PRESERVATION OF RuBP CARBOXYLASE WITHOUT OXYGENASE ACTIVITY DURING ANAEROBIOSIS

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1. Introduction

Fraction I-protein has not only ribulose-1,5-bisphosphate (RuBP) carboxylase but also RuBP oxygenase activity [1]. The enzyme catalyzes photosynthetic carbon dioxide fixation and photorespiratory oxidation of RuBP with the subsequent formation of glycolate. The key role of the enzyme in the regulation of these metabolic processes was recognized after the discovery of the stimulation of both enzyme activities by Mg^{2+}, CO_2 [2–6] and O_2 [7,8]; both substrates have to be present to grant full catalytic activities. Both O_2 and CO_2 compete for the same substrate RuBP, suggesting a common active site on the enzyme molecule [9,10].

However, these results show that conditions can be established for the isolated, homogenous enzyme under which the RuBP carboxylase is preserved, while the oxygenase activity is lost.

2. Materials and methods

RuBP carboxylase-oxygenase was isolated from spinach leaves and purified to homogeneity by ammonium sulfate fractionation, Sephadex G-200 chromatography and a sucrose density gradient centrifugation step [11]. The enzyme fractions with the highest spec. activity (1.2 μmol CO_2 fixed . mg protein^{-1} . min^{-1} and 0.11 μmol O_2 consumed . mg protein^{-1} . min^{-1}, respectively) were pooled and used throughout all the experiments.

The enzyme was dialysed against 50 mM Tris–SO_4, (pH 8.0), 10 mM $MgCl_2$ 10 mM $NaHCO_3$, and 4 mg protein in 0.5 ml; and gassed with argon to obtain almost complete inactivation. The inactivation procedure as well as the assay procedures for the carboxylase and the oxygenase reactions were described in [7,8].

The following chemicals were tested for altering the RuBP carboxylase-oxygenase activities: 2,2′-bipyridine (Fluka), neocuproin (Merck), 4-dimethylaminopyridine (EGA-Chemie), atebrin (Serva) anthrachinon-2-sulfonic acid (Merck). The diazaphenanthrene derivatives were synthesized by Dr Oettmeier, Ruhr-University, Bochum.

3. Results

RuBP carboxylase-oxygenase was preactivated with CO_2 and Mg^{2+} and deactivated by the removal of oxygen (table 1). We studied whether transition metal ions are involved in the deactivation procedure. Therefore, neocuproin and 2,2′-bipyridine were added to the enzyme solution as chelating agents prior to gassing with argon. After 3 h the enzyme activities were assayed under aerobic conditions. The results, summarized in table 1, indicate that the addition of these compounds to the enzyme solution caused a significant preservation of the carboxylase activity, whereas the oxygenase activity was lost.

The concentration of these compounds in the enzyme solution during the argon treatment was 2 mM, but in the assay it was diluted to 0.02 mM. This concentration was equivalent to a 50-fold excess of the reagent over the enzyme molecules.

The results obtained with 2,2′-bipyridine and neocuproin, a diazaphenanthrene derivative, promoted further experiments with chemically similar compounds but without chelating properties (as 4,7-diazaphenanthrene, or 2,9-diazaphenenanthrene). These compounds gave a similar effect: RuBP carboxylase

Table 1
RuBP carboxylase and oxygenase activities after anaerobic treatment

Experimental conditions	Enzyme activities in %	
	RuBP carboxylase	RuBP oxygenase
Aerobic control[a]	100[c]	100[c]
Anaerobic control[b]	11	5
+ 2 mM neocuproin	93	7
+ 2 mM 2,2'-bipyridine	90	6
+ 2 mM 4,7-diazaphenanthrene	88	5
+ 2 mM 2,9-diazaphenanthrene	84	5
+ 2 mM pyridine	53	6
+ 2 mM 4-dimethylaminopyridine	70	5
+ 2 mM atebrin	100	5
+ 2 mM 2-anthraquinonesulfonic acid	20	7

[a] The enzyme was dialyzed against 50 mM Tris–SO_4 buffer (pH 8.0) containing 10 mM $MgCl_2$ and 10 mM $NaHCO_3$
[b] The samples were gassed with argon for 3 h at 25°C
[c] The specific activity was equivalent to 1.2 U/mg protein (carboxylase), 0.11 U/mg protein (oxygenase)

activity was retained to 80–90%, whereas the RuBP oxygenase activity was as low as in the control. The effectiveness of other *N*-heterocyclic compounds was tested (table 1), and the best result was obtained with atebrin; rather small concentrations were already sufficient to yield full preservation of the RuBP carboxylase. Aromatic substances with negative charges, such as 2-anthraquinonesulfonic acid or 1-anilino-8-naphthalene sulfonic acid, were not able to preserve the RuBP carboxylase (table 1)

The dependence of the carboxylase preservation on the concentration of atebrin was analyzed (fig.1). Different amounts of atebrin were added to the fully activated enzyme, the solutions were gassed with argon for 3 h, and both enzyme activities were assayed. The

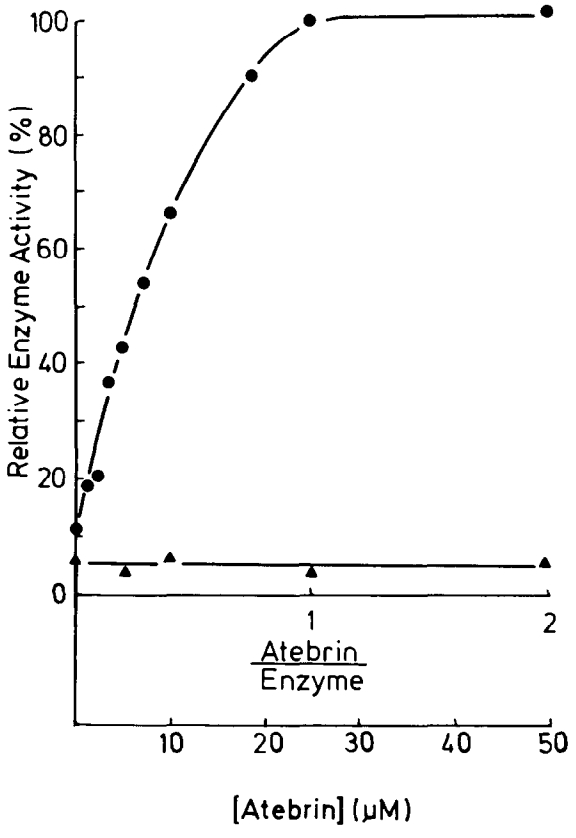

Fig.1. The dependence of RuBP carboxylase preservation on the concentration of atebrin. The enzyme solution, 13 mg protein in 1 ml 50 mM Tris–SO_4 buffer (pH 8.0) containing 10 mM $MgCl_2$ and 10 mM $NaHCO_3$, was gassed with argon for 3 h at 25°C in presence of atebrin (1–50 μM). The enzyme activities were assayed after this incubation period, 0.02 mg protein were used for the carboxylase assay (●——●) and 0.26 mg protein for the oxygenase assay (▲——▲). The relative enzyme activities are plotted against the ratio of atebrin to enzyme molecules and against the atebrin concentration in the incubation medium. The activity of the untreated enzyme corresponds to 100%.

Table 2
RuBP carboxylase and oxygenase activities in air–argon transition experiments

Buffer substance	Aerobic		Anaerobic	
	Carboxylase	Oxygenase	Carboxylase	Oxygenase
Tris	100[a]	100	8.5	6
Tricine	98	100	7	5
Bicine	89	84	7	5
Glycylglycine	91	92	6	5
Hepes	101	101	50	7.5
Mes	95	97	52	7

[a] Activity of the enzyme after dialysis against 50 mM buffer (pH 8.0) containing 10 mM $NaHCO_3$ and 10 mM $MgCl_2$; 100% was equivalent to the specific activity of the enzyme in Tris buffer (1.2 U/mg protein, carboxylase; 0.11 U/mg protein, oxygenase)

RuBP oxygenase activity was independent of the atebrin concentration with only 5% of the original activity. On the other hand, the RuBP carboxylase activity was dependent on the atebrin concentration; the highest activity was measured at atebrin >0.025 mM, or expressed in terms of an enzyme:atebrin ratio >1.

The preservation effect on RuBP carboxylase was further investigated in order to study whether the same results can be obtained when different buffer substances are used (table 2). The enzyme dialysed against 50 mM Hepes buffer (pH 8.0) containing 10 mM $NaHCO_3$ and 10 mM $MgCl_2$, gassed for 3 h with argon, still had 50% of the RuBP carboxylase activity, but only 5% RuBP oxygenase activity. Similar results were obtained with 50 mM MES buffer (pH 8.0) containing 10 mM $NaHCO_3$ and 10 mM $MgCl_2$.

Non-cyclic buffer substances, such as Tris, tricine, bicine or glycylglycine, could not prevent the deactivation of the RuBP carboxylase under similar conditions to the above.

We studied whether *N*-heterocyclic compounds, such as atebrin, have to be present during the removal of oxygen or whether addition to the anaerobic enzyme solution is sufficient to preserve the RuBP carboxylase. Activated enzyme was gassed with argon either before or after the addition of 1 mM atebrin. In absence of atebrin, both enzyme activities were lost simultaneously, as in [7,8]. The addition of 1 mM atebrin to the anaerobic enzyme solution did not affect the enzyme activities. In another set of experiments, 1 mM atebrin was added to the aerobic enzyme solution, and readdition had no influence on both activities. After gassing with argon in presence of atebrin, the RuBP carboxylase was preserved completely while the RuBP oxygenase was lost.

4. Discussion

The removal of oxygen from an aerobic enzyme solution was accompanied by the loss of both enzyme activities [7] and conformational changes [8]. The readdition of oxygen to a deactivated enzyme solution triggered reversible changes yielding fully activated enzyme molecules.

In [12] the mechanism of the deactivation and reactivation reactions was further investigated and the participation of superoxide anion ($O_2^{\cdot-}$) in both reactions could be elucidated.

The use of chelating agents (table 1) seemed conducive to the investigation of whether transition metal ions are also involved in the oxygen reduction. Our studies showed that neither iron nor copper ions could promote the deactivation reaction again after the addition of neocuproin or 2,2′-bipyridyl (data not shown).

On the other hand, these experiments revealed that such agents had a protective influence, but only on the RuBP carboxylase and not on the RuBP oxygenase. Therefore, a series of chemically similar compounds with no chelating properties were employed. The important constitutive property of these compounds for the preservation effect on the RuBP carboxylase seemed to be a *N*-heterocyclic ring system. The most effective substance was atebrin (6-chloro-9-

(4-(diethylamino)-1-methylbutyl)amino)-2-methoxy-acridine dihydrochloride).

Atebrin had to be present during the gassing procedure with argon to protect the RuBP carboxylase. The protective effect was lost after the removal of atebrin (not shown).

5. Conclusions

These results indicate a significant difference between RuBP carboxylase and RuBP oxygenase: the addition of atebrin or similar compounds preserved only the RuBP carboxylase function during the deactivation process when oxygen was removed. Considering the mechanism of both reactions, carboxylase and oxygenase, the common step in these reactions is proposed to be the binding and enolization of RuBP. In the presence of preserving agents the enzyme molecules were able to activate (enolize) RuBP and catalyze the reaction with CO_2 only.

Therefore, it can be concluded that oxygen does not directly react as the substrate for the oxidation of RuBP. It seems more likely that an additional step for the oxygenase reaction mechanism has to be considered, the activation of oxygen, which could be either a spin conversion process (triplet to singlet) or the reduction of oxygen to the superoxide anion ($O_2^{\cdot-}$). This part of the enzyme catalysis (oxygen activation) is lost due to the changes of the enzyme molecules during air–argon transitions.

These results suggest that the enzyme must have different sites for RuBP carboxylase and oxygenase, while sharing a common site for the enolization of RuBP.

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