

Immunological Study on the Structural Difference between Ribulose-1,5-bisphosphate Carboxylase/Oxygenase from *Nicotiana tabacum* var. John William's Broadleaf and the Tobacco Mutant Su/su

C. Nespoulous, P. Fabisch, A. Radunz, and G. H. Schmid

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

Z. Naturforsch. **43c**, 717–726 (1988); received July 4, 1988

RuBP Carboxylase/Oxygenase, Antibodies, Oxygenase Activity, Ratio RuBP Carboxylase/Oxygenase-Antibodies, Tobacco mutant Su/su

In the present paper we have attempted to characterize by means of immunological methods the molecular difference between ribulose 1,5-bisphosphate carboxylase/oxygenase from the wild type *N. tabacum* var. John William's Broadleaf and the tobacco mutant Su/su. The tobacco mutant Su/su exhibits in comparison to the wild type a higher photorespiratory activity. The reagents used in the present study are monospecific antisera to the two bifunctional enzymes to be compared. We have analyzed the oxygenase activity of the two enzymes in dependence on the binding of the amount of antibodies out of the homologous and the not homologous antiserum. These analyses have shown, that the enzymes of both phenotypes were 40% stronger inhibited in the equivalence regions with respect to their oxygenase function by antibodies of the antiserum to ribulose 1,5-bisphosphate carboxylase/oxygenase of the mutant than by antibodies of the wild type antiserum. It should be noted that the antiserum to the mutant enzyme exhibits a 25% lower antibody titer, than the antiserum to the wild type enzyme.

In the region of extreme antibody excess, *i.e.* when antibodies are mostly monovalently bound and most antigenic determinants are saturated with antibodies, the oxygenase activity of both enzymes decreases towards zero in the presence of the homologous as well as in the presence of non-homologous antibodies. In the region of excess of antigenic determinants, that is when only a few antibody molecules can bind onto the enzymes, only the oxygenase activity of the mutant enzyme is inhibited by its homologous antibodies by 40%. This apparent difference in the molecular structure of the two bifunctional enzymes to be compared is neither caught in the double immuno diffusion test nor in the tandem crossed immuno electrophoresis, using the two antisera as test reagents. In all cases only fused precipitation bands are observed.

Chemical modification of ribulose 1,5-bisphosphate carboxylase/oxygenase by hydroxylamine treatment or treatment with *o*-(*p*-nitrophenyl)hydroxylamine or simple heating of the enzyme to 50 °C are immunologically characterized. As a consequence of such treatment considerably less antibodies are adsorbed. The strongest influence exerts a treatment with *o*-(*p*-nitrophenyl)-hydroxylamine with simultaneous heat treatment of the enzyme for 20 min at 50 °C.

Introduction

In *Nicotiana tabacum* mutants are known such as the aurea mutant Su/su which exhibit a considerably higher activity of photorespiration than the wild type *N. tabacum* var. John William's Broadleaf [1–7]. This higher activity is due to a higher oxygenase activity of the bifunctional enzyme ribulose-1,5-bisphosphate carboxylase/oxygenase [6]. A correlation between a higher activity and a higher concentration

of the enzyme does not exist between such phenotypes as demonstrated by our own immunological quantitative analyses [8]. Moreover, by means of precipitation reactions in agarose gel, by means of the double immuno diffusion test as well as by means of the tandem crossed immuno electrophoresis with an antiserum to the RuBP carboxylase/oxygenase of the wild type no differences with respect to the molecular structure between the wild type and the mutant enzyme were detected [9]. Thus, it seems as if the large as well as the small subunits of both enzymes had the same structural composition.

In the present paper we demonstrate first, that also the use of a new antiserum, which is directed towards the mutant enzyme, confirms the above described results with respect to the precipitation reactions of the double immuno diffusion test as well as

Abbreviations: Tris buffer, tris (hydroxymethyl)-aminomethane; EDTA, ethylenediaminetetraacetic acid; DTT, dithiothreitol, RuBP carboxylase/oxygenase, ribulose 1,5-bisphosphate carboxylase/oxygenase.

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

Verlag der Zeitschrift für Naturforschung, D-7400 Tübingen
0341–0382/88/0900–0717 \$ 01.30/0



Dieses Werk wurde im Jahr 2013 vom Verlag Zeitschrift für Naturforschung in Zusammenarbeit mit der Max-Planck-Gesellschaft zur Förderung der Wissenschaften e.V. digitalisiert und unter folgender Lizenz veröffentlicht: Creative Commons Namensnennung-Keine Bearbeitung 3.0 Deutschland Lizenz.

Zum 01.01.2015 ist eine Anpassung der Lizenzbedingungen (Entfall der Creative Commons Lizenzbedingung „Keine Bearbeitung“) beabsichtigt, um eine Nachnutzung auch im Rahmen zukünftiger wissenschaftlicher Nutzungsformen zu ermöglichen.

This work has been digitalized and published in 2013 by Verlag Zeitschrift für Naturforschung in cooperation with the Max Planck Society for the Advancement of Science under a Creative Commons Attribution-NoDerivs 3.0 Germany License.

On 01.01.2015 it is planned to change the License Conditions (the removal of the Creative Commons License condition “no derivative works”). This is to allow reuse in the area of future scientific usage.

to the immuno electrophoretic methods of the tandem crossed immuno electrophoresis and the line rocket immuno electrophoresis. Thus, we can show that the new antiserum to the mutant *N. tabacum* Su/su as well as a mixed antiserum to the two enzymes to be compared yield only fusing bands, hence demonstrate serological identity of the two enzymes. Only the analysis of quantitative binding of antibodies onto the enzymes of the wild type and the mutant out of the homologous and non-homologous antisera lead to the conclusion that differences on the molecular level must exist between the two enzymes, since firstly differing amounts of antibodies are bound and secondly the different antisera inflict distinctly different inhibition effects upon the oxygenase activity.

Materials and Methods

Antiserum preparation

The antiserum to RuBP carboxylase/oxygenase of the wild type *N. tabacum* var. John William's Broadleaf (JWB) used was the monospecific antiserum described earlier [9–12]. The antiserum to RuBP carboxylase/oxygenase of the mutant *N. tabacum* Su/su was obtained according to the earlier described method by immunization of rabbits [10]. This antiserum, as demonstrated by Fig. 2, is monospecific.

Antigen-antibody precipitation and immuno electrophoresis in agarose gel

The methods of the double immuno diffusion test and of the tandem crossed immuno electrophoresis were described earlier [9, 12]. The combined line rocket immuno electrophoresis was carried out under the same conditions as the described rocket immuno electrophoresis [9, 12]. Here, points of the antigens were applied into neutral gel, containing no antibodies, and the antigens (RuBP carboxylase/oxygenase of *Spinacia oleracea* or of the mutant *N. tabacum* Su/su, Fig. 3) to be compared were also diffused into a gel strip of neutral gel. Electrophoresis of the antigens was carried out in direction of the antibody containing gel at 6 V/cm for 16 to 18 h.

Binding of antibodies onto the enzyme

According to the methods of Heidelberger and Kendall [13, 14] in parallel assays increasing amounts

the enzyme (3 to 120 µg) were added to 0.02 ml of the antiserum. After an incubation time of 5 h at room temperature under continuous shaking the precipitates were washed 4 times with 0.04 M Na₂HPO₄/KH₂PO₄-buffer, pH 7.4 in order to remove all soluble proteins. The precipitate was centrifuged for 20 min at 6000 × *g*, and the protein content determined according to Lowry *et al.* [15]. As control reactions the above described incubations were also carried out with the corresponding enzyme concentrations using a control serum (0.02 ml). The protein quantities thus obtained by unspecific precipitations were subtracted from the protein contents of the antiserum assays yielding the net amount of protein due to the enzyme antibody precipitation. In Fig. 4 the course of the Heidelberger-Kendall precipitation curve is shown in dependence on the amount of enzyme used. A curve was obtained with a maximum in the equivalence region. The amount of precipitated antibodies was determined from the precipitations in the antibody-antigen-equivalence region and in the region of antibody excess.

Furthermore, the content of precipitated antibodies was calculated according to an earlier described method [16] which followed equations established by Heidelberger and Kendall [13, 14] and Kabat and Mayer [17]. These calculated values correspond within an error of ±3% to the values derived from the curve. In Table I and II the calculated values for the molar ratios of antibodies to enzyme are summarized. The advantage of this method is that also values measured in the region of antibody excess, that is in the onset of the curve where enzyme concentrations are low, can be used. The enzyme preparation used were usually chloroplast preparations from which by means of two ammonium sulfate precipitations a protein mixture was obtained which consisted by 50% of RuBP carboxylase/oxygenase. The enzyme concentration was determined by means of the rocket immuno electrophoresis [8].

Influence of temperature and chemicals on the binding of antibodies onto RuBP carboxylase/oxygenase

The above described preparations of RuBP-carboxylase/oxygenase were tested for oxygenase activity under the following conditions according to the method of Okabe *et al.* [18]:

- a. heated to 50 °C;
- b. addition of 10 mM hydroxylamine;

- c. addition of 10 mM hydroxylamine and simultaneous heating to 50 °C;
- d. addition of 0.01 mM *o*-(*p*-Nitrophenyl)hydroxylamine;
- e. addition of 0.01 mM *o*-(*p*-Nitrophenyl)hydroxylamine and simultaneous heating to 50 °C.

Following a 10 min pre-treatment the preparations were immediately brought to reaction with the antisera or control sera as described above, with the amount of protein of the antibody-enzyme precipitate being determined.

Measurement of oxygenase activity

For the measurement of oxygenase activity RuBP-1,5-bisphosphate carboxylase/oxygenase was partially purified in order to remove at least the major part of other oxygen consuming activities such as that of phenoloxidase. 100 g of tobacco leaves (fresh weight, midribs removed) were homogenized with 400 ml 0.1 M Tris HCl buffer, pH 7.5, containing 25 mM MgCl₂, 1 mM EDTA, 5 mM DTT and 3% polyvinylpyrrolidone, in blender for 1 min at maximum speed. The resulting brei was filtered through 8 layers of cheese cloth and centrifuged for 15 min at 25000 × *g*. The supernatant was clarified by filtration through a Selecta paper filter no 595 and successively precipitated with ammonium sulfate at 35% and 55% saturation. The sediment obtained after the last centrifugation was resuspended in a small volume of Tris HCl 50 mM, pH 8 and MgSO₄ 5 mM.

All procedures were carried out at 4 °C. The final protein concentration was usually between 3 and 4 mg/ml. The protein solution, when stored at -20 °C, maintained its activity during several days. Before use aliquots were passed over a small Sephadex G 25 column (1 × 10 cm) equilibrated with the above described Tris HCl MgSO₄ buffer.

Antibodies used in the measurement of oxygenase activity were also purified from the crude antiserum by ammonium sulfate precipitation (50%) and passed over a small Sephadex G 25 column.

Activity measurements were carried out by measuring the ribulose 1,5-bisphosphate dependent oxygen uptake at 30 °C with a Clark type electrode (Rank Brothers, Bottisham, U.K.) RuBP carboxylase/oxygenase was activated at 30 °C in 0.1 M Tris HCl, pH 8.0, 15 min later antibodies were added into the activated assay. NaHCO₃ and MgCl₂ concentrations were kept at the above noted concentrations. Before measurement the mixture was incubated for 20 min at 25 °C. The assay was then divided, one part being used for the O₂-uptake measurement and the other for a quantitative protein determination. The final assay used for the oxygenase activity contained in a final volume of 1.5 ml 0.1 M Tris HCl, pH 9.3, 10 mM MgCl₂, 100 µl of enzyme preparation and 0.5 mM ribulose 1,5-bisphosphate (RuBP). The reaction was started by the addition of RuBP. The CO₂ and O₂ concentration in the reaction medium correspond to the concentrations which are in equilibrium with air.

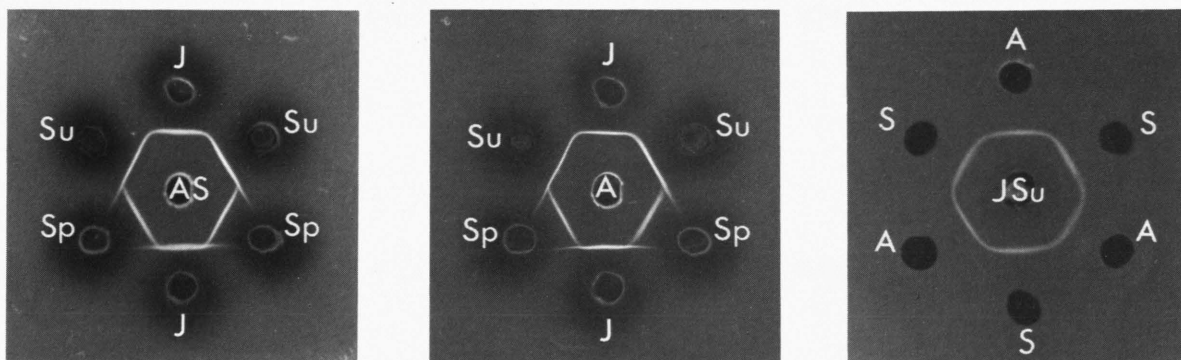


Fig. 1. Comparative analysis of RuBP carboxylase/oxygenase of *N. tabacum* var. John William's Broadleaf (JWB) with the enzymes of the tobacco mutant Su/su and *Spinacia oleracea* (spinach) with homologous and non-homologous antisera in the double immuno diffusion test.

Antigen: J, chloroplast preparation of JWB; Su, chloroplast preparation of Su/su; Sp, chloroplast preparation of spinach; J Su, chloroplast mixture of JWB and Su/su.

Antiserum: A, antiserum to RuBP carboxylase/oxygenase of JWB; S, antiserum to RuBP carboxylase/oxygenase of Su/su; AS, antiserum mixture of both antisera.

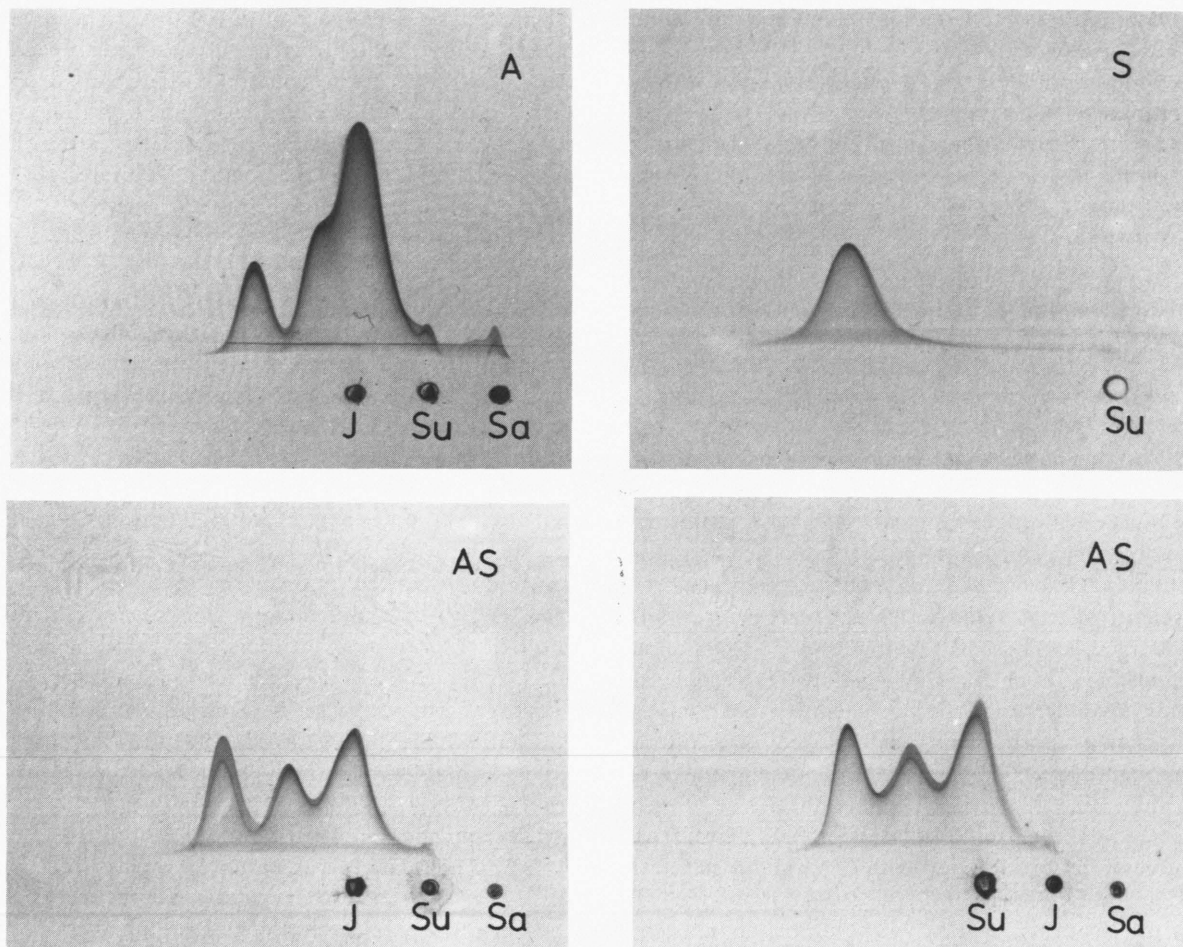


Fig. 2. Comparative analysis of RuBP carboxylase/oxygenase of *N. tabacum* var. JWB with the enzyme of the tobacco mutants *N. tabacum* Su/su and *N. tabacum* Su/su var. Aurea with homologous and non-homologous antisera in the tandem crossed immuno electrophoresis.

Antigen: J, chloroplast preparation of JWB; Su, chloroplast preparation of Su/su; Sa, chloroplast preparation of Su/su var. Aurea.

Antiserum: A, antiserum to RuBP carboxylase/oxygenase of JWB, 1% serum in the gel; S, antiserum to RuBP carboxylase/oxygenase of Su/su, 0.4% serum in the gel; AS, antiserum mixture of both antisera.

Results

The bifunctional enzyme RuBP-carboxylase/oxygenase of the tobacco mutant Su/su, which exhibits high photorespiratory activity is compared to that of the wild type *N. tabacum* var. John William's Broadleaf, a plant which according to the literature exhibits a normal photorespiratory activity [1–7]. The comparison is made serologically by means of the double immuno diffusions test and the tandem crossed immuno electrophoresis (Fig. 1 and 2). In both cases the test reagents were an antiserum to the wild type enzyme and one to the mutant enzyme as well as a

mixture of both antisera. The monospecificity of the antiserum to the wild type enzyme was already established earlier [9] whereas the monospecificity of the newly obtained antiserum to the mutant enzyme is shown in Fig. 2. The clear result is that both methods yield for the two enzymes to be compared only fusing precipitation bands, hence, in principal serological identity. On the other hand, if the enzyme of *Spinacia oleracea* is tested against these antisera or against the mixed antisera, as already shown in earlier studies, only partial identity with the tobacco enzyme is seen.

For these reason the same comparison was made in a further experiment with a combined rocket immuno electrophoresis and line immuno electrophoresis which is a sensitive detection method for antibody-antigen precipitations (Fig. 3). In this case also, the results obtained by double immuno diffusion and the tandem crossed immuno electrophoresis are confirmed, namely that between the two tobacco enzymes only fusing bands are observed which is not the case if the test is made between the tobacco and spinach enzyme.

In the following we determined the binding of antibodies onto the enzyme RuBP carboxylase/oxygenase according to the method of Heidelberger and Kendall [13, 14]. For this purpose in parallel assays,

constant amounts of sera were incubated with increasing quantities of antigen (Fig. 4). This method offers on the one hand not only the opportunity to determine in the region of antibody-antigen equivalence the molecule ratio of antigens to antibodies but on the other hand also the ratio of bound antibodies (Fig. 5) to antigen in the region of excess antibodies, the advantage in the latter case being that in the region of antibody excess the main portion of antibodies is monovalently bound. This way, most of the sterically accessible antigenic determinants can be covered with antibodies. According to this method the binding of antibodies onto the native untreated enzyme of the wild type and onto the enzyme of the tobacco mutant Su/su was determined using in each

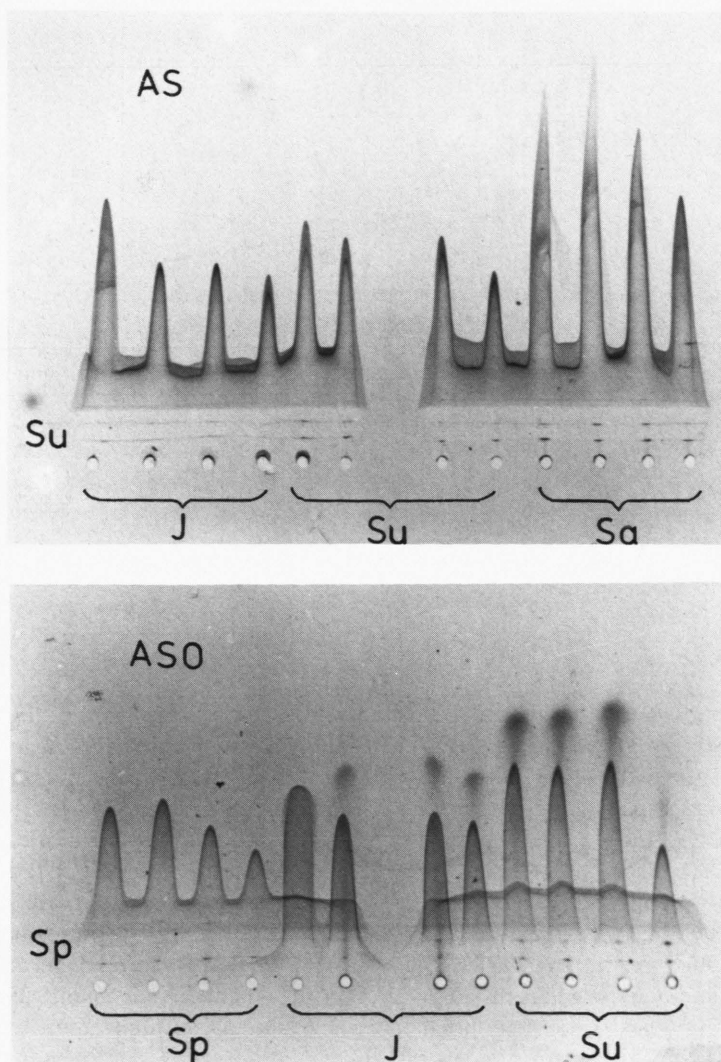


Fig. 3. Comparative analysis of RuBP carboxylase/oxygenase of *N. tabacum* var. JWB with the enzymes of the tobacco mutants *N. tabacum* Su/su and *N. tabacum* Su/su var. Aurea as well as with the enzyme of *Spinacia oleracea* in the line rocket immuno electrophoresis.

Antigen: J, chloroplast preparation of JWB; Su, chloroplast preparation of Su/su; Sa, chloroplast preparation of Su/su var. Aurea; Sp, chloroplast preparation of spinach.

Antiserum: AS, serum mixture of the antisera to RuBP carboxylase/oxygenase of *N. tabacum* var. JWB (1%) and of *N. tabacum* Su/su (1%); ASO, serum mixture of the antisera to RuBP carboxylase/oxygenase of *N. tabacum* var. JWB (1%), of *N. tabacum* Su/su (1%) and of *Spinacia oleracea* (1.5%).

Table I. Binding of specific antibodies onto RuBP carboxylase/oxygenase of *N. tabacum* var. JWB, *N. tabacum* Su/su and of *N. tabacum* Su/su var. Aurea out of antisera to RuBP carboxylase/oxygenase of *N. tabacum* var. JWB and *N. tabacum* Su/su in the region of antigen-antibody equivalence and in the region of extreme excess of antibodies.

Antiserum to	RuBP carboxylase/oxygenase of		
	<i>N. tabacum</i> var. JWB	<i>N. tabacum</i> Su/su	<i>N. tabacum</i> Su/su var. Aurea
RuBP carboxylase/oxygenase of JWB, in the equivalence region	5.9	5.7	5.9
in the region of extreme antibody excess	7.2	11.6	11.7
RuBP carboxylase/oxygenase of Su/su in the equivalence region	5.6	6.8	6.0
in the region of extreme antibody excess	11.2	13.7	12.1

The values give the number of antibody molecules bound onto one enzyme molecule.

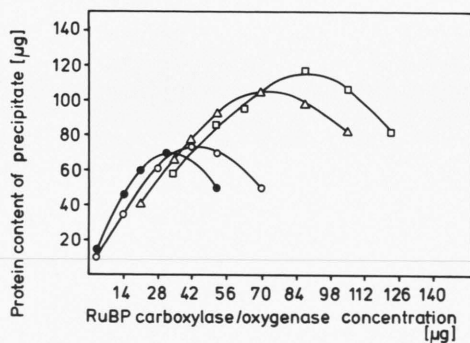


Fig. 4. Quantitative precipitations of RuBP carboxylase/oxygenase of *N. tabacum* var. John William's Broadleaf with the homologous antiserum after various treatments. The used amount of antiserum was 20 µl and the enzyme concentration 3.5–126 µg.

●—● Precipitation curve for the native enzyme; ○—○ precipitation curve after treatment of the enzyme with 10 mM hydroxylamine; △—△ precipitation curve after heating of the enzyme to 50 °C for 10 min; □—□ precipitation curve after treatment of the enzyme with 10 mM hydroxylamine with simultaneous heating for 10 min to 50 °C.

case the homologous as well as the not homologous antiserum. This analysis showed, as seen in Table I, that a substantial difference exists between the two enzymes. Whereas in the equivalence region of the antiserum to RuBP carboxylase/oxygenase of the wild type equal amounts of antibodies were bound onto both enzymes, approximately 20% more antibodies were adsorbed out of the antiserum to RuBP carboxylase/oxygenase of the mutant onto the homologous enzyme. In the region of extreme antibody excess approximately 60% more antibodies were bound out of the antiserum to RuBP carboxyl-

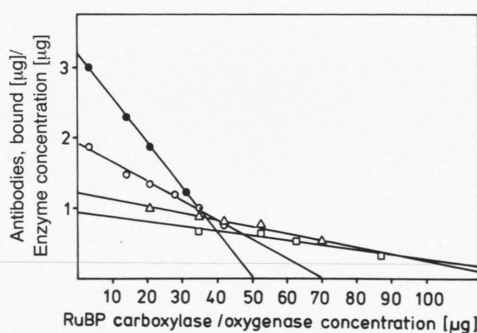


Fig. 5. Dependence of the ratio of amount of bound antibodies to RuBP carboxylase/oxygenase of *N. tabacum* var. JWB and its homologous enzyme on the amount of added enzyme.

ase/oxygenase of the wild type onto the mutant enzyme than onto the homologous enzyme of the wild type. In contrast to this, the enzyme of the mutant binds out of its homologous antiserum only 22% more antibodies than does the wild type enzyme. This clearly means that the enzyme molecule of the tobacco mutant *N. tabacum* Su/su either contains a larger number of homologous antigenic determinants than the wild type enzyme or that a more advantageous distribution ("better" spatial arrangement) of the determinants, exists in the mutant molecule.

In the context with this observation the question

Table II. Binding of specific antibodies onto RuBP carboxylase/oxygenase of *N. tabacum* var. JWB, *N. tabacum* Su/su and of *N. tabacum* Su/su var. Aurea out of the antisera to RuBP carboxylase/oxygenase of *N. tabacum* var. JWB and *N. tabacum* Su/su after treatment with hydroxylamine or a hydroxylamine derivative in the region of antigen-antibody equivalence and in the region of extreme antibody excess.

Antiserum to	RuBP carboxylase/oxygenase of														
	<i>N. tabacum</i> var. JWB					<i>N. tabacum</i> Su/su					<i>N. tabacum</i> Su/su var. Aurea				
	a	b	c	d	e	a	b	c	d	e	a	b	c	d	e
RuBP carboxylase/oxygenase of <i>N. tabacum</i> var. JWB in the region of equivalence	3.5	2.5	1.7	4.2	1.2	3.9	2.2	1.7	4.3	1.3	3.6	2.5	1.8	4.1	1.3
in the region of extreme antibody excess	7.0	4.5	3.5	8.3	2.4	7.7	4.5	3.5	8.6	2.6	7.2	4.6	3.6	8.2	2.5
RuBP carboxylase/oxygenase of <i>N. tabacum</i> Su/su in the region of equivalence	3.3	1.8	1.3			3.8	1.7	1.3			3.7	2.5	1.4		
in the region of extreme antibody excess	6.7	3.5	2.6			7.6	3.5	2.6			7.4	5.0	2.9		

The values give the number of antibody molecules bound per enzyme molecule. RuBP carboxylase/oxygenase was treated before incubation with the antiserum with

a. 10 mM hydroxylamine;

b. heated for 10 min to 50 °C;

c. 10 mM hydroxylamine with simultaneous heating for 10 min to 50 °C;

d. 0.01 mM *o*-(*p*-nitrophenyl)hydroxylamine;

e. 0.01 mM *o*-(*p*-nitrophenyl)hydroxylamine with simultaneous heating for 10 min to 50 °C.

arises whether chemical modifications of the enzyme induced by means of hydroxylamine (Okabe *et al.* [18]) or its derivatives (van Assche *et al.* [19], Schmid *et al.* [20]) or by the influence of heat treatments to 50 °C are immunochemically characterizable. Thus, Okabe *et al.* [18] have shown that under influence of a temperature treatment to 50 °C together with treatments with hydroxylamine the oxygenase activity is reduced in the cyanobacterium *Anabaena cylindrica* [18]. Binding of antibodies out of the homologous and non-homologous antisera by the thus treated enzymes showed that the enzymes have underwent such a drastic modification that both enzymes now seem to bind considerably less antibodies. Values for the respective antibody binding are listed in Table II. The largest molecular modification of the enzymes is not caused by the chemical treatment alone but by chemical treatment together with a simultaneous heating of the enzyme to 50 °C. In the region of extreme antibody excess, as expected and explained above, a relatively higher amount of antibodies is bound out of the two antisera onto the treated enzymes than in the region of equivalence. It should be emphasized again, that this chemical modification of RuBP carboxylase/oxygenase from the two tobacco

phenotypes was not seizeable with the methods of immuno precipitation and immuno electrophoresis in agarose gel which is apparently due to the fact that these molecular changes are reversible after more than 2 h whereas the above described precipitation reactions in agarose gel require more than 24 h.

In Fig. 6a–d the oxygenase activity of the bifunctional enzyme of the wild type *N. tabacum* var. John William's Broadleaf and the tobacco mutant Su/su, (which is the mutant with high photorespiration) [1–7] is plotted in dependance on the antibody binding out of the homologous and non-homologous antiserum. Curves (Δ – Δ) show the course of the quantitative enzyme antibody precipitation and curves (\circ – \circ) show the course of oxygenase activity of the enzymes. For this test the immunoglobulines of both antisera were purified by ammonium sulfate precipitation and subsequent chromatography on Sephadex G 25. RuBP carboxylase/oxygenase was 95% pure after ammonium sulfate precipitation and chromatography on Sephadex G 25.

Fig. 6a–d clearly shows that there exists a dependency between the degree of inhibition of the oxygenase function of the enzyme and the amount of bound antibodies. In the region of small antibody

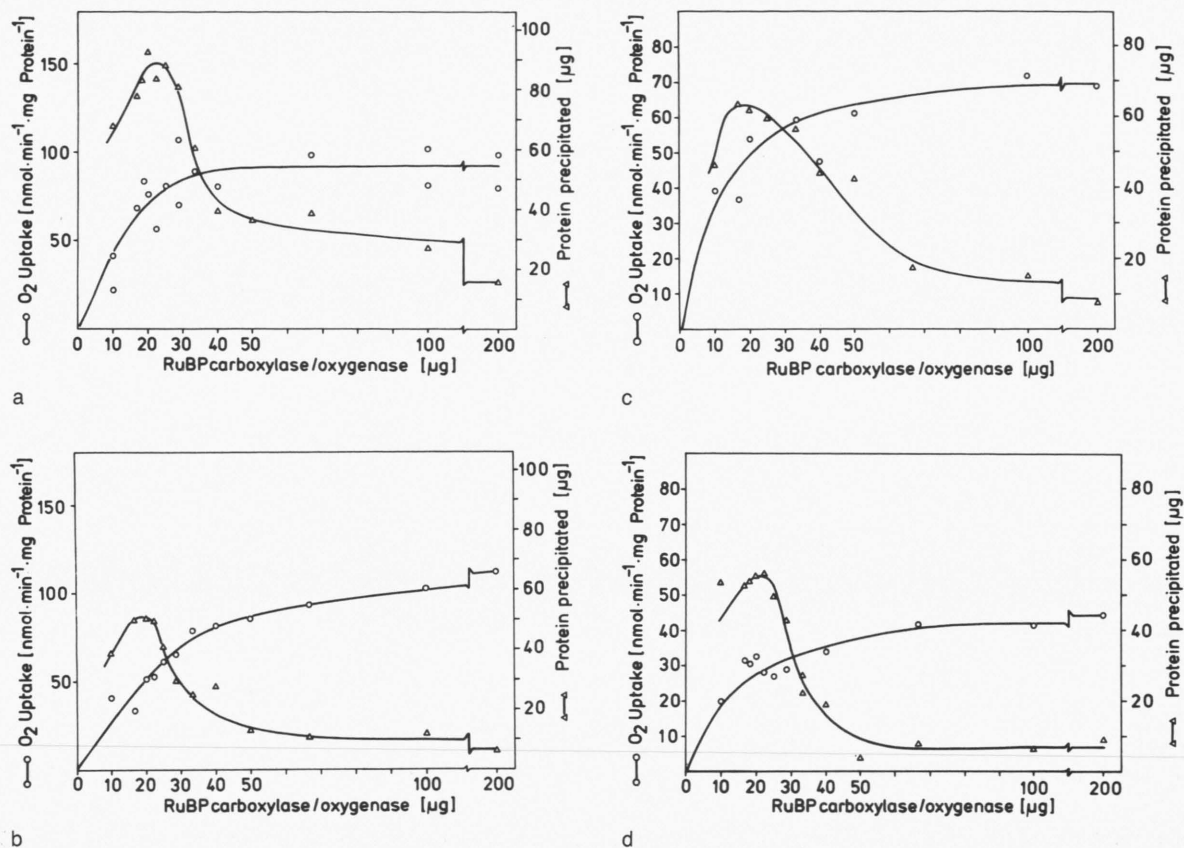


Fig. 6. Dependence of oxygenase activity of RuBP carboxylase/oxygenase of *N. tabacum* var. JWB and of the tobacco mutant Su/su on the binding of antibodies out of the homologous and the non-homologous antisera.

a. Treatment of RuBP carboxylase/oxygenase from the wild type with the homologous antiserum and b. with the non-homologous antiserum.

c. Treatment of RuBP carboxylase/oxygenase of the tobacco mutant *N. tabacum* Su/su with the non-homologous antiserum and

d. with the homologous antiserum.

The curves with ○—○ show the oxygenase activity of the enzyme incubated with antibodies whereas the curves with △—△ show the protein content of the enzyme-antibody precipitation.

additions (in the region of extreme excess of antigen) the enzyme maintains its high activity. In the course of higher antibody binding in the region of equivalence, the activity decreases strongly going in the region of extreme excess of antibodies towards zero. In this region the antibodies are mainly monovalently bound. This in turn clearly means that in the region of antibody excess more antigenic determinants are saturated or covered with antibodies.

It should be noted that the oxygenase activity of the enzyme was measured after incubation with antibodies as shown in Fig. 6a–d. The formed enzyme-antibody precipitations were not removed by cen-

trifugation and activity measurements were carried out with the precipitated enzymes. If instead the formed precipitates were removed by centrifugation, with also the supernatant of the reaction assay being assayed for oxygenase activities, it was shown that from the region of excess of antibodies to the equivalence region no activity was found. This clearly demonstrates that no soluble enzyme is present in the supernatant of the assay in the equivalence region. The experiment furthermore shows that RuBP carboxylase/oxygenase still exhibits oxygenase activity in a network, containing only few antibody molecules.

Moreover, we have noted a principal and most astonishing difference between the enzymes: If the oxygenase function is inhibited by an antiserum it really matters whether the antiserum of the wild type enzyme or whether that of the mutant is used. Antibodies to RuBP carboxylase/oxygenase of the mutant cause in the region of equivalence when used against both enzymes a 40% higher inhibition of the oxygenase activity than antibodies to the wild type enzyme (Fig. 6a–d). This is valid despite the fact that the antibody titer of the antiserum to the mutant enzyme is 25% lower than that of the wild type antiserum. Even with small antibody additions which is in the region of extreme antigenic excess the homologous antibodies cause in the mutant enzyme a 40% inhibition of oxygenase activity.

Discussion

In the present paper we were able to present the evidence that a very particular difference exists in the molecular structure of the bifunctional enzyme RuBP carboxylase/oxygenase of the wild type tobacco *N. tabacum* var. JWB and the tobacco mutant Su/su. It should be noted that the two phenotypes differ with respect to their photorespiratory activity [1–7]. It is generally assumed that the higher photorespiration of the mutant is due to a higher oxygenase activity of the bifunctional enzyme. In earlier papers we were able to show that the mutant enzyme exhibited a higher affinity for oxygen and a lower one to CO₂ when compared to the wild type enzyme [4]. Since with the conventional methods of double immuno diffusion and tandem crossed immuno electrophoresis, as well as with combined line rocket immuno electrophoresis the application of our two monospecific antisera only yielded serological identity, we have to conclude that the different oxygenase activities must be due to different conformational states of the enzyme. The problem to be solved is to find out how eventually in the two enzymes, which are

at least serologically identical, different conformational states are maintained. The notion that the different conformational states are linked to different enzyme activities is well known and is nearly trivial. However, chemical modification is particularly easy to obtain with RuBP carboxylase/oxygenase and these changes can be easily analyzed by immunological methods: Thus, we were able to show that treatment of the enzyme with hydroxylamine or *o*-(*p*-nitrophenyl)hydroxylamine or simply a heat treatment to 50 °C leads to a diminution of oxygenase activity, a situation in which more than half of the antigenic determinant groups are affected to such an extent that no further antibody binding can occur. Furthermore, we were able to show in the quantitative antigen-antibody precipitation reaction according to the method of Heidelberger and Kendall [13, 14] that a correlation exists between the amount of antibodies bound and the observed oxygenase activity. A small amount of bound antibodies does not cause yet any, or at most only a slight effect on the oxygenase activity. Only the binding of high amounts of antibodies causes a complete inhibition of oxygenase activity. Especially the observations contained in Fig. 6 lead to the immunochemical conclusion that in the two enzymes, which exhibit different oxygenase activities, certain molecular structures, which are represented by the antigenic determinants remain inaccessible or covered due to different conformational states. The only alternative could be that the two enzymes which appear serologically identical, when analyzed with conventional immunological methods, are nevertheless different in the sense that the same antigenic determinant types are arranged in such a close relationship or vicinity to each other, however, differently in the two enzymes, that binding of an antibody molecule sterically hinders further antibody binding.

Acknowledgements

C. N. thanks Dr. C. J. van Assche/Procida, Marseille for the gift of *o*-(*p*-nitrophenyl)hydroxylamine.

- [1] P. H. Homann and G. H. Schmid, *Plant Physiol.* **42**, 1619 (1967).
- [2] J. Zelitch and P. R. Day, *Plant Physiol.* **43**, 1838 (1968).
- [3] K. Okabe, G. H. Schmid, and J. Straub, *Plant Physiol.* **60**, 150 (1977).
- [4] K. Okabe and G. H. Schmid, *Chloroplast Development* (G. Akoyunoglou and J. H. Argyroudi-Akoyunoglou, eds.), p. 501, Elsevier Ass. Scientific Publishers, Amsterdam 1978.
- [5] G. H. Schmid, K. P. Bader, R. Gerster, C. Triantaphylides, and M. André, *Z. Naturforsch.* **36c**, 662 (1981).
- [6] R. Ishii and G. H. Schmid, *Z. Naturforsch.* **37c**, 93 (1982).
- [7] R. Ishii and G. H. Schmid, *Plant Cell Physiol.* **24**(8), 1525 (1983).
- [8] A. Radunz and G. H. Schmid, in: *Progress in Photosynthesis Research III* (J. Biggins, ed.), p. 9617, Martinus Nijhoff Publisher, Dordrecht, The Netherlands 1987.
- [9] A. Radunz and G. H. Schmid, *Z. Naturforsch.* **43c**, 554 (1988).
- [10] A. Radunz, *Z. Naturforsch.* **33c**, 731 (1978).
- [11] A. Radunz, G. H. Schmid, M. Bertrand, and E. Dujardin, in: *Regulation of chloroplast differentiation, Plant Biology 2* (G. Akoyunoglou and H. Senger, eds.), p. 197, Alan R. Liss Inc. New York 1987.
- [12] E. Dujardin, M. Bertrand, A. Radunz, and G. H. Schmid, *J. of Plant Physiol.* **128**, 95 (1987).
- [13] M. Heidelberger and F. Kendall, *J. Exp. Med.* **61**, 563 (1935).
- [14] M. Heidelberger and F. Kendall, *J. Exp. Med.* **62**, 697 (1935).
- [15] O. H. Lowry, N. J. Rosebrough, J. A. L. Farr, and R. J. Randall, *J. Biol. Chem.* **193**, 265 (1951).
- [16] A. Radunz, *Z. Naturforsch.* **38c**, 297 (1983).
- [17] E. Kabat and M. Mayer, *Exp. Immunochimistry 2*, Aufl. Thomas Springfield/I 1961.
- [18] K. Okabe, A. Codd, and W. D. P. Stewart, *Nature* **279**, 525 (1979).
- [19] C. J. van Assche, N. Reinier, B. Courtiade, A. Chancel, and S. Hübner, *Z. Naturforsch.* **42c**, 837 (1987).
- [20] G. H. Schmid, K. P. Bader, A. Radunz, C. J. van Assche, and B. Courtiade, *Z. Naturforsch.* **42c**, 965 (1987).