Effect of Antisera to RuBP-Carboxylase/Oxygenase of *N. tabacum* and *Spinacia oleracea* on the Oxygenase-Function of the Enzyme

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Dedicated to Professor Wilhelm Menke at the occasion of his 80th birthday

Monospecific Antisera, RuBP-Carboxylase/Oxygenase, Oxygenase Activity, Capacity of Antibody Binding, *N. tabacum* Wild Type, Tobacco Mutant, Su/su, Spinach

In the present paper we demonstrate that the conformational state of the bifunctional enzyme ribulose-1,5-bisphosphate carboxylase/oxygenase is different in the tobacco mutant Su/su when compared to the wild type tobacco or spinach. The conformational state of the tobacco mutant enzyme is characterized by the presence of a higher number of antigenic determinants accessible to antibody binding. This seems to be correlated to a higher oxygenase activity in the mutant. The bifunctional enzyme ribulose-1,5-bisphosphate carboxylase/oxygenase of the wild type tobacco *Nicotiana tabacum* var. John William's Broadleaf, of the tobacco mutant Su/su and of spinach (*Spinacia oleracea*) was characterized by comparative immunological methods. Although the enzyme of the tobacco mutant appears identical to the enzyme of the wild type, when analyzed in immunodiffusion tests and immuno electrophoretical analyses, it exhibits a higher oxygenase activity. On the other hand the spinach enzyme exhibits only partial serological identity to the two tobacco enzymes.

For the comparative studies pure IgG-fractions were prepared from the respective antisera. RuBP-carboxylase/oxygenase was used as a 70% purified enzyme preparation. Determination of the antibody binding capacity showed that the enzymes bind from the homologous antisera the highest amount of antibodies, which means that the antisera reflect the complementary picture of the enzyme structure. The enzyme molecules of *N. tabacum* var. JWB and of spinach bind 9 antibody molecules each. However, the binding capacity of the tobacco mutant enzyme exhibiting the higher oxygenase activity is 30% higher.

Measurement of the oxygenase function under the influence of the homologous as well as of the non-homologous antisera has led to the result that the oxygenase activity of all enzymes is inhibited. However, it is the degree of inhibition which differs. The antiserum to the mutant enzyme causes with the spinach as well as with the JWB-enzyme a higher degree of inhibition than that produced by the homologous antiserum. Therefore, a correlation between inhibitory effect brought about by this antiserum and the amount of antibodies bound does not exist. Whereas the enzyme of the tobacco wild type binds 20% less antibodies out of this antiserum its oxygenase activity is 60% more inhibited and the function of the spinach enzyme is 20% stronger inhibited although binding of antibodies from the antiserum to the tobacco enzyme is 50% lower.

These observations permit the conclusion that the antiserum towards the mutant enzyme contains more antibodies with a higher binding affinity towards reactive regions of the oxygenase function. This in turn means that the structure of this enzyme or its conformation must be different in comparison to the wild type enzyme.

Introduction

The *Nicotiana tabacum* mutant Su/su exhibits in comparison to the wild type *N. tabacum* var. John William's Broadleaf a 30% higher photorespiration [1–6]. This increased photorespiration is due to a higher oxygenase activity of the bifunctional enzyme ribulose-1,5-bisphosphate carboxylase/oxygenase [5, 6]. This functional difference of the enzyme can be characterized by immunological methods as shown by our laboratory in recent publications [7–9]. In the double immunodiffusion tests as well as in immunoelectrophoretical analyses in agarose gels RuBP carboxylase/oxygenase of the two phenotypes, namely of the *N. tabacum* mutant and the wild type are immunologi-

Abbreviations: EDTA, ethylenediamine tetraacetic acid; PVPP, polyvinyl-polypyrrolidone; Tris, tris-(hydroxymethyl)-aminomethane; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; RuBP, carboxylase/oxygenase: Ribulose-1.5-bisphosphate carboxylase/oxygenase.

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cally identical. Determination of the antibody binding capacity and measurement of the oxygenase activity under the influence of antisera has shown that structural differences or different conformational states between the enzyme species with higher and lower affinity towards oxygen exist [7–10].

In order to further characterize the structure of the bifunctional enzyme, comparative immunological studies of the two tobacco phenotypes with the enzyme of spinach are carried out, as it appears that the large subunits of the spinach and tobacco enzyme on which the reactive centers are located exhibit differences in their peptide composition. As shown by Kawashima and Wildman [11] 5 out of 22 peptide fragments do not match with respect to their mobility behaviour. Our own studies have shown that the spinach enzyme is only partially identical to that of tobacco and other Solanaceae [7, 10].

In order to carry out these investigations, pure IgG fractions were prepared from the monospecific antisera. RuBP-carboxylase was purified out of the respective chloroplast preparations by means of ammonium sulfate precipitation to a 70% pure preparation.

Materials and Methods

Preparation of the antisera

Antisera to RuBP carboxylase/oxygenase were obtained by immunization of rabbits according to earlier described methods [7, 12]. 0.2–0.4 mg enzyme were emulsified with Freund's adjuvant and injected subcutaneously at two spots of the back skin. For the preparation of the antisera to the large and small subunits the preparations of the respective subunits were emulsified with Freund's adjuvant and injected intramuscularly twice with a two weeks interval. After 24–27 days the same amount of enzyme in 2 ml buffer was intravenously injected. Blood was withdrawn after 8–10 days following this injection. Further blood withdrawals were made in 7 days intervals. These polyclonal antisera were monospecific as demonstrated in the agarose gel diffusion tests, rocket immuno- and crossed immunoelectrophoresis with chloroplast preparations [7, 10, 13] (Fig. 1).

Preparation of the IgG-Fractions

The immunoglobuline fraction (IgG) was isolated by affinity chromatography on CM-Affi-Gel-Blue (Bio Rad Laboratories, Technical Bulletin 1061/1092) from the respective sera. On a column with a volume of 100 ml (column dimensions 2.6 × 20 cm) up to 20 ml serum were given and the IgG-fraction eluted with a 0.01 M KH_2PO_4/K_2HPO_4 buffer, pH 7.25, containing 0.15 M NaCl. These IgG fractions were subjected to an ammonium sulfate precipitation (45% $(NH_4)_2SO_4$-saturation) and subsequently extensively dialyzed against a 0.06 M Na_2HPO_4/KH_2PO_4 buffer, pH 7.4 or for the inhibition experiments of the oxygenase activity the IgG fractions were dialyzed against 50 mM Tris-HCl, pH 8.0 in order to keep the pH and the ionic strength constant during the process of enzyme activation and incubation with antibodies. The determination of the IgG concentration was carried out according to the quantitative procedure of Mancini *et al.* [14] in the simple radial immunodiffusion in agarose gel. As antiserum we used an anti-rabbit-IgG from the goat.

Chloroplast preparation

Chloroplasts from the described *N. tabacum* species and of *Spinacia oleracea* were isolated by fractionating centrifugation in 0.05 M Tris buffer, containing 0.4 M sucrose, 0.28% pectinase, 0.2% bovine serum albumine and 0.01 M NaCl, pH 7.4. For the above described immuno diffusion tests and electrophoretical studies the chloroplasts were either suspended in a 0.06 M Na_2HPO_4/KH_2PO_4 buffer, pH 7.4 or in 0.03 M barbiturate buffer, pH 8.6. Both buffers contained for the dissolution of the chloroplast structure 2% Triton X 100.

Purification of RuBP carboxylase/oxygenase and separation of subunits

The deveined leaf material was ground in 50 mM Tris-HCl buffer, pH 7.8 containing 200 mM NaCl, 10 mM $MgCl_2$, 10 mM $NaHCO_3$, 1 mM EDTA, 10 mM β-mercaptoethanol and 3% (w/v) PVPP (buffer a) in a blender for 1 min at top speed. The leaf brei was filtered through 6 layers of cheese cloth and then through a paper filter (Schleicher & Schüll, 520 b). Subsequently the filtrate was centrifuged at 27,000 × *g* for 60 min. The supernatant was treated with ammonium sulfate to give 37%

saturation and allowed to stand for 30 min before centrifugation at 27,000 × *g* for 30 min. The resulting supernatant was brought to 55% saturation with ammonium sulfate and allowed to sedimentate before centrifugation. The supernatant was discarded and the precipitated proteins were gently resuspended [15] in a small volume of 50 mM Tris-HCl buffer, pH 7.6, containing 200 mM NaCl, 0.5 mM EDTA, 10 mM $MgCl_2$ and 10 mM $NaHCO_3$ (buffer b). The protein solution was clarified by centrifugation at 40,000 × *g* for 60 min and passed to gel chromatography on Sephadex G-25 fine (2.6 × 19 cm gel volume) equilibrated with buffer b to remove phenolic compounds [16] and for desalting. RuBP carboxylase/oxygenase containing fractions were pooled and directly applied for gel chromatography on Sepharose CL-4 B (2.6 × 93 cm gel bed) equilibrated with buffer b. The pooled RuBP carboxylase/oxygenase peak from this step was loaded onto a DEAE-Sephacel column (2.6 × 10 cm) and eluted with a linear gradient of 0.0–0.4 M $NaHCO_3$ in 50 mM Tris-HCl buffer, pH 8.0. For desalting, the pure RuBP carboxylase/oxygenase fraction was passed through a small Sephadex G-25 column (1 × 10 cm) equilibrated with 50 mM Tris-HCl buffer, pH 8.0 and tested for purity in the double immunodiffusion, in the crossed immunoelectrophoresis with polyspecific chloroplast antisera (Fig. 2) and in the SDS-PAGE according to the method of Laemmli [17]. All steps were carried out at 4 °C.

In order to separate the large and small subunits of RuBP carboxylase/oxygenase the pure enzyme was incubated in 25 mM Tris-HCl buffer, pH 8.0, containing 5% SDS and 0.3 M β-mercaptho-ethanol for 60 min at 22 °C and subsequently chromatographed on a Sephacryl S-200 column (1.6 × 65 cm gel bed) equilibrated with 50 mM Tris-HCl, pH 8.0 including 0.1% SDS. The small and large subunit containing peaks were examined for purity in the SDS-PAGE [17].

Enzyme-antibody precipitation in agarose gel

Qualitative and quantitative enzyme-antibody precipitations were carried out according to the earlier described procedures of double immunodiffusion, rocket-, tandem crossed and in the combined line rocket-immuno electrophoresis in 1% agarose gel [10].

Determination of binding of antibodies on the enzyme

Binding of antibodies onto the enzymes was carried out according to earlier described methods [7, 18–20]. The antisera used were the above described pure IgG fractions. The RuBP-carboxylase/oxygenase fraction used was a 70% pure preparation. The concentration of the enzyme was determined by means of the rocket immunoelectrophoresis. Protein determinations of the antigen-antibody precipitates were carried out according to Lowry *et al.* [21], using bovine serum albumine as a standard.

Inhibition of the oxygenase activity

Oxygenase activity and the effect of antibodies on RuBP carboxylase/oxygenase was measured according to earlier described methods [7] in an oxygen electrode (Rank Brothers, England). The measurement was carried out in 50 mM Tris-HCl buffer, pH 9.3. For catalytic activation the enzyme was incubated for 20 min at 30 °C in Tris-HCl buffer, pH 8.0, containing 20 mM $MgCl_2$ and 10 mM $NaHCO_3$ [22]. After addition of appropriate quantities of IgG fraction the concentrations of $MgCl_2$ and $NaHCO_3$ were kept at 20 mM or 10 mM, respectively. As substrate ribulose-1,5-bisphosphate from Sigma (R 8250) was used.

Results

Measurement of the oxygenase activity of the bifunctional enzyme of wild type tobacco and of *Spinacia oleracea* under the influence of the homologous and the non-homologous antisera to RuBP-carboxylase/oxygenase have led to the result that monospecific antisera (Fig. 1), which contain polyclonal antibodies exert an inhibitory effect on the oxygenase function of the enzyme. This inhibitory effect is dependent on the amount of the antibodies present as seen from Fig. 3a–f. With increasing antibody concentrations a decrease in the oxygenase activity is observed. This means that in the region of antigen excess, hence when only a few antibody molecules can be bound onto an enzyme molecule, oxygenase activity is only little influenced or not at all. Even in the equivalence region of enzyme-antibody, where a precipitation of the enzyme has already occurred, the enzyme still

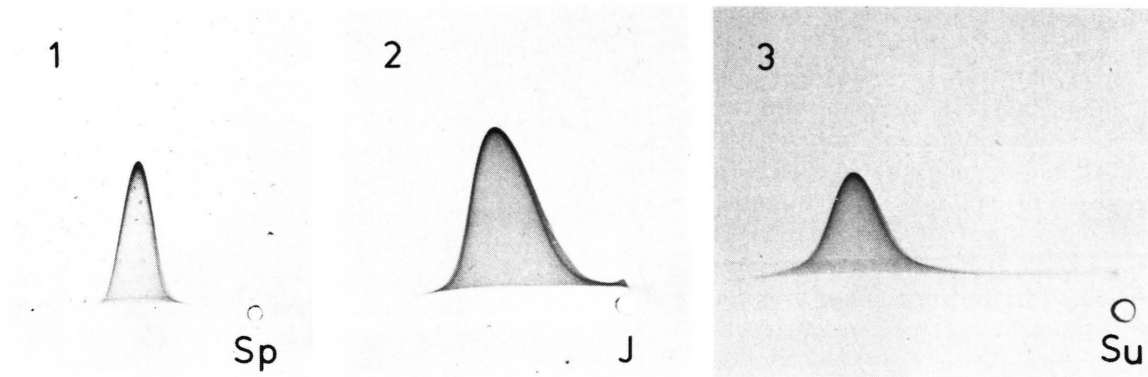

Fig. 1. Analysis for monospecifity of the antisera to RuBP carboxylase/oxygenase in the crossed immunoelectrophoresis.
Antisera: 1. Antiserum to RuBP carboxylase/oxygenase of *Sp. oleracea* (1% v/v in the gel); 2. antiserum to RuBP carboxylase/oxygenase of *N. tabacum* var. JWB (1% v/v in the agarose gel); 3. antiserum to RuBP carboxylase/oxygenase of *N. tabacum* mutant Su/su (1% v/v in the gel).
Antigen: J, chloroplast preparation of *N. tabacum* var. JWB; Su, chloroplast preparation of *N. tabacum* mutant Su/su; Sp, chloroplast preparation of *Sp. oleracea*.

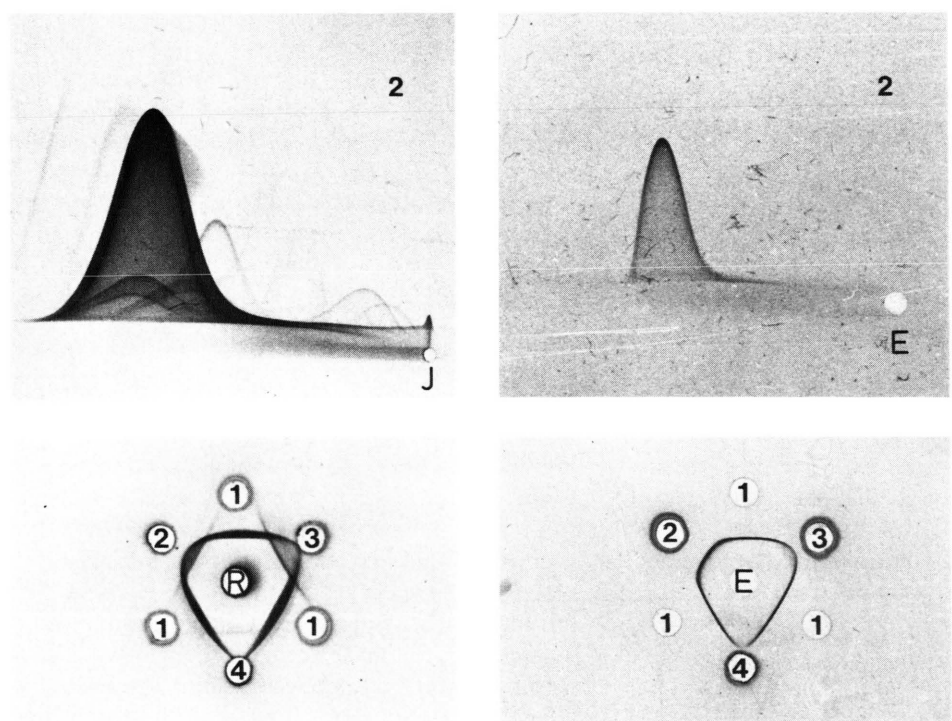

Fig. 2. Purity test of the isolated RuBP carboxylase/oxygenase of *Spinacia oleracea* in the double immunodiffusion and in the crossed immunoelectrophoresis.
Antisera: 1. to RuBP carboxylase/oxygenase of *N. tabacum* var. JWB; 2. to soluble chloroplast proteins (polyspecific); 3. to chloroplasts (polyspecific); 4. to the coupling factor of photophosphorylation (CF_1).
Antigen: J, chloroplast preparation of *N. tabacum* var. JWB; R, crude enzyme preparation after $(NH_4)_2SO_4$-precipitation and chromatography on Sephadex G-25; E, enzyme of *Spinacia oleracea* after twofold chromatography on Sepharose Cl-6 B.

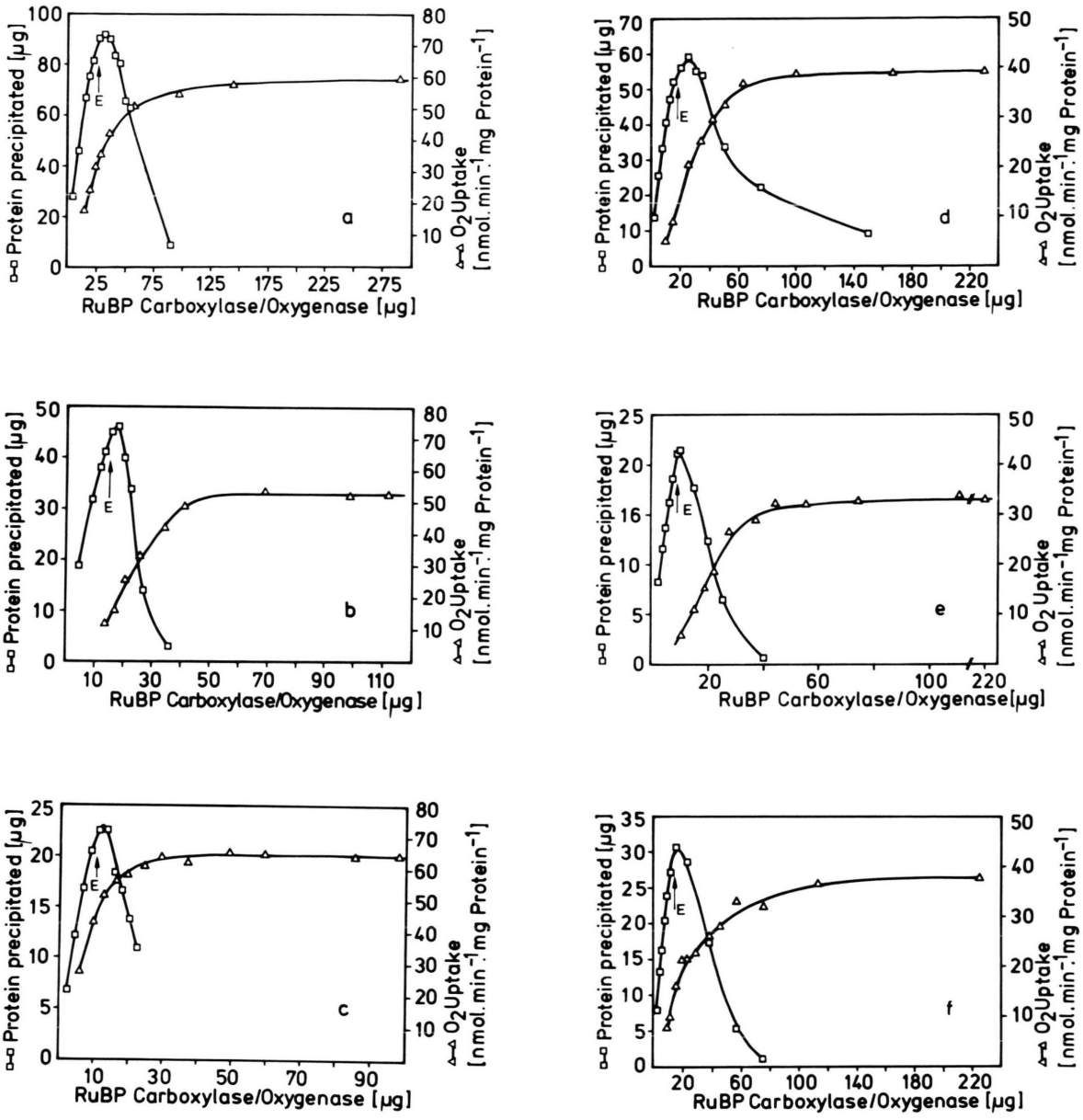

Fig. 3. Dependence of oxygenase activity of RuBP carboxylase/oxygenase of *N. tabacum* var. JWB and of *Sp. oleracea* on the binding of antibodies out of the homologous and non-homologous antisera and out of the antiserum to the enzyme of the *N. tabacum* mutant Su/su. a) Treatment of RuBP carboxylase/oxygenase from the wild type with the homologous antiserum; b) with the antiserum to the *N. tabacum* mutant Su/su enzyme; c) with the antiserum to the spinach enzyme; d) treatment of RuBP carboxylase/oxygenase from *Sp. oleracea* with the homologous antiserum; e) with the antiserum to the enzyme of the *N. tabacum* mutant Su/su; f) with the antiserum to the enzyme of the wild type of *N. tabacum* var. JWB. The curves △−△ shows the oxygenase activity of the enzyme incubated with antibodies whereas the curves with □−□ show the protein content of the enzyme-antibody precipitation.

exhibits a certain degree of activity. It is only in the region of antibody excess, that is when the major portion of the antibodies is monovalently bound, that oxygenase activity goes down towards zero. This means that a comparative study of the inhibitory action by the antibodies is only possible in a defined antigen-antibody region. Therefore, in Fig. 4 the inhibitory action on the oxygenase activity of the tobacco wild type enzyme and of *Spinacia oleracea* by different antisera is compared in a block diagram in the region of antigen-antibody equivalence. It was seen that not the homologous antisera caused the highest inhibitory effect but that it was the antiserum against the enzyme of the tobacco mutant Su/su (which exhibits the higher oxygenase activity) which caused the highest inhibitory effect on both, the enzyme of *N. tabacum* var. JWB as well as on the spinach enzyme. This inhibitory effect is with the JWB enzyme 60% higher and with the spinach enzyme 20% higher when compared to the inhibition caused by the respective homologous antisera. It is remarkable that the antisera against the tobacco RuBP-carboxylase/oxygenase exert on the spinach enzyme a 40% respectively a 20% higher inhibition than that which the antisera would cause on the enzyme of the tobacco wild type. On the other hand the inhibition caused by the antiserum to the spinach enzyme on the tobacco enzyme is only half as high.

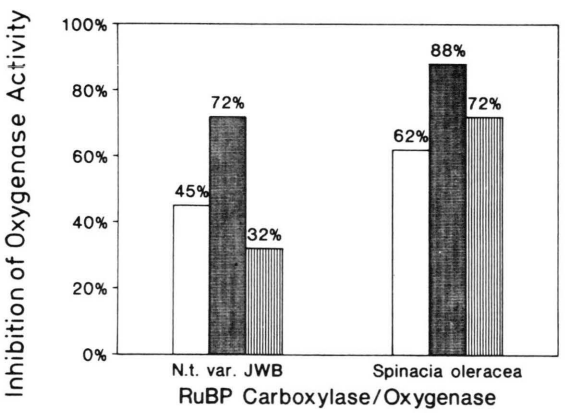

Fig. 4. Inhibition of the oxygenase activity of RuBP carboxylase/oxygenase of *N. tabacum* var. JWB and of *Sp. oleracea* in per cent activity in dependence on the antiserum to the enzyme of *N. tabacum* wild type, ☐; of the *N. tabacum* mutant Su/su ▤ and of *Sp. oleracea* ▥.

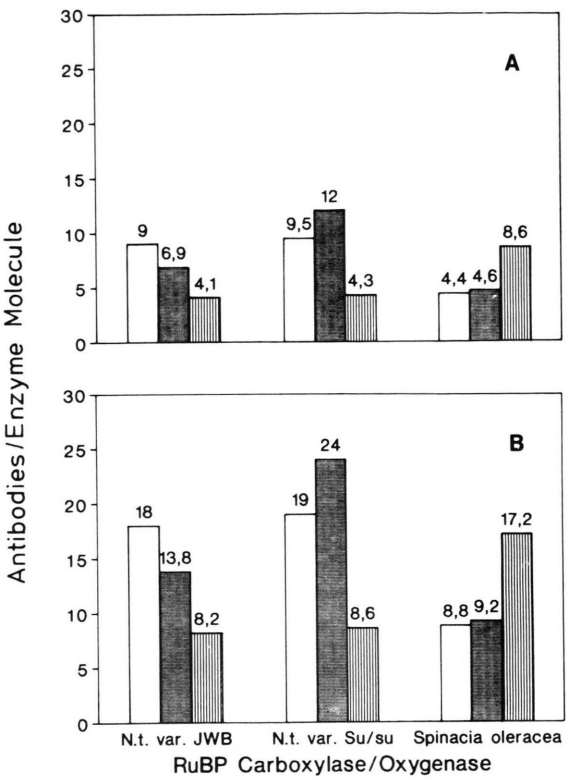

Fig. 5. Binding of antibodies onto RuBP carboxylase/oxygenase of *N. tabacum* var. JWB, the *N. tabacum* mutant Su/su and of *Sp. oleracea* out of the homologous and non-homologous antisera. The values give the number of antibody molecules bound per enzyme molecule in the equivalence region (A) and in the region of extreme antibody excess (B). Out of the antisera to the *N. tabacum* wild type enzyme ☐; to the *N. tabacum* Su/su mutant enzyme ▤; and to the *Sp. oleracea* enzyme ▥.

In a further experiment the antibody binding capacity of the enzymes out of the homologous and non-homologous antisera was determined in the quantitative immuno precipitation according to the method of Heidelberger and Kendall [18, 19]. As seen from the block diagram of Fig. 5A, the highest amount of antibodies is bound in the equivalence region by the respective homologous antisera. This amount corresponds to 9 antibody molecules bound onto the tobacco wild type enzyme, as well as onto the spinach enzyme. However, the enzyme of the tobacco mutant appears able to bind 30% more antibodies per enzyme molecule. From this we conclude that the obtained monospecific antisera contain antibodies against all an-

tigenic determinants. They thus reflect the complementary picture of the enzyme structure towards which they are directed. A difference in the antibody binding capacity further exists if the amount of antibody molecules is compared, which the tobacco and the spinach enzyme are able to bind out of the mutant antiserum. Whereas the tobacco wild type enzyme which in gel diffusion tests is identical to the mutant enzyme, binds 20% less antibodies, the spinach enzyme which is only partially identical to the mutant enzyme, binds only 50% of the antibodies out of the mutant antiserum in comparison to the homologous antiserum.

It should be noted that in the region of antibody excess twice the amount of antibodies is bound per enzyme molecule (Fig. 5 B). In this region in which antibodies are generally only monovalently bound, the same antibody binding situation is observed, as in the antigen-antibody equivalence region in which antibodies are bivalently bound.

The inhibitory effect of an antiserum is in general dependent on the amount of bound antibodies. To a certain extent this is also valid when the inhibitory effect of an antiserum to the non-homologous antigens is studied. Whereas the tobacco wild type enzyme binds 50% less antibodies out of the spinach antiserum, the inhibitory effect of the same antiserum appears 30% lower than that caused by the homologous antiserum. Correspondingly, the antiserum against the tobacco wild type enzyme exhibits with the spinach enzyme a 50% lower antibody binding capacity causing a 15% lower inhibitory effect than with the homologous antiserum. A proportionality between the de-

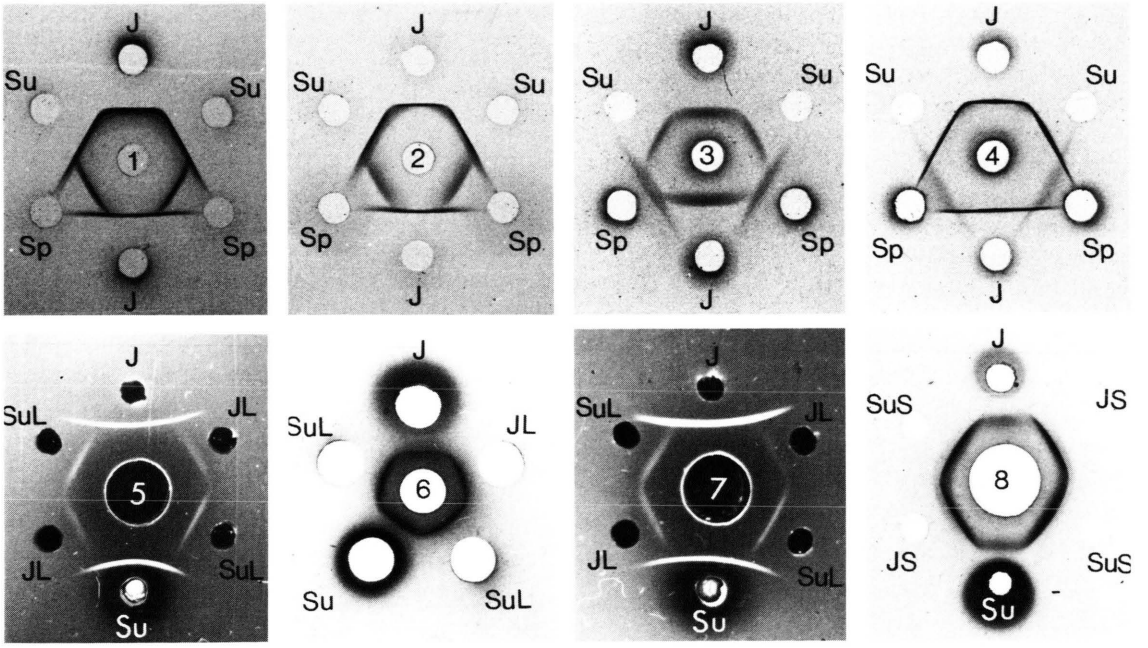

Fig. 6. Comparative analysis of RuBP carboxylase/oxygenase and of the large and small subunits of this enzyme of *N. tabacum* var. JWB with the enzymes of the *N. tabacum* mutant Su/su and *Sp. oleracea* with homologous and non-homologous antisera in the double immunodiffusions test.
Antisera: 1. to RuBP carboxylase/oxygenase of *N. tabacum* var. JWB; 2. to the *N. tabacum* mutant Su/su; 3. to spinach; 4. antiserum mixture of antisera 1–3; 5. antiserum mixture of antisera 1 and 2; 6. antiserum mixture of antisera to the large subunits of the enzyme of *N. tabacum* wild type and of the mutant Su/su; 7. antiserum mixture of antisera 1, 2, and 6; 8. antiserum mixture of antisera to the small subunits of the enzymes of *N. tabacum* wild type and of the mutant Su/su.
Antigen: J, chloroplast preparation of JWB; Su, chloroplast preparation of Su/su; Sp, chloroplast preparation of spinach; JL; large subunit of the enzyme of *N. tabacum* wild type; SuL, large subunit of the enzyme of the mutant Su/su; JS and SuS, small subunits of the two *N. tabacum* species.

gree of inhibition and the number of bound antibody molecules seems not to exist, when the effect of the antiserum to the tobacco mutant enzyme on the two non-homologous enzymes to be compared, is looked at. Although the wild type enzyme binds out of the antiserum to the mutant enzyme 20% less antibodies, the antiserum causes a 60% higher inhibition than that caused by the homologous antiserum. With the spinach enzyme the antiserum to the tobacco mutant enzyme causes a 20% higher inhibition, despite the fact that 50% less antibodies are bound out of this antiserum. From this result we might conclude that the antibody-induced inhibition of the oxygenase function of the bifunctional enzyme is not in every case dependent on the number of antibody molecules bound, but rather on the binding affinity of the antibody molecules and on the structure towards which the antibodies are directed. It appears that the antiserum to the tobacco mutant enzyme contains to a considerably higher extent antibodies with higher binding affinity towards the functional oxygen binding region. These structural differences would be seen neither in the double immuno diffusion tests (Fig. 6) nor in the combined line rocket immunoelectrophoresis (Fig. 7) nor in the tandem crossed immunoelectrophoresis (7) even when using the antisera to the enzymes to be compared. Between the tobacco species only fusing precipitation bands and between the tobacco species and spinach precipitation bands with one-sided spurs are observed.

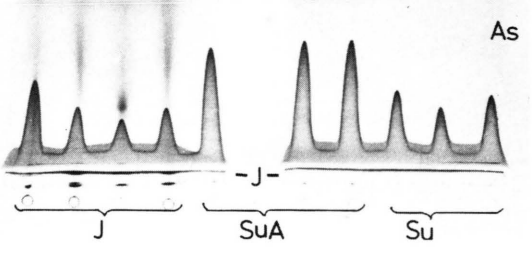

Fig. 7. Comparative analysis of the identity of the RuBP carboxylase/oxygenase of the *N. tabacum* mutants Su/su and *N. tabacum* Su/su var. Aurea in the combined line rocket immunoelectrophoresis.
Antisera: As, serum mixture of the antisera to RuBP carboxylase/oxygenase of *N. tabacum* var. JWB and of the mutant *N. tabacum* Su/su (1% v/v serum in the gel).
Antigen: J, chloroplast preparation of JWB; Su, chloroplast preparation of Su/su; SuA, chloroplast preparation of Su/su var. Aurea.

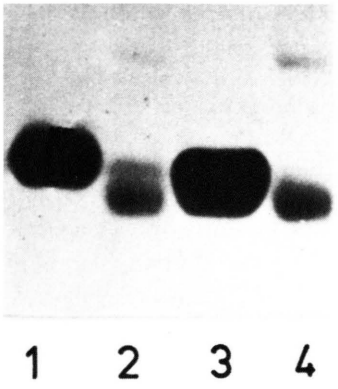

Fig. 8. Comparison of electrophoretic behaviour of native RuBP carboxylase/oxygenase in the polyacrylamide gel electrophoresis. Before electrophoresis the enzyme was activated in 50 mM Tris-HCl buffer, pH 8.0, containing 20 mM $MgCl_2$ and 10 mM $NaHCO_3$ for 20 min at 30 °C. Electrophoresis was carried out in 5% acrylamide gel (non-denaturing condition) at 5–6 mA, buffer 25 mM Tris-HCl, pH 8.0, containing 0.19 M glycine. Lane 1 and 3 activated RuBP carboxylase/oxygenase of *N. tabacum* var. JWB; lane 2 and 4 activated RuBP carboxylase/oxygenase of *N. tabacum* mutant Su/su; lane 3 and 4 activated enzyme plus 1 mM ribulose-1,5-bisphosphate (substrate).

Even the large subunits of the enzymes of the two *N. tabacum* species on which the catalytic centers are located, yield in the double immunodiffusion with the antisera to the native enzymes as well as with antisera to the large subunits fusing bands. The same is true for the reaction of the small subunits of the two tobacco species with their homologous antisera, showing immunological identity (Fig. 6). On the other hand analyses by gel electrophoresis have yielded the result that the native enzyme of the tobacco mutant Su/su in the activated condition, that is when a defined conformational state is present, exhibits a higher electrophoretical mobility than the enzyme of the wild type in the identical state (Fig. 8).

Discussion

The obtained antisera to the native RuBP-carboxylase/oxygenase of the tobacco species and of spinach exert an inhibitory action on the oxygenase activity of the enzymes. An inhibitory effect of these antisera on the carboxylase function has not been studied in context with the present studies. Nishimura and Akazawa [23] have studied the in-

fluence of the antisera to the native enzyme as well as of those to the large and small subunits of the spinach enzyme and found that the antisera, which are directed to the native enzyme and to the large subunit exert an inhibitory action on both the oxygenase as well as on the carboxylase function. However, the antiserum to the small subunit had no influence on the enzyme function. The same result was obtained by Gray and Kekwick [24] who compared the functions of the enzyme of *Phaseolus vulgaris* with those of spinach.

It should be noted that the inhibition of the function of an enzyme by reaction with antibodies depends on four very simple points:

1st, on the amount of bound and effective antibodies

2nd, on the specificity of the antiserum

3rd, on the binding affinity of the antibody

4th, on the antigenic determinants and on the molecular region towards which the antibodies are directed.

In the latter case one has to distinguish whether the antibody is directed towards functional groups in the region of the active site itself or to a site in the immediate vicinity of the active site or whether antibodies are bound to regions which are located in some distance from the enzyme's active center. In the first case a steric hindrance of the substrate molecules by the antibodies will occur and the inhibitory effect will be the stronger the higher the molecular weight of the substrate molecule will be. In the case of antibody binding onto regions situated away from the reaction center a conformational change will be induced which in general will lead to an inhibition of the function. In this context it should be noted, however, that also stimulations of enzyme functions due to conformational changes induced by antibody binding have been described [25]. As a rule the mechanism of an inhibitory action is determined by conformational changes of the enzyme molecule as well as by sterical hindrance of the substrate molecules. This means that comparative analyses of the structure-function relationship with antibodies as test reagents yield only informations, if antisera are used which contain antibodies with high binding affinities towards all antigenic determinants. This implies that the enzymes used for immunization are in a condition close to the native state and have not undergone denaturing.

Thus, as comparative antibody binding assays have shown, the highest amount of antibodies is bound out of the homologous antiserum onto the enzymes of the respective *N. tabacum* species as well as to that of spinach. This clearly demonstrates, that the obtained antisera are monospecific (Fig. 1) and represent a fully complementary picture of the enzyme structure. By binding of these antibodies an inhibition of the oxygenase function is induced. This inhibitory action depends on the amount of antibodies bound. Complete inhibition of the function occurs only in the region of excess antibodies, that is when antibodies are monovalently bound and thus block a maximum of antigenic determinants. If, however, the inhibitory action of the described antisera is compared in the region of equivalence of enzyme/antibody with the homologous and non-homologous enzymes it is seen, that the antiserum to the mutant enzyme which is the one with the highest affinity towards oxygen, is also the one which inhibits not only the enzyme of the tobacco wild type but also that of spinach strongest. Also, with this inhibitory action no proportionality to the amount of antibodies bound seems to exist. This means that the antiserum to the mutant enzyme contains more antibodies directed to the functional region of the oxygen binding site on the enzyme's large subunit, which have a higher binding affinity than those of the other two antisera. These results demonstrate that structural differences or differences in the conformation exist between these enzymes, exhibiting under natural conditions different degrees of oxygenase activity.

From the observation, that the antiserum to the tobacco mutant enzyme also inhibits 20% stronger the function of the spinach enzyme we may conclude, that the active centers of RuBP carboxylase/oxygenase of spinach and tobacco have a high degree of resemblance, despite the fact that the antigen-antibody reactions in gels always show only partial identity between the enzymes.

It should be noted that the present observations are only possible if an antiserum is used that contains antibodies to practically all antigenic determinants of the antigen. Hence, a monospecific polyclonal antiserum or a mixture of monoclonal antisera directed towards the same antigen must be used.

Restrictively we should note that the described

antisera have been obtained by immunization of just one rabbit and results might be interpreted in the sense that the differing reactions of the respective antisera are just due to the fact that the respective animals have been differently disposed to produce antibodies with higher or lower affinity towards certain antigenic regions. However, determination of the antibody binding capacity with another tobacco mutant namely with *N. tabacum* var. Consolation, has shown that the enzyme of the green phenotype, which also exhibits a 30% higher photorespiration than the wild type, also binds out of the homologous antiserum 30% more antibodies (8). On the other hand, the enzymes of the yellow- and yellow-green phenotypes derived from this mutant, which exhibit an oxygenase function comparable to that of the wild type, also show an antibody binding capacity corresponding to that of the wild type enzyme.

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