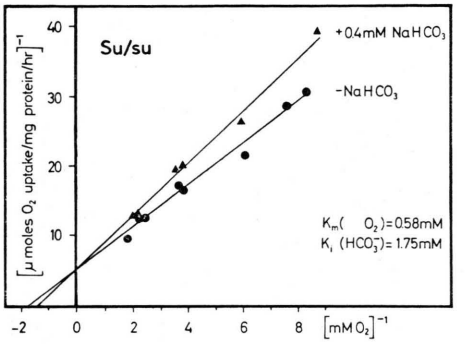

Fig. 3

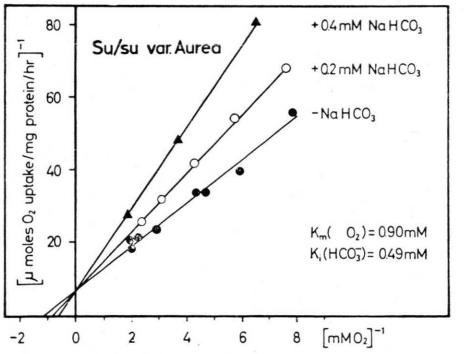

Fig. 4

Figs 2–4. Double reciprocal plots of the rate of RuDP dependent O_2-uptake as a function of O_2 and $NaHCO_3$ concentrations. $NaHCO_3$ was added 6 min before the addition of RuDP. The assay conditions are given in Materials and Methods.

in comparison to those of JWB and Su/su var. Aurea. These results demonstrate that in Su/su var. Aurea the affinity of the RuDP-oxygenase for oxygen is comparable to that of JWB but distinctly inferior than in Su/su. On the other hand, the affinity of the carboxylase for CO_2 of Su/su var. Aurea is also comparable to that of JWB but is distinctly

Table I. Properties of the RuDP carboxylase/oxygenase activity in leaf extracts of JWB, Su/su and Su/su var. Aurea.

Plant	JWB	Su/su	Su/su var. Aurea
Phenotype	green	yellow-green	yellow
Genomcomposition	(su/su Aur/aur) (su/su Aur/Aur)	(Su/su Aur/Aur)	(Su/su Aur/aur)
	[μM]	[μM]	[μM]
RuDP oxygenase (pH 8.34)			
K_m(RuDP)	25.9	26.0	27.6
$K_m(O_2)$	890 ± 50	630 ± 140	940 ± 40
$K_i(CO_2)$	7.4 ± 1.9	14.9 ± 5.1	5.8 ± 1.7
RuDP carboxylase			
$K_m(CO_2)$ (pH 7.80)	107 ± 11	143 ± 14	96 ± 11
$K_m(CO_2)$ (pH 7.98)	128 ± 25	160 ± 6	96

superior when compared to that of Su/su. This might offer an explanation why the photorespiratory activity in Su/su var. Aurea does not differ from that of JWB[1] whereas Zelitch and Day[2] had reported earlier that Su/su (the yellow-green type of plants) exhibited strong photorespiration.

In the previous genetical study[1] the genotypes of the three tobacco varieties were characterized as follows: JWB carried the gene constitution su/su Aur/Aur or su/su Aur/aur, the equivalent to the earlier described Su/su mutant[2] carried Su/su Aur/Aur and the new tobacco mutant Su/su Aur/aur. The comparison shows that the difference between Su/su and Su/su var. Aurea can only be due to the nuclear gene factor *aur*. Therefore, it was suggested that the factor *aur* might control photorespiration and/or the photosynthetic unit size. The present data suggest that the factor *aur* controls photorespiration at the level of the RuDP carboxylase/oxygenase activity which supports what has been proposed already earlier[1]. As to the role of the gene *aur* the possibility exists that 50% of the green phenotype plants carry the genotype Aur/aur. Leaves from the green wild type JWB were taken randomly from at least 30 plants. The observed $K_m(O_2)$ for RuDP-oxygenase in JWB was somewhat lower than in Su/su var. Aurea. Correspondingly, the $K_i(CO_2)$ of RuDP oxygenase and $K_m(CO_2)$ of RuDP-carboxylase were also somewhat higher than in Su/su var. Aurea, which is understandable if statistically half of the green plants carry Aur/aur and the other half Aur/Aur. To further investigate this point in detail it is obvious that one should use haploid plants derived from anther cultures of Su/su var. Aurea.

The new result presented in this paper is that there exists a difference with respect to the affinity towards CO_2 or O_2 of RuDP carboxylase/oxygenase among the mutants but not towards RuDP. From the literature it is known that carboxylase/oxygenase is composed of nonidentical multiple copies of eight large subunits with a mol. weight of approximately 55 000 and eight small subunits with a mol. weight of approximately 15 000[14–18]. Furthermore, it is thought that the large subunits are encoded and synthesized outside the chloroplasts[16, 19–23]. Since the three phenotypes, described in this paper, are derived from the same Su/su var. Aurea plant, all maternal influences must be expressed equally among them. Thus, the observed characteristic difference can not be ascribed to the large subunit (which is maternally encoded)[24]. Only the small subunit can be responsible. Consequently, this might lead to the idea that the gene *aur* controls photorespiration via the small subunits. Which in turn leads to an overcompensation of the expression of the nuclear gene *su*. The consequence of all this could be that the $K_m(O_2)$ of RuDP-oxygenase is decreased and the $K_m(CO_2)$ of RuDP-carboxylase is increased (Table I). This modification of the small subunit by the gene factor *aur* is likely to be so small that it cannot be detected by the isoelectric focussing technique as reported by Kung and Marsho in comparative work with crystallized RuDP carboxylase/oxygenase from Su/su and JWB[3]. This mode of control also supports the suggestion that the function of the small subunit regulates the activity of the catalytic site of the large subunit[3, 23].

The fact that the K_m (RuDP) values are alike in all three phenotypes (Table I) suggests that the two supposed binding sites for RuDP on the enzyme[25] are not modified by the factors *su* and *aur*.

Recently, it was reported that the kinetic properties of purified RuDP-carboxylase/oxygenase can be modified by the substrate and certain effectors

such as CO_2, Mg^{2+}, NADPH and a number of sugar phosphates [25]. In such experiments the order of the additions to the assay plays a role. Such experiments were not carried out in the present investigation as we used on purpose crude enzyme extracts. It appears from the present study that the determination of K_m values which does not require a purified enzyme preparation might be a useful tool for the selection of low photorespiratory phenotypes in an experimental seedling population.

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