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THE OXYGEN-DEPENDENT DEACTIVATION AND REACTIVATION OF SPINACH RIBULOSE-1,5-BISPHOSPHATE CARBOXYLASE

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Summary

Ribulose-1,5-bisphosphate carboxylase-oxygenase (3-phospho-D-glycerate carboxy-lyase (dimerizing), EC 4.1.1.39) is deactivated by the removal of oxygen, and reversibly reactivated by its readdition to the enzyme solution. A short pulse of oxygen to the anaerobic enzyme solution is sufficient to trigger the reactivation process; the K_a value for this reaction was estimated as 0.12 mM oxygen.

The enzyme could not be reactivated under anaerobic conditions by an organic oxidant (benzoylperoxide) or by sulfhydryl group reducing reagents (dithiothreitol or β-mercaptoethanol), suggesting that the process of reactivation was oxygen specific. Furthermore, the inhibition of the reactivation by superoxide anion scavengers such as Tiron (1,2-dihydroxybenzene-3,5-disulfonic acid), copper penicillamine, hydroxylamine, nitroblue tetrazolium, and ascorbate, indicated that the monovalent reduced oxygen was involved as the reacting species in this process.

The deactivation of the enzyme associated with the removal of oxygen was also sensitive to the presence of scavengers of $O_2^{\cdot}$, suggesting that superoxide anion was also involved in the deactivation process. Both the carboxylase and the oxygenase activities were similarly affected under all the experimental conditions studied.

On the basis of these results it is argued that the enzyme molecules are able to reduce oxygen and that superoxide anion causes the deactivation or reactivation of the enzyme.

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Introduction

Ribulose-1,5-bisphosphate carboxylase-oxygenase (3-phospho-D-glycerate carboxy-lyase (dimerizing), EC 4.1.1.39) catalyses either the CO_2 fixation in photosynthesis by yielding 3-phosphoglycerate as a product, or the photorespiratory oxidation of Rbu P_2 to phosphoglycolate and 3-phosphoglycerate [1—3]. The key role of this enzyme in the carbon metabolism of autotrophic organisms makes it understandable that it is regulated in several ways. A series of these regulatory effectors which control the enzymatic activities were studied, i.e. the pH value of the stroma [4], the concentrations of metabolites as 3-phosphoglycerate, fructose 1,6-bisphosphate, 6-phosphogluconate, NADPH [5—8].

The most important regulatory mechanism seems to be the activation of the enzyme molecules by its substrate CO_2 and Mg^{2+}. This effect has long been known and has been studied in more detail in recent years [9—14]. The activation of the enzyme by CO_2 may occur at a site different to the catalytic sites of the enzyme molecules [15,16]. The kinetic studies of the activation process and the binding studies led to the following model: CO_2 reacts with an ϵ-NH_2 group of Lys to yield a carbamate derivative which quickly binds Mg^{2+} [12,17].

We have recently demonstrated that for complete activation of the enzyme, the presence of oxygen is also necessary [18,19]. The removal of oxygen causes the loss of both ribulose-1,5-bisphosphate carboxylase and oxygenase activities [18]. Furthermore, these changes are paralleled by conformational changes as shown by intrinsic and extrinsic protein fluorescence and by the exposure of sulfhydryl groups [19]. These changes could be reversed by the addition of oxygen to the anaerobic solution.

In this communication we investigated how oxygen could interact with the enzyme molecule during the deactivation and reactivation process.

Materials and Methods

Materials

Ribulose-1,5-bisphosphate tetra sodium salt was synthesized enzymatically from ribose-5-phosphate [20]. Copper penicillamine was a gift from Dr. E. Elstner (München, F.R.G.), Tiron (1,2-dihydroxybenzene-3,5-disulfonic acid) was purchased from Merck (Darmstadt, F.R.G.) and 1,4-diazabicyclo(2,2,2)-octane was obtained from Fluka (Neu-Ulm, F.R.G.). $NaH^{14}CO_3$ was obtained from Amersham Buchler (Braunschweig, F.R.G.).

Methods

Purification of ribulose-1,5-bisphosphate carboxylase. The enzyme was purified from spinach leaves. The procedure has been reported earlier in detail [19,21] and included ammonium salt fractionation, gel chromatography on Sephadex G-200 and sucrose density gradient centrifugation. The enzyme was purified to homogeneity in presence of 10 mM $MgCl_2$ and 10 mM $NaHCO_3$ to guarantee complete activation, and stored with 30% sucrose at —20°C.

Ribulose-1,5-bisphosphate carboxylase-oxygenase assays. The enzyme was dialysed against 50 mM Tris-H_2SO_4 buffer, pH 8.0, containing 10 mM $MgCl_2$/

10 mM $NaHCO_3$ and gassed with argon in an oxygraph cell (Rank Brothers, Cambridge, U.K.) at 25° C with a gas flow of 0.5—1 l/h. The oxygen content of the cell was monitored continuously. In a control experiment with [^{14}C]-$NaHCO_3$, the loss of CO_2 during the gassing procedures was determined to be 4% after 3 h and therefore negligible. The assay mixture for the carboxylase assay (0.2 ml) was 50 mM Tricine-NaOH buffer, pH 8.0/10 mM $MgCl_2$/1 mM Rbu P_2/25 mM $NaH^{14}CO_3$ ($1.9 \cdot 10^{10}$ Bq/mol)/enzyme protein equivalent to 10—20 μg of protein. The reaction was stopped after 2 min by the addition of glacial acetic acid, and aliquots were removed for scintillation counting.

The oxygenase assay mixture (1.5 ml) was 50 mM Tris-H_2SO_4 buffer/pH 8.5/10 mM $MgCl_2$/0.7 mM RuBP/0.26 mM O_2/enzyme equivalent to 0.1 mg of protein and 0.35 mM bicarbonate which was carried over with the enzyme solution, and the oxygen uptake was measured polarographically (oxygraph by Rank Brothers, Cambridge, U.K.).

The enzyme samples used throughout the experiment had a specific carboxylase activity of 1—1.25 units/mg protein and a specific oxygenase activity of 0.1—0.12 units/mg protein.

Protein assay. Protein was determined by the method of Bradford [22] and bovine serum albumin was used as standard.

Nitrite determination. The amount of nitrite formed in the reaction mixture was determined as described by Elstner and Heupel [23].

Results

The interaction of the enzyme with oxygen during deactivation and reactivation was studied by two different approaches. First, the stoichiometry between the enzyme and oxygen molecules, the affinity for oxygen and the kinetic of reactivation were investigated. Additionally, the search for an inhibitor of these deactivation and reactivation processes was undertaken to characterize the mechanism of the enzyme-oxygen interaction.

Oxygen pulse experiments

The CO_2-Mg^{2+} activated enzyme was gassed with argon until all oxygen was removed from the oxygraph cell and the enzyme activities were reduced to about 5%. Then a short pulse of oxygen was added to the enzyme solution and the gassing with argon was continued until anaerobic conditions were achieved again. Aliquots were removed and the carboxylase activity of the enzyme samples was measured under aerobic and anaerobic conditions (Fig. 1). The carboxylase activity measured under aerobic conditions was observable 10 min after the pulse and reached a maximum after 40 min. The activity decreased again due to the removal of the oxygen after the pulse.

It was of special interest to assay the carboxylase activity of the enzyme after the oxygen pulse under anaerobic conditions to determine whether the presence of oxygen is required. The experiment was repeated as described above, except that the assay mixture was also gassed with argon in a oxygraph cell to ensure that any oxygen contamination did not occur. The data in Fig. 1 indicate that the oxygen was not required during the carboxylase assay. There-

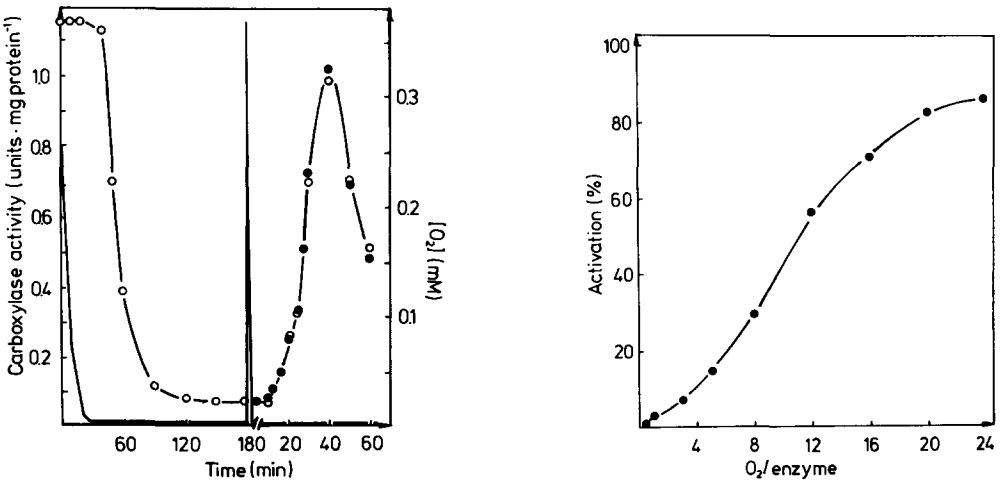


Fig. 1. Reactivation of anaerobic ribulose-1,5-bisphosphate carboxylase by an oxygen pulse. The enzyme solution, 15 mg protein/ml in 50 mM Tris-SO_4^{2-} buffer, pH 8.0, containing 10 mM $MgCl_2$ and 10 mM $NaHCO_3$, was gassed with argon for 3 h at 25°C. Aliquots containing 20 μg of enzyme protein were removed at the given times and analyzed for carboxylase activity. Then a short pulse was given, large enough to saturate the enzyme solution with oxygen, followed by a second argon treatment of the sample. At definite times aliquots were removed and the carboxylase activity was determined (○). In addition, the carboxylase activity was also measured anaerobically (●) by carrying out the test in an oxygraph cell with an argon gassed assay mixture. During the whole experiment, the oxygen concentration in the enzyme solution was monitored (solid line).

Fig. 2. Dependence of the reactivation of anaerobic ribulose-1,5-bisphosphate carboxylase on the oxygen concentration. The enzyme solution, 15 ml protein/mg in 50 mM Tris-SO_4^{2-} buffer, pH 8.0, containing 10 mM $MgCl_2$ and 10 mM $NaHCO_3$, was gassed for 3 h with argon at 25°C. The same buffer with a known amount of oxygen was added to the anaerobic enzyme, and the test tubes were sealed so that no gas phase was above the protein solutions. The carboxylase activity of all samples was measured with 20 μg of protein after 60 min incubation at 25°C. The activation of the enzyme measured for each oxygen concentration was plotted vs. the ratio of oxygen to enzyme molecules. The 100% value was equivalent to the activity of the untreated aerobic enzyme (1.13 units/mg protein).

fore, it can be concluded that the reactivation of the enzyme molecules with oxygen had already occurred during the oxygen pulse. It was noticed in earlier experiments that conformation changes, exposure of sulfhydryl groups and increase of carboxylase and oxygenase activities were parallel and that all appeared after a lag phase [19].

The question arose whether oxygen is specific for the activation reaction or whether it can be replaced by either another oxidant or even a reductant. The latter possibility has to be considered, since oxygen in the monovalent reduced state as the superoxide anion, $O_2^{\cdot-}$, is a potent reductant with a midpoint potential of −0.3 *V* [24].

Benzoylperoxide was chosen as an oxidant because it is stable and capable of reacting with both the hydrophilic and hydrophobic sites of proteins. The incubation of the enzyme under anaerobic conditions with 3 mM benzoylperoxide revealed that it could not reactivate the enzyme. The incubation with reductants such a dithiothreitol (1—5 mM) or β-mercaptoethanol (1—5 mM), also could not reactivate the enzyme under anaerobic conditions, even when the incubation period was prolonged for 90 min (data not shown).

The observation that oxygen alone could trigger the enzyme changes leads us to consider the stoichiometry of the enzyme-oxygen interaction. The amount of oxygen which was added to a constant amount of anaerobic enzyme was varied and the carboxylase activity was subsequently measured after the reactivation process was complete (Fig. 2). The experiment was carried out by adding the oxygen to the enzyme solution in a closed system (Fig. 2). The curve has sigmoidal shape; low concentrations of oxygen were not sufficient to yield optimal activation of the enzyme molecules. The highest percentage of activated enzyme molecules was obtained with a 24-fold excess of oxygen.

The K_a value for oxygen in the activation process was estimated as 0.12 mM (Fig. 3). This K_a value should not be confused with the K_m value for the ribulose-1,5-bisphosphate oxygenase reaction. The experiments for the determination of the K_a value were conducted in such a way that there was no gas phase above the enzyme solution, and therefore, the oxygen added was only in the solution. At oxygen concentrations lower than 0.05 mM the kinetics of the reactivation reaction changed, indicating a negative cooperative effect. The Hill coefficient was determined by replotting the data; at lower oxygen concentrations a Hill coefficient of 1.56 was measured, whereas at higher oxygen concentrations a value of 1 was observed.

The kinetic studies of the pulse experiments with varying oxygen content in the enzyme solution are presented in Fig. 4. After a short pulse of oxygen (in the range of 0.09—0.43 mM) to the anaerobic enzyme solution, the oxygen was removed again by gassing with argon. Aliquots were taken to determine the carboxylase activity, and the data of the lowest and highest oxygen concentration pulse are shown in Fig. 4a. The time period between the oxygen pulse and the point, at which the enzyme reached half-maximal reactivation was plotted

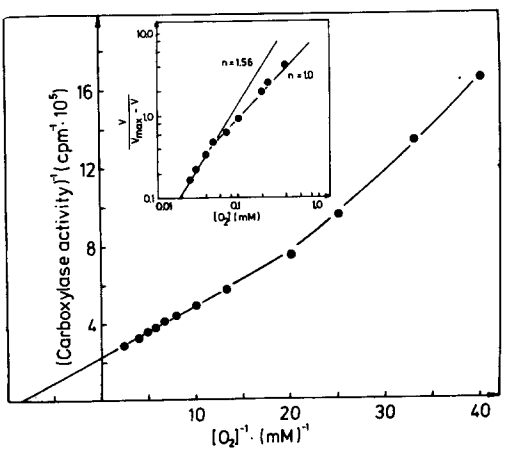


Fig. 3. The estimation of the K_a value for the oxygen dependent activation of the ribulose-1,5-bisphosphate carboxylase. The enzyme solution, 15 mg protein/ml in 50 mM Tris-SO_4^{2-} buffer, pH 8.0, containing 10 mM $MgCl_2$ and 10 mM $NaHCO_3$, was gassed for 3 h with argon at 25°C. Different mixtures of O_2 oversaturated and anaerobic 50 mM Tris-SO_4^{2-} buffer, pH 8.0, containing 10 mM $MgCl_2$ and 10 mM $NaHCO_3$, were added to 0.55 mg of anaerobic enzyme protein, giving a total volume of 1 ml, and the test tubes were sealed so that there was no gas phase above the enzyme samples. The tubes had different oxygen concentrations from 0.025 up to 0.4 mM. After an incubation period of 60 min at 25°C, the carboxylase activity was determined with 16.5 µg of enzyme protein.

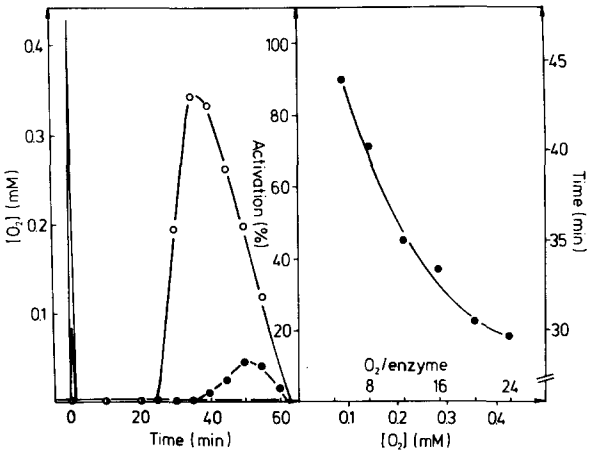


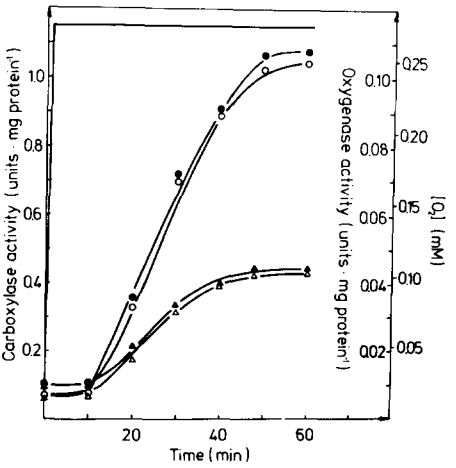
Fig. 4. Time dependence of the ribulose-1,5-bisphosphate carboxylase reactivation with oxygen. The enzyme solution, 15 mg protein/ml in 50 mM Tris-SO_4^{2-} buffer, pH 8.0, containing 10 mM $MgCl_2$ and 10 mM $NaHCO_3$, was gassed with argon for 3 h at 25°C. The anaerobic enzyme sample was oxygenated for a short period (about 1 min) with different concentrations of oxygen (O_2 per enzyme molecule ratios from 5 to 24), and gassed again with argon. The oxygen concentration in the enzyme solution during each of the pulse experiments was monitored. The carboxylase activity of all the samples was measured at the given times with 20 μg of the enzyme protein. The results for two of the pulse experiments with an O_2/enzyme ratio of 5 and 24, respectively, are shown in Fig. 4a. The time period between the oxygen pulse and half maximal activation of the enzyme was estimated for each different O_2/enzyme ratio and plotted against the oxygen concentration during the pulse and the ratio of oxygen to enzyme molecules, respectively (Fig. 4b).

against the ratio of oxygen to enzyme, and against the oxygen concentration in the reaction solution at the pulse respectively (Fig. 4b). The time period was longer (44 min) for lower oxygen concentrations during the pulse than for oxygen saturated enzyme solutions (29 min).

Inhibition of the oxygen activation

The results of the experiments described above indicate that oxygen is required for the activation process. A short pulse was sufficient to trigger the reactivation process by restoring active catalytic sites. The enzyme activity did not rise immediately after the oxygen stimulus, but the increase occurred after a significant lag phase (Fig. 1). This observation could be explained by assuming a primary reaction of the enzyme molecules with oxygen, followed by secondary conformational changes which finally restore functional sites. Since oxygen is not very reactive in the triplet state, it was more likely that the superoxide anion ($O_2^{\cdot-}$) could be the reactive species. Therefore, the involvement of $O_2^{\cdot-}$ in the reactivation reaction was examined by the use of the $O_2^{\cdot-}$ scavenger, Tiron (1,2-dihydroxybenzene-3,5-disulfonic acid) [25,26].

An anaerobic enzyme sample, activated with CO_2 and Mg^{2+}, was treated with 5 mM Tiron, the solution was immediately oxygenated, and the reactivation was measured (Fig. 5). The results show that the treatment with Tiron caused about 60% inhibition of both ribulose-1,5-bisphosphate carboxylase and oxygenase activities. It was noted that the samples with Tiron could not reach the same reactivation level as the control even after a prolonged time period of



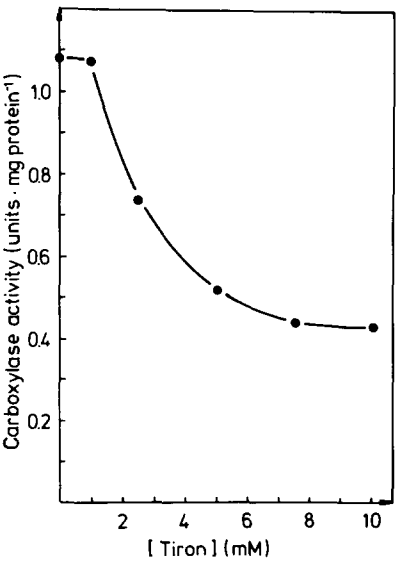


Fig. 5. The influence of Tiron on the oxygen reactivation process. The enzyme solution (12 mg protein/ml in 50 mM Tris-SO_4^{2-} buffer, pH 8.0, containing 10 mM $MgCl_2$ and 10 mM $NaHCO_3$) was gassed with argon for 3 h at 25°C. The anaerobic enzyme was treated with 5 mM Tiron (1,2-dihydroxybenzene-3,5-disulfonic acid) and immediately gassed with oxygen until the solution was saturated. The ribulose-1,5-bisphosphate carboxylase (▲) and ribulose-1,5-bisphosphate oxygenase (△) activities were measured every 10 min up to 1 h. The control samples were treated similarly but without Tiron (carboxylase ●, oxygenase ○). The specific activities, given in units/mg of protein, are plotted vs. time. The oxygen concentrations (solid line) was recorded during the whole experiment.

Fig. 6. Inhibition of the reactivation process of anaerobic ribulose-1,5-bisphosphate carboxylase depending on the Tiron concentration. The enzyme solution, 9 mg protein/ml in 50 mM Tris-SO_4^{2-} buffer, pH 8.0, was gassed with argon for 3 h at 25°C. Varying concentrations of Tiron were added to the anaerobic enzyme samples in the range of 1—10 mM, and the samples were immediately oxygenated until saturation of the solution. The carboxylase activity of each sample was determined after 60 min incubation at 25°C.

60 min in presence of oxygen (Fig. 5). The separation of the enzyme molecules from Tiron on a Sephadex G-25 column under aerobic conditions, however, yielded an enzyme fraction which was fully active, indicating that the presence of Tiron during the oxygen reactivation did not cause a permanent inhibition (data not shown).

Further control experiments with Tiron in the same concentration range in the enzyme activity assay as in those experiments described above revealed that there was no inhibition either of the carboxylase or of the oxygenase activity.

The concentration dependence of the inhibition of the reactivation process by Tiron is presented in Fig. 6. Maximal inhibition of 50% was obtained at a concentration above 7 mM, higher concentrations seem to have no additional effect.

The assumption that the superoxide anion ($O_2^{\cdot-}$) is involved in the reactivation process was further substantiated by the use of other scavengers of $O_2^{\cdot-}$ such as copper penicillamine [27], hydroxylamine [23,29], nitroblue tetrazolium [28] and ascorbate [30]; the results are summarized in Table I.

All the scavengers had the same effect as partial inhibitors of the reactivation process, even though their mode of action was different. Copper penicillamine

TABLE I

THE EFFECT OF $O_2^{\cdot}$ SCAVANGERS ON THE OXYGEN ACTIVATION PROCESS OF ANAEROBIC RIBULOSE 1,5-BISPHOSPHATE CARBOXYLASE

The enzyme solution, 12 mg protein/ml in 50 mM Tris-SO_4^{2-} buffer, pH 8.0, was gassed with argon for 3 h at 25°C. The listed $O_2^{\cdot}$ scavangers were added to anaerobic enzyme samples and the solutions were immediately gassed with oxygen unitl saturation. The carboxylase activity of the samples was determined after 60 min incubation at 25°C. The carboxylase activity of the $MgCl_2$-CO_2 activated aerobic enzyme was not affected by incubating the enzyme with the same amount of different reagents for 60 min at 25°C, as listed in the table. CuPA, copper penicillamine; NBT, nitroblue tetrazolium; Tiron, 1,2-dihydroxybenzene-3,5-disulfonic acid.

Treatment	Carboxylase activity (in %)
Aerobic enzyme	100 *
Anaerobic enzyme	10.5
+ oxygen	96.0
+ oxygen + 0.03 mM CuPA	61.0
+ oxygen + 5 mM NH_2OH	51.0
+ oxygen + 0.25 mM NBT	49.0
+ oxygen + 5 mM ascorbate	54.0
+ oxygen + 5 mM tiron	47.0

* The specific activity of the aerobic enzyme was 1.09 units/mg protein.

acted as a low molecular weight complex with superoxide dismutase activity [27]. Ascorbate and hydroxylamine are oxidized by $O_2^{\cdot}$ to dehydroascorbate [30] and nitrite [23,29], whereas nitroblue tetrazolium is reduced to the formazan derivative [28].

These observations suggest that superoxide anion is involved in the reactiva-

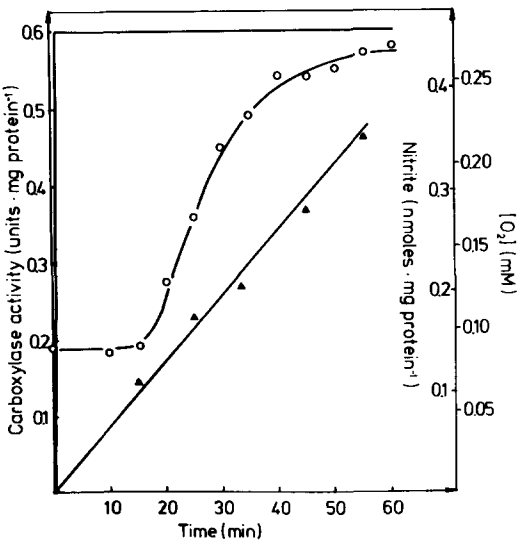


Fig. 7. The kinetics of the ribulose-1,5-bisphosphate carboxylase reactivation and of the production of nitrite in presence of hydroxylamine. The enzyme solution, 17.5 mg protein/ml in 50 mM Tris-SO_4^{2-} buffer, pH 8.0, containing 10 mM $MgCl_2$ and 10 mM $NaHCO_3$, was gassed for 3 h at 25°C. Hydroxylamine (pH 8.0) was added to yield a concentration of 10 mM and the solution was gassed with oxygen until it was saturated. The ribulose-1,5-bisphosphate carboxylase activity (○) was determined and the amount of the formed nitrite was analyzed with an aliquot containing 7 mg of protein (▲) according to the method of Elstner and Heupel [23]. The solid line shows the oxygen concentration in the sample.

tion mechanism. The kinetic of the oxygen reduction had to be elucidated, i.e. whether the production of $O_2^{\cdot-}$ started immediately after the addition of oxygen or whether a lag phase of the oxygen reduction could be observed. For this purpose anaerobic enzyme was stirred with oxygen in the presence of 10 mM hydroxylamine and the production of nitrite was measured. The results are presented in Fig. 7. After the addition of oxygen the enzyme started to oxidize hydroxylamine to nitrite immediately, but the reactivation of the ribulose-1,5-bisphosphate carboxylase was delayed for 15 min. As already observed in the experiments with Tiron (Fig. 5, and Table I), the enzyme was not completely activated in the presence of 10 mM hydroxylamine.

Inhibition of the deactivation process

The experiments described above indicated that the superoxide anion participates in the reactivation process of the anaerobic enzyme. Similar experiments were carried out to study whether $O_2^{\cdot-}$ is also involved in the deactivation process.

The CO_2-Mg^{2+}-activated enzyme was gassed with argon in the presence and absence of 5 mM Tiron and the decline of the ribulose-1,5-bisphosphate carboxylase-oxygenase activity was monitored (Fig. 8). In the presence of Tiron the decline of both activities was much slower than in the controls. It was noticed that both enzyme activities, carboxylase and oxygenase, are

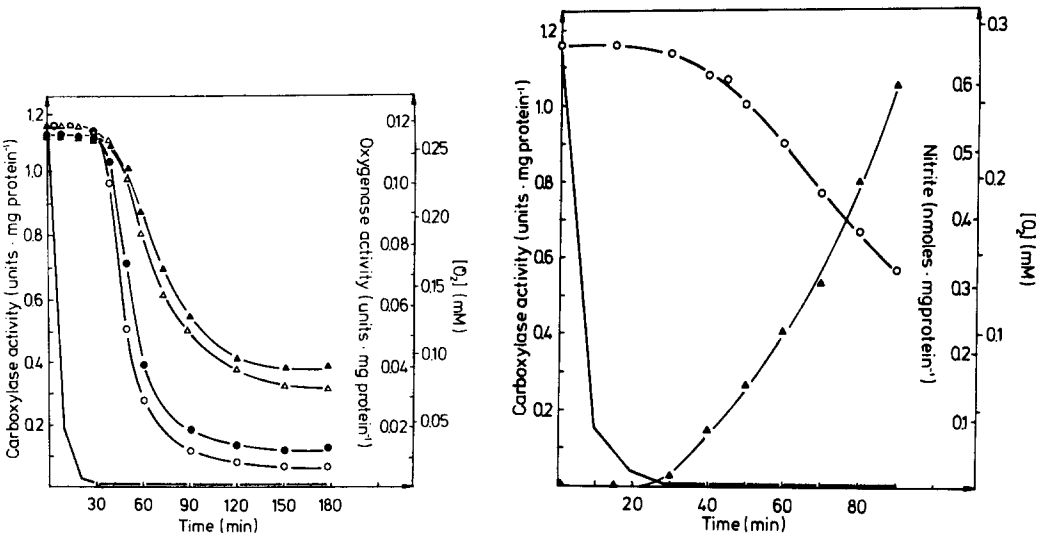


Fig. 8. Influence of Tiron on the deactivation process of ribulose-1,5-bisphosphate carboxylase. The enzyme solution, 13 mg protein/ml in 50 mM Tris-SO_4^{2-} buffer, pH 8.0, containing 10 mM $MgCl_2$ and 10 mM $NaHCO_3$, was gassed with argon for 3 h at 25°C either with or without 5 mM Tiron. The decline of ribulose-1,5-bisphosphate carboxylase activity in the control (●) and in the sample plus Tiron (▲), and of the ribulose-1,5-bisphosphate oxygenase in the control (○) and in the sample plus Tiron (△) were measured. The oxygen concentration in the solution was monitored (solid line).

Fig. 9. Deactivation of ribulose-1,5-bisphosphate carboxylase and formation of nitrite in presence of hydroxylamine. The enzyme solution (17.5 mg protein/ml in 50 mM Tris-SO_4^{2-} buffer, pH 8.0, containing 10 mM $MgCl_2$ and 10 mM $NaHCO_3$, was gassed with argon in presence of 10 mM hydroxylamine at 25°C. Aliquots were taken and analyzed for ribulose-1,5-bisphosphate carboxylase (20 µg protein, ○) and nitrite content (7 mg protein, ▲). The oxygen content of the solution was monitored (———).

equally affected in the presence of Tiron, as observed before in the reactivation experiment. These results indicate that $O_2^{\cdot}$ participates in the deactivation reaction and that the scavenging of the radicals inhibits this process.

The time course of oxygen removal, oxygen reduction (measured as nitrite formation) and the change of the ribulose-1,5-bisphosphate carboxylase activity is present in Fig. 9. Hydroxylamine (10 mM) was added to a fully activated enzyme sample and gassed with argon. Samples were taken and analyzed for carboxylase activity and the production of nitrite; the oxygen content of the solution was monitored simultaneously.

The onset of nitrite formation was observed only after the loss of oxygen in the enzyme solution. The activity remained constant for 30 min, even though a large part of the oxygen was lost from the solution. The sharp decline of the enzyme activity occurred at the same time as hydroxylamine was oxidized to nitrite.

Discussion

Oxygen interacts with ribulose-1,5-bisphosphate carboxylase-oxygenase in two ways: either as a substrate in the oxygenase reaction, or as an activator for both enzyme reactions. The interaction of the enzyme with oxygen in the oxygenase reaction is still not understood. Earlier studies by Lorimer et al. [32] concerning $^{18}O_2$ incorporation into the enzyme products with whole spinach leaves presented evidence that oxygen reacts with the C-2 carbon of Rbu P_2 yielding labelled phosphoglycolate. The oxidant of this reaction or the reaction site on the enzyme is not characterized so far.

Our studies [18,19] concerning possible interactions of oxygen with the enzyme molecules suggested that oxygen is, as are carbon dioxide and Mg^{2+}, necessary for complete activation of the enzyme. The removal of oxygen caused a similar decrease of the carboxylase and oxygenase activities and the reversibility of this effect indicated the regulatory role of oxygen [18].

The results of the preceding report [19] showed that the oxygen effect was linked with a series of changes of the enzyme molecules as measured by conformational changes and by exposure of sulfhydryl groups.

In this part of the studies, an attempt was made to determine the reaction which triggers these changes. The pulse experiments (Fig. 1, 4) revealed that oxygen needs to be present for only a short time during the reactivation process. It was not even required that oxygen was present during the ribulose-1,5-bisphosphate carboxylase assay, when an increase of enzyme activity was observed. The fact that the activity of the enzyme molecules appeared after a lag phase of 40 min following the oxygen pulse indicates that the reaction with oxygen triggers secondary, slower changes of the enzyme molecules leading to its reactivation.

This reactivation reaction could be inhibited by the addition of $O_2^{\cdot}$ scavengers. This observation was further substantiated by the kinetic study of nitrite formation from hydroxylamine during reactivation. Under anaerobic conditions the enzyme is able to oxidize hydroxylamine after the addition of oxygen, and this oxidation is associated with the inhibition of the reactivation. The superoxide anion has to be produced by the enzyme and participates in the reactivation reaction mechanism.

A similar involvement of superoxide anion in the deactivation of the enzyme is evident from the results presented in this communication (Fig. 9). However, the production of superoxide anion during inactivation is delayed until the detectable oxygen of the enzyme solution was removed. The transition of the enzyme molecules from the active (aerobic) state to the inactive (anaerobic) state seems directly related to the formation of superoxide anion (Fig. 9).

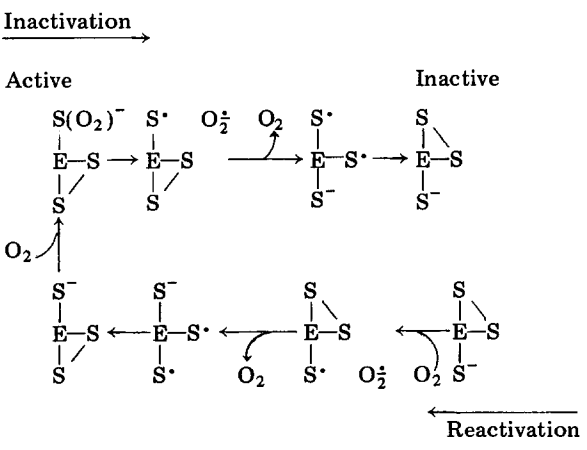
Apparently oxygen must react with one of the amino acid residues of the enzyme, since no other cofactors, able to react with oxygen were detected in purified ribulose-1,5-bisphosphate carboxylase preparations. Wishnick et al. [31] reported that the enzyme contains 1 gatom of tightly bound copper, but later investigations [32,33] demonstrated that only minor, non-stoichiometric amounts of iron or copper were detectable. We cannot rule out that traces of metal ions were part of the used buffer and do not know whether these ions play an important role in the deactivation and reactivation procedure. Analysis of the buffers used in the experiments described above revealed that the content of these metal ions was in the range of 10^{-7} M.

The participation of singlet oxygen in the deactivation and reactivation reaction could be ruled out because 100 mM 1,4-diazabicyclo-(2,2,2)-octane, a scavenger of singlet oxygen [34,35], had no effect during the transition from the aerobic to anaerobic conditions, or vice versa.

We think the cysteine residues of the enzyme may participate in the reactivation and deactivation mechanism, since only this amino acid would be able to reduce oxygen. The earlier work by Berger [38] demonstrating the binding of oxygen reversibly to sulfhydryl groups in an aprotic solvent system supports such an assumption. It is conceivable that oxygen is bound as peroxysulfenate in the triplet state [38] in a lipophilic region of the enzyme molecules. The loss of this bound oxygen occurs as $O_2^{\cdot-}$ if the enzyme solution contains no free oxygen. Following the production of $O_2^{\cdot-}$, a reaction takes place between $O_2^{\cdot-}$ and the enzyme molecules, probably the splitting of a structure determining Cys-S-S-Cys bridge. This reaction causes secondary changes which finally lead to the loss of enzyme activities.

A similar mechanism can be discussed for the reactivation process: oxygen is reduced to $O_2^{\cdot-}$ and restores the structure determining Cys-S-S-Cys bridge.

Therefore, we propose the following model:



Both deactivation and reactivation reactions affected the ribulose-1,5-bisphosphate carboxylase and the ribulose-1,5-bisphosphate oxygenase similarly indicating that these oxygen dependent reactions cause a general conformation change of the enzyme molecules. The production of superoxide anion during the transitions of the enzyme from one conformation to another is very interesting from the point of view that this enzyme is able to reduce oxygen. The involvement of superoxide anion as a possible oxidant in the ribulose-1,5-bisphosphate oxygenase reaction was discussed earlier [36,37]. It has to be elucidated whether there is a similar mechanism for the enzyme-oxygen interaction during the oxygenase reaction to the one presented in this paper.

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